ULPGC
Universidad de
Las Palmas de
Gran Canaria

Tesis Doctoral

Programa de Doctorado en Oceanografía y Cambio Global

DETERMINACIÓN DE POLIFENOLES, AMINOÁCIDOS Y OTROS METABOLITOS EN MICROALGAS Y EN SUS EXUDADOS

Determination of polyphenols, amino acids and other metabolites in microalgae and their exudates

Paula Santiago Díaz

Febrero 2024

Las Palmas de Gran Canaria

UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA

Programa de doctorado en Oceanografía y Cambio Global

Escuela de doctorado de la Universidad de Las Palmas de Gran Canaria

Título de la Tesis: Determinación de polifenoles, aminoácidos y otros metabolitos en microalgas y en sus exudados

Tesis Doctoral presentada por Dª Paula Santiago Díaz, investigadora contratada predoctoral en formación del Programa Predoctoral de Formación del Personal Investigador de la Universidad de Las Palmas de Gran Canaria

Dirigida por la Dra. Milagros Rico Santos

Codirigida por el Dr. Argimiro Rivero Rosales

Las Palmas de Gran Canaria, a fecha de la firma digital

La Directora, El Codirector, La doctoranda,

REGLAMENTO 1/2023, DE ESTUDIOS DE DOCTORADO DE LA UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA aprobado por el Consejo de Gobierno el 26 de enero de 2023 (BOULPGC nº2 (Extraordinario) de 27 de enero de 2023)

Artículo 24. Tesis por compendio de publicaciones

1. Para la presentación de tesis por compendio de publicaciones será necesario:

a) Un mínimo de tres publicaciones, con unidad temática, indexadas en el *Journal Citations Reports*, *Arts and Humanities Citation Index* o equivalentes, de las que el doctorando sea el primer autor o autor principal. Al menos una de ellas deberá haber sido publicada en una revista cuyo índice de impacto la sitúe dentro de la primera mitad en orden decreciente de índice de impacto entre las revistas del área. Se considerarán publicaciones equivalentes aquellas que se recojan en los procedimientos en la última convocatoria de obtención de sexenios de investigación en cada campo.

b) Para acreditar la condición de autor principal, esta deberá ser reconocida por el resto de autores de las publicaciones presentadas como núcleo de la tesis doctoral al mismo tiempo que estos deberán renunciar a utilizar estas publicaciones como núcleo principal de otras tesis doctorales, sin perjuicio de que dichas publicaciones puedan ser presentadas como méritos complementarios en las tesis doctorales que pudieran presentar los otros autores de dichas publicaciones.

c) En las áreas de especial incidencia tecnológica dos de estas publicaciones podrán ser sustituidas por patentes en explotación o publicaciones en congresos reconocidos por la CNEAI en sus baremos para la obtención de sexenios.

d) Que en las publicaciones o patentes conste la Universidad de Las Palmas de Gran Canaria a través de la afiliación del doctorando y del director en su caso.

ULPGC Universidad de Las Palmas de Gran Canaria | Escuela de Doctorado

REGLAMENTO 1/2023, DE ESTUDIOS DE DOCTORADO DE LA UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA aprobado por el Consejo de Gobierno el 26 de enero de 2023 (BOULPGC nº2 (Extraordinario) de 27 de enero de 2023)

Artículo 40. Mención Internacional en el título de Doctor

1. En el anverso del título de Doctor podrá incluirse la mención «Doctor internacional», cuando en el desarrollo de los estudios de doctorado y en la tesis concurran los siguientes requisitos:

a) Que, durante el periodo de formación necesario para la obtención del título de Doctor, el doctorando haya realizado una estancia mínima de tres meses fuera de España en una institución de enseñanza superior o centro de investigación de prestigio, cursando estudios o realizando trabajos de investigación. La estancia y las actividades han de ser avaladas por el director y autorizadas por la Comisión Académica, incorporándolas al documento de actividades del doctorando.

b) Que parte de la tesis doctoral, al menos el resumen y las conclusiones, se haya redactado y sea presentada en una de las lenguas habituales para la comunicación científica en su campo de conocimiento, distinta a cualquiera de las lenguas oficiales de España. Esta norma no será de aplicación cuando las estancias, informes y expertos procedan de un país de habla hispana.

c) Que la tesis haya sido informada por un mínimo de dos expertos, doctores pertenecientes a alguna institución de educación superior o instituto de investigación no española.

d) Que, al menos, un experto perteneciente a alguna institución de educación superior o centro de investigación no española, con el título de doctor, y distinto del responsable de la estancia mencionada en el apartado 1, haya formado parte del tribunal evaluador de la tesis.

"La verdadera ciencia enseña, por encima de todo, a dudar y a ser ignorante"

-Miguel de Unamuno (1864-1936) escritor y filósofo español

AGRADECIMIENTOS

Quiero comenzar agradeciendo a todas aquellas personas que, de manera directa o indirecta, han formado parte de una de las etapas más desafiantes y enriquecedoras de mi vida que ha supuesto este período como doctoranda.

A mis padres, por acompañarme en cada paso, en cada exposición de TFG, TFM y por supuesto en esta Tesis. Gracias por enseñarme el valor del trabajo, por motivar mi curiosidad científica desde mis primeros años y por apoyarme en cada decisión que he tomado en la vida.

A mi hermano Álvaro, por esas charlas de desconexión que hemos compartido cuando ha sido necesario, por el apoyo y el consejo mutuo (aunque a veces no lo sigamos) y por esperarme para almorzar todos los días.

A mis abuelos, que han compartido todas mis etapas como estudiante y me han sido público de todos los ensayos de mis exposiciones. A mi abuela, que siempre ha confiado en que podría lograr todo lo que me propusiera con su energía positiva y su visión optimista hacia la vida. Y a mi abuelo, que, a pesar de todo, siempre recuerda que su nieta es Ingeniera Química.

A mis tíos Fati y Javi por verme crecer y apoyarme todos estos años, independientemente del lugar del mundo en el que me encontrase, siempre he sentido su apoyo y su cariño.

A mis directores de tesis, por el tiempo que han invertido en mi formación como investigadora y como docente, sin su liderazgo no habría sido posible. A Milagros por todos esos fines de semana, vacaciones, tardes y noches que ha empleado en mi desarrollo durante estos casi 8 años y por confiar en mí, y en mi trabajo, desde aquel TFG de 2017. A Argimiro, por aparecer en el laboratorio para ser no sólo un mentor, sino un compañero, por tratarme como un igual y por todas esas horas que hemos compartido hablando de química y de la vida.

A Miguel por estar ahí siempre para orientarme, aconsejarme, ayudarme y confiar en mí durante todos estos años. Siempre te estaré agradecida.

Al grupo del café Sarah, Rayco y Miriam, por escuchar las muchas veces que amenacé con dejarlo y por las muchas veces que me animaron a no hacerlo. El café de las 9 se convirtió en el momento más esperado del día.

A todos los miembros del grupo QUIMA, en especial a Magdalena y a Melchor por acogerme como una más y por permitirme disponer de sus recursos y experiencia

para llevar a cabo esta Tesis Doctoral. Su labor como investigadores es y será siempre un ejemplo a seguir para mí.

A mis amigos que han sido apoyo fundamental en esta aventura. Por compartir todos los momentos de incertidumbre y por ayudarme a desconectar para volver a conectar con más ganas. Por esas cenas de psicólogo-paciente que hemos compartido y que espero sigamos compartiendo en todos los sitios que nos quedan por descubrir de la isla. A mi triángulo por estar siempre, por soportar todas mis quejas y por celebrar todos mis logros.

A René, mi compañero de vida, por todos estos años que hemos crecido juntos como personas y como profesionales. Gracias por creer en mí mucho más de lo que yo he creído nunca, por soportar todos los altibajos que ha supuesto este camino y por quererme incondicionalmente.

La presente Tesis Doctoral se ha realizado en el Departamento de Química de la Universidad de Las Palmas de Gran Canaria, en el Edificio de Ciencias Básicas del Campus Universitario de Tafira. Su desarrollo ha tenido lugar dentro del grupo de Química Marina (QUIMA) como investigadora contratada predoctoral en formación del Programa Predoctoral de Formación del Personal Investigador de la Universidad de Las Palmas de Gran Canaria (PIFULPGC-2019).

Los trabajos de la presente tesis se enmarcan dentro de dos proyectos de investigación siendo Juana Magdalena Santana Casiano la investigadora principal de ambos proyectos:

Efecto de la acidificación oceánica, la temperatura y la materia orgánica en la persistencia de Fe(II) en el Océano Atlántico (ATOPFe) (Referencia CTM2017-83476-P), proyecto de excelencia I+D de convocatoria pública competitiva financiado por el Ministerio de Economía, Industria y Competitividad. Fecha de Inicio: 01-01-2018; Fecha de Finalización: 30-09-2021.

Respuesta del Fe en un Océano Acifidificado (FeRIA) (Referencia: PID2021-123997NB-100), proyecto de convocatoria pública competitiva financiado por el Ministerio de Ciencia e Innovación. Fecha de Inicio: 01-09-2022; Fecha de Finalización: 31-08-2025.

ÍNDICE

RESUMEN

El planeta Tierra se enfrenta a un cambio global que incluye transformaciones en el clima, la biodiversidad, los ciclos biogeoquímicos, los ecosistemas y las interacciones humanas con el medio. Estas alteraciones tienen su origen tanto en procesos naturales como en actividades antropogénicas y representan un desafío para los ecosistemas marinos. Los cambios ambientales derivados de la contaminación o de la acidificación oceánica constituyen condiciones de estrés para la vida marina, afectando desde los niveles más básicos de la cadena alimentaria, como el fitoplancton, hasta los sistemas ecológicos más complejos.

La comprensión de la repercusión de estas transformaciones en la distribución, composición y productividad del fitoplancton marino es crucial para evaluar el impacto general del cambio global en la biodiversidad marina, la pesca y la capacidad de los océanos para actuar como sumideros de carbono. En especial, el estudio de las variaciones en la composición bioquímica de estos microorganismos, tanto a nivel intracelular como extracelular, es relevante debido a su relación directa con los ciclos biogeoquímicos y con la tolerancia a factores de estrés ambiental.

La presente Tesis Doctoral expone a través de los diferentes capítulos los resultados del estudio del impacto de factores de estrés ambiental en las microalgas marinas *Phaeodactylum tricornutum* y *Emiliania huxleyi*, tanto en la producción de metabolitos orgánicos intra- y extracelulares, como en la capacidad antioxidante de los mismos.

En el Primer Capítulo se establece una contextualización y estado del arte que describe la relevancia del estudio de los factores de estrés seleccionados, el papel biológico de los metabolitos orgánicos analizados (aminoácidos, compuestos fenólicos y carbohidratos) y la relación de la capacidad antioxidante de dichos metabolitos frente a las condiciones de cultivo.

En el Segundo Capítulo se definen los objetivos de la Tesis Doctoral y las futuras líneas de investigación en las que se aplicarán y se desarrollarán los resultados obtenidos en la misma.

En el Tercer Capítulo se detalla la metodología para la determinación de los compuestos orgánicos en los extractos de las diferentes microalgas, así como en las aguas enriquecidas con sus exudados. Para el análisis de aminoácidos y polifenoles se

optimizaron métodos de cromatografía líquida de alta resolución en fase reversa con detector de diodo array, con los que se consiguió la identificación y cuantificación de ambos tipos de compuestos de manera sencilla, exacta y reproducible. Para la determinación de carbohidratos y de la capacidad antioxidante se empleó la espectrofotometría UV-Vis.

En el Cuarto Capítulo se presentan y discuten los resultados de la aplicación de la metodología desarrollada para el análisis de las especies de microalgas citadas anteriormente cultivadas bajo diferentes condiciones, así como su empleo en el análisis de otros materiales vegetales de origen terrestre. Además, se incluyen los resultados obtenidos en las dos estancias de investigación realizadas en centros internacionales.

Por último, en el Quinto Capítulo se recogen las conclusiones de esta Tesis, donde queda de manifiesto la importancia del estudio de los metabolitos orgánicos en el fitoplancton marino ante diferentes condiciones de estrés ambiental, ayudando a comprender el impacto del cambio global en la salud de los océanos.

ABSTRACT

Planet Earth is facing a global change that includes transformations in climate, biodiversity, biogeochemical cycles, ecosystems, and human interactions with the environment. These alterations originate from both natural processes and anthropogenic activities and represent a challenge for marine ecosystems. Environmental changes derived from pollution or ocean acidification constitute stress conditions for marine life, affecting from the most basic levels of the food chain, such as phytoplankton, to the more complex ecological systems.

Understanding the effect of these transformations on the distribution, composition, and productivity of marine phytoplankton is crucial to assess the overall impact of global change on marine biodiversity, fisheries, and the oceans' ability to act as carbon sinks. Particularly, the study of variations in the biochemical composition of these microorganisms, both intracellularly and extracellularly, is relevant due to their direct relationship with biogeochemical cycles and tolerance to environmental stress factors.

This Doctoral Thesis presents, through different chapters, the results of the study on the impact of environmental stress factors on the marine microalgae *Phaeodactylum tricornutum* and *Emiliania huxleyi* in the production of intra- and extracellular organic metabolites and their antioxidant capacity.

The First Chapter establishes a contextualization and state of the art that describes the relevance of studying the selected stress factors, the biological role of the analyzed organic metabolites (amino acids, phenolic compounds, and carbohydrates), and the relationship of the antioxidant capacity of these metabolites with the culture conditions.

The Second Chapter defines the objectives of the Doctoral Thesis and future research lines that will be developed with the methods and the results obtained in it.

The Third Chapter details the methodology for the determination of organic compounds in extracts from different microalgae and their exudates. Different methods of reverse-phase high-performance liquid chromatography with a diode-array detector were optimized for the analysis of amino acids and polyphenols, in a simple, accurate, and reproducible manner. For the determination of carbohydrates and antioxidant capacity, UV-Vis spectrophotometry was employed.

The Fourth Chapter presents and discusses the results of applying the developed methodology for the analysis of the microalgae species cultivated under different

conditions, as well as its use in the analysis of other terrestrial plant materials. Additionally, the results obtained during the two research stays at international centers are included.

Finally, the Fifth Chapter draws the conclusions of this Thesis, highlighting the importance of the study of organic metabolites in marine phytoplankton under different environmental stress conditions, helping to understand the impact of global change on the health of the oceans.

CAPÍTULO 1.
INTRODUCCIÓN

1.1. Fitoplancton en el ecosistema marino

El fitoplancton constituye el conjunto de microorganismos autótrofos que, mediante la fotosíntesis, son capaces de convertir el dióxido de carbono y otros compuestos inorgánicos en oxígeno y moléculas orgánicas. Esta característica los convierte en productores primarios y en la base de la cadena trófica de los ecosistemas marinos y, por tanto, cambios en su estructura pueden afectar a niveles tróficos superiores. Aunque el fitoplancton constituye menos del 1% de la biomasa fotosintética de la Tierra, es responsable de cerca del 50% de la producción primaria global y la primera fuente de energía de los ecosistemas acuáticos (Field et al., 1998).

El fitoplancton marino se puede clasificar en cinco grupos de acuerdo con su estructura celular: diatomeas, dinoflagelados, cianobacterias, algas verdes y cocolitofóridos (Santhanam et al., 2018). De estos, todos exceptuando las cianobacterias, pertenecen a las denominadas microalgas marinas. Las microalgas marinas son una fuente de compuestos bioactivos entre los que se encuentran proteínas, lípidos, polisacáridos, pigmentos, vitaminas y polifenoles (Anusree et al., 2023; Cichoński y Chrzanowski, 2022; Wu et al., 2021). Su composición varía aproximadamente entre un 12-35% de proteína, 4,6-23% de carbohidratos y 7,2-23% de lípidos (Brown et al., 1993) y es importante para las cadenas tróficas superiores, así como una fuente alternativa de moléculas con potenciales aplicaciones en nutrición, medicina o cosmética. Además, su composición bioquímica es un indicador del cambio en su estado fisiológico en respuesta a cambios en el medio (Bhavya et al., 2019).

Las microalgas marinas tienen la capacidad de liberar una gran variedad de compuestos orgánicos como carbohidratos simples y polisacáridos, aminoácidos, lípidos, ácidos orgánicos, y compuestos fenólicos, contribuyendo a la materia orgánica disuelta en el medio (DOM por sus siglas en inglés “Dissolved Organic Matter”) (Myklestad, 2001). Estas moléculas orgánicas disueltas intervienen en el ciclo del carbono marino y en la biodisponibilidad del hierro (Fe) (Engel et al., 2004; Hassler et al., 2011). La DOM es un sustrato esencial para los microrganismos heterotróficos en el océano y, por tanto, su variación afectará al resto de organismos del medio (Kahler et al., 1997).

1.2. El cambio global y su efecto en el fitoplancton marino

El cambio global se puede definir como aquellas alteraciones de los procesos fundamentales que regulan el funcionamiento del sistema Tierra. Está provocado tanto por actividades antropogénicas, como por procesos naturales, alterando la homeostasis de los ecosistemas.

Los océanos, como el mayor bioma de la Tierra, se ven significativamente afectados tanto directa como indirectamente por el cambio producido por las actividades del ser humano. De hecho, algunos de sus efectos son específicos de los océanos, como la acidificación oceánica, mientras que otros, los comparte con los sistemas terrestres como la contaminación, el calentamiento o la eutrofización (Duarte, 2014).

Procesos naturales como las erupciones volcánicas submarinas son capaces de modificar dramáticamente la química del medio marino, disminuyendo el pH, afectando al sistema de carbonato, reduciendo las concentraciones de oxígeno disuelto e incluso liberando metales y nutrientes (Santana-Casiano et al., 2013).

Este estado resultante de factores externos ambientales que ejercen una presión sobre un ecosistema u organismo es el denominado estrés ambiental, y las condiciones que lo generan se denominan condiciones de estrés.

El fitoplancton marino juega un papel determinante en los procesos de cambio global y en el ciclo biogeoquímico de los océanos, y constituye un indicador idóneo del cambio climático por varias razones (Hays et al., 2005):

(i) No es un recurso explotado comercialmente, por lo que sus cambios a largo plazo se pueden atribuir al cambio climático.

(ii) Su relativamente corto ciclo de vida hace que su respuesta a los cambios ambientales sea más rápida y refleje las condiciones actuales del medio.

(iii) Tiene la capacidad de permanecer en el medio de manera libre lo que les permite responder a cambios en condiciones como temperatura y corrientes afectando a su distribución geográfica.

Es por ello, que entender los factores que controlan las dinámicas de estos microorganismos es importante para poder predecir el impacto de futuras variaciones del clima en el ecosistema marino (Winder y Sommer, 2012).

En los siguientes apartados se describen dos de los factores de estrés ambiental derivados de actividades antropogénicas que se han estudiado en la presente Tesis Doctoral.

1.2.1. Función del hierro y del cobre en el medio marino

Los metales traza (manganeso, hierro, cobalto, cobre, zinc y cadmio), es decir, aquellos presentes en cantidades muy bajas en la naturaleza, tienen un papel biológico significativo para el fitoplancton en la superficie del océano (Morel y Price, 2003). El hierro (Fe) es un micronutriente esencial involucrado en procesos celulares fundamentales, incluyendo la respiración, la fotosíntesis, la absorción y la fijación de nitrógeno (Hogle et al., 2014; Raven et al., 1999). Controla la productividad, la composición de las especies y la estructura trófica de las comunidades microbianas en grandes regiones del océano (Hunter y Boyd, 2007). El cobre (Cu) también un micronutriente esencial para el desarrollo del fitoplancton ya que forma parte de proteínas encargadas del proceso fotosintético (Yruela, 2013), está relacionado con el ciclo del Fe y su asimilación (Maldonado et al., 2006) y con el ciclo del nitrógeno oceánico (Jacquot et al., 2013).

Eventos naturales como las erupciones volcánicas, particularmente en situaciones donde la lava interactúa con el agua de mar, pueden contribuir significativamente al aumento de las concentraciones de metales como el Fe y el Cu en el medio marino (Mason et al., 2021). Sin embargo, en las últimas décadas han sido las actividades industriales las que han aumentado los niveles de estos metales en zonas costeras, descargando al océano hasta 9 millones de toneladas anuales en el caso del Cu (Pinto et al., 2003).

Si la fracción biodisponible de Cu es elevada, este puede acumularse en el interior de las células de las microalgas, lo que reduce su crecimiento y contenido en pigmentos fotosintéticos (Sáez et al., 2015). Además, este exceso de Cu puede catalizar la producción de especies reactivas de oxígeno (ROS, por sus siglas en inglés “Reactive Oxygen Species”) que producen daños en ácidos nucleicos, proteínas, carbohidratos y lípidos dando lugar a alteraciones en el metabolismo e incluso la muerte celular (Gill y Tuteja, 2010). Estos efectos, así como la tolerancia frente al Cu dependerán de la especie de microalga y de su concentración (Franklin et al., 2001). En el caso del Fe ocurre lo contrario, ya que estimula la floración de fitoplancton, mejorando la productividad biológica del océano (Hunter y Boyd, 2007).

Para hacer frente a los efectos generados por metales, las algas poseen diferentes mecanismos de detoxificación y tolerancia (Priyadarshini et al., 2019):

(i) Reducción del metal por unión a la superficie de las células por adsorción física a través de grupos cargados negativamente en la superficie celular (-COOH, -OH-, -NH_2, -SH…etc.).

(ii) Inclusión del metal en orgánulos en el interior de la célula (compartimentalización).

(iii) Producción de moléculas capaces de formar complejos con metales en el interior celular como péptidos, enzimas, proteínas, moléculas con grupos tioles, ácidos orgánicos, entre otros.

(iv) Exclusión de metales del citoplasma por transporte activo o sistema de eflujo.

Además de esta capacidad de regulación intracelular, las microalgas pueden liberar al medio compuestos orgánicos capaces de formar complejos con los metales (Fisher y Fabris, 1982; Koukal et al., 2007; Lombardi et al., 2005; Lombardi y Vieira, 1999; López et al., 2015), siendo uno de los mecanismos para mantener las concentraciones de Fe disuelto por encima de su solubilidad inorgánica (Shaked y Lis, 2012), y para disminuir la toxicidad del Cu en aguas naturales (Nielsen y Wium-Andersen, 1970). En especial, el Cu(II) es el estado de oxidación que presenta toxicidad para los microorganismos y una gran afinidad por compuestos orgánicos (Lombardi et al., 2005).

Por tanto, la naturaleza de estos exudados está relacionada con la persistencia de los metales en el medio, aumentando su solubilidad y biodisponibilidad lo que facilita su transporte en la cadena alimentaria a través de la ingestión de los complejos metal-metabolito orgánico por parte de organismos filtradores (Lombardi et al., 2005).

Más del 99% del Cu y Fe total disuelto en los océanos se encuentra unido a una gran variedad de ligandos orgánicos fuertes y débiles, aunque su estructura química sigue siendo desconocida (Arnone et al., 2022; Coale y Bruland, 1988; Dupont et al., 2004; Jacquot y Moffett, 2015). Por ello, la caracterización de los compuestos orgánicos que interactúan con los iones metálicos resulta de gran relevancia tanto a nivel celular, para profundizar en su relación con la tolerancia del fitoplancton; como a nivel extracelular, para establecer el papel de estos metabolitos orgánicos en la biodisponibilidad, persistencia y toxicidad de dichos metales en el entorno marino.

1.2.2. Impacto de la acidificación oceánica

El aumento de los niveles de CO_2 atmosférico sigue siendo uno de los principales causantes del calentamiento global. Los océanos son fundamentales en este fenómeno, ya que absorben el CO_2 de la atmósfera. Sin embargo, este proceso conlleva a su vez su acidificación, ya que el CO_2 se combina con el agua para dar lugar a ácido carbónico (H_2CO_3) que, a su vez, se disocia en iones oxidanio (H_3O^+) e iones hidrogenocarbonato (HCO_3^-) y, en menor medida en iones carbonato (CO_3^{2-}). La concentración de iones oxidanio está relacionada con el pH del medio, de tal manera que una disminución de una unidad en el pH corresponde a un incremento de 10 veces en la concentración de estos. Esta alteración del pH conlleva a su vez cambios en la química del entorno marino con graves consecuencias para muchos organismos.

A pesar de que los efectos fisiológicos y ecológicos del cambio climático en el fitoplancton oceánico están ampliamente estudiados (Hays et al., 2005; Van de Waal y Litchman, 2020; Winder y Sommer, 2012), sus implicaciones a nivel molecular y bioquímico en condiciones de acidificación no se ha investigado en profundidad.

Estudios realizados por Jin et al. (2015) demostraron que la acidificación oceánica aumenta la producción de compuestos fenólicos en *Emiliana huxleyi.* Este tipo de microalgas denominadas cocolitofóridos son especialmente sensibles al aumento del CO_2 en el medio, ya que disminuye su calcificación y debilita la estructura de carbonato cálcico (cocolitos) que las protege. La acumulación de polifenoles actuaría como mecanismo de protección ya que pueden ser tóxicos para los potenciales depredadores. Por otro lado, la acidificación del medio de cultivo aumenta la producción de ROS en el interior de las células de *E. huxleyi*, por lo que la acumulación y liberación de metabolitos orgánicos con capacidad antioxidante podría minimizar los efectos dañinos de estas especies reactivas (Vázquez et al., 2023).

El aumento en la producción de polifenoles ante la acidificación no solo se produce a nivel intracelular, sino que también son liberados al medio externo por el fitoplancton como respuesta a esta disminución del pH. En estudios de Samperio-Ramos et al. (2017) se comprobó cómo el descenso del pH intensifica la exudación de polifenoles y ácidos urónicos por parte del alga *E. huxleyi*. Además de compuestos fenólicos, la producción de otros metabolitos orgánicos se puede ver alterada. Es el caso de la diatomea *Skeletonema costatum,* en la que un descenso en el pH del medio de cultivo produce

alteraciones en el perfil y contenido de aminoácidos libres intracelulares, así como en los carbohidratos extracelulares (Taraldsvik y Myklestad, 2000).

Especies químicas como los iones hidroxilo y carbonato (OH^- y CO_3^{2-}) capaces de formar complejos fuertes con metales divalentes y trivalentes en el océano, disminuirán su concentración en condiciones de acidificación, aumentando la fracción de estos metales en su forma libre. Un descenso del pH del mar de 8,1 a 7,4 implica un aumento en la solubilidad de Fe(III) en un 40%, lo que podría tener un gran impacto en los ciclos biogeoquímicos (Millero et al., 2009; Morel y Price, 2003). Mientras que el mismo descenso aproximadamente, incrementaría en un 30% el Cu(II) disuelto favoreciendo su biodisponiblidad y toxicidad (Millero et al., 2009). Por tanto, la disminución del pH aumenta la solubilidad de los metales y disminuye su capacidad de interacción con la superficie de partículas orgánicas favoreciendo la persistencia de las formas más tóxicas para la biota marina. Especies como *Desmodesmus* sp. MAS1 y *Heterochlorella* sp. MAS3 modificaron la síntesis de carbohidratos, proteínas y lípidos al exponerse a un pH más ácido en presencia de Cu, Fe, Mg y Zn en comparación con los cambios producidos a pH más básicos (Abinandan et al., 2021). Estos cambios bioquímicos y fisiológicos consiguieron minimizar la toxicidad de los metales, mejorando su tolerancia.

Por otro lado, la acidificación de los océanos aumenta el tiempo de residencia de las formas reducidas del Fe y del Cu, al disminuir su velocidad de oxidación (Millero et al., 2009). La reducción de Fe(III) a Fe(II) debido a la presencia de compuestos orgánicos de origen planctónico como el ácido gálico fue observada por Pérez-Almeida et al. (2022).

La identificación de estos ligandos orgánicos en agua de mar representa un desafío por las bajas concentraciones en las que se encuentran (rango de nM), y por la relevancia de sus efectos en la biodisponibilidad y toxicidad de metales, y en la cadena trófica de los ecosistemas marinos, que afectaría a la biodiversidad de mares y océanos.

1.3. Metabolitos orgánicos y su papel biológico

1.3.1. Aminoácidos

Desde el aislamiento por primera vez de un aminoácido, la asparagina, por Vauquelin y Robiquet extraída del *Asparagus sativus* en 1806 (Vickery y Schmidt, 1931), el conocimiento sobre su funcionalidad tanto en humanos como en plantas se ha ampliado considerablemente. Los aminoácidos se caracterizan por ser compuestos químicos

abundantes que contienen y transportan nitrógeno en la mayoría de plantas (Tegeder, 2014). En su estructura poseen al menos un grupo amino (-NH_2) y un grupo carboxilo (- COOH) que los convierte en zwitteriones (derivado del alemán Zwitter, "híbrido"), es decir, pueden presentar grupos con carga positiva (-NH_3^+) y negativa (-COO^-). Su estado variará en función de su punto isoeléctrico (pH en el que el aminoácido posee una carga neta de cero) y del pH del medio.

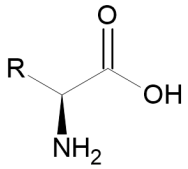


Figura 1. Estructura básica de un L-aminoácido

Los aminoácidos pueden existir como isómeros L o D (excepto la glicina al carecer de un carbono asimétrico) siendo los L-aminoácidos, los más abundantes en la naturaleza y los constituyentes de proteínas vegetales. Estos a su vez se pueden clasificar en dos grupos: esenciales (leucina, isoleucina, metionina, fenilalanina, arginina, histidina, triptófano, valina, treonina y lisina) y no esenciales (alanina, asparagina, cisteína, glutamina, ácido aspártico, ácido glutámico, glicina, prolina, serina y tirosina). Los aminoácidos no esenciales son aquellos que el ser humano es capaz de sintetizar, mientras que los esenciales deben ser incorporados al organismo en la dieta. Las plantas son capaces de sintetizar tanto aminoácidos esenciales como no esenciales a partir de carbono inorgánico.

Los aminoácidos son metabolitos primarios y la base estructural de las proteínas interviniendo en una gran variedad de procesos vitales en los organismos. Además de los pertenecientes a las proteínas y péptidos, los aminoácidos se pueden encontrar en su forma libre en las células, siendo importantes en el desarrollo celular de organismos vegetales.

Entre los factores de estrés abiótico más dañino tanto en plantas como en animales se encuentra la presencia de metales pesados (Cu, Cd, Pb, Hg…). La relación de los aminoácidos con la respuesta a este tipo de estrés abiótico se conoce desde el estudio de la acumulación de prolina en célula vegetales de plantas como *Lemna minor*, *Oriza sativa* o *Cajanus cajan* (Alia y Saradhi, 1991; Bassi y Sharma, 1993; Chen et al., 2001; Shah y Dubey, 1997). En la actualidad, la acción de la prolina como respuesta a la presencia de

metales está ampliamente estudiada. Este aminoácido puede actuar como agente quelante al unirse a los iones metálicos, neutralizando sus efectos (Sharma et al., 1998) y puede participar como captador de ROS producidos por la presencia de altas concentraciones de metales, mejorando así la tolerancia de las plantas a la exposición a estos (Matysik et al., 2002).

Sharma y Dietz (2006) concluyen que además de la prolina, aminoácidos como la histidina, asparagina, glutamina, ácido aspártico, ácido glutámico, alanina o cisteína también podrían intervenir en la respuesta de las plantas al estrés por metales como Cd, Cu, Ni y Zn. Esto se debe a que diversos aminoácidos (arginina, ácido aspártico, valina, triptófano, ácido glutámico, glicina, alanina, leucina, isoleucina, fenilalanina, serina, histidina) poseen la capacidad de complejar iones metálicos (Bathula y Kotra, 2019; Bougherra et al., 2018; Cuevas et al., 1998; Hossain et al., 2015; Kryukova et al., 2005; Mesu et al., 2006). Por tanto, la acumulación de aminoácidos y sus derivados podría ser considerada como una respuesta activa y no sólo como consecuencia de una desregulación del metabolismo.

La acumulación de aminoácidos como respuesta al estrés por metales también se ha manifestado en microalgas. Afkar et al. (2010) comprobaron cómo un aumento en la concentración de Co, Cu y Zn producía un incremento del contenido total de aminoácidos libres intracelulares en *Chlorella vulgaris*. Estos autores proponen esta acumulación como consecuencia de la supresión de la biosíntesis de proteínas para disminuir los niveles de metales libres por algún mecanismo quelante que contrarresta su toxicidad. Çelekli et al. (2013) encontraron resultados similares en *Scenedesmus quadricauda var. longispina* cultivada bajo diferentes concentraciones de Cd(II). El aumento en la concentración de este metal en el medio dio lugar a un incremento en el contenido de prolina intracelular.

Kováčik et al. (2010) llevaron a cabo un análisis más detallado del perfil de aminoácidos identificando 17 aminoácidos diferentes en extractos de *Scenedesmus quadricauda* cultivada en presencia de concentraciones letales de Cu. Como resultado, observaron un mayor incremento en el contenido de arginina, histidina, metionina y prolina en sus células.

Chia et al. (2015) propusieron la variación en la composición de aminoácidos totales (libres e hidrolizados de proteínas y péptidos) de las microalgas como

biomarcadores sensibles ante el estrés por metales. Además, autores como Huang et al. (2022) emplearon la determinación aminoácidos intracelulares como una forma más de evaluar los efectos de metales como el Cu en microalgas marinas. Por otra parte, la producción de aminoácidos también puede ser inducida por una limitación en el contenido de algún metal, como es el caso del Fe, cuyo déficit promueve la síntesis de ácido domoico en la diatomea *Pseudonitzschia* (Maldonado et al., 2002; Rue y Bruland, 2001).

La liberación de aminoácidos al medio de cultivo por microalgas ante variaciones de las concentraciones de metales traza no ha sido tan ampliamente investigada. La interacción de estos aminoácidos con metales esenciales como el Fe o el Mn podría influir en su biodisponibilidad. En las aguas del Ártico se detectaron ligandos orgánicos del Fe con características fluorescentes similares a los aminoácidos aromáticos triptófano y tirosina, cuya procedencia se relaciona con materia orgánica derivada del fitoplancton (Williford et al., 2021). Estos aminoácidos también han sido identificados en el material extracelular liberado por el alga *Scenedesmus acutus* (McIntyre y Guéguen, 2013). Algunos estudios han relacionado esta materia orgánica exudada en presencia de metales con grupos específicos pertenecientes a aminoácidos (Dupont et al., 2004; Dupont y Ahner, 2005). Por ejemplo, aminoácidos que contienen el grupo tiol como la cisteína son capaces de formar un complejo con Fe(III) mediante la transferencia de un electrón del grupo tiol del dando lugar a la reducción de Fe(III) a Fe(II), y a la consecuente oxidación de cisteína a cistina facilitando la biodisponibilidad del Fe(II) (Santana-Casiano et al., 2000).

La caracterización de la naturaleza de los aminoácidos, tanto acumulados en las células algales como liberados al medio, es importante ya que puede ser un factor determinante y selectivo en la complejación de metales (Tonietto et al., 2014).

1.3.2. Compuestos fenólicos

Los “polifenoles” o “compuestos fenólicos” mencionados por primera vez en el siglo IV a.C. como “taninos vegetales”, se empleaban en el curtido de pieles con extractos vegetales. Durante el siglo XX, con el avance de las técnicas analíticas, se amplió el conocimiento de la estructura y función de estos compuestos en plantas. En la actualidad, estos “taninos vegetales” se conocen como polifenoles capaces de establecer enlaces de hidrógeno con el colágeno (acción de *tanning*). Existen numerosos tipos y estructuras de polifenoles caracterizadas por poseer un anillo aromático con uno o más grupos hidroxilos

como sustituyentes (Quideau et al., 2011). Atendiendo a las estructuras más comunes entre ellos, se pueden clasificar en: ácidos fenólicos (hidroxibenzoicos e hidroxicinámicos), flavonoides, estibenos y lignanos. A su vez, dentro de los flavonoides se distinguen los flavonoles, flavonas, isoflavonas, flavanonas, antocianinas y flavanos (Manach et al., 2004).

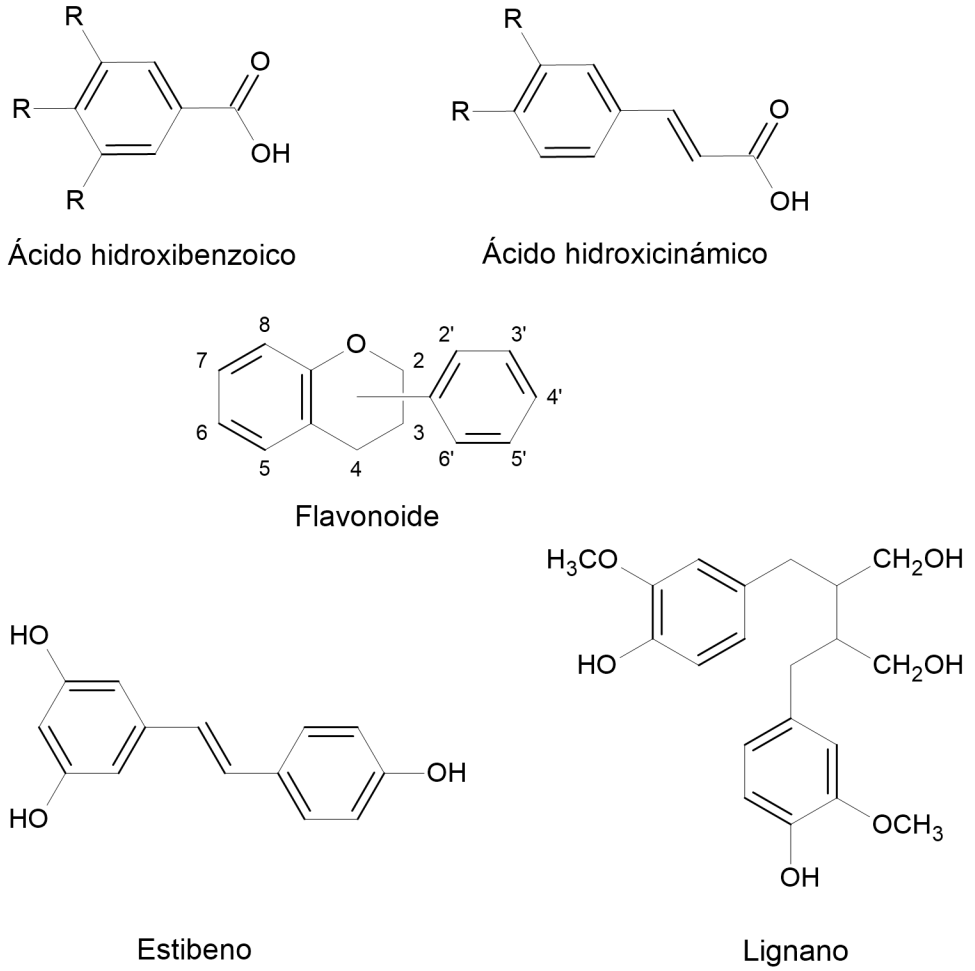


Figura 2. Estructuras básicas de los diferentes grupos de polifenoles. R= OH, H, OCH_3

Los polifenoles se consideraban productos de desecho del metabolismo primario de plantas. Sin embargo, hoy en día se reconoce su importancia como uno de los metabolitos secundarios vegetales que intervienen en procesos como la pigmentación, reproducción, crecimiento y desarrollo, resistencia a los patógenos, capacidad antioxidante y complejación de metales (Lattanzio et al., 2008).

A pesar de que en la segunda mitad de los años ochenta los polifenoles se seguían considerando exclusivos de plantas terrestres, en la actualidad es conocida su presencia

en algas. Aunque la mayoría de la literatura sobre polifenoles se centra en macroalgas, en los últimos años el estudio de estos compuestos en microalgas se ha visto incrementado.

El contenido total en polifenoles varía entre especies, e incluso entre microalgas de la misma especie en función de las condiciones de cultivo (Cardoso et al., 2020). De la misma forma, el perfil polifenólico de cada microalga es diferente, siendo algunos exclusivos de especies concretas (Kapoor et al., 2022). Aun así, existen determinados ácidos fenólicos que coinciden en gran parte de las microalgas como el ácido gálico, ferúlico, cafeico, clorogénico, sinápico, coumárico, vanílico y protocatecuico (Del Mondo et al., 2021).

Gran parte de las investigaciones de polifenoles en microalgas se centran en el estudio de su capacidad antioxidante y complejante, actuando como protección ante diferentes condiciones de estrés relacionadas con la luz, salinidad, cantidad de nitrógeno, toxicidad de metales (Chokshi et al., 2017; Curcuraci et al., 2022; Fal et al., 2022; Roy et al., 2021; Smerilli et al., 2019; Strejckova et al., 2019).

La respuesta ante el estrés metálico ha sido ampliamente estudiada mediante la cuantificación de polifenoles totales intracelulares en microalgas por el método de Folin-Ciocalteu. Hamed, Selim, et al. (2017) expusieron a dos especies de microalgas *Chlorella sorokiniana* y *Scenedesmus acuminatus* a dosis subletales de Cu, dando como resultado un incremento de los polifenoles (cuantificados mediante el método Folin-Ciocalteu) y flavonoides totales intracelulares. Este autor realiza la misma investigación con Zn, analizando nuevamente los polifenoles y flavonoides totales (Hamed, Zinta, et al., 2017). En ambos estudios, la cantidad de polifenoles acumulados en el interior de las células aumenta con respecto a aquellas microalgas que no han sido cultivadas en presencia de estos metales. De la misma forma, los compuestos fenólicos totales (cuantificados mediante el método Folin-Ciocalteu) aumentaron en la microalga *Scenedesmus quadricauda* expuesta a Cd, Ni y Cu, en comparación con cultivos sin presencia de metales (Strejckova et al., 2019).

A pesar de que la determinación de polifenoles totales mediante el reactivo de Folin-Ciocalteu es ampliamente utilizado, dicho reactivo no es específico, y es capaz de reaccionar de manera simultánea con polifenoles, aminoácidos o vitaminas (Huang et al., 2005) por lo que no refleja el contenido específico de polifenoles.

El método desarrollado por López et al. (2011) permite la determinación de 14 polifenoles diferentes. Estudios llevados a cabo por el grupo QUIMA aplicando esta metodología permitieron identificar los polifenoles que experimentaban cambios ante el estrés por metales en diferentes microalgas (*Phaeodactylum tricornutum* y *Dunaliella tertiolecta*) (López et al., 2015; Rico et al., 2013). El aumento o disminución de los polifenoles acumulados y exudados al medio, así como el perfil polifenólico, dependerá del mecanismo propio de la especie de microalga y del metal que genere ese estrés oxidativo. El alga *D. tertiolecta* es capaz de excluir el Cu del interior de sus células de manera más eficiente que *P. tricornutum,* por lo que la acumulación de polifenoles en su interior no es necesaria y es menor.

Como se ha comentado anteriormente, los polifenoles exudados por algas son capaces de formar complejos con otros metales divalentes, de esta forma pueden aumentar o disminuir su disponibilidad beneficiando al fitoplancton y a otros organismos (Ragan et al., 1979). El ácido gálico, el ácido sinápico y la catequina forman complejos con el Fe(III), reducen el Fe(III) a Fe(II) y retrasan la oxidación de Fe(II) a Fe(III), prolongando su biodisponibilidad en el medio marino (González et al., 2012, 2019; Santana-Casiano et al., 2014).

1.3.3. Carbohidratos

Los carbohidratos constituyen el grupo de moléculas orgánicas más abundantes en el planeta Tierra, formando parte de tejidos vegetales, animales, bacterias y hongos. Comprenden una amplia categoría de azúcares simples (monosacáridos, entre tres y siete átomos de carbono) y sus polímeros (di-, oligo-, y polisacáridos), así como sus derivados ácidos urónicos y aminoazúcares. Son esenciales en plantas ya que constituyen los productos primarios de la fotosíntesis y desempeñan múltiples funciones: transporte de energía y carbono, estructurales, moléculas señales, reguladores osmóticos, y precursores de otras moléculas (Halford et al., 2011).

De la misma forma que en las plantas, la síntesis de carbohidratos en algas tiene dos funciones principales: (i) actúan como componentes estructurales en las paredes celulares y (ii) sirven como componentes de almacenamiento en el interior de la célula. Su proporción puede variar en función de la especie de alga marina, en algas rojas y pardas representan un 74 % de la materia orgánica, mientras que en microalgas se encuentran en menor proporción (Romankevich, 1984). El tipo de carbohidrato

acumulado también dependerá de la especie, por ejemplo, las algas rojas sintetizan un híbrido entre almidón y glucógeno, las algas verdes sintetizan polisacáridos de tipo amilopectina (almidón), y, en algas como *Emiliania huxleyi*, predomina el manitol (Markou et al., 2012; Obata et al., 2013).

Los carbohidratos no sólo proporcionan la energía necesaria para los procesos metabólicos de los organismos, sino que también permiten a las microalgas sobrellevar condiciones ambientales adversas como la limitación de nutrientes (N, P, S, Fe, Mn y K); el exceso de metales pesados; el estrés osmótico por variaciones en la concentración de sales; o las variaciones en la intensidad lumínica y en la temperatura (Markou et al., 2012).

Como ocurre con los aminoácidos y polifenoles, su capacidad de complejación les permite unirse a metales esenciales aumentando su biodisponibilidad. Esto ha sido demostrado por Hassler et al. (2011) al añadir tres carbohidratos diferentes en cultivos y en poblaciones naturales de fitoplancton eucariótico, comprobando un aumento de la biodisponibilidad de Fe y sugiriendo que este fenómeno podría ser generalizable. Además, su concentración en la superficie del mar es mayor que la concentración de Fe y la de sideróforos con grupos carboxílicos, por lo que es probable que formen complejos con una fracción importante de Fe disuelto incluso en presencia de ligandos fuertes, como los sideróforos (Benner, 2011).

Como se ha mencionado anteriormente, ante la toxicidad de metales, las microalgas son capaces de aumentar la síntesis de carbohidratos como mecanismo de defensa (Afkar et al., 2010; Pistocchi et al., 1997). Especies como *Scenedesmus quadricauda, Scenedesmus acutus, Scenedesmus obliquus* y *Chlorella* sp. experimentaron un aumento en el contenido de carbohidratos intracelulares al ser cultivadas en presencia de metales, en comparación con aquellas cultivadas en ausencia de estos (Dao et al., 2017; He et al., 2017; Silva et al., 2018).

Sin embargo, en los estudios llevados a cabo por Thabet et al. (2023) tanto los carbohidratos totales como el almidón de las células de la microalga marina *Chlorococcum dorsiventrale,* aumentaron significativamente tras 24 horas de exposición a concentraciones tóxicas de Hg, pero disminuyeron tras 72 horas de tratamiento. Este metal es conocido por causar daños graves en la cadena transportadora de electrones del proceso fotosintético, además de cambios en el metabolismo de carbono lo que puede

producir un consumo de almidón por parte de la célula en favor de la biosíntesis de lípidos. Por el contrario, la cantidad de carbohidratos en la microalga *Selenastrum gracile* expuesta a diferentes concentraciones de Cu no se vio afectada significativamente (p>0,05) con respecto al control (Rocha et al., 2016).

Los carbohidratos extracelulares liberados al medio pueden contribuir en mayor medida que los intracelulares a mitigar el estrés por metales (Pistocchi et al., 1997). Concentraciones tóxicas de Cu, Cd, Zn o Pb en el cultivo de diferentes especies de microalgas da lugar a un incremento en los carbohidratos extracelulares, favoreciendo su tolerancia a estos iones metálicos (Pistocchi et al., 2000). De hecho, la extracción del material polimérico exudado (60% polisacáridos) de los cultivos agravó la toxicidad y redujo la capacidad de eliminación de metales de la solución (Li et al., 2023).

1.4. Capacidad antioxidante de los metabolitos orgánicos

La capacidad antioxidante de los metabolitos orgánicos sintetizados por el fitoplancton marino participa en la tolerancia a las diferentes condiciones de estrés ambiental producidas por el cambio global. Estas condiciones de estrés oxidativo producen alteraciones en la bioquímica de las células planctónicas y por tanto en las comunidades del ecosistema marino. La principal consecuencia del estrés oxidativo es la producción de radicales libres y especies reactivas de oxígeno.

Un radical libre es aquel compuesto capaz de existir independientemente con uno o más electrones desapareados. Cuando este reacciona con una especie que no es un radical, se genera un nuevo radical comenzando así una reacción en cadena. Aquellos que contienen oxígeno son los anteriormente mencionados ROS que incluyen tanto radicales de oxígeno ($O_2^{\bullet}$, $OH^{\bullet}$, $LOO^{\bullet}$, y $LO^{\bullet}$) como moléculas que contienen oxígeno pero que no son radicales (HClO, H_2O_2, y O_3) (Halliwell et al., 1995). Estas especies reactivas se generan como subproductos o intermedios durante los procesos normales del metabolismo celular. Sin embargo, la acumulación excesiva de ROS en el interior de las células puede producir daños y alteraciones importantes en biomoléculas (He et al., 2017).

El aumento en las concentraciones de ROS intracelulares y otros marcadores de estrés (ADN dañado, o acumulación de MDA) puede ser consecuencia de diversas condiciones en el medio marino como cambios osmóticos, de temperatura, deshidratación (Ross y Alstyne, 2007; Suggett et al., 2008), exposición a metales pesados,

microplásticos, acidificación del medio y contaminantes orgánicos (Danouche et al., 2022; Keyer y Imlay, 1996; Lin et al., 2020; Liu et al., 2019; Luz et al., 2018).

Aquellas sustancias químicas capaces de inhibir o minimizar la oxidación de otras moléculas en un sistema biológico se denominan antioxidantes. Rice-Evans et al. (1997) establecen que la capacidad antioxidante de una especie química viene determinada por:

(i) Su reactividad como agente donante de hidrógeno o de electrones (relacionado con su potencial de reducción).
(ii) La capacidad de estabilizar y deslocalizar el electrón desapareado del derivado del antioxidante resultante.
(iii) Su reactividad con otros antioxidantes.
(iv) El potencial de quelación de metales de transición.

Existen diferentes moléculas que forman parte del sistema de defensa antioxidante de los organismos vegetales, principalmente enzimas y compuestos no enzimáticos. Entre los enzimáticos se incluyen superóxido dismutasa, catalasa, ascorbato peroxidasa, glutatión reductasa, entre otros. De los antioxidantes no enzimáticos se destacan el ácido ascórbico (Vitamina C), glutatión (tripéptido), prolina, α-tocoferoles (vitamina E), carotenoides, polifenoles, entre otros (Gill y Tuteja, 2010).

Los polifenoles son considerados uno de los mayores contribuyentes a la actividad antioxidante de las plantas. Estas propiedades antioxidantes se deben a su capacidad de donar un electrón o un átomo de hidrógeno. El electrón desapareado resultante es capaz de deslocalizarse a lo largo de todo el anillo aromático, dando lugar a un radical estable (Rice-Evans et al., 1997). Por otro lado, la capacidad complejante de los compuestos fenólicos es proporcionada por la presencia de al menos dos grupos hidroxilos adyacentes en un anillo aromático capaces de establecer enlaces de coordinación con cationes metálicos (Andjelković et al., 2006). De esta manera, actúan como antioxidantes preventivos ya que evitan la formación de radicales libres catalizada por la presencia de metales de transición.

Los estudios publicados sobre la correlación entre la cantidad de compuestos fenólicos en microalgas y su actividad antioxidante son contradictorios. Mientras que en algunos han encontrado una correlación significativa (Goiris et al., 2012; Morowvat y Ghasemi, 2016; Strejckova et al., 2019), en otros llevados a cabo en varias especies

diferentes de microalgas, se ha determinado que no hay correlación significativa con su contenido en polifenoles (Li et al., 2007). Esto puede deberse a que la capacidad de complejación de compuestos fenólicos como los flavonoides depende de factores como el elemento metálico, el pH del medio y la cantidad de grupos hidroxilos de la molécula (Mira et al., 2002), además de la presencia de otros compuestos antioxidantes, y la interacción entre ellos (con efectos aditivos, sinérgicos o antagonistas).

Lee et al. (2016) concluyeron que gran parte de la capacidad antioxidante de material vegetal de trigo y arroz se atribuye al contenido de metabolitos primarios como los aminoácidos, a pesar, de que el contenido en flavonoides también estaba correlacionado significativamente con la actividad antioxidante. Aminoácidos como el triptófano, histidina, tirosina y cisteína poseen capacidad antioxidante, en concreto, capacidad inhibitoria de radicales (Aliaga y Lissi, 2000). Sus anillos aromáticos y la presencia del grupo tiol les confiere la capacidad de reducir los radicales presentes en el medio. Por otro lado, sus grupos amino y carboxílicos participan en la complejación de iones metálicos, convirtiéndose en un mecanismo de prevención de la oxidación producida por dichos metales (Bathula y Kotra, 2019; Bougherra et al., 2018; Cuevas et al., 1998; Hossain et al., 2015; Kryukova et al., 2005; Mesu et al., 2006). Además, cabe tener en cuenta que la combinación de aminoácidos y polifenoles puede producir un efecto sinérgico en la capacidad antioxidante aumentando la capacidad protectora (Ran et al., 2020; Zhang et al., 2019).

Ajisaka et al. (2009) evaluaron la capacidad antioxidante de 12 carbohidratos distintos mediante diferentes ensayos: capacidad inhibitoria de radicales DPPH (por sus siglas en inglés de “2,2-diphenyl-1-picrylhydrazyl”), capacidad reductora de Fe(III) a Fe(II), ensayo del superóxido dismutasa y el método de la desoxirribosa. Sus resultados demostraron que la presencia de los grupos amino, carboxilo, carbonilo o sulfonilo es necesaria para que los carbohidratos presenten capacidad antioxidante, pero no es suficiente. De hecho, Fernandes y Coimbra (2023) concluyen que es la unión de los carbohidratos a grupos peptídicos o fenólicos lo que contribuyen en mayor medida a la capacidad antioxidante de los polisacáridos. También la presencia de átomos de nitrógeno, azufre o fósforo en los carbohidratos (pueden donar sus pares de electrones y formar complejos con iones metálicos como Cu, Fe, Co, Ni, Zn, Cr, entre otros) les permite actuar como mecanismo antioxidante preventivo (Gyurcsik y Nagy, 2000).

En la actualidad no existe un método estándar aceptado para evaluar la capacidad antioxidante de diferentes compuestos en matrices variables. Por lo que la aplicación de varios métodos de análisis permite evaluar y comparar los diferentes mecanismos de actividad antioxidante. La elección del método de determinación es importante. Por ejemplo, Hajimahmoodi et al. (2010) observaron que los compuestos fenólicos son los mayores contribuyentes a la capacidad antioxidante evaluada por diferentes ensayos, pero su correlación solo es significativa en los ensayos FRAP.

La capacidad de las microalgas marinas para producir compuestos con actividad antioxidante tiene un impacto directo en la resistencia de estos organismos frente a las condiciones de estrés que se pueden producir en el entorno marino. Comprender como varía la síntesis de estos metabolitos orgánicos ante el estrés oxidativo intracelular y extracelular es esencial para evaluar su contribución a mitigación de los efectos negativos que estos factores pudieran provocar en el medio.

CAPÍTULO 2. OBJETIVOS

2.1 Objetivos de la tesis

El principal objetivo de la presente Tesis Doctoral es la identificación de los diferentes metabolitos orgánicos implicados en aumentar la tolerancia de las microalgas marinas a factores de estrés ambiental, debido al impacto que estos compuestos tienen en la química de metales traza como Fe y Cu. El trabajo se centra en el estudio de metabolitos orgánicos como aminoácidos, polifenoles y carbohidratos acumulados en células de microalgas marinas, y en sus exudados al medio, cultivadas en presencia de concentraciones subletales y letales de metales y en diferentes condiciones de pH, simulando futuros escenarios de acidificación oceánica. Para ello, se desarrollan técnicas analíticas adecuadas para cada matriz (células y exudados) y se estudia la viabilidad de la aplicación de esta metodología en el análisis del potencial uso de diferentes materiales vegetales en industrias como la alimentaria, farmacéutica o cosmética, entre otras.

Este objetivo general se ha estructurado en 4 objetivos específicos:

Objetivo 1. Desarrollo y optimización de una metodología sensible y reproducible para la determinación de compuestos orgánicos según su naturaleza, en células de microalgas y en el medio de cultivo enriquecido con sus exudados.

- Extracción de los diferentes compuestos orgánicos intracelulares seleccionados de las microalgas.
- Desarrollo del tratamiento previo de las aguas de cultivo que contienen los exudados orgánicos de interés, mediante técnicas de preconcentración para su posterior determinación.
- Optimización de un método de análisis de aminoácidos por cromatografía líquida de alta resolución en fase reversa (RP-HPLC) acoplada a un detector de Diodo Array (DAD).
- Optimización de un método de análisis de polifenoles por cromatografía líquida de alta resolución en fase reversa (RP-HPLC) acoplada a un detector de Diodo Array (DAD).
- Optimización de la metodología de cuantificación de carbohidratos totales y ácidos urónicos totales por espectrofotometría de UV-Vis.
- Determinación de la capacidad antioxidante de los extractos y los exudados mediante el empleo de diferentes ensayos.

- Determinación del estado de estrés oxidativo de las células mediante el ensayo de determinación de maldondialdehído.

Objetivo 2. Vinculación del papel de los compuestos orgánicos exudados y acumulados por microalgas marinas cultivadas bajo diferentes condiciones de concentración de metales, mediante la aplicación de la metodología desarrollada en el Objetivo 1. La consecución de este objetivo se evidencia con la publicación de los resultados en el siguiente artículo:

- Estudio de la acumulación de los aminoácidos libres y polifenoles intracelulares en la diatomea *Phaeodactylum tricornutum* cultivada bajo diferentes condiciones de estrés oxidativo por presencia de Cu y en agua de mar en ausencia de Cu.
    - Santiago-Díaz, P., Rivero, A., Rico, M., González-González, A., González-Dávila, M., y Santana-Casiano, M. (2023). Copper toxicity leads to accumulation of free amino acids and polyphenols in *Phaeodactylum tricornutum* diatoms. *Environmental Science and Pollution Research*, 1-10.
- Artículo enviado a la revista *Scientific Reports*: Caracterización de los compuestos orgánicos liberados al medio por la diatomea *Phaeodactylum tricornutum* cultivada bajo condiciones de estrés oxidativo por la presencia de diferentes concentraciones de Cu.
    - Rico, M., Santiago-Díaz, P., Rivero, A., Santana-Casiano, M. Characterization of organic compounds exuded by *Phaeodactylum tricornutum* diatom grown under Cu stress.

Objetivo 3. Determinación de los compuestos orgánicos exudados y acumulados por microalgas marinas cultivadas bajo diferentes condiciones de acidificación del medio, mediante la aplicación de la metodología desarrollada en el Objetivo 1.

- Artículo enviado a la revista *Biogeoscience*: Estudio de las variaciones en polifenoles y carbohidratos de la microalga *Emiliania huxleyi* bajo condiciones simuladas de acidificación oceánica.
    - Rico, M., Santiago-Díaz, P., Samperio-Ramos, G., González-Dávila, M., Santana-Casiano, J.M. Variations of polyphenols and carbohydrates of *Emiliania huxleyi* grown under simulated ocean acidification conditions.

Objetivo 4. Aplicación de la metodología desarrollada en el Objetivo 1 a los extractos de diferentes especies de microalgas de agua dulce identificadas y suministradas por el Banco Español de Algas y de plantas terrestres autóctonas. Este objetivo queda recogido en los siguientes artículos:

- Estudio de metabolitos bioactivos de diferentes especies de microalgas recogidas en las Islas Canarias (*Spirogyra* sp., *Cosmarium* sp., y *Cosmarium* blytii) con potenciales aplicaciones biotecnológicas.
    - Santiago-Díaz, P., Rico, M., Rivero, A., y Santana-Casiano, M. (2022). Bioactive metabolites of microalgae from Canary Islands for functional food and feed uses. *Chemistry y Biodiversity,* 19(9), e202200230.
- Estudio de diferentes compuestos orgánicos en tres especies distintas de microalgas (*Pseudopediastrum boryanum*, *Chloromonas* cf. *reticulata*, y *Chloroidium saccharophilum*) y su capacidad antioxidante.
    - Santiago-Díaz, P., Rivero, A., Rico, M., y Gómez-Pinchetti, J. L. (2021). Characterization of novel selected microalgae for antioxidant activity and polyphenols, amino acids, and carbohydrates. *Marine Drugs*, 20(1), 40.
- Artículo enviado a la revista *Food Frontiers*: Estudio del potencial nutricional y farmacéutico de la planta herbácea *Artemisia thuscula*.
    - Nutritional and pharmaceutical potential of *Artemisia thuscula.* Rico, M., Santiago-Díaz, P.

Los objetivos 1,2 y 3 anteriores se alinean con los contemplados en los siguientes proyectos de investigación:

Título: *Efecto de la acidificación oceánica, la temperatura y el contenido de materia orgánica en la persistencia de Fe(II) en el Océano Atlántico (ATOPFe)* (Referencia: CTM2017-83476-P). Organismo financiador: Ministerio de Economía, Industria y Competitividad. Ámbito de financiación: Nacional. Convocatoria: EXCELENCIA: PROY I+D. Tipo: Proyecto de Convocatoria Pública Competitiva. Duración: Enero 2018- Septiembre 2021.

Título: *Respuesta Del Fe en Un Océano Acidificado (FERIA)* (Referencia: PID2021-123997NB-I00). Organismo financiador: Ministerio de Ciencia e Innovación. Ámbito de financiación: Nacional. Convocatoria Pública Competitiva. Duración: septiembre 2022-31 de agosto 2025.

El objetivo 4 se alinea con los objetivos del Banco Español de Algas (BEA): aislamiento, caracterización, conservación, suministro y desarrollo de técnicas de cultivo y aplicaciones de microalgas y cianobacterias desde el punto de vista científico-tecnológico. El BEA es una infraestructura de la Universidad de Las Palmas de Gran Canaria, miembro de la Organización Europea de Colecciones de Cultivos (ECCO), de la Federación Mundial de Colecciones de Cultivo (WFCC) y está incluido en el Centro Mundial de Datos sobre Microorganismos (WFCC-MIRCEN). Está acreditado ante el Gobierno de España como autoridad internacional para el depósito de microorganismos, conforme a las disposiciones establecidas en el Tratado de Budapest. El grupo QUIMA mantiene una estrecha colaboración con el BEA, que suministra las microalgas objeto de estudio en función del interés biotecnológico, y de los estudios previos sobre las condiciones de cultivo sostenible que proporcionan un crecimiento óptimo de biomasa. Estos trabajos se alinean con los objetivos de los proyectos: REBECA-CCT (MAC/1.1.B/269) y H2020-INFRADEV-03-2019 *Project* IS_MIRRI21 (No. 871129) en el marco del programa *European Territorial Cooperation Program* PCT-MAC 2014-2020.

2.2 Futuras líneas de investigación

Los primeros trabajos desarrollados por el grupo QUIMA estudiando la variación del perfil fenólico de la microalga *Dunaliella tertiolecta* cultivada en presencia de altas concentraciones de Cu y Fe fueron seleccionados por la Global Medical Discovery como un "***Key Scientific Article contributing to excellence in biomedical research***". Siguiendo esta línea de investigación, la metodología desarrollada, así como los resultados de esta Tesis Doctoral, se están aplicando en la consecución de los fines propuestos en los siguientes proyectos vigentes en la actualidad:

- **Respuesta del Fe en un océano acidificado (FeRIA) (Referencia: PID2021-123997NB-I00)**

Este proyecto se centra en analizar los efectos de la acidificación y el calentamiento en los océanos, así como su impacto en los ciclos biogeoquímicos de metales traza, desde la perspectiva del cambio climático causado por la actividad humana o por fenómenos naturales, como las emisiones volcánicas.

Como se ha comentado anteriormente, el Fe es crucial para el crecimiento de organismos marinos, existiendo en formas solubles asimilables Fe(II) e insolubles y estables Fe(III). La acidificación oceánica reduce la oxidación del Fe soluble, mientras que el aumento de la temperatura acelera este proceso. La materia orgánica según sus características y grupos funcionales también juega un papel importante en la oxidación y biodisponibilidad del hierro.

Los objetivos del proyecto incluyen estudiar la especiación del Fe en áreas afectadas por ambos tipos de procesos, analizar la cinética de oxidación del hierro en entornos volcánicos y caracterizar la materia orgánica en esas áreas.

La identificación y caracterización de la materia orgánica (ligandos del Fe, aminoácidos, polisacáridos y exopolímeros) se llevará a cabo con las técnicas analíticas optimizadas en el presente trabajo. Con ello se podrá realizar el estudio de la interacción del Fe con compuestos orgánicos individuales identificados.

Estos estudios permitirán determinar si las áreas afectadas por emisiones volcánicas pueden ser modelos para comprender los efectos de la acidificación y el calentamiento causados por la actividad humana. Además, se busca comprender cómo la persistencia del Fe en el entorno marino está influenciada por diferentes factores.

- **Análisis multidisciplinar de sumideros de carbono azul en aguas costeras (MultiCoast) (Referencia: TED2021-130892B-I00)**

El objetivo principal de este proyecto es llevar a cabo un análisis exhaustivo y multidisciplinario para comprender el comportamiento del carbono en los ecosistemas costeros, específicamente en la región de Canarias.

Estas zonas actúan como interfaces entre sistemas terrestres y oceánicos, donde ocurren diversas reacciones químico-físicas que influyen en el ciclo del carbono, afectando la interacción de metales esenciales, nutrientes y materia orgánica con los ecosistemas.

Canarias, una región ultraperiférica con una rica biodiversidad, ofrece un entorno ideal para investigar la captación de carbono en sistemas costeros. El proyecto busca analizar cómo el CO_2 se transfiere y transforma en materia orgánica en aguas costeras, considerando su relación con otros parámetros químico-físicos. Para ello se aplicarán las

técnicas analíticas de determinación de compuestos orgánicos (aminoácidos, polifenoles, carbohidratos) desarrolladas en este trabajo.

Este proyecto proporcionará información sobre el almacenamiento de CO_2 en sistemas costeros en Canarias, permitiendo el diseño de estrategias y soluciones basadas en la naturaleza, así como orientando las decisiones de conservación de ecosistemas con mayor capacidad de captura de carbono.

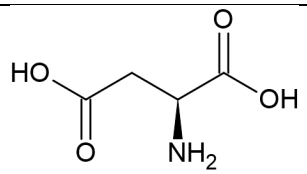
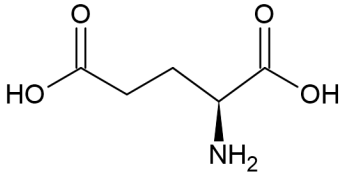
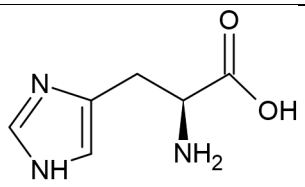
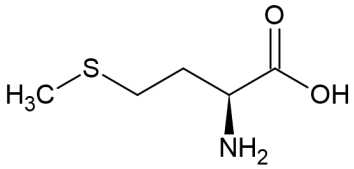
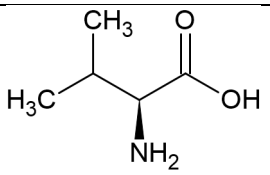
CAPÍTULO 3. MATERIALES Y MÉTODOS

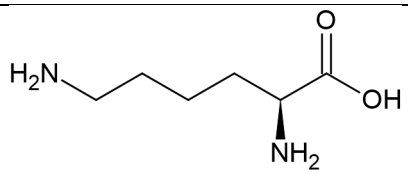
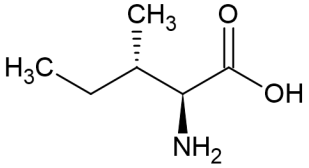
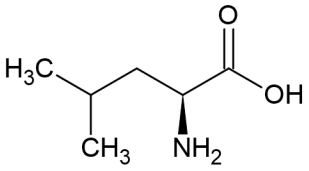
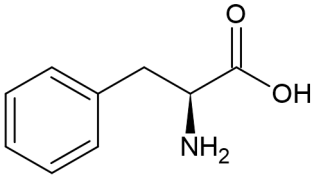
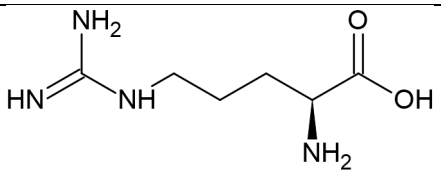
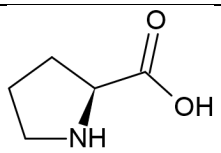
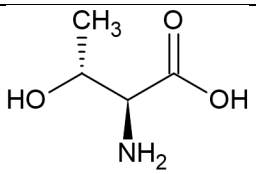
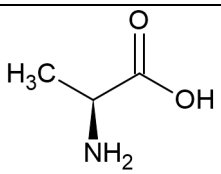
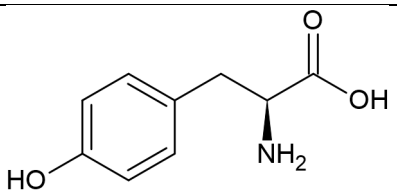
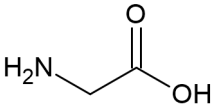
3.1. Metabolitos orgánicos estudiados

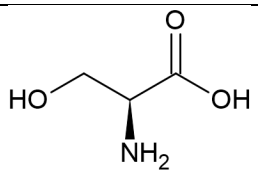
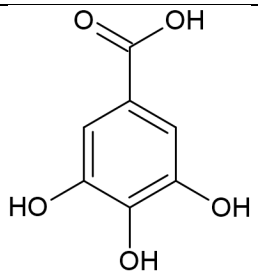
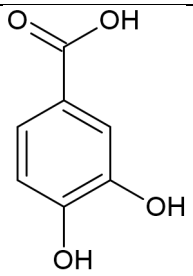
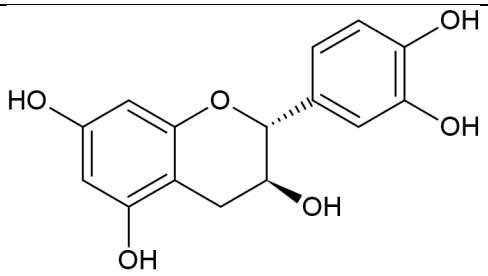
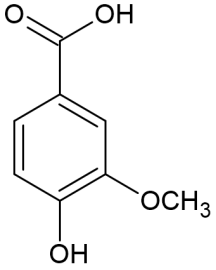
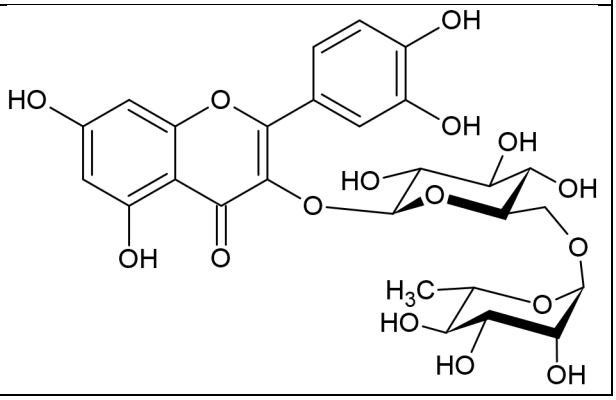
En el presente trabajo se desarrollaron y optimizaron técnicas de determinación de diferentes metabolitos orgánicos en extractos de células de microalgas y en sus aguas de cultivo, así como en otros materiales vegetales de origen terrestre. La identificación de metabolitos se llevó a cabo para 16 aminoácidos (L-aminoácidos) (ácido aspártico, ácido glutámico, histidina, metionina, valina, lisina, isoleucina, leucina, fenilalanina, arginina, prolina, treonina, alanina, tirosina, glicina, serina) y 10 polifenoles (ácido gálico, ácido protocatecuico, catequina, ácido vanílico, rutina, epicatequina, ácido siríngico, ácido gentísico, ácido cumárico y ácido ferúlico).

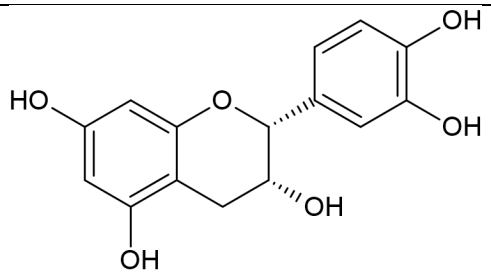
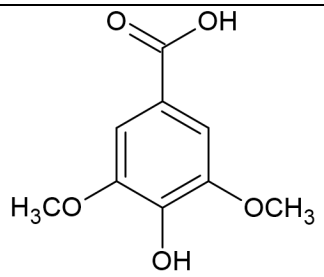
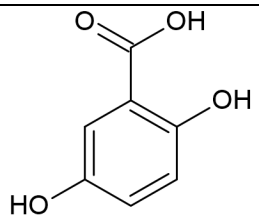
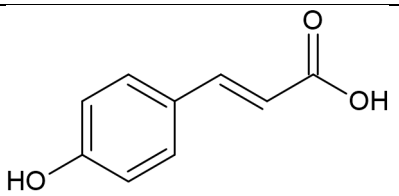
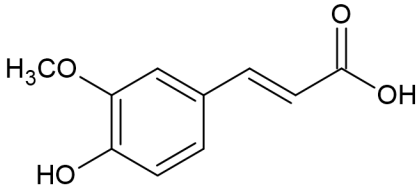
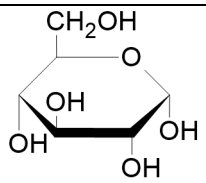
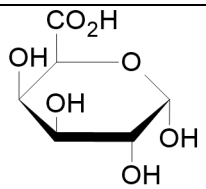
Los compuestos analizados, su clasificación y su estructura se muestran en la siguiente tabla:

Tabla 1. Clasificación y estructura de los metabolitos estudiados

Compuesto	Clasificación	Estructura
Aminoácidos		
Ácido aspártico	No esencial	
Ácido glutámico	No esencial	
Histidina	Esencial	
Metionina	Esencial	
Valina	Esencial	

Lisina	Esencial	
Isoleucina	Esencial	
Leucina	Esencial	
Fenilalanina	Esencial	
Arginina	Esencial	
Prolina	No esencial	
Treonina	Esencial	
Alanina	No esencial	
Tirosina	No esencial	
Glicina	No esencial	

Serina	No esencial	
Polifenoles		
Ácido gálico	Ácido fenólico	
Ácido protocatecuico	Ácido fenólico	
(+)-Catequina	Flavonoide	
Ácido vanílico	Ácido fenólico	
Rutina	Flavonoide	

(-)-Epicatequina	Flavonoide	
Ácido siríngico	Ácido fenólico	
Ácido gentísico	Ácido fenólico	
Ácido p-cumárico	Ácido fenólico	
Ácido ferúlico	Ácido fenólico	
Carbohidrato		
D-Glucosa	Monosacárido	
Ácido galacturónico	Ácido urónico	

3.2. Metodología para la determinación de aminoácidos

3.2.1. Análisis por cromatografía líquida de alta resolución en fase reversa con detector de diodo array (RP-HPLC-DAD)

La cromatografía líquida de alta resolución en fase reversa (RP-HPLC, por sus siglas en inglés "Reverse Phase High Performance Cromatography") es una técnica analítica ampliamente utilizada por su eficiencia, sensibilidad y reproducibilidad. Entre los detectores más comunes se encuentra el detector de diodo array (DAD, por sus siglas en inglés "Diode Array Detector"). Su funcionamiento se basa en la capacidad de los compuestos de absorber radiación en la región ultravioleta (UV) o visible (Vis). Se caracteriza por ser un detector avanzado de UV-Vis capaz de adquirir simultánea y continuamente datos de absorción de los compuestos a analizar.

Los aminoácidos carecen de grupos cromóforos capaces de absorber radiación, por lo que su determinación mediante este tipo de detectores requiere de un paso previo de reacción del aminoácido con un agente derivatizante, dando lugar a un derivado cuya detección es posible. Este proceso se conoce como derivatización y se puede llevar a cabo anterior o posteriormente a la separación cromatográfica. Entre los reactivos derivatizantes se distinguen diferentes compuestos (ninhidrina, cloruro de dansilo, 1-fluoro-2,4-dinitrobenceno, fenilisotiocianato, orto-ftalaldehído, 9H-fluoren-9-ilmetil cloroformiato, dietil 2(etoximetiliden)propanodioato y 6-aminoquinolil-N-hidroxisuccinimidil carbamato) (Callejón et al., 2010). En este estudio se escoge el fenilisotiocianato (PITC, por sus siglas en inglés "Phenylthioisocyanate") capaz de reaccionar con aminas primarias y secundarias con niveles de detección de picomoles (Dai et al., 2014). Tanto la reacción con PITC como el método de separación cromatográfico se desarrollaron inicialmente para su aplicación en la determinación de 3 aminoácidos (ácido glutámico, ácido aspártico y prolina) en las microalgas de agua dulce *Spirogyra* sp., *Cosmarium* sp., y *Cosmarium blytii*. Posteriormente, el método de derivatización y el de separación cromatográfica se optimizaron incluyendo el análisis de un total de 10 aminoácidos en células de microalgas *Pseudopediastrum boryanum, Chloromonas* cf. *reticulata* y *Chloridium saccharophilum*. Finalmente, se incorporaron 5 aminoácidos más que hicieron posible la identificación y cuantificación de 15 aminoácidos diferentes (8 esenciales y 7 no esenciales) en extractos de *Artemisia*

thuscula, permitiendo determinar la calidad de las proteínas en función del contenido de aminoácidos esenciales.

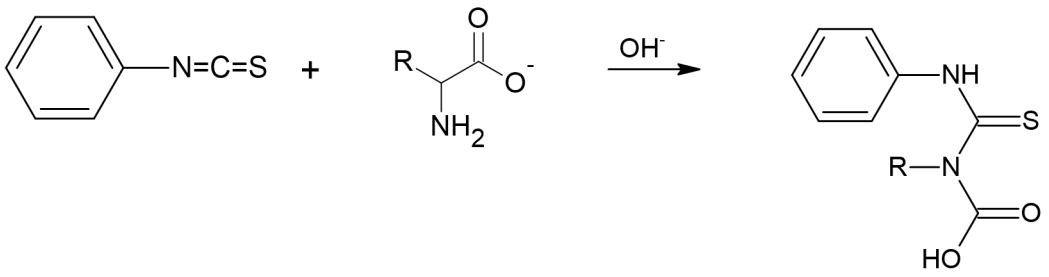


Figura 3. Reacción de derivatización de aminoácidos con PITC

La reacción de derivatización del PITC con los aminoácidos (Figura 3) contenidos en los extractos se lleva a cabo como paso previo en medio acuoso y fuera del equipo cromatográfico. Una vez han reaccionado, los derivados y el PITC en exceso se separan por extracción líquido-líquido con tetracloroetileno (C_2Cl_4) y hexano (C_6H_{14}).

El primer método de determinación de 3 aminoácidos consistió en una modificación del método empleado por Shi et al. (2013) y los datos obtenidos para dicho método se encuentran recogidos en la siguiente tabla:

Tabla 2. Datos de las curvas de calibración de los 3 aminoácidos analizados

Aminoácido	Ecuación de regresión	Coeficiente de correlación (r)	Rango de linealidad (mg L^{-1})	Tiempo de retención (min)
Ácido glutámico	y=3282,3x-45100	0,9991	40-800	3,12
Ácido aspártico	y=4757,8x-144564	0,9982	40-800	2,91
Prolina	y=20896x-627078	0,9986	40-800	14,94

La validación del método se llevó a cabo mediante la determinación de la recuperación y la desviación estándar relativa (RSD, por sus siglas en inglés "Relative Standard Deviation") y se muestran en la siguiente tabla:

Tabla 3. Datos de validación del método de análisis de 3 aminoácidos

Aminoácido	Recuperación (%)[a]	RSD (%)[b]
Ácido glutámico	107,2 ± 5,1	2,31
Ácido aspártico	107,9 ± 2,4	2,25
Prolina	100,9 ± 8,7	0,73

[a]Media ± Desviación estándar de tres mediciones

[b]n=6

Como se puede comprobar, este método permite la determinación exacta y precisa de tres aminoácidos no esenciales. Estos son de relevancia ya que los ácidos glutámico y aspártico son los aminoácidos encontrados en mayor cantidad en las microalgas, mientras que el aminoácido prolina desempeña un papel importante en la mitigación del estrés abiótico en plantas, macro y microalgas. Sin embargo, para poder realizar un análisis más completo del perfil de aminoácidos contenido en células vegetales, con el fin de determinar la calidad nutricional de las proteínas, así como el comportamiento de esos aminoácidos en células sometidas a condiciones de estrés, se optimizó el método para la identificación de hasta 10 aminoácidos (6 esenciales y 4 no esenciales).

Este nuevo método de análisis cromatográfico consigue la separación de los 10 aminoácidos en menos de 40 minutos. Los datos sobre la curva de calibración, coeficiente de correlación, rango de linealidad y tiempo de retención se muestran en la tabla 4.

Tabla 4. Datos de las curvas de calibración de los 10 aminoácidos analizados

Aminoácido	Ecuación de regresión	Coeficiente de correlación (r)	Rango de linealidad (mg L^{-1})	Tiempo de retención (min)
Histidina	y=25997x-14121	0,9996	1-40	6,19
Arginina	y=19662x-12550	0,9994	1-40	6,86
Ácido glutámico	y=26298x-28054	0,9995	1-40	14,41
Ácido aspártico	y=28322x-39034	0,9983	1-40	14,82
Prolina	y=31791x-17999	0,9996	1-40	15,40
Metionina	y=28102x-27903	0,9995	1-40	27,22
Valina	y=31304x-22630	0,9996	1-40	29,39
Lisina	y=47494x-52354	0,9999	1-40	31,09
Isoleucina	y=36650x-34008	0,9995	1-40	34,42
Fenilalanina	y=27738x-44705	0,9976	1-40	34,87

La validación del método se realiza mediante la evaluación del porcentaje de recuperación, la desviación estándar relativa, los límites de detección y cuantificación (LOD y LOQ, respectivamente, por sus siglas en inglés “Limit Of Detection” y “Limit Of Quantification”). Los datos se detallan a continuación:

Tabla 5. Datos de validación del método de análisis de 10 aminoácidos

Aminoácido	LOD (μg mL^{-1})[a]	LOQ (μg mL^{-1})[a]	Recuperación (%)[b]	RSD (%)[c]
Histidina	0,0019	0,0064	96,3 ± 1,7	1,73
Arginina	0,0020	0,0065	92,4 ± 2,9	2,47
Ácido glutámico	0,0007	0,0025	95,5 ± 1,5	2,51
Ácido aspártico	0,0006	0,0021	96,0 ± 1,9	2,50
Prolina	0,0033	0,0110	96,6 ± 1,0	3,88
Metionina	0,0008	0,0028	96,5 ± 1,9	3,00
Valina	0,0005	0,0015	96,8 ± 1,3	3,18
Lisina	0,0100	0,0335	96,4 ± 1,3	2,19
Isoleucina	0,0008	0,0026	101,8 ± 3,6	2,78
Fenilalanina	0,0011	0,0037	97,4 ± 3,1	3,10

[a]LOD y LOQ se calculó como la señal-ruido de seis replicados

[b]Media ± Desviación estándar de tres mediciones

[c]n=6

Tal y como se puede comprobar, se consigue desarrollar un método rápido, lineal, preciso y exacto que permite la determinación de 10 aminoácidos con una buena sensibilidad y reproducibilidad.

Partiendo de este método, se realizó una ampliación a cinco aminoácidos más, así como una calibración y validación del nuevo método (Tablas 6 y 7). Las condiciones cromatográficas (eluyentes, temperatura, flujo, longitudes de onda…) se mantuvieron.

Tabla 6. Datos de las curvas de calibración de los 15 aminoácidos analizados

Aminoácido	Ecuación de regresión	Coeficiente de correlación (r)	Rango de linealidad (mg L^{-1})	Tiempo de retención (min)
Histidina	y=17180x-5026,6	0,9998	1-50	6,19
Arginina	y=6305,5x+700.73	1	1-50	6,63
Asparagina	y=5816,2x+590,24	1	1-50	10,01
Glutamina	y=6330,2x+771,77	1	1-50	10,57
Serina	y=9666,9x+1487,8	1	1-50	12,51
Ácido glutámico	y=5744,6x+248,58	0,9999	1-50	14,78
Ácido aspártico	y=5288,3x+314,37	0,9999	1-50	15,26
Prolina	y=27233x+3977,4	1	1-50	15,62
Treonina	y=8228.9x +2393,5	0,9999	1-50	16,27
Metionina	y=10822x+1874,4	1	1-50	27,64
Valina	y=13579x+1891,1	1	1-50	29,72
Lisina	y=21013x-4622,5	1	1-50	31,76
Leucina	y=13531x+1208,3	0,9999	1-50	34,31
Isoleucina	y=14820x+1124,6	1	1-50	34,73
Fenilalanina	y=14323x-240,47	1	1-50	35,27

La validación se realiza mediante la evaluación de los mismos parámetros que el método anterior, que se muestran en la siguiente tabla:

Tabla 7. Datos de validación del método de análisis de 10 aminoácidos

Aminoácido	LOD (μg mL^{-1})[a]	LOQ (μg mL^{-1})[a]	Recuperación (%)[b]	RSD (%)[c]
Histidina	0,0157	0,0524	108,6 ± 1,6	3,20
Arginina	0,0168	0,0558	104,3 ± 22,8	2,96
Asparagina	0,0172	0,0574	108,0 ± 3,5	2,16
Glutamina	0,0073	0,0245	109,3 ± 1,0	1,92
Serina	0,0155	0,0516	107,7 ± 3,6	1,86
Ácido glutámico	0,0052	0,0172	109,6 ± 5,3	3,63
Ácido aspártico	0,0045	0,0149	104,0 ± 1,6	3,59
Prolina	0,0026	0,0087	97,6 ± 1,4	0,95
Treonina	0,0085	0,0282	114,6 ± 14,6	4,81
Metionina	0,0055	0,0185	114,6 ± 13,7	1,61
Valina	0,0022	0,0075	110,3 ± 5,8	1,32
Lisina	0,0025	0,0085	113,0 ± 5,0	3,23
Leucina	0,0051	0,0168	108,3 ± 4,7	2,40
Isoleucina	0,0045	0,0148	109,2 ± 4,9	2,87
Fenilalanina	0,0035	0,0116	108,6 ± 5,1	3,06

[a]LOD y LOQ se calculó como la señal-ruido de seis replicados

[b]Media ± Desviación estándar de tres mediciones

[c]n=6

La metodología de extracción de aminoácidos en células de microalgas y de la planta terrestre *Artemisia thuscula*, las condiciones de derivatización con PITC y el método cromatográfico empleado se encuentran detalladamente descritos en el Capítulo 4 destinado a la discusión de los resultados, dentro del apartado "materials and methods" de los artículos incluidos en esta Tesis Doctoral.

3.2.2. Extracción de aminoácidos en exudados mediante resinas para su análisis por RP-HPLC-DAD

La baja concentración de aminoácidos en agua de cultivo enriquecida con exudados de microalgas y en muestras de agua de mar, dificulta su análisis de manera directa por RP-HPLC-DAD. La concentración de las muestras por liofilización fue descartada por la elevada cantidad de sales que posee el agua de mar. Sin embargo, el aislamiento y pre-concentración de aminoácidos en este tipo de muestras se llevó a cabo

en la presente Tesis Doctoral con resinas de intercambio iónico, consiguiendo eliminar la sal de acuerdo con el método desarrollado por Takano et al. (2010).

Para ello, se empleó la resina DOWEX 50WX8, que contiene un gel formado por un copolímero de estireno y divinilbenceno. Además, este copolímero posee el grupo sulfonato ($-SO_3^-$) como grupo funcional iónico activo, responsable de la retención de los cationes. El procedimiento de extracción de aminoácidos sigue las siguientes etapas:

1. **Prelavado de la resina**

   Antes de utilizar la resina se debe realizar la siguiente secuencia de prelavado con ácido clorhídrico (1M), agua destilada, hidróxido de sodio (1M) y finalmente agua destilada, para asegurar la completa elución de posibles compuestos retenidos en la resina.

2. **Acondicionamiento de la resina**

   El siguiente paso es el acondicionamiento con el que se consigue que la resina quede en las mismas condiciones que la matriz de la muestra. Para ello se hace pasar ácido clorhídrico ocupando los protones todos los lugares cargados negativamente en la resina. Este paso se denomina *activación de la resina*, y una vez llevado a cabo, debe mantenerse siempre húmeda para que este proceso de solvatación no sea revertido.

3. **Paso de la muestra**

   La muestra que contiene los aminoácidos (en este caso agua de mar con exudados de microalgas) se acidifica a pH 1 y se hace pasar por la resina. De esta forma los aminoácidos quedan cargados positivamente, y a medida que pasan por la resina intercambian su lugar con el de los protones retenidos en los grupos sulfonatos. Tanto los aminoácidos como los cationes de las sales son retenidos por lo que es necesario tener en cuenta la cantidad de resina necesaria para que ambos cationes se retengan y no compitan entre sí.

4. **Lavado de la resina**

   La resina se lava con agua destilada para la elución de aquellos compuestos que no han quedado retenidos (moléculas neutras, negativas, etc.). En este paso, los cationes que se hayan podido retener se eluyen con el agua.

5. Elución de los aminoácidos

La elución se puede realizar cambiando la fuerza iónica del eluyente o cambiando el pH. En este caso, al pasar amoníaco (NH_3) al 10% con un pH aproximado de 12, los aminoácidos se cargan negativamente y se desprenden de la resina.

Una vez eluídos los aminoácidos en amoníaco, las muestras se llevan a sequedad y el residuo resultante se disuelve en la mínima cantidad de agua ultrapura necesaria para su posterior reacción de derivatización con PITC. De esta forma, los aminoácidos se pueden analizar por RP-HPLC-DAD con la misma metodología utilizada para los extraídos de células de microalgas.

Para comprobar la exactitud del método, calculada como el porcentaje de analitos recuperados en extracciones de mezclas de 15 aminoácidos a una concentración inicial de 10 mg L^{-1}, se realiza el procedimiento de aislamiento y concentración en la resina, la derivatización con PITC y el análisis por RP-HPLC-DAD. Los resultados de recuperación en % se muestran en la siguiente tabla:

Tabla 8. Recuperaciones (%) de los aminoácidos retenidos por la resina de intercambio iónico

Aminoácido	Concentración teórica (mg L^{-1})	Concentración experimental (mg L^{-1})	Recuperación (%)
Histidina	10	5,23	52
Arginina	10	6,33	63
Asparagina	10	6,25	63
Glutamina	10	7,39	74
Serina	10	5,01	50
Ácido glutámico	10	4,24	42
Ácido aspártico	10	2,80	28
Prolina	10	5,81	58
Treonina	10	4,45	44
Metionina	10	4,05	41
Valina	10	5,89	59
Lisina	10	2,39	24
Leucina	10	4,83	48
Isoleucina	10	3,64	36
Fenilalanina	10	5,64	56

No se obtuvieron recuperaciones mayores del 74%, por lo que este método de aislamiento y concentración de aminoácidos no parece el más apropiado para el análisis de muestras de exudados y agua de mar. Además, la metodología desarrollada presenta dos inconvenientes para el tratamiento de un elevado número de muestras: el tiempo necesario para llevar a cabo todo el procedimiento y el volumen de disolventes necesario.

3.3. Metodología para la determinación de polifenoles

3.3.1. Preparación de muestras

La preparación de las muestras, tanto extractos de células, como aguas de cultivo enriquecidas con exudados y muestras de agua de mar, es un paso previo muy importante en el análisis de polifenoles, así como de cualquier otro metabolito. Una de las técnicas de extracción más empleadas es la extracción con disolventes debido a su eficiencia, facilidad de uso y amplio rango aplicabilidad (Stalikas, 2007). Entre los factores que influyen en la eficacia de la extracción se encuentran el pH, la temperatura, la ratio de volumen de muestra-disolvente y el número e intervalos de tiempo de los pasos de extracción individuales (Stalikas, 2007). Sin embargo, el factor más relevante es el tipo de disolvente, y en concreto su polaridad. Como se ha podido comprobar en investigaciones del grupo QUIMA, la naturaleza del disolvente afecta a la eficiencia de extracción de compuestos fenólicos (López et al., 2011). Además, se demostró que el perfil fenólico depende del disolvente de extracción debido a su diversa estructura química en cuanto a polaridad, acidez, grupos hidroxilos y/o anillos aromáticos. Por tanto, resulta complicado la elección de un disolvente eficaz para la extracción de la totalidad de estos.

En los experimentos de esta Tesis Doctoral se emplea como disolvente de extracción el metanol, ya que estudios previos de Jerez-Martel et al. (2017) demostraron una mayor eficiencia para extraer compuestos fenólicos con elevada capacidad antioxidante en microalgas. Además, se combina con métodos de extracción mecánicos como el baño de ultrasonido y la agitación, para facilitar dicha extracción.

La baja concentración de polifenoles en aguas de cultivo enriquecidas con exudados de microalgas precisa de una etapa previa de extracción en fase sólida (SPE, por sus siglas en inglés “Solid-Phase Extraction”). Esta técnica permite el aislamiento y concentración de polifenoles, ya que quedan retenidos por afinidad con el adsorbente de

los cartuchos SPE. En este trabajo se ha utilizado cartuchos Chromabond-Easy (Macherey-Nagel^{TM}) rellenos de un copolímero de poliestireno-divinilbencceno modificado, que es muy hidrofílico y confiere una gran humectabilidad, por lo que están especialmente recomendados para análisis de fenoles en muestras acuosas. Los polifenoles presentes en agua de mar quedan retenidos en los cartuchos, que son lavados con agua destilada para eliminar las sales. La posterior elución de los polifenoles retenidos se realiza con metanol, permitiendo su purificación y concentración para su determinación por RP-HPLC-DAD.

3.3.2. Determinación de polifenoles por cromatografía líquida de alta resolución en fase reversa con detector de Diodo Array (RP-HPLC-DAD)

La cromatografía líquida de alta resolución se ha empleado para el análisis de polifenoles desde los años 70 (Molderez et al., 1978), siendo una de las preferidas en la separación y cuantificación de estos compuestos. El uso de detectores UV-Vis o DAD para el análisis de polifenoles es sencillo, puesto que no es necesario un paso previo de derivatización ya que los polifenoles son capaces de absorber radiación UV.

El método cromatográfico empleado para la identificación y cuantificación de polifenoles en esta Tesis Doctoral se basa en uno desarrollado anteriormente por López et al. (2011) con modificaciones. Este se optimizó para identificar 10 polifenoles en 30 minutos. Se escogieron dos longitudes de onda diferentes en función de las máximas absorbancias de cada polifenol: 270 nm para el ácido gálico, ácido protocatecuico, catequina, ácido vanílico, epicatequina, ácido siríngico y rutina; y 324 nm para el ácido gentísico, ácido cumárico y el ácido ferúlico.

Para la cuantificación de estos polifenoles, se realizó una curva de calibración de cada uno con una buena linealidad dentro del rango de concentraciones seleccionado. Los datos de calibración se muestran en la siguiente tabla:

Tabla 9. Datos de las curvas de calibración de los 10 polifenoles analizados

Compuesto fenólico	Ecuación de regresión	Coeficiente de correlación (r)	Rango de linealidad (mg L^{-1})	Tiempo de retención (min)
Ácido gálico	y=26175x+4101,2	0,9999	1-50	4,69
Ácido protocatecuico	y=21559x+3642,3	0,9999	1-50	8,58
Catequina	y=4565,6x-885,01	0,9999	1-50	11,56
Ácido vanílico	y=24618x-5725,6	0,9998	1-50	16,05
Epicatequina	y=4576,4x+21,971	1	1-50	16,77
Ácido siríngico	y=27108x-1745,8	1	1-50	17,27
Rutina	y=4923,4x-1820,9	0,9995	1-50	25,87
Ácido gentísico	y=12419x-4477,1	0,9999	1-50	14,30
Ácido coumárico	y=47113x-3127,6	0,9999	1-50	21,16
Ácido ferúlico	y=47286x+12501	0,9995	1-50	22,22

Este método cromatográfico consigue cuantificar e identificar 10 polifenoles de manera rápida, lineal, precisa y exacta tal y como se puede observar en los datos de validación del método recogidos en la siguiente tabla:

Tabla 10.Validación del método para la determinación de polifenoles por RP-HPLC-DAD

Compuesto fenólico	LOD (µg mL^{-1})[a]	LOQ (µg mL^{-1})[a]	Recuperación (%)[b]	RSD (%)[c]
Ácido gálico	0,0576	0,1920	98,4 ± 2,7	1,80
Ácido protocatecuico	0,0808	0,2695	100,5 ± 1,2	2,74
Catequina	0,1094	0,3648	103,1 ± 1,0	3,85
Ácido vanílico	0,0551	0,1836	109,0 ± 4,7	2,59
Epicatequina	0,2003	0,6676	109,2 ± 1,4	2,55
Ácido siríngico	0,0221	0,0736	102,8 ± 3,3	2,34
Rutina	0,1206	0,4019	97,6 ± 4,7	2,49
Ácido gentísico	0,0955	0,3183	98,3 ± 2,9	3,35
Ácido coumárico	0,0888	0,2960	101,4 ± 4,8	2,46
Ácido ferúlico	0,0299	0,0998	91,8 ± 3,6	2,12

[a]LOD y LOQ se calculó como la señal-ruido de seis replicados

[b]Media ± Desviación estándar de tres mediciones

[c]n=6

Una descripción más detallada del método se puede encontrar en el Capítulo 4, destinado a la discusión de los resultados, dentro del apartado "Materials and methods" en cada uno de los artículos correspondientes en esta Tesis Doctoral.

3.3.3. Determinación de flavonoides totales

Dentro de los compuestos fenólicos se ha empleado un método de determinación de flavonoides totales basado en uno previamente descrito por Chang y Kim (2018) donde las proporciones de los reactivos empleados fueron adaptadas. Este es el método colorimétrico del cloruro de aluminio ($AlCl_3$) que se basa en la capacidad de los flavonoides para formar complejos con iones de aluminio por reacción con el nitrito de sodio ($NaNO_2$) en medio alcalino. El complejo formado presenta absorción a una longitud de onda de 510 nm. Este análisis se caracteriza por su rapidez, bajo coste y por ser ampliamente aceptado y empleado para la determinación de flavonoides totales.

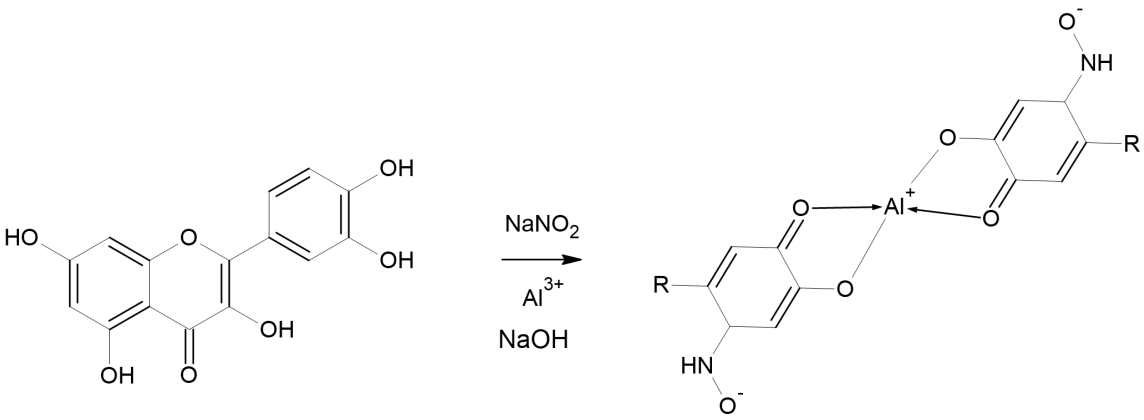


Figura 4. Complejación del flavonoide quercetina con Al(III)

Previamente a la aplicación del método en muestras vegetales, se realizó un estudio donde se evaluó la recuperación de los flavonoides totales de una disolución de concentración conocida de quercetina. El porcentaje de recuperación se encontró entre 99,8 y 101,2 %, confirmando la exactitud del método. El procedimiento se encuentra descrito en el Capítulo 4 (apartado 4.3.3) del presente documento, destinado a la discusión de los resultados.

3.4. Determinación de carbohidratos en células de microalgas y sus aguas de cultivo enriquecidas con exudados

Los métodos colorimétricos para la determinación de carbohidratos se emplean desde hace más de 50 años. Uno de los más utilizados fue desarrollado por Dubois et al.

(1956), también conocido como método del fenol-ácido sulfúrico (*phenol-sulfuric acid method,* en inglés). Es un método sencillo y sensible capaz de cuantificar azúcares simples por reacción con el ácido sulfúrico concentrado, dando lugar a un derivado furfural, que, en combinación con el fenol da un tono amarillento a la disolución, capaz de absorber radiación en el espectro visible a 490 nm. Este método es adecuado tanto para muestras de microalgas como para el agua de mar de cultivo, ya que se puede emplear en presencia de sales (Pokrzywnicka y Koncki, 2018).

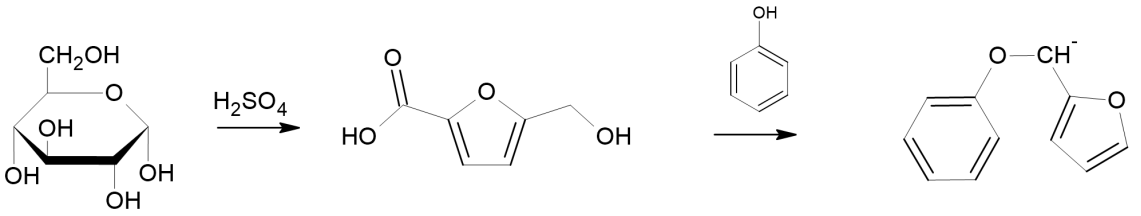


Figura 5. Reacción de Dubois con D-glucosa

El método de la antrona se emplea también para la cuantificación de carbohidratos totales. Es semejante al método de Dubois con la diferencia del empleo del reactivo de antrona (9,10-dihidro-9-oxoantraceno) para dar los derivados furfurales de coloración azul-verdosa con absorbancia a 625 nm. Presenta el inconveniente de una disminución del color de la disolución resultante en presencia de ácidos urónicos y hexosaminas (Kurzyna-Szklarek et al., 2022).

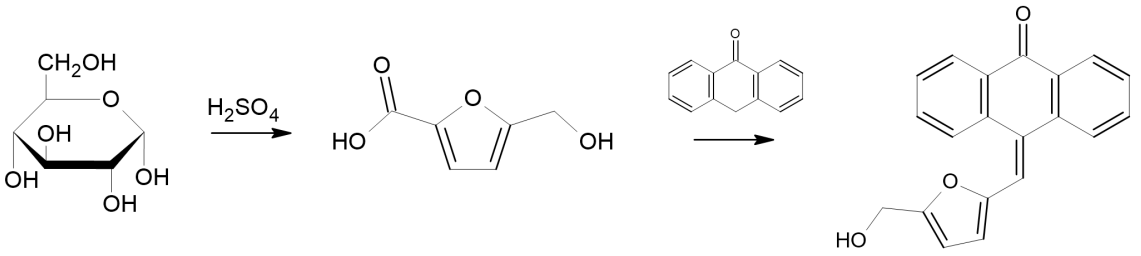


Figura 6. Reacción de antrona con D-glucosa

En el presente trabajo se ha adaptado el método de Dubois, para el análisis de agua enriquecida con exudados de microalgas y extractos de células. La necesidad de concentrar las muestras de aguas y los extractos de células para alcanzar una concentración cuantificable hizo que los volúmenes fueran proporcionalmente reducidos, tal y como se describe en el Capítulo 4, en el apartado de metodología de los artículos incluidos en esta Tesis Doctoral.

3.5. Determinación de ácidos urónicos en células de microalgas y sus aguas de cultivo enriquecidas con exudados

La determinación de ácidos urónicos intracelulares y exudados al medio por las microalgas, se llevó a cabo mediante el método que emplea el reactivo m-fenilfenol desarrollado por Blumenkrantz y Asboe-Hansen (1973).

Hasta ese momento, la determinación de ácidos urónicos se realizaba con carbazol en ácido sulfúrico concentrado y la adición de iones de tetraborato. Sin embargo, el empleo de ácido sulfúrico concentrado en muestras con una gran cantidad de azúcares neutros da lugar a un producto de color pardo que disminuye la especificidad de la reacción. La sustitución del carbazol por m-fenilfenol aumentó la sensibilidad y especificidad de la reacción, disminuyendo la interferencia de los azúcares neutros.

El derivado formado por reacción con m-fenilfenol da lugar a una coloración rosa de la disolución, midiendo su absorbancia a 520 nm.

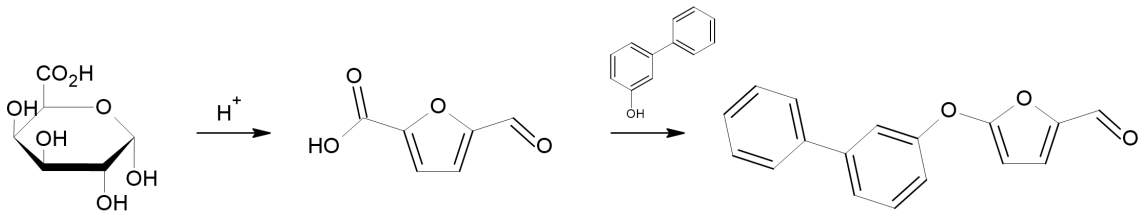


Figura 7. Reacción del ácido D-Galacturónico con m-fenilfenol

Siguiendo las modificaciones propuestas por Bastos et al. (2015) el método de determinación de ácidos urónicos se adaptó a volúmenes más pequeños para poder aplicarlo a muestras de agua de mar de cultivo de microalgas. El procedimiento se describe a continuación:

1. Se preparan 3 tubos de ensayo: 1 corresponderá a la corrección del blanco y 2 a las réplicas del ensayo con las muestras.
2. A cada tubo de ensayo de añaden 125 µL de muestra.
1. Se colocan los tubos en un baño de hielo y se añade 1 mL de borato de sodio 200 mM en H_2SO_4 concentrado.
2. Se lleva a un baño termostático a 100ºC durante 10 minutos.
3. Una vez pasado ese tiempo, se enfrían en un baño de hielo.
4. Se añaden 20 µL de m-fenilfenol 0,15 % (m/v) en 0,5% (m/v) NaOH, sólo a las muestras.

5. Se homogeniza bien y se deja reposar la disolución resultante en oscuridad durante 15 minutos.
6. Se mide la absorbancia en un espectrofotómetro a una longitud de onda de 520 nm.

Los resultados de la aplicación de este método se encuentran recogidos en el Capítulo 4, en el apartado correspondiente los resultados de las estancias doctorales (apartado 4.4.1) de la presente Tesis Doctoral.

3.6. Separación de compuestos orgánicos por peso molecular

Determinadas técnicas analíticas como la cromatografía de alta resolución de intercambio aniónico (HPAEC-PAD, por sus siglas en inglés "High Performance Anion-Exchange Chromatography/Pulsed Amperometric Detection") requieren de un paso previo de eliminación de sal en muestras de agua de mar. Autores como Borchard y Engel (2015) emplean unos dispositivos de filtración que consisten en unos tubos de centrífuga cónicos (Amicon® Macrosep) con una membrana de polietersulfona de 1kDa para la desalación y separación de carbohidratos de la muestra por tamaños. De esta manera, se consigue eliminar la sal de muestras, así como la separación por peso molecular de los analitos de interés. Esto último, permite distinguir entre compuestos orgánicos disueltos como los azúcares (monosacáridos, disacáridos) y los pertenecientes a moléculas mayores como los azúcares procedentes de polisacáridos.

Para poder llevarlo a cabo en muestras de agua de mar enriquecidas con exudados, se optimiza un procedimiento experimental descrito a continuación:

1. **Preconcentración y eliminación de sal**
    a. Se acondiciona el tubo de centrífuga con 20 mL de agua destilada y NaOH 0,1N (5000xg, 40 min) dos veces. De esta manera se evita la interferencia de glicerina y azida de sodio en el análisis posterior.
    b. Se añaden 20 mL de muestra al tubo de centrífuga inmediatamente después, sin dejar secar la membrana.
    c. Se calibran los tubos de centrífuga en la balanza.
    d. Se centrifuga a 5000xg durante 40 min.
    e. Este primer filtrado se recoge y se guarda para un análisis posterior.

f. Se añaden otros 20 mL de agua destilada, se tapa, agita, y centrifuga de nuevo (5000xg, 40 min). El filtrado se desecha. Se repite este paso 4 veces, para eliminar el 95-99% de la sal.
g. Se traspasa lo que no se ha filtrado (material mayor de 1 kDa) para análisis posteriores.

2. Reutilización de las membranas

a. Añadir 20 mL de NaOH 0,1N y centrifugar 5000xg durante 40 min.
b. Almacenar las membranas en agua:etanol 1:1.

3. Análisis del compuesto

a. Llevar a cabo el análisis del analito a estudiar.
b. En esta Tesis Doctoral se ha aplicado la separación al análisis de ácidos urónicos en muestras de agua de mar enriquecida con exudados de dos microalgas diferentes cultivadas bajo distintas condiciones de estrés ambiental (Capítulo 4, apartado 4.4.1).

3.7. Ensayos de determinación de la capacidad antioxidante

Dado que ningún ensayo puede caracterizar el perfil antioxidante total de una muestra, generalmente se utiliza más de un método para evaluar su poder antioxidante.

Los ensayos de determinación de la capacidad antioxidante pueden clasificarse de manera general en función del tipo de reacción que tiene lugar. Se distinguen dos tipos: ensayos de transferencia de electrones (ET, por sus siglas en inglés "electron transfer") y de transferencia de átomos de hidrógeno (HAT, por sus siglas en inglés "hydrogen atom transfer"). Los ensayos ET miden la capacidad reductora del antioxidante mientras que los ensayos HAT cuantifican la capacidad de donar un átomo de hidrógeno. Además, la actividad antioxidante se puede evaluar mediante la capacidad complejante de iones metálicos. En este, los antioxidantes inactivan a los iones metálicos impidiendo su reacción con H_2O_2 y la generación de $OH^{\bullet}$.

Los métodos de cuantificación *in vitro* de la capacidad antioxidante empleados en los trabajos de investigación de esta Tesis Doctoral son todos métodos colorimétricos, donde se mide un cambio de intensidad en el color debido a la reacción del antioxidante presente en la muestra, de manera que la pérdida de color es proporcional a la concentración de dicho antioxidante.

El procedimiento experimental de cada ensayo se encuentra descrito en el Capítulo 4 destinado a la discusión de resultados, en el apartado "Materials and methods" correspondiente de los artículos incluidos en esta Tesis Doctoral.

3.7.1. Capacidad inhibitoria de radicales libres

La determinación de la capacidad antioxidante mediante el ensayo de inhibición del radical DPPH es ampliamente conocido y empleado desde su desarrollo en los años cincuenta (Blois, 1958) al tratarse de un método colorimétrico sencillo y eficaz. El DPPH$^{\bullet}$ es un radical estable que en disolución con metanol posee un color violeta, capaz de absorber radiación a 515 nm. Es un proceso de transferencia de un átomo de hidrógeno, donde, en presencia de un antioxidante se produce la reducción de DPPH$^{\bullet}$, perdiendo la coloración violeta original, disminuyendo así la absorbancia.

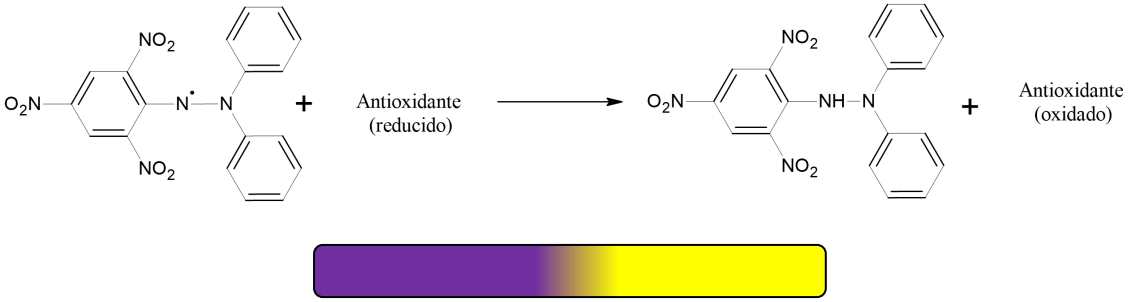


Figura 8. Reacción del ensayo de capacidad antioxidante con el radical DPPH y cambio de color

La monitorización de la disminución de la absorbancia a lo largo del tiempo permite el cálculo de la capacidad inhibitoria de radicales mediante la siguiente ecuación:

$$\text{RSA (\%)} = \left[\text{Absorbancia del DPPH} - \frac{\text{Absorbancia de la muestra}}{\text{Absorbancia del DPPH}}\right] \text{x100}$$

Si se realiza una curva de calibrado en función de la concentración de DPPH$^{\bullet}$ y se monitoriza de la misma forma la reacción de reducción del radical, se puede obtener la concentración de DPPH$^{\bullet}$ neutralizado. El resultado final se puede expresar como porcentaje de DPPH inhibido, de acuerdo con la ecuación anterior, o como cantidad de radical DPPH inhibido por unidad de volumen o por unidad de biomasa de muestra algal.

3.7.2. Capacidad de reducción de Fe (III) a Fe (II)

El ensayo para determinar la capacidad reductora de Fe(III) a Fe(II) de los antioxidantes en la muestra (FRAP assay, por sus siglas en inglés "Ferric Reducing

Antioxidant Power") proporciona resultados rápidos y reproducibles. Se trata de una reacción de transferencia de electrones en la que se produce la reducción del Fe(III) contenido en el reactivo FRAP, un complejo férrico de tripiridiltriazina (TPTZ, por sus siglas en inglés derivadas de su nombre "2,4,6-Tris(2-pyridyl)-s-triazine"), que da lugar a una coloración azulada de la disolución con un máximo de absorción a 593 nm. La presencia de Fe(III) en exceso en el reactivo FRAP, previene la interferencia de sustancias capaces de complejar este metal en el ensayo, asegurando que la capacidad antioxidante procede exclusivamente del mecanismo reductor de Fe(III) (Benzie y Strain, 1996).

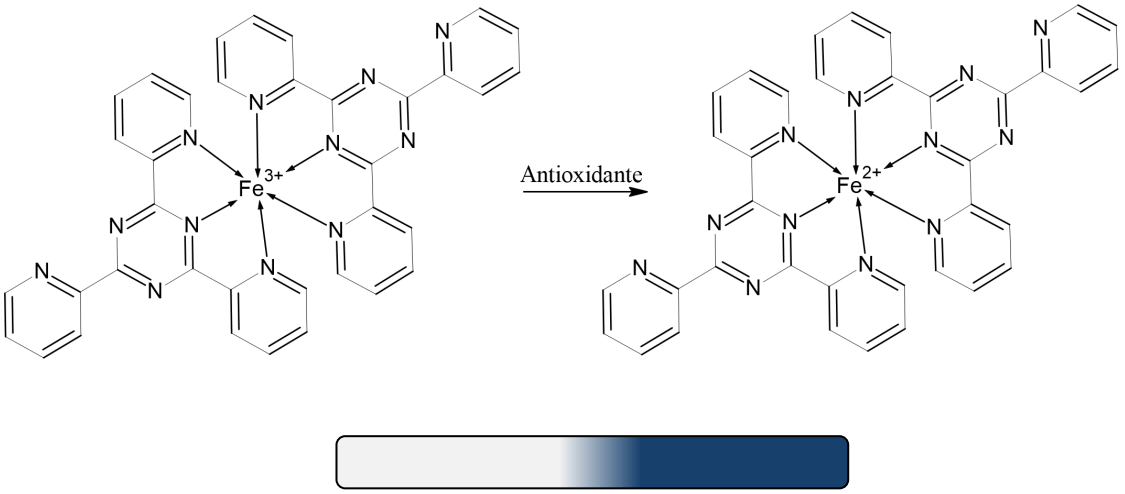


Figura 9. Reacción del ensayo de capacidad reductora de Fe(III) (FRAP) y cambio de color

De la misma forma que en el ensayo de DPPH, si se realiza una curva de calibrado con sulfato ferroso ($FeSO_4$) los resultados se pueden expresar como cantidad de Fe(II) por litro o por unidad de biomasa.

3.7.3. Capacidad de reducción de Cu(II) a Cu(I)

La determinación de la capacidad de reducción de Cu(II) a Cu(I) también conocida como ensayo CUPRAC (por sus siglas en inglés "Cupric Reducing Antioxidant Capacity") es un método simple y versátil. Al igual que para el ensayo FRAP, se trata de una reacción de transferencia de electrones, donde se produce un cambio en la coloración de la solución de azul a amarillo con máxima absorción a 450 nm, producto de la reacción de reducción entre el antioxidante y el reactivo CUPRAC (bis(neocuproína) y Cu(II)).

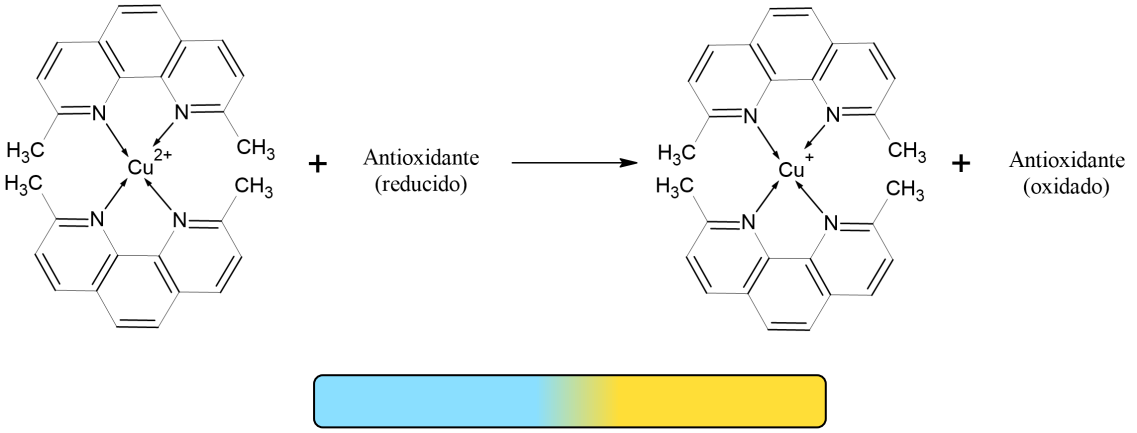


Figura 10. Reacción del ensayo de capacidad reductora de Cu(II) (CUPRAC) y cambio de color

Mediante la relación de las absorbancias de las muestras y las absorbancias para el ensayo empleando trolox®, los resultados se expresan en equivalentes de trolox® por unidad de volumen o por unidad de biomasa de muestra algal.

3.7.4. Capacidad complejante de Fe(II)

La capacidad complejante de Fe(II) se evalúa mediante la medida de inhibición de la formación del complejo ferrocina-Fe(II) de color azul con un pico de absorción a 562 nm. En presencia de compuestos capaces de formar complejos con Fe(II), la formación del complejo ferrocina-Fe(II) disminuye, reduciendo su absorción y la coloración azul de la disolución.

Por tanto, al adicionar Fe(II) a la muestra, se forma el complejo correspondiente con los antioxidantes presentes. Posteriormente, al añadir a esta disolución el reactivo ferrocina, este formará complejo ferrocina-Fe(II) con los iones del metal que han quedado libres en disolución en el paso anterior.

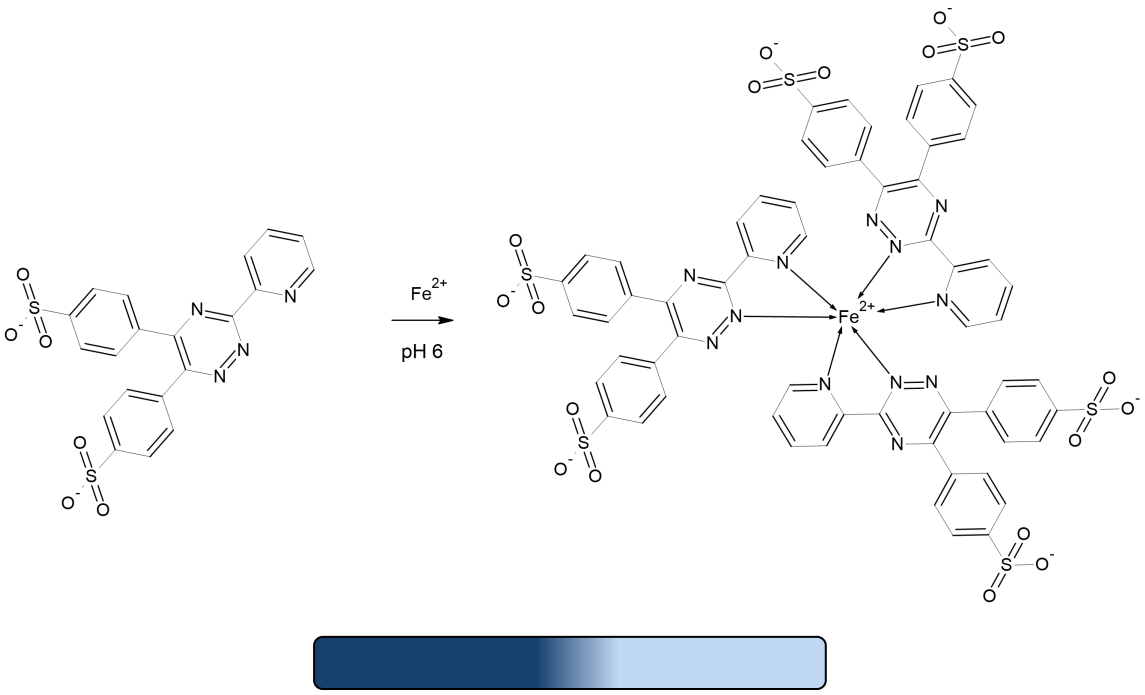


Figura 11. Formación del complejo ferrocina-Fe(II) y pérdida de color

Si se compara con un ensayo control donde se emplea agua destilada en lugar de la muestra, la capacidad complejante será máxima y corresponderá a la del complejo ferrocina-Fe(II). Por diferencia se puede determinar la inhibición de la formación del complejo con la siguiente ecuación:

$$\text{Inhibición de ferrocina-Fe(II) (\%)} = \frac{\text{Absorbancia del control - Absorbancia de la muestra}}{\text{Absorbancia del control}} \text{ x100}$$

3.7.5. Capacidad complejante de Cu(II)

La determinación de la capacidad complejante de Cu(II), al igual que la de Fe(II), se basa en la inhibición de la formación de un complejo de pirocatecol violeta (PV) (ácido 3,3',4-trihidroxifucsina-2"-sulfónico) con Cu(II) por parte de las moléculas con capacidad complejante de la muestra. De esta manera se compara la absorbancia del complejo PV-Cu(II) formado en ausencia de la muestra (control con agua destilada) que alcanza un color oscuro, y en presencia de la muestra, cuyo color pasa a amarillo. Los compuestos complejantes de Cu(II) formarán el complejo con los iones metálicos disueltos, mientras que los restantes formarán el complejo con el PV. La capacidad complejante se estima por la medida de la reducción del color y, por tanto, la reducción de la absorbancia de la disolución resultante.

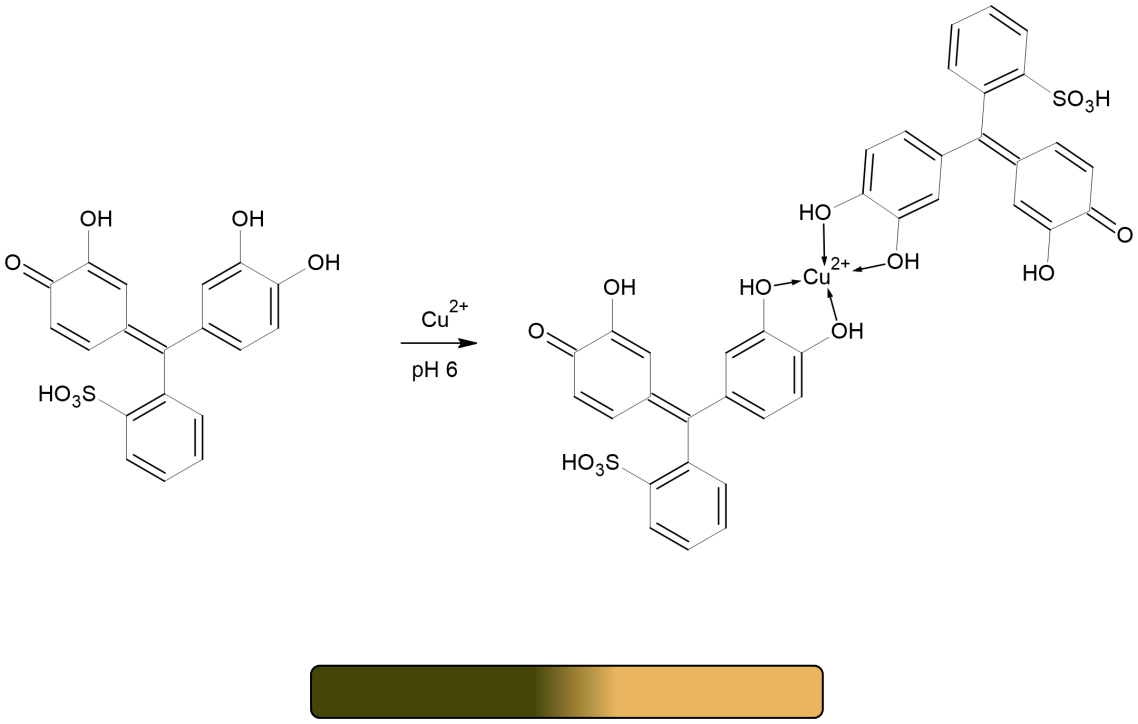


Figura 12. Formación del complejo PV-Cu(II) y pérdida de color

La capacidad complejante de Cu(II) de la muestra se expresa como porcentaje de inhibición del complejo PV-Cu(II) y se calcula de la siguiente forma:

$$\text{Inhibición de PV- Cu(II) (\%)} = \frac{\text{Absorbancia del control - Absorbancia de la muestra}}{\text{Absorbancia del control}} \text{ x100}$$

3.8. Determinación de malondialdehído

El malondialdehído (MDA) es un producto secundario de la peroxidación de lípidos e indicador de los efectos de las ROS generadas por estrés oxidativo (Janero, 1990). La reacción del MDA con el ácido tiobarbitúrico (TBA, por sus siglas en inglés "Thiobarbituric Acid") da lugar a un compuesto rosa que puede ser determinado espectrofotométricamente.

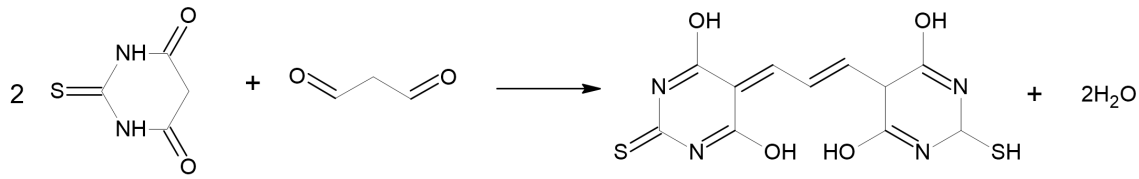


Figura 13. Reacción de MDA con TBA

Para eliminar las interferencias de carbohidratos y pigmentos, se mide la absorbancia de la disolución resultante a tres longitudes de onda diferentes (440, 532 y

600 nm) y los equivalentes de MDA se calculan mediante la siguiente ecuación (Hodges et al., 1999).

$$\text{MDA (nmol mL}^{-1}) = [(A - B)/157000]10^6$$

Donde:

$A = (Abs532_{+TBA} - Abs600_{+TBA}) - (Abs532_{-TBA} - Abs600_{-TBA})$

$B = [(Abs440_{+TBA} - Abs600_{+TBA})0.0571]$

CAPÍTULO 4. RESULTADOS Y DISCUSIÓN

4.1. *Phaeodactylum tricornutum*

4.1.1. Efecto de la toxicidad del cobre en la acumulación de aminoácidos libres y polifenoles en células de la diatomea *Phaeodactylum tricornutum*

El Cu es un metal esencial para la vida de los microorganismos marinos que en determinadas concentraciones puede ser tóxico, causando alteraciones importantes en las células. En aguas costeras, la contaminación por Cu se puede producir por factores antropogénicos, poniendo en riesgo la biodiversidad del fitoplancton que habita estas aguas.

El fitoplancton, y en particular las microalgas marinas, han desarrollado mecanismos relacionados con los metabolitos orgánicos para contrarrestar los efectos dañinos que puedan producir los metales pesados. Los polifenoles han sido ampliamente estudiados por su papel en la protección ante la toxicidad del Cu. Sin embargo, la implicación de otros compuestos orgánicos como los aminoácidos, no ha recibido especial atención.

Para comprobar la implicación de estos metabolitos en la tolerancia de la diatomea marina *Phaeodactylum tricornutum* a la presencia de Cu en el medio, se expuso a la diatomea a diferentes concentraciones de este metal durante distintos ciclos de crecimiento (0,31, 0,79 y 1,31 µM de Cu(II) y a un control que consistía en agua de mar sin adición de Cu). Con una densidad celular inicial de $1x10^7$ célula L^{-1}, la presencia de 0,31 µM de Cu(II) resultó subletal para la diatomea, cuya densidad celular se redujo un 37% y su crecimiento celular coincidía aproximadamente al del control. Por otro lado, la exposición a 0,79 y 1,31 µM de Cu(II) produjo una reducción de 82 y 91% respectivamente, siendo estos niveles letales para el crecimiento del alga. La densidad celular utilizada en el estudio se ha encontrado en determinados casos en aguas costeras (*blooms*), sin embargo, la densidad celular habitual en esas zonas es mucho más baja (10^3-10^6). Por ello, los efectos tóxicos del Cu se pueden intensificar causando daños aún mayores en el fitoplancton marino y, por tanto, en todo el ecosistema costero.

La monitorización de la densidad celular a lo largo del tiempo en el control permitió la realización de la curva de crecimiento donde se observó los diferentes estados de crecimiento del alga. La fase exponencial y estacionaria terminaron después de 12 y

16 días respectivamente, comenzando la fase de muerte celular a los 16 días cuyo seguimiento finalizó a los 21 días. A partir de esta información las células se recolectaron y analizaron al principio y al final de la fase estacionaria (12 y 18 días) y en la fase de muerte celular (21 días).

La identificación de polifenoles y aminoácidos en extractos de células de *P. tricornutum* se llevó a cabo aplicando el método optimizado de RP-HPLC-DAD. Además, se evaluó el estado de estrés oxidativo de la célula mediante el análisis de MDA y la capacidad antioxidante de los extractos por diferentes ensayos: RSA, FRAP y CUPRAC.

El perfil de aminoácidos analizado difiere entre los extractos de células en función de la concentración de Cu y el ciclo. La variación temporal de aminoácidos libres totales en el control se explica por una disminución de los 12 a los 18 días, debido al empleo de los aminoácidos por parte de la célula para la síntesis de proteínas, como ya se ha comprobado en otros estudios. A partir de los 21 días, las células se encuentran en fase de muerte celular por lo que el aumento en el contenido de aminoácidos se puede deber a la degradación de las proteínas. El mismo comportamiento se detectó en las células expuestas a niveles subletales de Cu, con la diferencia de un incremento en el contenido total de aminoácidos libres acumulados intracelularmente, lo que podría suponer un mecanismo de defensa para minimizar la toxicidad del Cu.

En los cultivos con concentraciones letales de Cu, la cantidad de aminoácidos intracelulares libres experimenta un gran aumento en comparación con el control. Bajo estas condiciones, las microalgas en fase estacionaria entran en un "estado de supervivencia", cesan su crecimiento y centran su actividad metabólica en activar mecanismos para adaptarse y sobrevivir, en detrimento de la síntesis de proteínas, por lo que la cantidad de aminoácidos libres intracelulares es mayor.

Entre los aminoácidos acumulados en mayor cantidad, destacan la metionina y la histidina, cuyos niveles aumentaron en las células hasta un 66,8 y 24,5 veces más que en el control. Ambos aminoácidos son capaces de unirse al Cu, pudiendo interaccionar con este ion tanto en la membrana celular como en el interior de la célula, previniendo así su toxicidad. Además, el ácido glutámico y la prolina también experimentaron un incremento de 20,5 y 22 veces respectivamente la cantidad en el control. El ácido glutámico es precursor de la prolina, siendo esta última ampliamente conocida por su

efecto como inhibidor de radicales libres y complejante de iones metálicos. Su acumulación es un mecanismo de tolerancia al estrés que genera la presencia de determinadas concentraciones de metales como el Cu. Por otro lado, la isoleucina y la valina no se detectaron en ninguna de las condiciones experimentales.

La misma tendencia se encontró en el perfil polifenólico de los extractos del alga recolectada a los 18 días, donde su contenido estuvo estrechamente relacionado con la concentración de Cu en el medio. El contenido total en polifenoles llegó a ser 11,3 veces mayor en las células cultivadas con la concentración más letal de Cu comparado con el control. En especial, el ácido gálico, el ácido vanílico y la epicatequina experimentaron un aumento considerable bajo estas condiciones. La producción de polifenoles en las células como respuesta a la presencia de Cu ha sido estudiada previamente en el grupo QUIMA. Estos compuestos son reconocidos por su capacidad antioxidante. En particular, el ácido gálico ha demostrado ser muy eficaz en la inhibición del radical DPPH. La acumulación de polifenoles tiene lugar como consecuencia del estado de estrés oxidativo de la célula, que emplea la producción de estos compuestos para sobrellevar las condiciones de toxicidad del medio y la excesiva producción de ROS que conlleva. Este estado oxidativo de las células se manifiesta en el incremento de hasta 14,5 veces del contenido celular de MDA, al aumentar las concentraciones de Cu en el medio.

En cuanto a la capacidad antioxidante, tanto el ensayo de RSA, como el FRAP y el CUPRAC presentan la misma tendencia: a medida que aumenta la concentración de Cu a la que se encuentran expuestas las células de *P. tricornutum,* la actividad antioxidante de los extractos también aumenta. Por tanto, la capacidad antioxidante por célula es mayor para los niveles letales de Cu debido a la mayor necesidad de la diatomea de contrarrestar el estrés incrementado bajo esas condiciones.

Este estudio puede contribuir a la comprensión del papel de los aminoácidos y los polifenoles en el mecanismo de tolerancia y adaptación de la microalga marina *P. tricornutum* contra la toxicidad de metales pesados como el Cu. Además, estos resultados facilitarán la identificación de moléculas orgánicas concretas para el estudio de su papel en la regulación de metales pesados en el agua de mar como componentes de la materia orgánica disuelta.

Este trabajo fue publicado en la revista *Environmental Science and Pollution Research* en 2023.

Environmental Science and Pollution Research
https://doi.org/10.1007/s11356-023-25939-0

RESEARCH ARTICLE

Copper toxicity leads to accumulation of free amino acids and polyphenols in *Phaeodactylum tricornutum* diatoms

Paula Santiago-Díaz[1,2] · Argimiro Rivero[1,2] · Milagros Rico[1,2] · Aridane González González[1,2] · Melchor González-Dávila[1,2] · Magdalena Santana-Casiano[1,2]

Received: 27 September 2022 / Accepted: 10 February 2023

Abstract
This work is focused on the effect of lethal and sub-lethal copper (Cu) concentrations on the free amino acid and polyphenol production by the marine diatom *Phaeodactylum tricornutum* (*P. tricornutum*) after 12, 18, and 21 days of exposure. The concentrations of 10 amino acids (arginine, aspartic acid, glutamic acid, histidine, lysine, methionine, proline, valine, isoleucine, and phenylalanine) and 10 polyphenols (gallic acid, protocatechuic acid, *p*-coumaric acid, ferulic acid, catechin, vanillic acid, epicatechin syringic acid, rutin, and gentisic acid) were measured by RP-HPLC. Under lethal doses of Cu, free amino acids reached levels significantly higher than those in the control cells (up to 21.9 times higher), where histidine and methionine showed the highest increases (up to 37.4 and 65.8 times higher, respectively). The total phenolic content also increased up to 11.3 and 5.59 times higher compared to the reference cells, showing gallic acid the highest increase (45.8 times greater). The antioxidant activities of cells exposed to Cu were also enhanced with increasing doses of Cu(II). They were evaluated by 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging ability (RSA), cupric ion reducing antioxidant capacity (CUPRAC), and ferric reducing antioxidant power (FRAP) assays. Malonaldehyde (MDA) exhibited the same tendency: cells grown at the highest lethal Cu concentration yielded the highest MDA level. These findings reflect the involvement of amino acids and polyphenols in protective mechanisms to overcome the toxicity of copper in marine microalgae.

Keywords Amino acid · Polyphenol · Microalgae · Antioxidant activity · Copper toxicity · *Phaeodactylum tricornutum*

Introduction

The presence of heavy metals in the coastal seawater at high concentrations has a direct impact on marine microorganisms. The uptake of metals by the cells can cause a range of toxic alterations such as enhanced production of reactive oxygen species (ROS) inducing oxidative stress and breaking the oxidative balance of microalgae; disruption of protein structures affecting enzymes and nucleic acids functions by metal binding or displacement of an essential element, among others (Shivaji and Dronamaraju 2019; Wei et al. 2014). Coastal waters are critical habitats for many marine species, where metal toxicity can change the start and end timing of the blooms and their amplitude, affecting the survival and hatching time of commercially important species (Trombetta et al. 2019).

Copper (Cu) is one of those trace metals that becomes toxic when exceeding certain levels. Natural concentrations of Cu in coastal seawater are generally low (0.008 and 0.050 μM); however, local anthropogenic sources principally related with industrial activities and domestic wastes can result in local increase in Cu concentrations above 3.0 μM (Leal et al. 2018; Pérez-Cid et al. 2021). According to the aquatic life criteria from Environmental Protection Agency (EPA), the Criterion Maximum Concentration (CMC) for Cu acute toxicology is 0.075 μM and the Criterion Continuous Concentration (CCC) for Cu chronic toxicology is 0.049 μM.

Responsible Editor: Gangrong Shi

✉ Milagros Rico
milagros.ricosantos@ulpgc.es

[1] Departamento de Química, Facultad de Ciencias del Mar, Universidad de Las Palmas de Gran Canaria, Campus de Tafira, 35017 Las Palmas de Gran Canaria, Spain

[2] Instituto de Oceanografía Y Cambio Global (IOCAG), Universidad de Las Palmas de Gran Canaria, Las Palmas de Gran Canaria, Spain

Published online: 21 February 2023

There is considerable evidence with respect to the involvement of phenolic compounds in protection mechanisms against copper toxicity in plants and algae growing in conditions of metal stress (Kováčik et al. 2010; López et al. 2015; Lwalaba et al. 2020; Sharma et al. 2019; Stiller et al. 2021). However, little is known about the implication of amino acids. Therefore, this study is mainly focused on the impact of copper toxicity on the amino acid profile of microalgae cells during growth. Some studies have evidenced accumulation of free amino acids in microalgae cells cultivated under Cu concentrations as a response to heavy metal stress (Hamed et al. 2017; Seregin and Kozhevnikova 2021).

Amino acids like histidine (His) and methionine (Met) have been found to play a significant role in heavy metal detoxification and tolerance (Hall 2002; Murphy et al. 2011; Patrick 2003). His, Met, and cysteine (Cys) are the predominant amino acids in several transmembrane proteins to facilitate Cu transport across the cell membrane at different pH and oxidative environments (Rubino et al. 2011; Rubino and Franz 2012; Öhrvik and Thiele 2014). After cell wall metal adsorption, the transport toward chloroplasts, mitochondria, and vacuoles is mediated by small glutamic acid (Glu) and Cys-rich polypeptides, namely, metallothioneins and phytochelatins (Balzano et al. 2020; Blaby-Haas and Merchant 2012; Cobbett 2000). These polypeptides have been evidenced in *P. tricornutum* growing under exposure to metals (Morelli et al. 2005; Torres et al. 1997; Wei et al. 2014).

Microalgae can provide information on the potential impacts of Cu. The marine diatom *P. tricornutum* is commonly used in toxicity tests (Moreno-Garrido et al. 2000; Singh and Shrivastava 2016). Its sensitivity makes them ideal to study the influence of Cu levels on the phenolic and amino acid profiles variations at different phases of the diatom growth. However, the biochemical response of these microorganisms cultivated under Cu stress is still not fully understood, neither their role in regulating the speciation of trace metals contaminants in seawater as components of the dissolved organic matter.

The aim of this work was to study the chemical response of the diatom *P. tricornutum* to high Cu concentrations at different stages of growth (12, 18, and 21 days) by analyzing the contents of 10 amino acids and 10 polyphenols in cells. The changes in composition could be used by cells as an intracellular mechanism of reaction to the Cu toxicity. Moreover, the antioxidant activities of *P. tricornutum* cells extracts were evaluated in terms of radical scavenging ability (RSA), the total reduction power by the ferric reducing antioxidant power (FRAP) assay, and the cupric ion reducing antioxidant capacity with (CUPRAC) assay. In addition, malonaldehyde (MDA) was also measured as a biomarker for oxidative stress (Hodges et al. 1999).

Materials and methods

Chemicals

Methanol (HPLC gradient grade), ethanol, and tetrachloroethylene (synthesis grade) were purchased from Scharlab (Barcelona, Spain). $CuSO_4 \cdot 7H_2O$, phenylisothiocyanate (PITC), 2,4,6-tri(2-pyridyl)-triazine (TPTZ), neocuproine, and DPPH were supplied by Sigma-Aldrich (St. Louis, MO, USA). Triethylamine (TEA), hydrochloric acid (37%), sodium acetate, glacial acetic acid, $Fe_3Cl \cdot 6H_2O$ and $FeSO_4 \cdot 7H_2O$, trichloroacetic acid (TCA), butylated hydroxytoluene (BHT), and 2-thiobarbituric acid (TBA) (analysis quality) were supplied by Panreac (Barcelona, Spain). Formic acid (synthesis grade) and amino acid standards Glu, His, Met, arginine (Arg), aspartic acid (Asp), lysine (Lys), proline (Pro), valine (Val), isoleucine (Ile), and phenylalanine (Phe) were provided by Merck (Darmstadt, Germany). Polyphenol standards were supplied as follows: gallic acid (GAL), protocatechuic acid (PCA), p-coumaric acid (COU), ferulic acid (FA), catechin (CAT), vanillic acid (VAN), epicatechin (ECAT), syringic acid (SYR), and Trolox (TR) by Sigma-Aldrich Chemie (Steinheim, Germany); rutin (RU) and gentisic acid (GA) by Merck (Darmstadt, Germany). Ultrapure water was obtained from a Milli-Q system from Millipore (Bedford, MA, USA).

Algae cultivation

Axenic strains of *P. tricornutum* (REC 001B) were provided by the Spanish Bank of Algae (Taliarte, Spain). The diatoms were harvested in a clean culture chamber (Friocell FC111) with permanent illumination (24 h at 8000 lx) under a constant temperature of 24 °C.

Seawater used for cultures was sampled off the coast of Gran Canaria, treated with ultraviolet radiation and passed through 0.45-µm filters. The seawater was enriched with nutrients (SWn) at the concentrations used in a f/2 medium without added trace metals and EDTA. The nutrient concentrations were $[NO_3^-] = 883$ µM, $[HPO_4^{2-}] = 29.3$ µM, and $[SiO_3^{2-}] = 142$ µM (González et al. 2012).

Cu experiments

For the Cu exposure treatments, cultures of SWn with an initial cell density of 1×10^7 cells L^{-1} were spiked with 0.31 µM, 0.79 µM, and 1.57 µM Cu (II). The Cu concentrations selected were lower than those described in coastal seawater (Leal et al. 2018) but higher than the CMC and

CCC defined by EPA for acute and chronic toxicology, respectively.

The control was a SWn culture with the same initial cell density without Cu addition. To separate the biomass, the cells were filtered by gravity to avoid rupture using 1.2-µm filters (trace metal acid clean pore-size nitrocellulose, Sartorius™). This process was carried out after 12, 18, and 21 days of growth. The cell concentration was determined daily with a light microscope (Microbiotest, Inc.) with a hemocytometer counter and by measuring the absorbance (Abs) at 670 nm with a spectrophotometer (USB4000).

Free amino acid extraction and quantification

The following 10 amino acids were examined in the cells: Glu, His, Met, Arg, Asp, Lys, Pro, Val, Ile, and Phe. They were selected because of their antioxidant properties (Hwang et al. 2019) and their role in the response of plants to several types of abiotic stress (Marquis et al. 2020; Zhang et al. 2020a, b).

Samples (1 L) of control and Cu-exposed diatom cultures were filtered as described above, and the biomass was extracted with deionized water (50 mL) by sonication for 30 min. The mixture was freeze-dried, and the residue was dissolved in 5 mL of water. Amino acids were derivatized according to Shi et al. (2013). In brief, samples or amino acid standard solutions (5 mL) were mixed with 2.5 mL of PITC 0.1 M and 2.5 mL of TEA 1 M, and the resulting solutions were stirred for 1 h at room temperature. Then, 5 mL of tetrachloroethylene was added and vigorously shaken, and the upper layer was collected. This step was performed twice. The final solution was filtered through a 0.22-µm nylon and stored at − 20 °C until analysis.

Chromatographic analysis was carried out with a Jasco LC-4000 HPLC equipment provided with a PU-4180 quaternary pump, an AS-4150 autosampler, an MD-4015 photodiode array detector and an LC-Net ll interface. Amino acids were separated with a Phenomenex C18 column (250 mm × 4.6 mm, 5 µm) and a Phenomenex guard column maintained at 30 °C. The gradient elution was made with ultrapure water with 0.1% formic acid as mobile phase A and methanol as mobile phase B. The flow rate was 1 mL min^{-1} and the injection volume was 10 µL. The following program for eluent A was applied: 0 min, 75%; 30 min, 40%; 40 min, 40%; and finally, column was washed and reconditioned (Santiago-Díaz et al. 2022). Data acquisition was carried out with ChromNav software, and the statistical analysis (available as supplementary data) was made using Past software provided with an ANOVA test and a Tukey's test. Significant differences between Cu treatments for each amino acid after 12 and 18 days were found. The determination of statistically significant differences (considered at $p < 0.05$) between each treatment and the control for each individual amino acid was carried out with the Tukey's test. Algae samples were analyzed by triplicate and the results were expressed as fmol $cell^{-1}$.

Polyphenols contents, antioxidant activities, and MDA determinations

Samples (1 L) exposed to Cu for 18 days were filtered as described above and cells were freeze-dried and extracted twice with 10 mL of methanol combining sonication and stirring. The samples were centrifuged at 2700 g for 15 min, the supernatants were collected and evaporated to dryness, and the residue was dissolved in 5 mL of methanol for determining polyphenols contents and antioxidant activities. For MDA analysis, freeze-dried cells were extracted twice with deionized water (3 mL).

The concentrations of the 10 polyphenols GAL, PCA, CAT, VAN, RU, ECAT, SYR, GA, CA, and FA were evaluated. They were selected because their involvement in protection mechanisms against copper toxicity has been widely evidenced (Kováčik et al. 2010; López et al. 2015; Lwalaba et al. 2020; Rico et al. 2013; Santana-Casiano et al. 2014). Methanol extracts described above (2 mL) were concentrated to dryness and the residue was dissolved in methanol (200 µL) and filtered with a syringe filter (0.2 µm). The chromatographic analysis was made with the equipment and columns described in the "Free amino acid extraction and quantification" section. The elution was also performed with the same eluents, flow rate, injection volume, and column temperature (30 °C). The gradient elution method for A was from 0 to 5 min, 80% isocratic; from 5 to 30 min, linear gradient from 80 to 40%; and the column was washed with a mixture of A and B (1:9) and conditioned for the next analysis. Simultaneous monitoring was set at 270 nm (GAL, PA, CAT, VA, RU, ECAT, and SA) and 324 nm (GA, CA, and FA) for quantification (Santiago-Díaz et al. 2022). Algae samples were analyzed by triplicate, and the results were expressed as attomol (amol) $cell^{-1}$.

The RSA was evaluated according to Bondet et al. (1997) with modifications. Methanol extracts (70 µL) were mixed with 1 mL of free radical DPPH solution (0.067 mM) for 10 min. The Abs was recorded at 515 nm, and the results obtained from a calibration curve ($y = 11.987x—0.1352$, $R^2 = 0.9996$) were expressed as fmol of inhibited DPPH $cell^{-1}$. Measurements were taken in triplicate and the results were averaged.

The FRAP reagent was freshly prepared by mixing 100 mL of acetate buffer solution 0.3 M (pH 3.6) with 10 mL of TPTZ (10 mM) in HCl (40 mM) and 10 mL of $FeCl_3 \cdot 6H_2O$ solution (20 mM) (Benzie and Strain 1996). Methanol extracts (200 µL) and 1.4 mL of pre-warmed FRAP reagent (37 °C) were mixed for 10 min. The mixture was cooled and the Abs recorded at 593 nm. Results

calculated from a calibration curve constructed with $FeSO_4 \cdot 7H_2O$ solutions ranging from 0.38 to 2.69 μM ($y = 0.6265x + 0.1882$, $R^2 = 0.9988$) were expressed as pmol of Fe(II) cell^{-1}.

The CUPRAC reagent was freshly prepared with equal volumes of $CuSO_4 \cdot 7H_2O$ (10 mM), neocuproine ethanolic solution (7.5 mM), and NH_4Ac buffer solution (1 M) (Apak et al. 2006). This reagent (570 μL) was diluted with water (930 μL) and mixed with 200 μL of methanol extracts for 30 min, and the Abs was recorded at 450 nm. The results were expressed as fmol TR cell^{-1} and calculated from a standard curve prepared with solutions in the range of concentrations from 11.6 to 77.5 μM ($y = 0.6215x + 0.0004$; $R^2 = 0.9996$). The estimation was carried out in triplicate, and the results were averaged.

Aqueous extracts of cells (0.5 mL) were mixed with either (i) 0.5 mL of aqueous solution of TCA (20%) and BHT (0.01%) or (ii) 0.5 mL of the above solution with 0.5% TBA added. The mixtures were heated at 95 °C for 25 min, cooled to 5 °C, and centrifuged at 1300 rpm for 10 min. MDA reacts with TBA to produce a pinkish-red adduct. The Abs was measured at three wavelengths to correct the interferences of carbohydrates and pigments (440, 532, and 600 nm) and the equivalents of MDA were calculated according to Hodges et al. (1999), and expressed as amol of MDA cell^{-1}.

Results

Effects of Cu levels on growth of P. tricornutum

The growth curves of *P. tricornutum* in controls and exposures of 0.31, 0.79, and 1.57 μM of Cu are shown in Fig. 1. In the controls, the mean absolute growth rate was

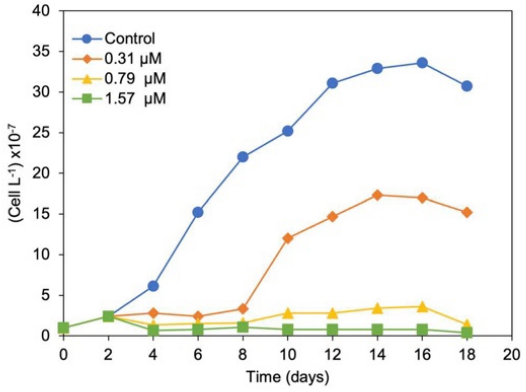


Fig. 1 Growth curve of *P. tricornutum* exposed to different copper concentrations in seawater enriched with f/2 nutrients (24 h at 8000 lx, 24 °C) with an initial cell density of 1×10^7 cells L^{-1}

2.11×10^7 cell L^{-1} day^{-1}. The stationary phase ended after 16 days, achieving during this phase a maximum cell density of 33.6×10^7 cell L^{-1}. From 16 to 21 days, the diatoms cells were in the death phase. Therefore, the cell density was daily measured until day 18 and subsequently measured on day 21. According to the evolution of the control cultures, the days 12, 18, and 21 were selected for the determination of free amino acid profiles and the day 18 for the studies of antioxidant activity, MDA, and phenolic contents.

When the initial concentration of Cu was 0.31 μM, a prolonged lag phase was found compared to the control culture. Although the mean absolute growth rate in 0.31 μM Cu cultures (0.783×10^7 cell L^{-1} day^{-1}) was significantly different ($\rho < 0.05$) from the control culture, biomass was reduced only by 37%, suggesting that this concentration is a sub-lethal dose to the microalga. However, for cultures enriched with 0.79 and 1.57 μM of Cu, lack of exponential phase of growth was observed and cell density decreased by 82 and 91% respectively, indicating these concentrations are lethal for *P. tricornutum* under the experimental conditions.

Free amino acid profile in P. tricornutum

The amino acid profiles of *P. tricornutum* cells collected after 12, 18 and 21 days are summarized in Table 1. Under lethal Cu concentrations, the total sum of free amino acid contents quantified in diatom cells increased with the addition of Cu to the culture medium, reaching levels up to 21.9 times higher compared to the control culture. In these conditions, Met and His were the predominant amino acids, showing the greatest increase in concentration up to 66.8 and 24.5 times higher respectively. Increased concentrations of Glu (up to 20.5 times higher) and Pro (up to 22 times higher) were also observed.

Among the analyzed free amino acids, His, Glu, Pro, and Met were detected in all the extracts. However, Ile and Phe were below detection limit under the experimental conditions (not presented in the Table 1). Val was only detected after 12 and 18 days of exposure to sub-lethal Cu concentrations, and detected in all the cultures after 21 days; Lys was not detected in cells harvested after 12 days at 0.79 μM of Cu, and after 18 and 21 days of exposure to the highest Cu concentration (1.57 μM).

Phenolic profile in P. tricornutum cells

Profile of polyphenols, measured as content of ten polyphenols in cell extracts of *P. tricornutum* biomass exposed to copper for 18 days, was strongly affected by the metal concentration (Table 2). Under our experimental conditions, ECAT was not detected in the seawater enriched with 0.79 μM of Cu and CAT was only detected

Table 1 Free amino acid contents in extracts of the diatom *P. tricornutum* exposed to different Cu concentrations after 12, 18, and 21 days

Amino acid[a]	Control	[Cu(II)] 0.31 µM	[Cu(II)] 0.79 µM	[Cu(II)] 1.57 µM
After 12 days				
His	4.64 ± 0.54	9.04 ± 0.12[b]	28.3 ± 0.8[b]	57.5 ± 0.7[b]
Arg	0.41 ± 0.01	1.05 ± 0.00[b]	1.45 ± 0.33[b]	n.d
Glu	0.54 ± 0.01	0.96 ± 0.00[b]	2.16 ± 0.01[b]	4.43 ± 0.05[b]
Asp	0.58 ± 0.00	1.18 ± 0.01[b]	2.94 ± 0.02[b]	7.54 ± 0.04[b]
Pro	0.38 ± 0.00	0.83 ± 0.03[b]	1.71 ± 0.27[b]	3.08 ± 0.06[b]
Met	2.08 ± 0.02	8.33 ± 0.69	84.9 ± 1.7[b]	136.9 ± 8.6[b]
Val	0.56 ± 0.00	1.28 ± 0.01[b]	n.d	n.d
Lys	0.92 ± 0.04	1.04 ± 0.03[b]	n.d	n.d
Sum	10.11 ± 0.62	23.71 ± 0.89	121.40 ± 3.13	209.40 ± 9.45
After 18 days				
His	2.31 ± 0.09	6.21 ± 0.06[b]	56.5 ± 0.3[b]	86.4 ± 2.0[b]
Arg	1.96 ± 0.06	4.14 ± 0.10[b]	20.6 ± 0.2[b]	n.d
Glu	0.42 ± 0.02	0.87 ± 0.01[b]	4.94 ± 0.08[b]	8.62 ± 0.03[b]
Asp	n.d	0.52 ± 0.01	12.4 ± 0.1	11.7 ± 0.1
Pro	0.38 ± 0.02	0.63 ± 0.01	3.31 ± 0.10	8.41 ± 0.04[b]
Met	1.22 ± 0.01	3.25 ± 0.13	69.2 ± 4.7[b]	29.9 ± 3.9[b]
Val	1.16 ± 0.02	0.33 ± 0.00[b]	n.d	n.d
Lys	0.38 ± 0.02	0.65 ± 0.03[b]	4.85 ± 0.17[b]	n.d
Sum	7.83 ± 0.24	16.6 ± 0.3	171.8 ± 5.7	145.0 ± 6.1
After 21 days				
His	11.8 ± 0.0	24.7 ± 0.1[b]	37.4 ± 0.4[b]	45.0 ± 0.2[b]
Arg	1.14 ± 0.04	1.97 ± 0.04[b]	2.82 ± 0.04[b]	2.22 ± 0.18[b]
Glu	1.75 ± 0.03	2.26 ± 0.01[b]	3.11 ± 0.08[b]	3.29 ± 0.02[b]
Asp	1.84 ± 0.02	2.53 ± 0.03[b]	3.90 ± 0.04[b]	4.70 ± 0.02[b]
Pro	1.12 ± 0.05	1.83 ± 0.09[b]	2.62 ± 0.14[b]	2.26 ± 0.05[b]
Met	8.32 ± 0.11	16.9 ± 0.93[b]	22.5 ± 1.5[b]	84.5 ± 5.7[b]
Val	1.61 ± 0.01	2.66 ± 0.07[b]	3.86 ± 0.05[b]	3.55 ± 0.05[b]
Lys	1.43 ± 0.00	2.00 ± 0.00[b]	2.82 ± 0.03[b]	n.d
Sum	29.0 ± 0.3	54.9 ± 1.3	79.0 ± 2.3	145.6 ± 6.2

n.d. means not detected

[a]Fmol of amino acid per cell (means ± standard deviation of three measurements)

[b]Significant differences to the control (Tukey's test, $p < 0.05$)

Table 2 Polyphenol contents in cells of diatom *P. tricornutum* exposed to different copper levels for 18 days (expressed as amol cell^{-1})

Phenolic compound*	Control	Cu(II) 0.31 µM	Cu(II) 0.79 µM	Cu(II) 1.57 µM
Gallic acid	10.2 ± 4.0	122.8 ± 34.0	283.3 ± 16.5	467.0 ± 31.5
Catechin	11.8 ± 4.1	n.d	n.d	n.d
Vanillic acid	24.0 ± 1.6	14.2 ± 2.5	53.3 ± 3.8	132.0 ± 5.6
Syringic acid	10.0 ± 1.9	8.27 ± 1.99	20.6 ± 3.1	23.7 ± 0.4
Epicatechin	7.59 ± 3.95	3.13 ± 0.32	n.d	100.6 ± 17.4
Sum	63.9 ± 15.5	148.4 ± 38.7	357.2 ± 23.4	723.2 ± 55.0

n.d. means not detected

*Results are expressed as amol cell^{-1} (means ± standard deviation of three measurements)

in the control cells. GA, VAN, and SYR were detected in all cultures. The other 5 polyphenols investigated were below detection limit. As compared to the control, the amounts of individual phenolic compounds GA, VAN, and SYR increased strongly at the lethal Cu concentration of 1.57 µM. The same behavior was observed in the

Table 3 Antioxidant activities and MDA contents per cell of *P. tricornutum* cultured under copper exposition for 18 days

Cu concentration (μM)	FRAP* (fmol of Fe(II))	CUPRAC* (pmol of TR)	Inhibited DPPH* (fmol)		MDA* (amol)
0	10.44 ± 0.02	6.38 ± 0.02	1.4 ± 0.2	4.5 ± 0.4	
0.31	11.08 ± 0.01	10.55 ± 0.02	26.5 ± 0.5	10.25 ± 0.07	
0.79	29.06 ± 0.07	29.53 ± 0.05	59 ± 2	23.7 ± 0.1	
1.57	31.1 ± 0.2	41.8 ± 0.2	79 ± 1	65.8 ± 0	

*All results are expressed per cell as means ± standard deviation of three measurements

total sum of the identified polyphenols, which was 11.3 times higher.

Antioxidant activities

The antioxidant activities of cells exposed to Cu for 18 days and evaluated by DPPH scavenging activity, FRAP and CUPRAC tests were enhanced as the concentration of Cu increased (Table 3). The reference cells yielded enough antioxidants to inhibit 1.4 fmol of DPPH cell^{-1} followed by cells cultured at 0.31, 0.79, and 1.57 μM of Cu. FRAP and CUPRAC results showed the same tendency: cells grown at the highest lethal Cu level yielded the highest antioxidant capacities.

MDA contents

The amount of cellular MDA increased up to 14.5-fold higher than that of the control at the highest lethal level of Cu, reflecting increased oxidative stress (Table 3).

Discussion

Effects of Cu levels on growth of P. tricornutum

The studies carried out showed that the increase in Cu concentration produced a delay in the lag phase and a lower absolute growth rate in the exponential phase that resulted in a lower number of cells in the stationary phase under sub-lethal Cu doses (Fig. 1). The average cell number was close to the starting cell concentration when the Cu levels exceeded the toxicity threshold (0.79 uM and 1.57 uM), indicating an inhibitory effect, as has been previously reported (López et al. 2015; Osborn and Hook 2013; Renzi et al. 2014; Rico et al. 2013). These results agree with the study of Markina and Aizdaicher (2006), who found a decrease in *P. tricornutum* cell concentration grown on Goldberg medium prepared from seawater and an extended lag phase with increasing levels of Cu (2.04 and 3.93 μM of Cu). This behavior was also reported by Cid et al. (1995), and only Cu concentrations higher than 15.74 μM did not fit the logistic function.

The initial cell density used in this study has been reached in some coastal areas (Dursun and Tas 2019) and in lake ecosystems (Zhang et al. 2020a, b). Lower phytoplankton abundances commonly observed in coastal waters (Effendi et al. 2016; Fehling et al. 2012) could intensify the toxic effects of Cu exposition (Moreno-Garrido et al. 2000; Singh and Shrivastava 2016) affecting the physiological processes of coastal marine organisms and their communities (Leal et al. 2018; Trombetta et al. 2019).

Free amino acids profile of cells

The evolution of each single free amino acid content in the control cells during growth is shown in Table 1. The maximum cell density was achieved on the stationary phase, where cells showed a lower total content of amino acids (7.83 fmol cell^{-1}) than that found at the end of the exponential phase (10.11 fmol cell^{-1}). Several studies do agree with this temporal variability of free amino acids observed here, indicating that this reduction of amino acids is due to the formation of proteins involved in all cell functions when the maximum biomass is reached (Lourenço et al. 2004; Vendruscolo et al. 2019). Diatoms *Rhizosolenia delicatula* have shown the same behavior during a spring bloom, where the total free amino acids decreased when the maximum biomass was achieved (Martin-Jézéquel et al. 1992). These same results were also observed by Sakevich and Klochenko (1998), who studied the variability of the free amino acids profile of cyanobacteria *Microcystis aeruginosa* and green microalgae *Scenedesmus acuminatus* during growth, and reported higher amino acid contents at the early growth exponential phase, which decreased at the stationary phase. In the present study, the free amino acid concentrations increased after 21 culture days probably due to the degradation of proteins in dead cells (Bidle 2016).

Free amino acids in cells grown in seawater enriched with a sub-lethal dose of Cu exhibited similar behavior than the control cells. Reduction of free amino acids was observed during the highest biomass accumulation growth phase, followed by an increase in the death phase after 21 days (Table 1), which could be due to proteolysis according to Sakevich and Klochenko (1998) and Martin-Jézéquel et al. (1992). However, the free amino acid levels at sub-lethal Cu

dose were much higher than those found in the control cells (Table 1). We hypothesize that this accumulation of free amino acids could be a self-defense mechanism to minimize Cu toxicity as has been previously reported (Djoko et al. 2017; Kovács et al. 2012; Lwalaba et al. 2020).

In contrast to the free amino acids behavior described above for control cells, a dramatic increase of free amino acids was observed in cells exposed to both lethal Cu levels (Table 1). These results agree with the findings of Jaishankar and Srivastava (2017), who reported that the entry of microorganisms to the stationary phase survival caused by the presence of toxicants or other stress factors ceases growth but cells remain metabolically active. These authors indicated that cells activate the mechanisms to adapt and survive at this stage, reprograming the gene expression pattern and diverting their resources to increase the amino acids production rather than cell division until environmental conditions improve, with an overall decrease in protein synthesis. This reduction of protein synthesis has been reported as a common consequence of stress, remaining the amino acids free and increasing their concentration in the intracellular medium (Kovács et al. 2012; Lwalaba et al. 2020; Sui et al. 2019). Accumulation of free amino acids was also reported by Afkar et al. (2010), who evaluated the effect of cobalt, copper, and zinc on the physiological response of green microalgae *Chlorella vulgaris*, where cells exposed to Cu below a concentration of 0.1 μM showed the most pronounced stimulation of the total free amino acid production.

Among the tested free amino acids here, the concentration of Met and His experienced the major increase at the lethal Cu doses (Table 1). These increases could be linked to their ability of binding metal ions to transport them across the cell membrane and to prevent metals entry into the cell (Narayanan and Natarajan 2018; Öhrvik and Thiele 2014). Combinations of Met/His or Met/Cys can facilitate Cu regulation under different scenarios of pH and redox environments (Rubino et al. 2011; Rubino and Franz 2012). Kováčik et al. (2010) also reported accumulation of amino acids Met, His, Arg, and Pro in microalgae *Scenedesmus quadricauda* exposed to 25 μM of Cu.

Cells exposed to Cu also showed higher concentrations of free Glu and Pro than those found in the control cells (Table 1). These increased levels of free Pro (osmolyte, free radical scavenger, and metal chelator) and its precursor Glu can also contribute to mitigate Cu stress (Çelekli et al. 2013; Tripathi and Gaur 2004). Pro detoxifies the ROS excess produced under stress, and its accumulation has been considered an important index for stress tolerance capacity in plants, bacteria, algae, and other organisms (Hamed et al. 2017; Sharma and Dietz 2006). Accumulation of intracellular Pro has also been detected in microalgae *Chlorella sorokiniana* and *Scenedesmus acuminatus* exposed to sub-lethal doses of Cu (Hamed et al. 2017), in cyanobacteria *Westiellopsis prolifica* under several heavy metal stresses (Fatma et al. 2007), and in plants treated with Cu and Co, among others (Lwalaba et al. 2020).

Polyphenols profile of cells, antioxidant activities, and MDA content

After 18 days of exposition to lethal Cu concentrations, the contents of all identified polyphenols increased (Table 2). These results are consistent with previous inhibition tests performed in our laboratory (Rico et al. 2013; Santana-Casiano et al. 2014), where *P. tricornutum* cells were exposed to 0.31 and 0.79 μM of Cu under the same conditions described above, but with an initial cell density twice higher (2×10^7 cells L^{-1}). These studies showed lower growth inhibition (20 and 47.5% respectively) than those found in the present study, lower increases of polyphenol contents with respect to the control cells (only 1.3 and 2.4 times higher, respectively) and enhanced radical scavenging activities as the Cu level increased. In the current study, the highest antioxidant activities were also observed in cells exposed to increasing doses of Cu, where overproduction of MDA, commonly caused by an increase of free radicals (Danouche et al. 2022), was also detected (Table 3). Therefore, these cells must produce relevant amounts of antioxidants (such as polyphenols) in order to minimize the Cu stress by neutralizing free radicals to prevent oxidation. In fact, the MDA production is linked to the increase of phenolic compounds through a linear correlation ($r=0.9999$; $p<0.05$). In the same sense, Hamed et al. (2017) also observed FRAP values significantly higher in microalgae *Chlorella sorokiniana* and *Scenedesmus acuminatus* exposed to sub-lethal doses of Cu (25 and 50 μM) compared to those found in the control cells.

Accordingly, we hypothesize that the presence of high levels of Cu in the culture seawater modifies the cellular metabolism to enhance the production of polyphenols to try to minimize the toxic effect of Cu (Rocha et al. 2016; Santana-Casiano et al. 2014; Yan and Pan 2002). Among the polyphenols detected here, GAL, the most active in inhibiting DPPH (Jerez-Martel et al. 2017), experienced the maximum increase in cells exposed to Cu (up to 45.8 times greater than that found in control cells).

The increases in the production of a selected group of amino acids and polyphenols could also be the diatom cellular strategy of defense, adaptation, and tolerance to metal toxicity through their joint action. Several amino acids have shown to enhance the antioxidant effect of phenolic compounds (Ran et al. 2020; Zhang et al. 2019). In fact, more accumulation of both metabolites in barley (*Hordeum vulgare*) produced higher tolerance to the combined stress of Co and Cu (Lwalaba et al. 2020). Accumulation of amino acids and organic acids has also been reported by Jain and Chen (2018) as a defense strategy of α-proteobacterium *Caulobacter crescentus* cells stressed

by Ni(II). The inhibitory effect of Ni(II) observed on the cell division rate was completely nullified by supplementation with combined amino acids and organic acids Pro, Ala, malic acid, and citric acid, with restoration of growth.

Conclusion

This study evidenced a great accumulation of amino acids and polyphenols in *P. tricornutum* cells exposed to Cu toxicity. This accumulation was strongly influenced by the Cu concentration and could be a cellular protective mechanism for toxicity, adaptation, and tolerance. The antioxidant capacity of cells was also enhanced with increasing doses of Cu in the culture seawater, where cells also showed higher levels of MDA, indicating that these diatoms produced relevant amounts of antioxidants in response to oxidative stress, which was corroborated by the correlation between MDA and polyphenols. Pro increase observed in this study is known to confer stress tolerance by detoxifying the excess level of ROS, and Met and His accumulation may be due to their significant role in chelating and transporting of metal ions and in regulating the biosynthesis of other metabolites involved in different defense strategies. The results of the current manuscript will help to understand how the marine diatoms respond to Cu toxicity by producing amino acids and polyphenols and will support potential candidates to study and understand their role in regulating heavy metals in seawater as components of the dissolved organic matter.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11356-023-25939-0.

Author contribution Santiago-Díaz: Amino acids and polyphenols analysis, validation, investigation. A. Rivero: Conceptualization, methodology, supervision. M. Rico: Conceptualization, methodology, supervision, FRAP, DPPH, and CUPRAC assays, and MDA contents. All the three authors performed the formal analysis and wrote the paper.

A. G. González: validation, investigation. M. González-Dávila: cell culture, conceptualization, methodology, resources. J. M. Santana-Casiano: cells culture, investigation, project administration, funding acquisition and provision of resources.

All authors critically revised the manuscript.

Funding Open Access funding provided thanks to the CRUE-CSIC agreement with Springer Nature. This study received financial support from the ATOPFe project (CTM2017-83476-P) by the Spanish Ministerio de Economía, Industria y Competitividad. The participation of Paula Santiago was funded through a PhD scholarship from the Universidad de Las Palmas de Gran Canaria (PIFULPGC-2019) to join the Ph.D. Program in Oceanography and Global Change (DOYCAG). This Ph.D. Program is promoted by the Institute of Oceanography and Global Change (IOCAG).

Data availability Data are available from the authors upon reasonable request. The analysis of variance (ANOVA) table for concentration of amino acids in *Phaeodactylum tricornutum* cells exposed to different Cu treatments during different times is available as supplementary data.

Declarations

Ethics approval We declare that all ethical guidelines for authors have been followed by all authors.

Consent to participate All authors have given their consent to participate in submitting this manuscript to this journal. This research does not involve human participants and/or animals.

Consent to publish All authors have given their consent to publish this paper in this journal.

Competing interests The authors declare no competing interests.

References

Afkar E, Ababna H, Fathi AA (2010) Toxicological response of the green alga Chlorella vulgaris, to some heavy metals. Am J Environ Sci 6:230–237. https://doi.org/10.3844/ajessp.2010.230.237

Apak R, Güçlü K, Özyürek M, Esin Karademir S, Erçağ E (2006) The cupric ion reducing antioxidant capacity and polyphenolic content of some herbal teas. Int J Food Sci Nutr 57:292–304

Balzano S, Sardo A, Blasio M, Chahine TB, Dell'Anno F, Sansone C, Brunet C (2020) Microalgal metallothioneins and phytochelatins and their potential use in bioremediation. Front Microbiol 11:1–16. https://doi.org/10.3389/fmicb.2020.00517

Benzie IFF, Strain JJ (1996) The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. Anal Biochem 239:70–76. https://doi.org/10.1006/abio.1996.0292

Bidle KD (2016) Programmed cell death in unicellular phytoplankton. Curr Biol 26:R594–R607. https://doi.org/10.1016/j.cub.2016.05.056

Blaby-Haas CE, Merchant SS (2012) The ins and outs of algal metal transport. Biochim Biophys Acta Mol Cell Res 1823:1531–1552. https://doi.org/10.1016/j.bbamcr.2012.04.010

Bondet V, Brand-Williams W, Berset C (1997) Kinetics and mechanisms of antioxidant activity using the DPPH• free radical method. LWT Food Sci Technol 30:609–615. https://doi.org/10.1006/fstl.1997.0240

Çelekli A, Kapi M, Bozkurt H (2013) Effect of cadmium on biomass, pigmentation, malondialdehyde, and proline of scenedesmus quadricauda var. longispina. Bull Environ Contam Toxicol 91:571–576. https://doi.org/10.1007/s00128-013-1100-x

Cid A, Herrero C, Torres E, Abalde J (1995) Copper toxicity on the marine microalga Phaeodactylum tricornutum: effects on photosynthesis related parameters. Aquat Toxicol 31:165–174. https://doi.org/10.1016/0166-445X(94)00071-W

Cobbett CS (2000) Phytochelatins and their roles in heavy metal detoxification. Plant Physiol 123:825–832. https://doi.org/10.1104/pp.123.3.825

Danouche M, El Ghatchouli N, Arroussi H (2022) Overview of the management of heavy metals toxicity by microalgae. J Appl Phycol 34:475–488. https://doi.org/10.1007/s10811-021-02668-w

Djoko KY, Phan M-D, Peters KM, Walker MJ, Schembri MA, McEwan AG (2017) Interplay between tolerance mechanisms to copper and acid stress in Escherichia coli. Proc Natl Acad Sci 114:6818–6823. https://doi.org/10.1073/pnas.1620232114

Dursun F, Tas S (2019) Variations in abundance and diversity of phytoplankton in the surface waters of the Golden Horn Estuary (Sea of Marmara). J Mar Biolog Assoc UK 99:279–290. https://doi.org/10.1017/S0025315418000073

Effendi H, Kawaroe M, Lestari DF, Mursalin PT (2016) Distribution of phytoplankton diversity and abundance in Mahakam Delta, East Kalimantan. Procedia Environ Sci 33:496–504. https://doi.org/10.1016/j.proenv.2016.03.102

Fatma T, Khan MA, Choudhary M (2007) Impact of environmental pollution on cyanobacterial proline content. J Appl Phycol 19:625–629. https://doi.org/10.1007/s10811-007-9195-2

Fehling J, Davidson K, Bolch CJS, Brand TD, Narayanaswamy BE (2012) The relationship between phytoplankton distribution and water column characteristics in North West European Shelf Sea Waters. PLoS ONE 7:1–16. https://doi.org/10.1371/journal.pone.0034098

González AG, Santana-Casiano JM, González-Dávila M, Pérez N (2012) Effect of organic exudates of Phaeodactylum tricornutum on the Fe (II) oxidation rate constant. Cienc Mar 38:245–261. https://doi.org/10.7773/cm.v38i1B.1808

Hall JL (2002) Cellular mechanisms for heavy metal detoxification and tolerance. J Exp Bot 53:1–11. https://doi.org/10.1093/jexbot/53.366.1

Hamed SM, Selim S, Klöck G, AbdElgawad H (2017) Sensitivity of two green microalgae to copper stress: Growth, oxidative and antioxidants analyses. Ecotoxicol Environ Saf 144:19–25. https://doi.org/10.1016/j.ecoenv.2017.05.048

Hodges DM, DeLong JM, Forney CF, Prange RK (1999) Improving the thiobarbituric acid-reactive-substances assay for estimating lipid peroxidation in plant tissues containing anthocyanin and other interfering compounds. Planta 207:604–611. https://doi.org/10.1007/s004250050524

Hwang H-S, Winkler-Moser JK, Liu SX (2019) Study on antioxidant activity of amino acids at frying temperatures and their interaction with rosemary extract, green tea extract, and ascorbic acid. J Food Sci 84:3614–3623. https://doi.org/10.1111/1750-3841.14963

Jain A, Chen WN (2018) Involvement of organic acids and amino acids in ameliorating Ni(II) toxicity induced cell cycle dysregulation in Caulobacter crescentus: a metabolomics analysis. Appl Microbiol and Biotechnol 102:4563–4575. https://doi.org/10.1007/s00253-018-8938-0

Jaishankar J, Srivastava P (2017) Molecular basis of stationary phase survival and applications. Front Microbiol 8:2000. https://doi.org/10.3389/fmicb.2017.02000

Jerez-Martel I, García-Poza S, Rodríguez-Martel G, Rico M, Afonso-Olivares C, Gómez-Pinchetti JL (2017) Phenolic profile and antioxidant activity of crude extracts from microalgae and cyanobacteria strains. J Food Qual 2017:2924508. https://doi.org/10.1155/2017/2924508

Kováčik J, Klejdus B, Hedbavny J, Bačkor M (2010) Effect of copper and salicylic acid on phenolic metabolites and free amino acids in Scenedesmus quadricauda (Chlorophyceae). Plant Sci 178:307–311. https://doi.org/10.1016/j.plantsci.2010.01.009

Kovács Z, Simon-Sarkadi L, Vashegyi I, Kocsy G (2012) Different accumulation of free amino acids during short- and long-term osmotic stress in Wheat. Sci World J 2012:216521. https://doi.org/10.1100/2012/216521

Leal PP, Hurd CL, Sander SG, Armstrong E, Fernández PA, Suhrhoff TJ, Roleda MY (2018) Copper pollution exacerbates the effects of ocean acidification and warming on kelp microscopic early life stages. Sci Rep 8:14763. https://doi.org/10.1038/s41598-018-32899-w

López A, Rico M, Santana-Casiano JM, González AG (2015) Phenolic profile of Dunaliella tertiolecta growing under high levels of copper and iron. Environ Sci Pollut Res 22:14820–14828

Lourenço SO, Barbarino E, Lavín PL, Lanfer Marquez UM, Aidar E (2004) Distribution of intracellular nitrogen in marine microalgae: calculation of new nitrogen-to-protein conversion factors. Eur J Phycol 39:17–32. https://doi.org/10.1080/0967026032000157156

Lwalaba JLW, Zvobgo G, Mwamba TM, Louis LT, Fu L, Kirika BA, Tshibangu AK, Adil MF, Sehar S, Mukobo RP, Zhang G (2020) High accumulation of phenolics and amino acids confers tolerance to the combined stress of cobalt and copper in barley (Hordeum vulagare). Plant Physiol Biochem 155:927–937. https://doi.org/10.1016/j.plaphy.2020.08.038

Markina ZV, Aizdaicher NA (2006) Content of photosynthetic pigments, growth, and cell size of microalga Phaeodactylum tricornutum in the copper-polluted environment. Russ J Plant Physiol 53:305–309. https://doi.org/10.1134/S1021443706030034

Marquis V, Smirnova E, Poirier L, Zumsteg J, Schweizer F, Reymond P, Heitz T (2020) Stress- and pathway-specific impacts of impaired jasmonoyl-isoleucine (JA-Ile) catabolism on defense signalling and biotic stress resistance. Plant Cell Environ 43:1558–1570. https://doi.org/10.1111/pce.13753

Martin-Jézéquel V, Sournia A, Birrien J-L (1992) A daily study of the diatom spring bloom at Roscoff (France) in 1985. III. Free amino acids composition studied by HPLC analysis. J Plankton Res 14:409–421. https://doi.org/10.1093/plankt/14.3.409

Morelli E, Mascherpa MC, Scarano GO (2005) Biosynthesis of phytochelatins and arsenic accumulation in the marine microalga Phaeodactylum tricornutum in response to arsenate exposure. Biometals 18:587–593. https://doi.org/10.1007/s10534-005-2998-1

Moreno-Garrido I, Lubián LM, Soares AMVM (2000) Influence of cellular density on determination of EC50 in microalgal growth inhibition tests. Ecotoxicol Environ Saf 47:112–116. https://doi.org/10.1006/eesa.2000.1953

Murphy JT, Bruinsma JJ, Schneider DL, Collier S, Guthrie J, Chinwalla A, Robertson JD, Mardis ER, Kornfeld K (2011) Histidine protects against zinc and nickel toxicity in Caenorhabditis elegans. PLoS Genet 7:e1002013. https://doi.org/10.1371/journal.pgen.1002013

Narayanan IG, Natarajan SK (2018) Peptides derived from histidine and methionine-rich regions of copper transporter 1 exhibit anti-angiogenic property by chelating extracellular Cu. Chem Biol Drug Des 91:797–804. https://doi.org/10.1111/cbdd.13145

Öhrvik H, Thiele DJ (2014) How copper traverses cellular membranes through the mammalian copper transporter 1, Ctr1. Ann NY Acad Sci 1314:32–41. https://doi.org/10.1111/nyas.12371

Osborn HL, Hook SE (2013) Using transcriptomic profiles in the diatom Phaeodactylum tricornutum to identify and prioritize stressors. Aquat Toxicol 138–139:12–25. https://doi.org/10.1016/j.aquatox.2013.04.002

Patrick L (2003) Toxic metals and antioxidants: Part II. The role of antioxidants in arsenic and cadmium toxicity. Altern Med Rev 8(2):106–128

Pérez-Cid B, Falqué E, Simal-Gandara J (2021) Coastline levels of dissolved heavy metals in the estuarine water–system of Vigo. Int J Environ Res Public Health 18:1–12. https://doi.org/10.3390/ijerph18042136

Ran L, Chi Y, Huang Y, He Q, Ren Y (2020) Synergistic antioxidant effect of glutathione and edible phenolic acids and improvement of the activity protection by coencapsulation into chitosan-coated liposomes. LWT 127:109409. https://doi.org/10.1016/j.lwt.2020.109409

Renzi M, Roselli L, Giovani A, Focardi SE, Basset A (2014) Early warning tools for ecotoxicity assessment based on Phaeodactylum tricornutum. Ecotoxicology 23:1055–1072. https://doi.org/10.1007/s10646-014-1249-z

Rico M, López A, Santana-Casiano JM, González AG, González-Dávila M (2013) Variability of the phenolic profile in the diatom Phaeodactylum tricornutum growing under copper and iron stress. Limnol Oceanogr 58:144–152. https://doi.org/10.4319/lo.2013.58.1.0144

Rocha GS, Parrish CC, Lombardi AT, Melão MGG (2016) Copper affects biochemical and physiological responses of Selenastrum gracile (Reinsch). Ecotoxicology 25:1468–1477. https://doi.org/10.1007/s10646-016-1698-7

Rubino JT, Franz KJ (2012) Coordination chemistry of copper proteins: how nature handles a toxic cargo for essential function. J Inorg Biochem 107:129–143. https://doi.org/10.1016/j.jinorgbio.2011.11.024

Rubino JT, Chenkin MP, Keller M, Riggs-Gelasco P, Franz KJ (2011) A comparison of methionine, histidine and cysteine in copper(i)-binding peptides reveals differences relevant to copper uptake by organisms in diverse environments. Metallomics 3:61–73. https://doi.org/10.1039/c0mt00044b

Sakevich AI, Klochenko PD (1998) Free amino acids in ecological metabolism of algae. Hydrobiol J 34:70–79

Santana-Casiano JM, González-Dávila M, González AG, Rico M, López A, Martel A (2014) Characterization of phenolic exudates from Phaeodactylum tricornutum and their effects on the chemistry of Fe(II)-Fe(III). Mar Chem 158:10–16. https://doi.org/10.1016/j.marchem.2013.11.001

Santiago-Díaz P, Rivero A, Rico M, Gómez-Pinchetti JL (2022) Characterization of novel selected microalgae for antioxidant activity and polyphenols, amino acids, and carbohydrates. Mar Drugs 20:40. https://doi.org/10.3390/md20010040

Seregin IV, Kozhevnikova AD (2021) Low-molecular-weight ligands in plants: role in metal homeostasis and hyperaccumulation. Photosyn Res 150:51–96. https://doi.org/10.1007/s11120-020-00768-1

Sharma SS, Dietz KJ (2006) The significance of amino acids and amino acid-derived molecules in plant responses and adaptation to heavy metal stress. J Exp Bot 57:711–726. https://doi.org/10.1093/jxb/erj073

Sharma A, Shahzad B, Rehman A, Bhardwaj R, Landi M, Zheng B (2019) Response of phenylpropanoid pathway and the role of polyphenols in plants under abiotic stress. Molecules 24(13):2452. https://doi.org/10.3390/molecules24132452

Shi Z, Li H, Li Z, Hu J, Zhang H (2013) Pre-column Derivatization RP-HPLC Determination of amino acids in Asparagi radix before and after heating process. IERI Procedia 5:351–356. https://doi.org/10.1016/j.ieri.2013.11.115

Shivaji S, Dronamaraju VL (2019) Scenedesnus rotundus isolated from the petroleum effluent employs alternate mechanisms of tolerance to elevated levels of cadmium and zinc. Sci Rep 9:8485. https://doi.org/10.1038/s41598-019-44374-1

Singh PK, Shrivastava AK (2016) Role of initial cell density of algal bioassay of toxic chemicals. J Basic Microbiol 56:812–819. https://doi.org/10.1002/jobm.201500597

Stiller A, Garrison K, Gurdyumov K, Kenner J, Yasmin F, Yates P, Song B-H (2021) From fighting critters to saving lives: polyphenols in plant defense and human health. Int J Mol Sci 22(16):8995. https://doi.org/10.3390/ijms22168995

Sui Y, Muys M, Vermeir P, D'Adamo S, Vlaeminck SE (2019) Light regime and growth phase affect the microalgal production of protein quantity and quality with Dunaliella salina. Bioresour Technol 275:145–152. https://doi.org/10.1016/j.biortech.2018.12.046

Torres E, Cid A, Fidalgo P, Abalde HC, J, (1997) Long-chain class III metallothioneins as a mechanism of cadmium tolerance in the marine diatom Phaeodactylum tricornutum Bohlin. Aquat Toxicol 39:231–246

Tripathi BN, Gaur JP (2004) Relationship between copper- and zinc-induced oxidative stress and proline accumulation in Scenedesmus sp. Planta 219:397–404. https://doi.org/10.1007/s00425-004-1237-2

Trombetta T, Vidussi F, Mas S, Parin D, Simier M, Mostajir B (2019) Water temperature drives phytoplankton blooms in coastal waters. PLoS ONE 14:1–28. https://doi.org/10.1371/journal.pone.0214933

Vendruscolo RG, Fagundes MB, Maroneze MM, do Nascimentode MenezesBarinZepkaJacob-LopesWagner TCCRJSLQLR (2019) Scenedesmus obliquus metabolomics: effect of photoperiods and cell growth phases. Bioprocess Biosyst Eng 42:727–739. https://doi.org/10.1007/s00449-019-02076-y

Wei Y, Zhu N, Lavoie M, Wang J, Qian H, Fu Z (2014) Copper toxicity to Phaeodactylum tricornutum: a survey of the sensitivity of various toxicity endpoints at the physiological, biochemical, molecular and structural levels. Biometals 27:527–537. https://doi.org/10.1007/s10534-014-9727-6

Yan H, Pan G (2002) Toxicity and bioaccumulation of copper in three green microalgal species. Chemosphere 49:471–476. https://doi.org/10.1016/S0045-6535(02)00285-0

Zhang B, Xia T, Duan W, Zhang Z, Li Y, Fang B, Xia M, Wang M (2019) Effects of organic acids, amino acids and phenolic compounds on antioxidant characteristic of Zhenjiang Aromatic Vinegar. Molecules 24:3799. https://doi.org/10.3390/molecules24203799

Zhang J-S, Wang Y-Q, Song J-N, Xu J-P, Yang H-B (2020a) Effect of aspartic acid on physiological characteristics and gene expression of salt exclusion in Tartary buckwheat under salt stress. J Plant Biochem Biotechnol 29:94–101. https://doi.org/10.1007/s13562-019-00518-y

Zhang Y, Zuo J, Salimova A, Li A, Li L, Li D (2020b) Phytoplankton distribution characteristics and its relationship with bacterioplankton in Dianchi Lake. Environ Sci Pollut Res 27:40592–40603. https://doi.org/10.1007/s11356-020-10033-6

Copper toxicity leads to accumulation of free amino acids and polyphenols in Phaeodactylum tricornutum diatoms

Citation Data: Environmental Science and Pollution Research, ISSN: 1614-7499, Vol: 30, Issue: 17, Page: 51261-51270
Publication Year: 2023

Scopus metrics

3 84th percentile
Citations in Scopus

1,13
Field-Weighted citation impact

PlumX metrics

Captures

6
Readers

Journal Citation Reports™

ENVIRONMENTAL SCIENCE AND POLLUTION RESEARCH

Publisher name: SPRINGER HEIDELBERG

Journal Impact Factor ™

2022	Five Year
5.8	5.4

JCR Category	Category Rank	Category Quartile
ENVIRONMENTAL SCIENCES *in SCIE edition*	67/275	Q1

4.1.2. Caracterización de compuestos orgánicos exudados por la diatomea *Phaeodactylum tricornutum* cultivada bajo condiciones de estrés por cobre

La acumulación de compuestos orgánicos en el interior de las células de microalgas constituye un mecanismo de defensa ante la toxicidad de metales pesados en el medio, sin embargo, no es el único recurso del fitoplancton para combatir esta toxicidad. La liberación extracelular de determinadas moléculas se presenta también como una respuesta para sobrellevar los efectos dañinos provocados por la presencia de estos metales.

Aunque es sabido que diversos compuestos orgánicos actúan como ligandos de los metales en el medio marino, el conocimiento sobre su naturaleza química es limitado. La caracterización de moléculas orgánicas en agua de mar no es sencilla, y requiere de técnicas y equipos especializados. Además, su baja concentración en el medio hace necesario el empleo de métodos de concentración previos al análisis.

Para poder identificar los compuestos orgánicos exudados, se lleva a cabo un cultivo de la diatomea marina *Phaeodactylum tricornutum* bajo diferentes concentraciones de Cu durante diferentes períodos de tiempo. Los exudados analizados en este trabajo pertenecen a los experimentos con células de *P. tricornutum* expuestos en el apartado anterior de la presente Tesis Doctoral. Por ello, el medio de cultivo consistió de la misma manera en agua de mar como control y agua de mar enriquecida a una concentración letal y dos subletales de Cu(II) (0,31, 0,79 y 1,31 μM respectivamente). Los exudados se analizaron después de 12 y 18 días del ciclo de crecimiento del alga, al inicio y al final de la fase estacionaria respectivamente. Durante esta fase se acumula una mayor cantidad de polifenoles y carbohidratos, además de obtener una mayor capacidad complejante de Cu, en comparación con la fase exponencial.

Los compuestos orgánicos que se determinaron en las aguas enriquecidas con exudados de la diatomea fueron polifenoles (mediante RP-HPLC-DAD) previa concentración mediante SPE (cartuchos ChromaBond Easy) y carbohidratos totales (por espectrofotometría UV-VIS). Además, se evaluó su capacidad antioxidante por medio de diferentes ensayos: RSA, FRAP, CUPRAC, y capacidad complejante de Cu(II).

El perfil de compuestos fenólicos extracelulares varía en función de las condiciones experimentales y del período de análisis, de la misma manera que en el interior de la célula. Sin embargo, la liberación de polifenoles no aumenta con la concentración de Cu, como ocurre intracelularmente. A los 12 días el contenido total de polifenoles disminuye en cultivos con concentraciones subletales, se mantiene a la concentración letal más baja y aumenta a la concentración más alta de Cu en comparación con el control. A los 18 días los polifenoles totales se mantienen a niveles subletales de Cu, mientras que aumenta bastante para las concentraciones letales, comparados con el control. Además, de los polifenoles identificados destaca la cantidad de ácido siríngico y de epicatequina, siendo mayor que los intracelulares tras 18 días de cultivo. Estas diferencias en la naturaleza y cantidad de los compuestos fenólicos acumulados y liberados al medio responden al mecanismo de defensa particular del alga ante la presencia de Cu.

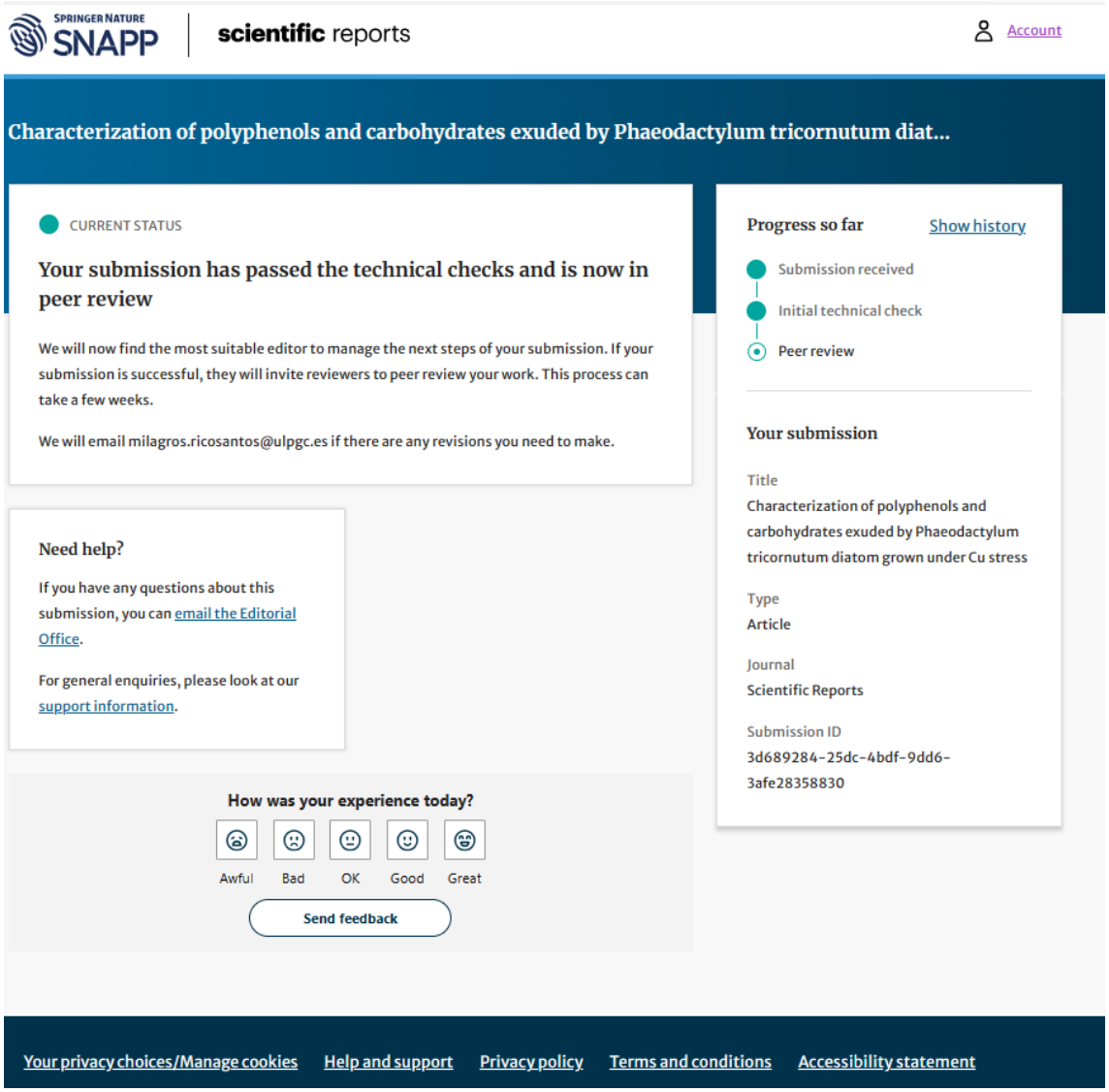
En los carbohidratos totales disueltos extracelulares se observó un aumento a medida que se incrementa el nivel de Cu en el medio, siendo mayor a los 18 días que a los 12 días para las concentraciones letales de Cu. Los carbohidratos son metabolitos primarios, al igual que los aminoácidos, esenciales en el crecimiento y desarrollo del alga. Su variación en el tiempo ante concentraciones letales de Cu en el medio es similar al encontrado en el interior de las células para los aminoácidos, es decir, la actividad celular primaria de la microalga disminuye para activar los mecanismos de defensa necesarios para sobrellevar las nuevas condiciones como puede ser la exudación de estos carbohidratos al medio.

La capacidad antioxidante de los exudados se analizó a los 18 días de cultivo. En todos los ensayos se obtuvo un aumento de la capacidad antioxidante a medida que aumentan los niveles de Cu en el medio, comparado con el cultivo sin Cu. Esto se cumplió en todos los experimentos, excepto en el FRAP y CUPRAC para las dosis subletales de Cu. La máxima actividad antioxidante se alcanzó a la mayor concentración de Cu tanto en la neutralización del radical DPPH, como en la reducción de Fe(III) a Fe(II), con 5,43 fmol de DPPH neutralizado y 5,34 fmol de Fe(II) reducido por el exudado de una célula, respectivamente. También destaca la capacidad complejante de Cu(II) de los exudados, 32 veces mayor que el control a niveles letales de Cu.

Este aumento de la capacidad antioxidante de los exudados podría ser parte del mecanismo de defensa de la diatomea ante la toxicidad del Cu, ya sea por la inhibición de los radicales libres producidos, o por la disminución de Cu(II) libre en el medio por complejación. De hecho, se encontró una correlación significativa entre los carbohidratos y los polifenoles identificados con la capacidad complejante de Cu.

Este estudio evidencia la liberación al medio de compuestos orgánicos como los polifenoles y los carbohidratos por parte de la diatomea marina *P. tricornutum,* que podría ser considerada como una estrategia extracelular de tolerancia, adaptación y reducción de los efectos tóxicos del Cu. Esta exudación de compuestos al medio marino puede cambiar la composición bioquímica del mismo afectando a las comunidades de fitoplancton y a la biodiversidad.

Este trabajo ha sido enviado a la revista *Scientific Reports* y se encuentra actualmente bajo revisión.


SPRINGER NATURE
SNAPP
scientific reports
Account
Characterization of polyphenols and carbohydrates exuded by Phaeodactylum tricornutum diat...
CURRENT STATUS
Your submission has passed the technical checks and is now in peer review
We will now find the most suitable editor to manage the next steps of your submission. If your submission is successful, they will invite reviewers to peer review your work. This process can take a few weeks.
We will email milagros.ricosantos@ulpgc.es if there are any revisions you need to make.
Need help?
If you have any questions about this submission, you can email the Editorial Office.
For general enquiries, please look at our support information.
How was your experience today?
Awful
Bad
OK
Good
Great
Send feedback
Progress so far
Show history
Submission received
Initial technical check
Peer review
Your submission
Title
Characterization of polyphenols and carbohydrates exuded by Phaeodactylum tricornutum diatom grown under Cu stress
Type
Article
Journal
Scientific Reports
Submission ID
3d689284-25dc-4bdf-9dd6-3afe28358830
Your privacy choices/Manage cookies
Help and support
Privacy policy
Terms and conditions
Accessibility statement

Characterization of polyphenols and carbohydrates exuded by *Phaeodactylum tricornutum* diatom grown under Cu stress

Milagros Rico[a,b*], Paula Santiago-Díaz[a,b], Argimiro Rivero [a,b], Magdalena Santana-Casiano [a,b]

[a]Departamento de Química, Universidad de Las Palmas de Gran Canaria, Campus de Tafira, 35017 Las Palmas de Gran Canaria, Spain.

[b]Instituto de Oceanografía y Cambio Global (IOCAG), Universidad de Las Palmas de Gran Canaria, Unidad Asociada ULPGC-CSIC, Las Palmas de Gran Canaria, Spain.

*Corresponding author at: Departamento de Química, Facultad de Ciencias del Mar, Universidad de Las Palmas de Gran Canaria, Campus de Tafira, 35017 Las Palmas de Gran Canaria, Canary Islands, Spain

Corresponding author: milagros.ricosantos@ulpgc.es (Milagros Rico)

Abstract

This study is focused on analysing polyphenols and carbohydrates released by *Phaeodactylum tricornutum* (*P. tricornutum*) diatoms cultured in natural seawater enriched with sublethal and lethal Cu doses. Cu concentrations of 0.31, 0.79 and 1.57 µM reduced cell densities by 37, 82 and 91%, respectively, compared to the control. The total sum of all identified polyphenols and total carbohydrates released by cells grown under lethal Cu levels increased up to 18.8 and 107.4 times, respectively, compared to data from a control experiment. Four different in vitro assays were used to estimate the antioxidant activities of the extracellular compounds: 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical inhibition, cupric ion reducing antioxidant capacity (CUPRAC), ferric reducing antioxidant power (FRAP) and Cu complexing ability (CCA). The highest antioxidant activities were observed in the Cu lethal treatments, where the CCA assay exhibited a

greater increase (up to 32.2 times higher than that found in the control experiment) to reduce the concentration of free Cu in the medium and its toxicity. The presence of Cu stimulated the release of polyphenols and carbohydrates to the medium as a detoxification mechanism to survive under lethal levels of Cu regulating its speciation.

1. Introduction

Marine microorganisms are known to release organic ligands that can regulate and modify the speciation of trace metals in the surrounding environment, affecting the bioavailability of these trace metals needed in a vast array of enzymatic reactions, and their potential toxicity [1,2,3]. Thus, chemical speciation may aid biosorption capacity and cellular uptake of metals in regions of low concentration and hinder toxicity in the presence of high concentration [4]. Relatively little is known about the chemical characteristics of extracellular organic compounds present in seawater due to their complex chemical structures and molecular sizes, and their low concentration [5,6]. These organic ligands bind more than 99.6% of the total iron and copper in natural waters [7] and include a wide range of organic compounds such as proteins, lipids, carbohydrates, uronic acids and polyphenols [8,9,10].

Copper is an essential nutrient that can become toxic at certain concentrations and exhibits affinity for a wide range of ligands containing sulfur, nitrogen and oxygen [11,12]. The level of this trace metal varies from 0.008 to 0.05 μM in natural environments and is increased by local anthropogenic activities or natural activities such as volcanic episodes up to 3.0 μM [13,14,15]. The presence of Cu in coastal seawater at high concentrations has a direct impact on marine microorganisms, causing serious damage, such as disruption of important proteins or alteration of the oxidative balance of cells, modifying the composition of the extracellular matter in the surrounding medium [16,17]. As a

consequence, Cu toxicity induces mortality in phytoplankton, modifies the start and end timing of blooms and their amplitude, changes the phytoplankton community structure and affects the survival of important species, contributing to biodiversity loss [18,19].

The role of extracellular organic matter exuded by marine phytoplankton exposed to toxic levels of Cu is not entirely clear. Previous research reported defence strategies of *Phaeodactylum tricornutum* (*P. tricornutum*) diatom that consist of exuding various types of metabolites [20]. Determining how the concentrations of extracellular substances secreted by phytoplankton change in response to elevated levels of Cu could provide a better understanding of their role with respect to regulatory mechanisms for metals that are potentially toxic, and could be useful in helping to explain the metal bioavailability.

The aim of this work was to evaluate the content of polyphenols and carbohydrates released by the diatom *P. tricornutum* grown for 12 and 18 days in natural seawater as a control, and in seawater enriched with two lethal and one sublethal copper concentrations (1.57, 0.79 and 0.31µM, respectively). The effects of Cu toxicity on the growth and intracellular productivity of these diatoms have been previously reported [21]. The present study is focused on the characterization of their exudates. Therefore, the total extracellular carbohydrates were evaluated by using the phenol-sulfuric acid reaction [22], and 10 selected phenolic compounds were identified and quantified by reversed-phase high-performance liquid chromatography (RP-HPLC) due to their Cu chelating ability [23,24,25]. In addition, the antioxidant activities of the organic compounds isolated by solid phase extraction (SPE) from seawater samples enriched with exudates were determined through four different tests: free radical scavenging ability (RSA) against DPPH radical; FRAP, CUPRAC and CCA assays [26].

2. Results

2.1. Cell growth and productivity under Cu pressure

High concentrations of metals such as copper induce stress in phytoplankton that changes their growth patterns as well as their interaction with the environment. Fig. 1 summarizes the effects of Cu toxicity on *P. tricornutum* diatom growth, previously reported by Santiago-Díaz et al. [21] In brief, a reduction in both the growth curve and the biomass generated was observed. The mean absolute growth rate was $2.11x10^7$ cell L^{-1} day^{-1} achieving a maximum cell density of $33.6x10^7$ cell L^{-1} during the stationary phase, which ended after 16 days. A Cu concentration of 0.31 μM was a sublethal dose for the microalga, with a biomass reduction of 37% compared to the control. A lack of an exponential phase of growth and cell density decreases of 82 and 91% were observed at the highest Cu concentrations (0.79 and 1.57 μM, respectively), indicating that these Cu levels were lethal for *P. tricornutum* under our experimental conditions. A more detailed description is given by Santiago-Díaz et al. [21].

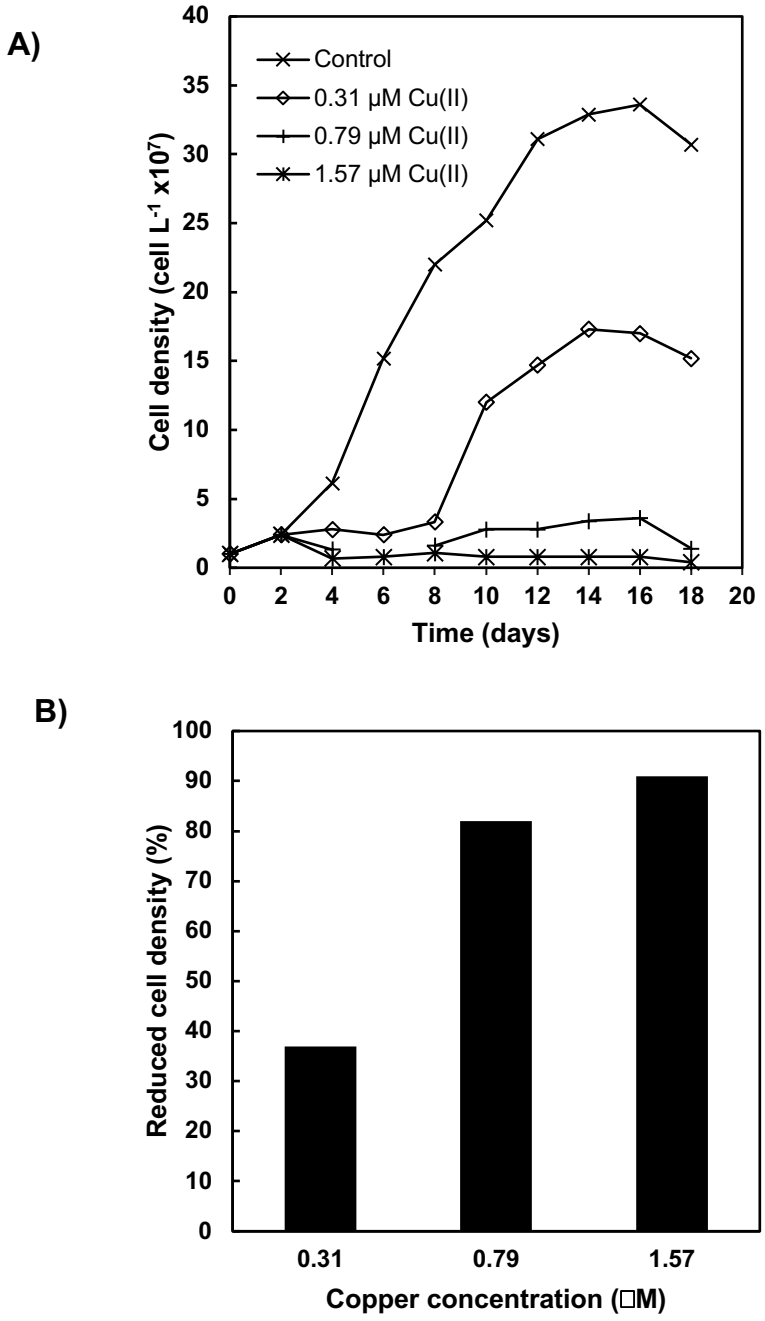


Fig 1. Cell density of *P. tricornutum* grown under different Cu(II) concentrations: A) Cell growth curves; B) Decrease in cell density.

Santiago-Díaz et al. [21] reported the accumulation of intracellular free amino acids and polyphenols after 18 days of exposure to lethal levels of Cu (Table 1). Under these conditions, the total sum of all polyphenols identified in the cells increased up to 11.3

times compared to the control (Fig. 2), and the highest antioxidant activities were also observed in cells exposed to lethal Cu treatments. The production of malondialdehyde (MDA), commonly caused by an increase in free radicals [27], evidenced enhanced oxidative stress in cells cultured under lethal Cu levels by extending its concentration up to 14.5-fold, and presented a linear correlation ($r=0.9999$; $p \square 0.05$) with the production of phenolic compounds (Fig. 2).

Table 1. Productivity data of *P. tricornutum* cells exposed to different Cu concentrations (extracted from Santiago-Díaz et al. [21])

	Control	Cu(II) 0.31µM	Cu(II) 0.79 µM	Cu(II) 1.57 µM
Total sum of the identified intracellular amino acids[a]				
After 12 days	10.11 ± 0.62	23.71 ± 0.89	121.40 ± 3.13	209.40 ± 9.45
After 18 days	7.83 ± 0.24	16.6 ± 0.3	171.8 ± 5.7	145.0 ± 6.1
Total sum of identified intracellular phenolics described in section 2.1[b]				
After 18 days	63.9±15.5	148.4±38.7	357.2±23.4	723.2 ± 55.0
Intracellular MDA contents[b]				
After 18 days	4.5 ± 0.4	10.25 ± 0.07	23.7 ± 0.1	65.8 ± 0

[a] Results expressed as femtomol of amino acid $cell^{-1}$

[b] Results expressed as attomol $cell^{-1}$

All results are expressed as means ± standard deviations of three measurements

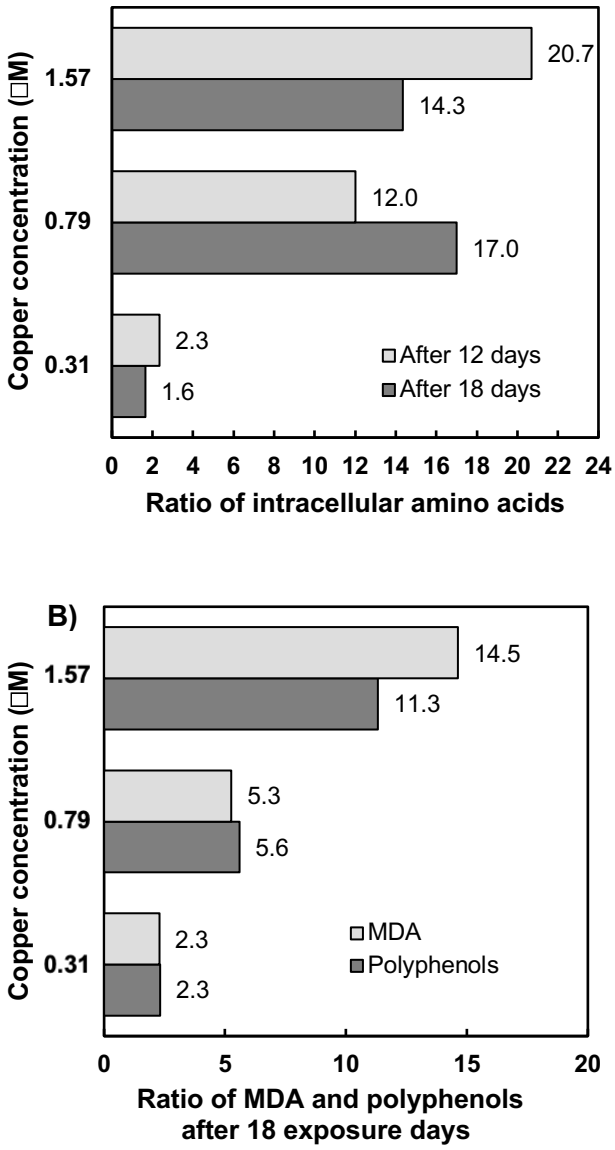


Fig. 2. Ratio of intracellular amino acids (A), polyphenols (B) and MDA (B) in *P. tricornutum* diatoms exposed to Cu compared to those in control cells (data extracted from Santiago-Díaz et al., 2023).

2.2 Characterization of phenolic compounds in exudates

The results of the identification and quantification of 10 different phenolics in *P. tricornutum* exudates are summarized in Table 2. After 12 days, the total amount of all phenolic compounds identified was lower than that of control cells at the sublethal dose of Cu, was similar at the lowest lethal dose, and was higher under the highest Cu level.

After 18 days, the total phenolic content remained similar at the sublethal Cu dose and was 6.7- and 18.8- fold higher at the lethal Cu levels compared with the control experiment (Fig. 3). However, longer culture periods reduced exuded phenolics from 71.0 (after 12 days) to 30.84 (after 18 days) in the control experiment and increased them under lethal Cu concentrations.

Table 2. Phenolic compounds exuded by the diatom *P. tricornutum* grown under different copper concentrations and exposure times

Phenolic compound	Control	Cu(II) 0.31 µM	Cu(II) 0.79 µM	Cu(II) 1.57 µM
After Cu exposure periods of 12 days				
GAL	4.57 ± 0.74	< LOQ	< LOQ	< LOQ
PCA	10.7 ± 2.8	<LOQ	2.33 ± 0.01	< LOQ
SYR	30.9 ± 1.9	1.92 ± 0.98	41.27 ± 0.79	107.32 ± 2.20
ECAT	20.9 ± 0.46	3.60 ± 0.40	19.77 ± 6.04	23.41 ± 7.85
RU	3.9 ± 0.7	22.30 ± 4.39	<LOQ	< LOQ
Sum	71.0 ± 6.6	27.81 ± 5.77	63.37 ± 6.84	130.72 ± 10.04
After Cu exposure periods of 18 days				
PCA	<LOQ	<LOQ	<LOQ	15.07 ± 1.86
CAT	<LOQ	2.43 ± 0.41	<LOQ	<LOQ
VAN	1.31 ± 0.08	<LOQ	<LOQ	<LOQ
SYR	19.22 ± 0.39	15.81 ± 0.31	101.33 ± 3.43	335.08 ± 0.06
ECAT	10.12 ± 0.21	7.85 ± 0.99	106.77 ± 32.31	228.16 ± 11.74
RU	0.19 ± 0.01	1.53 ± 0.40	<LOQ	<LOQ
Sum	30.84 ± 0.69	27.62 ± 2.11	208.09 ± 35.75	578.31 ± 13.66

The results are expressed as attomol $cell^{-1}$ (means ± standard deviations of three measurements)

SYR and ECAT were found in all the experiments at maximal concentrations compared to the other phenolic compounds tested. SYR content was 3.5 and 17.4 times higher than

that of the control (after 12 and 18 days, respectively). GA, COU and FA were below the limit of quantification (LOQ) in all diatom exudates.

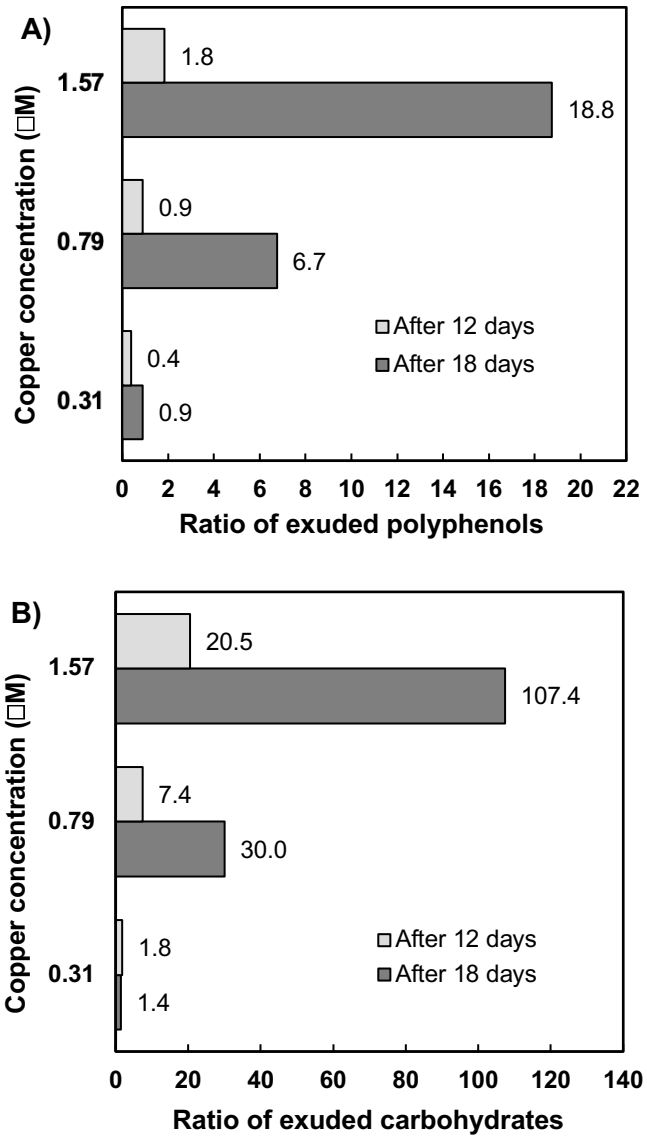


Fig. 3. Ratio of exuded polyphenols (A) and carbohydrates (B) by *P. tricornutum* diatoms exposed to Cu for 12 and 18 days compared to those released by control cells.

2.3 Total carbohydrates

Table 3 summarizes the carbohydrates released by diatoms after a culture period of 12 and 18 days. Compared with control cells, extracellular carbohydrates levels increased with the addition of Cu to the medium after both culture periods, being up to 20.5- and 107.4-fold higher in cells grown under lethal levels of Cu after 12 and 18 days,

respectively (Fig. 3). However, cells exuded more carbohydrates after 12 days than after 18 days in the control experiment. The same behavior was observed in the Cu sublethal enrichment assay, whereas cells exposed to lethal levels of Cu showed a higher content of extracellular carbohydrates after 18 days of culture periods than those quantified after 12 days.

Table 3. Total carbohydrates exuded by *P. tricornutum* diatom grown in seawater enriched with different copper concentrations and incubated for 12 and 18 days.

Time (days)	Control	Cu(II) 0.31 µM	Cu(II) 0.79 µM	Cu(II) 1.57 µM
12	0.57 ± 0.04	1.03 ± 0.02	4.2 ± 0.2	11.7 ± 0.6
18	0.34 ± 0.06	0.47 ± 0.03	10.2 ± 1.0	36.5 ± 2

The results are expressed as pmol Glc eq $cell^{-1}$ (means ± standard deviations of three measurements).

2.4 Antioxidant capacity assays

Table 4 shows the antioxidant activities of *P. tricornutum* exudates analysed by DPPH, FRAP, CUPRAC and CCA assays after Cu exposure for 18 days. An enhanced antioxidant capacity was found with increasing Cu concentration compared to reference cultures, except for sublethal levels of Cu in FRAP and CUPRAC tests, where a slight decrease was observed. Under lethal Cu doses, compounds from diatom exudate showed higher DPPH radical inhibition ability than those exuded by control cells (up to 5.43-fold higher), higher Fe(III) to Fe(II) reduction capacity (up to 5-fold higher), higher Cu(II) to Cu(I) reduction capacity (up to 3-fold higher than the control) and much higher Cu-complexation capacity (up to 32.2 times higher).

Table 4. Antioxidant activities of *P. tricornutum* exudates cultured under different copper concentrations and collected after 18 days.

Antioxidant assay (units)	Control	Cu(II) 0.31 µM	Cu(II) 0.79 µM	Cu(II) 1.57 µM
DPPH (fmol DPPH cell^{-1})	1.00 ± 0.02	1.12 ± 0.02	4.04 ± 0.00	5.43 ± 0.00
FRAP (fmol Fe(II) cell^{-1})	1.64 ± 0.05	1.53 ± 0.09	4.24 ± 0.03	5.34 ± 0.13
CUPRAC (fmol TR cell^{-1})	1.35 ± 0.01	1.12 ± 0.01	3.78 ± 0.03	3.28 ± 0.08
CCA (fmol Cu cell^{-1})	0.234 ± 0.006	1.646 ± 0.004	3.228 ± 0.004	7.53 ± 0.02

The results are expressed as fmol cell^{-1} (means ± standard deviations of three measurements).

Significantly positive correlations ($p < 0.05$) were observed between the CCA values and the total polyphenol content (R=0.9820) and total carbohydrate content (R=0.9806).

3. Discussion

This study focuses on analyzing polyphenols and total carbohydrates produced by *P. tricornutum* cells exposed to sublethal and lethal doses of Cu to identify organic compounds involved in the extracellular mechanisms of Cu detoxification. The entry of microorganisms into the stationary phase activates the mechanisms to adapt and survive at this stage, reprogramming the gene expression pattern until environmental conditions improve. Therefore, organic metabolites were quantified at the beginning and at the end of this growth phase and compared with those secreted by cells grown in the absence of added Cu [21]. In addition, Rahman et al. reported that *P. tricornutum* showed a higher total phenolic content in the stationary stage than in the exponential growth phase [28], and a higher carbohydrates accumulation during this growth stage was also exhibited by diatoms and dinoflagellate species exposed to different Cu treatments (from 0.16 to 0.79

□M) [29]. Furthermore, Lombardi et al. found strong copper complexing agents released by freshwater Cyanophyta mainly in the stationary growth phase [30].

P. tricornutum diatoms exposed to 1.57 μM Cu for 18 days exuded total levels of all identified polyphenols up to 18.8-fold higher than those exuded by control cells (Table 2), and reached 11.3-fold higher amounts inside the cell (Table 1 and Fig. 2) [21]. The increased production of polyphenols, well known antioxidants, could be explained by their ability to interrupt radical chain reactions and thus prevent or limit cell damage [25,31]. Our results agree with those reported by López et al. [10], who found that marine microalgae *Dunaliella tertiolecta* grown in the presence of high levels of Cu exuded most of the polyphenols into the medium to alleviate Cu toxicity. Li et al. [1] studied Cu and Zn accumulation and detoxification strategies in the freshwater green microalga *Chlamydomonas reinhardtii* (*C. reinhardtii*) , showing that extracellular matter played a major role in Cu sorption and resistance, which was not observed in the Zn tolerance strategy, where extracellular substances played a minor role.

Relevant differences were found between intra- and extracellular phenolic profiles as a function of metal level and culture period, i.e., GAL was below the LOQ in exudates (Table 2), while it was the most abundant phenolic compound in cells, reaching an amount up to 45.8 times higher than in control cells [21]. Moreover, the phenolic amounts released in the Cu sublethal dose experiment were lower than those exuded by control cells, while the content inside the cells in the same experiment increased. Diatoms have multiple mechanisms to fight against metal toxicity inside the cell, such as gene regulation, chelation, transport into compartments, vacuoles or other organelles, causing heavy metal immobilization, etc. [32,33,34,35], but only exuded and cell wall-associated compounds seem to participate in detoxification mechanisms externally [1]. Therefore,

the differences observed in phenolic profiles appear to be the cellular response to different requirements for survival inside and outside of Cu-exposed diatoms.

We observed that the amount of extracellular carbohydrates exuded by *P. tricornutum* cells was also strongly affected by the Cu concentration in the medium after 12 and 18 days of culture, increasing their production upon addition of Cu. However, prolonged exposure periods decreased extracellular carbohydrates in control and sublethal Cu level experiments and increased them under lethal conditions (Table 3). These results are consistent with those previously reported by Santiago-Díaz et al. for amino acids [21], primary metabolites essential in the growth and development functions of microorganisms [36], which decreased during the growth phase of highest biomass accumulation in control cells and under sublethal Cu concentrations and increased under lethal conditions. Under the latter conditions, cells survive by decreasing various cellular activities, such as growth, and focusing on defense mechanisms [37,38].

Our study showed that carbohydrates exuded by cells increased up to 30- and 107.4-fold in the medium enriched with lethal Cu concentrations after 18 days compared to the control (Table 3). This increased carbohydrate exudation may reduce Cu ion activities in the surrounding medium and may facilitate Cu detoxification mechanisms by changing its speciation, improving the tolerance of *P. tricornutum* to Cu stress [36,39,40,41]. In addition, carbohydrates have also been reported to be involved in other metal detoxification mechanisms such as direct quenching of ROS [42] and biotransformation of Cu(II) into less soluble and less toxic CuS [43].

Our results agree with those of Tonietto et al., who found that carbohydrates were the main components of the exudates of the cyanobateria *Cylindrospermopsis raciborskii* and were strongly correlated with the ligand concentration for Cu (R=0.997) [44], concluding

that a high diversity of ligands increases the metal buffering capacity of the medium and thus, enhances the tolerance of the biota to metal toxicity. Li et al. also reported increases in extracellular polysaccharides exuded by *C. reinhardtii* under Cu toxicity conditions [1], with polysaccharides being the main substances bound to Cu on the cell surface*, and enhanced* sorption of *C. reinhardtii* cells with increasing Cu concentration. In addition, a significant correlation of extracellular polysaccharides, proteins and DNA with Cu accumulation was found. Li et al. suggested increased production and secretion of extracellular substances to form a biofilm to improve Cu removal and tolerance of *C. reinhardtii* [1]. In extreme environments, several microorganisms have been found to survive by forming a network of biofilms mainly composed of exopolysaccharides (EPS) with functional groups such as uronic acids, sulfated units, and phosphates. These EPSs are often polyanionic, and act as ligands towards toxic metals increasing cell tolerance [23,45,46]. The potent antioxidant activity of several polysaccharides has been related to the uronic acid content [47,48,49], which has been quantified in EPS produced by *P. tricornutum* diatoms in the range of 1.4–6.3%, depending on the extraction conditions [50].

The radical scavenging ability of the exuded compounds against DPPH and their FRAP, CUPRAC and CCA capacities increased under lethal Cu concentrations (Table 4). Under these conditions, the increased production of free radicals, corroborated by the higher levels of intracellular MDA detected (Table 1), must be equalized by a similar rate of antioxidant production to neutralize them or repair the damage they cause. Furthermore, the ability of the exuded compounds to complex Cu(II) showed the greatest increase (up to 32.2-fold higher than that found in the control experiment). We hypothesize that this increased copper complexing activity of the exudates could be the cellular response to the excess of free Cu(II) outside the cells, decreasing its concentration. In fact, significant

positive correlations (p□0.05) were found between the exuded carbohydrates and the total content of the identified polyphenols with CCA values, indicating that both types of metabolites could have an important role in reducing the level of free Cu(II). González-Dávila et al. studied the interaction between exudates released by the marine phytoplankton species *Dunaliella tertiolecta* and copper ions [4], including adsorption to the cell surface, concluding that exudates are involved in reducing the concentration of free Cu.

No correlation was found between carbohydrate and polyphenol contents and DPPH, CUPRAC and FRAP activities, probably due to the presence of other antioxidants, and the dependency of the antioxidant activities on the assay mechanism/kinetics, the profile of the antioxidants, their structures and mix ratios, and the joint action of the compounds, with either synergistic or additive effects [26,51]. Rahman et al. reported that carotenoids and phenolics were the major contributors to the antioxidant capacity of *P. tricornutm* cells [28], which correlated to phenolics in the exponential phase and to carotenoids fucoxanthin and β-carotene in the stationary phase. In any case, a more pronounced antioxidant content/activity (radical inhibition, metal reduction or complexation) associated with higher heavy metal concentrations in the environment has been reported as mechanism providing tolerance to metal ions [33].

The important role of exudates in Cu accumulation and removal has recently been demonstrated by Li et al. [1], who found that elimination of these extracellular substances from cultures of *C. reinhardtii* exposed to Cu and Zn intensified metal toxicity and decreased the removal of these two metals, the effect being more pronounced for Cu. In fact, the EC50 (metal concentration (mg/L) required to reduce the cell growth rate by 50%) decreased 28.6 ± 3.4 and 19.1 ± 2.6% for Cu and Zn, respectively, compared to cells with intact exudates. Cu adsorption and its maximum accumulation capacities by *C.*

reinhardtii exposed to Cu decreased by 60.0 ± 0.1% and 68.5 ± 0.1%, respectively, when extracellular substances were removed.

Our results are in agreement with previous studies reporting increased contents of intra- and extracellular polyphenol content in *P. tricornutum* diatoms exposed to sublethal doses of Cu (0.31 and 0.79 □M with growth inhibitions of 20 and 47.5%, respectively) [20], suggesting the accumulation of these compounds as a protective action of cells to decline metal toxicity. In addition, accumulation of amino acids and carbohydrates has also been reported as a mechanism of tolerance to elevated levels of heavy metals [45,52,53]. Therefore, we hypothesize that these increases observed in the present study could be a self-defense mechanism to try to minimize the toxic effect of Cu, as well as an adaptation and tolerance strategy of cells [10,37].

Cu speciation plays an important role in regulating phytoplankton community structure affecting ocean primary production [17]. Vasconcelos et al. [19] investigated the biological behaviour of the coccolithophore *Emiliania huxleyi* (*E. huxleyi*) grown in seawater enriched with its own exudates and those of *P. tricornutum*, *Porphyra* spp. and *Enteromorpha* spp. and found that *P. tricornutum exudates induced growth inhibition and stimulated the* highest cellular release of Cu-complexing organic ligands of all media (160±5 nM followed by 127±4 nM for *Enteromorpha* spp.; 114±3 and 92±4 nM for *E. huxleyi* and *Porphyra* spp. respectively). They concluded that *P. tricornutum* exudates caused a toxic effect on *E. huxleyi* microalgae. The effects of Cu toxicity described here for similar cell densities as those found during phytoplankton blooms ($1x10^7$ cells L^{-1}) [54,55] could be intensified at lower abundances commonly observed in coastal environmental contexts [56], where Cu could also be enhanced over the concentrations tested here. Under these conditions, Cu availability per cell remains higher, and phytoplankton could release compounds that inhibit microalgal cell division, modifying

microalgal community composition and affecting physiological processes of coastal marine organisms, which could change biodiversity [19].

Analysis of *P. tricornutum* diatoms grown under Cu stress evidenced a large accumulation and exudation of phenolic and carbohydrate compounds strongly affected by the Cu level in the culture seawater, indicating that these compounds are involved in detoxification mechanisms in the extracellular medium. The antioxidant activities of the exudates corroborated that these diatoms produced relevant amounts of antioxidants in response to metal stress, where the Cu-complexing compounds showed the highest increase. This study helps to partially understand the response of marine diatoms to Cu toxicity through the production of carbohydrates and polyphenols, which may play an important role in regulating the speciation of contaminating trace metals. In addition, the evidenced change in the composition of exudates in the presence of toxicants could cause a toxic effect on coastal phytoplankton communities changing their structure and contributing to the loss of biodiversity.

4. Materials and methods

4.1 Chemicals

Methanol (HPLC gradient grade), ethanol and tetrachloroethylene (synthesis grade) were purchased from Scharlab (Barcelona, Spain), D-glucose (Glc), m-hydroxyphenyl, $CuSO_4 \cdot 7H_2O$, DPPH, 2,4,6-tri(2-pyridyl)-triazine (TPTZ), neocuproine (of reagent grade), pyrocatechol violet (PV) and Trolox (TR) were supplied by Sigma-Aldrich (St. Louis, MO, USA). Formic acid (synthesis grade), $Fe_3Cl \cdot 6H_2O$ and $FeSO_4 \cdot 7H_2O$ were supplied by Panreac (Barcelona, Spain). Ultrapure water was obtained from a Milli-Q system from Millipore (Bedford, MA, USA).

Polyphenol standards were supplied as follows: gallic acid (GAL), protocatechuic acid (PCA), p-coumaric acid (COU), ferulic acid (FA), catechin (CAT), vanillic acid (VAN), epicatechin (ECAT), syringic acid (SYR) and by Sigma–Aldrich Chemie (Steinheim, Germany); rutin (RU) and gentisic acid (GA) by Merck (Darmstadt, Germany).

4.2 Cultures

Axenic strains of *P. tricornutum* (REC 001B) were provided by the Spanish Bank of Algae (Taliarte, Spain). Microalgae were cultured according to Santiago-Díaz et al. in a clean culture chamber (Friocell FC111) under a complete photoperiod (24 h at 8000 lx)[21], a temperature of 24ºC and an initial cell density of $1x10^7$ cells L^{-1}.

Seawater used for cultures was sampled off the coast of Gran Canaria, treated with ultraviolet radiation and passed through 0.45 µm filters. The experimental cultures were carried out with seawater enriched with NO_3^- (883 µM), HPO_4^{2-} (29.3 µM), and SiO_3^{2-} (142 µM) [57]. For Cu exposure treatments, seawater was enriched with 0.31 µM, 0.79 µM and 1.57 µM Cu (II). Controls were prepared as described above but without Cu addition. After 12 and 18 days of growth, cells were filtered by gravity to avoid rupture using 1.2 µm filters (trace metal acid clean pore-size nitrocellulose, Sarthorius™). The cell density was calculated daily with a light microscope (Microbiotest, Inc.) equipped with a hemocytometer counter, and spectrophotometrically (USB4000) by measuring the absorbance (Abs) at 670 nm. The effect of Cu toxicity on cell growth, and amino acid and phenolic contents was studied and previously reported [21]. Seawater enriched with exudates produced by these diatoms was also analysed, and the results are summarized in the present study.

4.3 Phenolic compounds identification and quantification

For isolation and concentration of phenolic compounds in cultures of *P. tricornutum,* 700 mL of each sample was passed through SPE cartridges (Macherey-Nagel Chromabond Easy, 500 mg) at a flow rate of 2 mL min^{-1}. The retained analytes were eluted with 15 mL of methanol and evaporated to dryness. The residue was resolved in 300 µL of methanol, and the solution was filtered through a 0.22 µm filter to be injected into the HPLC equipment.

The determination of the phenolic compounds by RP-HPLC was carried out with Jasco LC-4000 HPLC equipment provided with a PU-4180 quaternary pump, an AS-4150 autosampler, an MD-4015 photodiode array detector, LC-Net ll interface, a Phenomenex C18 column (250 mm x 4.6 mm, 5 µm) and a Phenomenex guard column maintained at 30ºC. The elution was performed with ultrapure water containing 0.1% formic acid (phase A) and methanol (phase B). The flow rate was 1 mL min^{-1}, and the injection volume was 10 µL. The gradient elution method for A was as follows: 0 min, 75%; 30 min, 40%; 40 min, 40%. Finally, the column was washed and reconditioned. Simultaneous monitoring was set at 270 nm (GAL, PCA, CAT, VAN, RU, ECAT, and SYR) and 324 nm (GA, COU, and FA) for quantification [21]. Algae samples were analysed in triplicate and the results were expressed as attomol (amol) $cell^{-1}$.

4.4 Total carbohydrates

The total sugar content in *P. tricornutum* cell cultures was determined following the phenol-sulfuric acid method [22] with some modifications. One milliliter of seawater was freeze-dried, and the residue was dissolved in 340 □L of distilled water. The resulting solution (100 □L) was mixed with 100 □L of phenol (5%) and 0.9 mL of cold concentrated sulfuric acid (98%), stirred and heated in a water bath at 100ºC for 10 min and cooled in an ice-water bath. The absorbance was measured at 490 nm in a UV-VIS

spectrophotometer. The total carbohydrate content was determined from a calibration curve prepared using Glc as a standard (y=0.0049x + 0.0742; R^2=0.9906), and expressed as picomole (pmol) of Glc equivalent $cell^{-1}$.

4.5 Antioxidant capacity assays

The antioxidant activities of the compounds exuded by cells were evaluated by pre-concentration of 780 mL of seawater enriched with *P. tricornutum* exudates collected after 18 days of culture following the same SPE procedure described in section 2.3. The analytes were resolved with 300 µL of methanol, and the antioxidant activity assays were performed according to Sethi et al. with modifications [26].

The radical scavenging activity was evaluated by reaction of 30 µL of sample with 800 µL of DPPH solution (0.044 mM) for 15 min. The absorbance was measured at 515 nm, and the results were obtained from a calibration curve prepared with different concentrations of DPPH (y=11.987x-0.1352; R^2=0.9996) and expressed as femtomol (fmol) of inhibited DPPH per cell.

The ferric reducing capacity was determined by mixing for 10 min at 37ºC, 10 µL of samples and 1 mL of freshly prepared FRAP-reagent consisting of 100 mL of 0.3 M acetate buffer solution (pH 3.6) with 10 mL of TPTZ (10 mM) in HCl (40 mM), and 10 mL of $FeCl_3 \cdot 6H_2O$ solution (20 mM). The mixture was cooled and the absorbance was read at 593 nm. The results obtained from a standard curve prepared with solutions of $FeSO_4 \cdot 7H_2O$ in distilled water (y=0.5378x+0.2275; R^2=0.9969) are expressed as fmol of reduced Fe(III) per cell.

The cupric ion reducing capacity assay was carried out with fresh CUPRAC reagent prepared by mixing equal volumes of $CuSO_4 \cdot 7H_2O$ (10 mM), neocuproine ethanolic

solution (7.5 mM) and NH_4Ac buffer solution (1 M). This reagent (1 mL) was mixed with 20 µL of sample for 30 min. Then, the absorbance was recorded at 450 nm, and the results from a calibration curve prepared with Trolox (TR) ($y=0.2859x + 0.0762$; $R^2=0.9998$) are expressed as fmol of TR per cell.

The Cu(II)-chelating activity was evaluated with PV according to Sánchez-Vioque et al. with modifications [58]. Samples (50 µL) were mixed with 700 µL of sodium acetate buffer (50 mM, pH 6.0) and 20 µL of $CuSO_4$ (5 mM) by stirring for 30 min at room temperature. After that, 50 µL of PV (4 mM) was added and the mixture was stirred for 30 min. The absorbance was measured at 632 nm. The results are expressed as fmol of complexed Cu^{2+} per cell.

To measure the statistical relationship between the content of polyphenols and carbohydrates and antioxidant activities, the Pearson correlation test was used. Tests were accepted as statistically significant with p values < 0.05.

References

1. Li, C. *et al.* A comparative study of the accumulation and detoxification of copper and zinc in *Chlamydomonas reinhardtii*: The role of extracellular polymeric substances. *Sci. Total Environ.* **871,** 161995 (2023). https://doi.org/10.1016/j.scitotenv.2023.161995.
2. Santana-Casiano, J. M. *et al.* Characterization of phenolic exudates from *Phaeodactylum tricornutum* and their effects on the chemistry of Fe(II)-Fe(III). *Mar. Chem.* **158**, 10–16 (2014). https://doi.org/10.1016/j.marchem.2013.11.001
3. Vraspir, J. M. & Butler, A. Chemistry of marine ligands and siderophores. *Ann. Rev. Mar. Sci.* **1**, 43–63 (2009). https://doi.org/10.1146/annurev.marine.010908.163712

4. González-Dávila, M., Santana-Casiano, J. M., Pérez-Pena, J. & Millero, F. J. Binding of Cu(II) to the surface and exudates of the alga *Dunaliella tertiolecta* in seawater *Environ. Sci. Technol.* **29**, 289–301 (1995).

5. Lv, J., Zhang, S., Luo, L. & Cao, D. Solid-phase extraction-stepwise elution (SPE-SE) procedure for isolation of dissolved organic matter prior to ESI-FT-ICR-MS analysis. *Anal. Chim. Acta* **948**, 55–61 (2016). https://doi.org/https://doi.org/10.1016/j.aca.2016.10.038

6. Kaiser, K. & Benner, R. Biochemical composition and size distribution of organic matter at the Pacific and Atlantic time-series stations. *Mar. Chem.* **113**, 63–77 (2009). https://doi.org/10.1016/j.marchem.2008.12.004

7. Strmečki, S., Plavšić, M., Steigenberger, S. & Passow, U. Characterization of phytoplankton exudates and carbohydrates in relation to their complexation of copper, cadmium and iron. *Mar. Ecol. Prog. Ser.* **408**, 33–46 (2010).

8. Chen, X. *et al.* The nature of dissolved organic matter determines the biosorption capacity of Cu by algae. *Chemosphere* **252**, 126465 (2020). https://doi.org/10.1016/j.chemosphere.2020.126465

9. Hansell, D. A. & Orellana, M. V. Dissolved Organic Matter in the Global Ocean: A Primer. *Gels* **7**, 128 (2021). https://doi.org/10.3390/gels7030128

10. López, A., Rico, M., Santana-Casiano, J. M., González, A. G. & González-Dávila, M. Phenolic profile of *Dunaliella tertiolecta* growing under high levels of copper and iron. *Environ. Sci. Pollut. Res.* **22**, 14820–14828 (2015). https://doi.org/10.1007/s11356-015-4717-y

11. Duffus, J. H. "Heavy metals" – a meaningless term? (IUPAC technical report). *Pure App. Chem.* **74**, 793–807 (2002). https://doi.org/10.1351/pac200274050793

12. Whitby, H., Posacka, A. M., Maldonado, M. T. & van den Berg, C. M. G. Copper-binding ligands in the NE Pacific. *Mar. Chem.* **204**, 36-48 (2018). https://doi.org/10.1016/j.marchem.2018.05.008

13. Leal, P. P. *et al*. Copper pollution exacerbates the effects of ocean acidification and warming on kelp microscopic early life stages. *Sci. Rep.* **8**, 14763 (2018). https://doi.org/10.1038/s41598-018-32899-w

14. Li, J. *et al.* Copper mobilization via seawater-volcanic rock interactions: New experimental constraints for the formation of the iron oxide Cu-Au (IOCG) mineralization. *Geochim. Cosmochim. Acta* **330**, 209–229 (2022). https://doi.org/10.1016/j.gca.2022.06.004

15. Pérez-Cid, B., Falqué, E. & Simal-Gandara, J. Coastline levels of dissolved heavy metals in the estuarine water–system of Vigo. *Int. J. Environ. Res. Public. Health* **18**, 1–12 (2021). https://doi.org/10.3390/ijerph18042136

16. Eriksen, R. S., Mackey, D. J., van Dam, R. & Nowak B. Copper speciation and toxicity in Macquarie Harbour, Tasmania: an investigation using a copper ion selective electrode. *Mar. Chem.* **74**, 99–113 (2001). https://doi.org/10.1016/S0304-4203(00)00117-1

17. Kong, L. Copper Requirement and Acquisition by Marine Microalgae. *Microorganisms* **10**, 1853 (2022). https://doi.org/10.3390/microorganisms10091853

18. Trombetta, T. *et al*. Water temperature drives phytoplankton blooms in coastal waters. *PLoS One* **14**, 1–28 (2019). https://doi.org/10.1371/journal.pone.0214933

19. Vasconcelos, M. T. S. D., Leal, M. F. C. & van den Berg, C. M. G. Influence of the nature of the exudates released by different marine algae on the growth, trace metal uptake and exudation of *Emiliania huxleyi* in natural seawater. *Mar. Chem.* **77,** 187–210 (2002). https://doi.org/10.1016/S0304-4203(01)00087-1

20. Rico, M., López, A., Santana-Casiano, J. M., González, A. G. & González-Dávila, M. Variability of the phenolic profile in the diatom *Phaeodactylum tricornutum* growing under copper and iron stress. *Limnol. Oceanogr.* **58**, 144–152 (2013). https://doi.org/10.4319/lo.2013.58.1.0144

21. Santiago-Díaz, P. *et al*. Copper toxicity leads to accumulation of free amino acids and polyphenols in *Phaeodactylum tricornutum* diatoms. *Environ. Sci. Pollut. Res.* **30**, 51261–51270 (2023). https://doi.org/10.1007/s11356-023-25939-0

22. Dubois, K. M., Gilles, K. A., Hamilton, G. J. K., Rebers, P. A. & Smith, F. Colorimetric method for determination of sugars and related substances. *Analyt. Chem*. **28**, 350–356 (1956). https://doi.org/10.1021/ac60111a017

23. Banerjee, A. *et al.* Extremophilic Exopolysaccharides: Biotechnologies and Wastewater Remediation. Extremophilic Exopolysaccharides: Biotechnologies and Wastewater Remediation. *Front. Microbiol.* **12**, (2021). https://doi.org/10.3389/fmicb.2021.721365

24. Norkus, E. *et al.* Environmentally friendly natural polyhydroxylic compounds in electroless copper plating baths: application of xylitol, D-mannitol and D-sorbitol as copper(II) ligands. *J. Appl. Electrochem.* **35**, 41–47 (2005). https://doi.org/10.1007/s10800-004-2056-7

25. Quideau, S., Deffieux, D., Douat-Casassus, C. & Pouységu, L. Plant polyphenols: chemical properties, biological activities, and synthesis. *Angew. Chem. Int. Ed. Engl.* **50**, 586–621 (2011). https://doi.org/10.1002/anie.201000044

26. Sethi, S. *et al.* Significance of FRAP, DPPH, and CUPRAC assays for antioxidant activity determination in apple fruit extracts. *Eur. Food Res. Technol.* **246**, 591–598 (2020). https://doi.org/10.1007/s00217-020-03432-z

27. Danouche, M., El Ghatchouli, N. & Arroussi, H. Overview of the management of heavy metals toxicity by microalgae. *J. Appl. Phycol.* **34**, 475–488 (2022). https://doi.org/10.1007/s10811-021-02668-w

28. Rahman, N. A. *et al.* Taxon- and Growth Phase-Specific Antioxidant Production by Chlorophyte, Bacillariophyte, and Haptophyte Strains Isolated From Tropical Waters. *Front. Bioeng. Biotechnol.* **8**, 581628 (2020). https://doi.org/10.3389/fbioe.2020.581628

29. Pistocchi, R., Mormile, A. M., Guerrini, F., Isani, G. & Boni, L. Increased production of extra- and intracellular metal-ligands in phytoplankton exposed to copper and cadmium. *J. Applied Phycol.* **12**, 469–477 (2000). https://doi.org/10.1080/09670269710001737049

30. Lombardi, A. T. & Vieira, A. A. H. Copper complexation by Cyanophyta and Chlorophyta exudates. *Phycologia* **39**, 118–125, (2000). https://doi.org/10.2216/i0031-8884-39-2-118.1

31. Bešlo, D. *et al.* Antioxidant Activity, Metabolism, and Bioavailability of Polyphenols in the Diet of Animals. *Antioxidants* **12**, 1141 (2023). https://doi.org/10.3390/antiox12061141

32. Nikookar, K., Moradshahi, A. & Hosseini, L. Physiological responses of *Dunaliella salina* and *Dunaliella tertiolecta* to copper toxicity. *Biomol. Eng.* **22**, 141–146 (2005). doi:10.1016/j.bioeng.2005.07.001

33. Nowicka, B. Heavy metal–induced stress in eukaryotic algae—mechanisms of heavy metal toxicity and tolerance with particular emphasis on oxidative stress in exposed cells and the role of antioxidant response. *Environ. Sci. Pollut. Res.* **29**, 16860–16911 (2022). https://doi.org/10.1007/s11356-021-18419-w

34. Morelli, E., Mascherpa, M. C. & Scarano, G. Biosynthesis of phytochelatins and arsenic accumulation in the marine microalga *Phaeodactylum tricornutum* in response to arsenate exposure. *BioMetals* **18**, 587–593 (2005). https://doi.org/10.1007/s10534-005-2998-1

35. Torres, E., Cid, A., Fidalgo, P., Herrero, C. & Abalde, J. Long-chain class III metallothioneins as a mechanism of cadmium tolerance in the marine diatom *Phaeodactylum tricornutum* Bohlin. *Aquatic Toxicol.* **39**, 231–246 (1997). https://doi.org/10.1016/S0166-445X(97)00034-9

36. Salam, U. *et al.* Plant Metabolomics: An Overview of the Role of Primary and Secondary Metabolites against Different Environmental Stress Factors. *Life* **13**, 706 (2023). https://doi.org/ 10.3390/life13030706

37. Bernard, E. & Guécuen, C. Molecular changes in phenolic compounds in *Euglena gracilis* cells grown under metal stress. *Front. Plant Sci.* **14**, 1099375 (2023). https://doi.org/10.3389/fpls.2023.1099375

38. Jaishankar, J. & Srivastava, P. Molecular Basis of Stationary Phase Survival and Applications. *Front. Microbiol.* **8**, 2000 (2017). https://doi.org/10.3389/fmicb.2017.02000

39. Khademian, E., Salehi, E., Sanaeepur, H., Galiano, F. & Figoli, A. A systematic review on carbohydrate biopolymers for adsorptive remediation of copper ions from aqueous environments-part A: Classification and modification strategies. *Sci. Total Environ.* **738**, 139829 (2020). https://doi.org/10.1016/j.scitotenv.2020.139829

40. Liu, S., Ni, L., Ma, F. & Fu, H. Analysis of intracellular copper ions content and distribution in algae by gold nanorods method. *Chemosphere* **259**, 127512 (2020). https://doi.org/10.1016/j.chemosphere.2020.127512

41. Lombardi, A. T., Hidalgo, T. M. R. & Vieira, A. A. H. Copper complexing properties of dissolved organic materials exuded by the freshwater microalgae *Scenedesmus acuminatus* (Chlorophyceae). *Chemosphere* **60**, 453–459 (2005). https://doi.org/10.1016/j.chemosphere.2004.12.071

42. Keunen, E., Peshev, D., Vangronsveld, J., Van Den Ende, W. & Cuypers, A. Plant sugars are crucial players in the oxidative challenge during abiotic stress: Extending the traditional concept. *Plant Cell Environ.* **36**, 1242–1255 (2013). https://doi.org/10.1111/pce.12061

43. Mathivanan, K. *et al.* Production and functionality of exopolysaccharides in bacteria exposed to a toxic metal environment. *Ecotoxicol. Environ. Saf.* 111567 (2021). https://doi.org/10.1016/j.ecoenv.2020.111567

44. Tonietto, A. E., Lombardi, A. E., Vieira, A. A. H., Parrish, C. C. & Choueri, R. B. *Cylindrospermopsis raciborskii* (Cyanobacteria) exudates: Chemical characterization and complexation capacity for Cu, Zn, Cd and Pb. *Water Res.* **49**, 381–390 (2014). https://doi.org/10.1016/j.watres.2013.10.025

45. Kazy, S. K., Sar, P., Singh, S. P., Sen, A. K. & D'Souza, S. F. Extracellular polysaccharides of a copper-sensitive and a copper-resistant *Pseudomonas aeruginosa* strain: synthesis, chemical nature and copper binding. *World J. Microb. Biotechnol.* **18**, 583–588 (2002). https://doi.org/10.1023/A:1016354713289

46. Sasaki, M., Takagi, A., Sasaki, D., Nakamura, A. & Asayama, M. Characteristics and function of an extracellular polysaccharide from a green alga *Parachlorella*. *Carbohydr. Polymers* **254**, 117252 (2021). https://doi.org/10.1016/j.carbpol.2020.117252

47. Chen, H., Zhang, M. & Xie, B. Quantification of uronic acids in tea polysaccharide conjugates and their antioxidant properties. *J. Agric. Food Chem.* **52**, 3333–3336 (2004). https://doi.org/10.1021/jf0349679

48. Yang, M., *et al*. Marine-derived uronic acid-containing polysaccharides: Structures, sources, production, and nutritional functions. *Trends Food Sci. Technol.* **122**, 1–12, (2022). https://doi.org/10.1016/j.tifs.2022.02.013

49. Zhou, J., Hu, N., Wu, Y. L., Pan, Y. J. & Sun, C. R. Preliminary studies on the chemical characterization and antioxidant properties of acidic polysaccharides from *Sargassum fusiforme*. *J. Zhejiang Univ. Sci. B.* **9**, 721–727 (2008). https://doi.org/10.1631/jzus.B0820025

50. Guzmán, S., Gato, A., Lamela, M., Freire-Garabal, M. & Calleja, J. M. Anti-inflammatory and immunomodulatory activities of polysaccharide from *Chlorella stigmatophora* and *Phaeodactylum tricornutum*. *Phytother. Res.* **17**, 665–670 (2003). https://doi.org/10.1002/ptr.1227

51. Šamec, D., Karalija, E., Šola, I., Vujčić-Bok, V. & Salopek-Sond, B. The role of polyphenols in abiotic stress response: the influence of molecular structure. *Plants* **10**, 118 (2021). https://doi.org/10.3390/plants10010118

52. Jiang, Z. *et al.* Contributions of polysaccharides to arsenate resistance in *Chlamydomonas reinhardtii. Ecotoxicol. Environ. Saf.* **229**, 113091 (2022). https://doi.org/10.1016/j.ecoenv.2021.113091

53. Lwalaba, J. L. W. *et al*. High accumulation of phenolics and amino acids confers tolerance to the combined stress of cobalt and copper in barley (*Hordeum vulagare*). *Plant Physiol. Biochem.* **155**, 927–937 (2020). https://doi.org/10.1016/j.plaphy.2020.08.038

54. Dursun, F. & Tas, S. Variations in abundance and diversity of phytoplankton in the surface waters of the Golden Horn Estuary (Sea of Marmara). *J. Mar. Biol. Assoc. U. K.* **99**, 279–290 (2019). https://doi.org/10.1017/S0025315418000073

55. Zhang, Y. *et al.* Phytoplankton distribution characteristics and its relationship with bacterioplankton in Dianchi Lake. *Environ. Sci. Pollut. Res.* **27**, 40592–40603 (2020). https://doi.org/10.1007/s11356-020-10033-6

56. Singh, P. K. & Shrivastava, A. K. Role of initial cell density of algal bioassay of toxic chemicals. *J. Basic Microbiol.* **56**, 812–819 (2016). https://doi.org/https://doi.org/10.1002/jobm.201500597

57. González, A. G., Santana-Casiano, J. M., González-Dávila, M. & Pérez, N. Effect of organic exudates of *Phaeodactylum tricornutum* on the Fe (II) oxidation rate constant. *Cienc. Mar.* **38**, 245–261 (2012). https://doi.org/10.7773/cm.v38i1B.1808

58. Sánchez-Vioque, R. *et al.* Polyphenol composition and antioxidant and metal chelating activities of the solid residues from the essential oil industry. *Ind. Crops Prod.* **49**, 150-159 (2013). http://dx.doi.org/10.1016/j.indcrop.2013.04.053

4.2. Variación del contenido en polifenoles y carbohidratos del alga *Emiliania huxleyi* cultivada bajo diferentes condiciones de acidificación oceánica simulada

Las microalgas son esenciales en los ecosistemas marinos, ya que suponen la base de la cadena alimentaria y son responsables de casi la mitad de la producción primaria global. Entre estos organismos, los cocolitofóridos como el alga *Emiliania huxleyi* caracterizados por poseer una pared celular compuesta por carbonato de calcio, juegan un papel importante en ciclo biogeoquímico del carbono y son particularmente sensibles a la acidificación oceánica.

Modificaciones en el pH del medio influyen en el crecimiento de las microalgas, así como en su composición bioquímica y en los compuestos que liberan al medio. Estos cambios afectan no sólo a la estructura, composición y distribución del fitoplancton en los océanos, sino también a niveles superiores de la cadena trófica.

Para estudiar cómo afecta la acidificación oceánica a la composición bioquímica intracelular y extracelular del alga *E. huxleyi,* se han llevado a cabo cultivos de esta microalga a diferentes pH: 7,75, 7,9, 8,1 y 8,25 alcanzados mediante el burbujeo de CO_2 en el medio de cultivo. Estos pH representan 4 escenarios de acidificación diferentes: el de la época pre-industrial (8,25); el pH actual de los océanos (8,1); y dos posibles condiciones de acidificación oceánica futuras (7,9 y 7,75).

Los polifenoles y carbohidratos se han determinado en células del alga y en el agua de mar de cultivo enriquecida con sus exudados. La cuantificación de carbohidratos totales se realizó por espectrofotometría UV-VIS y la caracterización de polifenoles por RP-HPLC-DAD previa concentración a través de SPE Además, se evaluó la capacidad antioxidante mediante dos ensayos diferentes: DPPH y FRAP.

El alga *E. huxleyi* no mostró diferencias significativas en su densidad celular al crecer a diferentes condiciones de pH, posiblemente debido a su capacidad de adaptación. A pH intermedios (7,9 y 8,1) se alcanzó un ligero aumento en el número de células. Por otro lado, sí se observaron variaciones en los perfiles de compuestos orgánicos intracelulares acumulados y exudados por este microorganismo.

La cantidad de polifenoles acumulados en el interior de la célula fue máxima en el menor pH, es decir, a 7,75. Este comportamiento se ha podido observar previamente

en fitoplancton y en plantas terrestres, ya que el aumento del CO_2 en el medio induce la producción de ROS pudiendo aumentar la acumulación de antioxidantes como los polifenoles como mecanismo de defensa.

No se observó una correlación significativa entre los polifenoles intracelulares y extracelulares. En los pH más extremos 7,75 y 8,25 la cantidad de polifenoles extracelulares fue mayor que en los pH intermedios. Estas diferencias en el perfil fenólico dentro y fuera de las células pueden deberse a los diversos mecanismos para contrarrestar la acidificación del pH en el interior (buffers, intercambiadores de Na^+-H^+, cambios metabólicos, entre otros) y en el exterior, limitados a la permeabilidad de la membrana y al material exudado.

El contenido de carbohidratos totales intracelulares se mantuvo prácticamente constante en el rango de pH de 8,25 a 7,9, con un ligero descenso a pH 7,75, mientras que la cantidad de carbohidratos extracelulares no experimentó ningún cambio a medida que disminuía el pH hasta 7,75, no habiendo correlación significativa entre ellos. Estos resultados coinciden con los observados en macroalgas como *Ulva rigida* y *Ulva fasciata* cultivadas en condiciones de acidificación del medio. Por otro lado, en otras especies de diatomeas los carbohidratos tanto intracelulares como extracelulares varían según las condiciones de pH del medio de cultivo.

Los extractos de las células cultivadas en el medio más acidificado (pH 7,75) mostraron la mayor capacidad de inhibir radicales libres y de reducir Fe(III) a Fe(II), siendo también las que producen mayor cantidad de polifenoles intracelulares. A medida que disminuye el pH, la capacidad para neutralizar radicales libres de los extractos celulares aumenta, así como los polifenoles intracelulares acumulados. Su producción puede estar relacionada con la capacidad de los polifenoles para inhibir las ROS generados por la acidificación del medio. Por otro lado, a pH 7,75 se observa una correlación significativa entre la capacidad de reducción de Fe(III) a Fe(II) con los polifenoles producidos por *E. huxleyi*. Esto puede explicar por qué la liberación de polifenoles exudados a pH más bajos disminuye ya que su poder reductor no es necesario por el aumento de la persistencia de metales esenciales como el Fe(II) a menor pH, aumentando su concentración y poder reductor dentro de la célula para compensar el estrés oxidativo.

En los exudados, la capacidad antioxidante aumenta al disminuir el pH (hasta 7,9), disminuyendo de nuevo en las condiciones de mayor acidificación (pH 7,75). Esta tendencia no coincide con la observada en los polifenoles liberados al medio por *E. huxleyi*, mayor en los pH intermedios y menor en los extremos.

La actividad antioxidante dependerá de los compuestos que se acumulen o se liberen al medio y de las interacciones entre estos. En este estudio solo se han determinado un grupo de polifenoles y carbohidratos, excluyendo otros compuestos que puedan contribuir al efecto antioxidante del alga bajo las condiciones de acidificación.

Los resultados de este trabajo han permitido comprobar el efecto de la acidificación oceánica en la composición bioquímica del alga *E. huxleyi.* Las variaciones en compuestos como carbohidratos y polifenoles podrían modificar la estructura y composición de sus comunidades y, por tanto, afectar al resto del fitoplancton y como consecuencia al ecosistema marino.

Este trabajo ha sido enviado a la revista *Biogeoscience*.

Variations of polyphenols and carbohydrates of *Emiliania huxleyi* grown under simulated ocean acidification conditions

M. Rico*[1,2], P. Santiago-Díaz[1,2], G. Samperio-Ramos[3], M. González-Dávila[1,2], J. M. Santana-Casiano[1,2]

[1]Departamento de Química, Universidad de Las Palmas de Gran Canaria, Campus de Tafira, 35017 Las Palmas de Gran Canaria, Spain

[2]Instituto de Oceanografía y Cambio Global (IOCAG), Universidad de Las Palmas de Gran Canaria, Unidad Asociada ULPGC-CSIC, Las Palmas de Gran Canaria, Spain

[3]Instituto de Investigaciones Oceanológicas. Universidad Autónoma de Baja California. Carretera Ensenada-Tijuana nº 3917, Fracc. Playitas, Ensenada Baja California. C.P. 22860.

*Corresponding author at: Departamento de Química, Facultad de Ciencias del Mar, Universidad de Las Palmas de Gran Canaria, Campus de Tafira, 35017 Las Palmas de Gran Canaria, Canary Islands, Spain

Corresponding author: milagros.ricosantos@ulpgc.es

Abstract

Global environmental changes strongly affect the growth and biochemical composition of microalgae. Cultures of the coccolithophore *Emiliania huxleyi* were grown under four different CO_2-controlled pH conditions (7.75, 7.90, 8.10, and 8.25) to improve understanding of the adaptive mechanisms of these organisms through changes in phenolic compounds and carbohydrate content and composition under ocean acidification (OA) scenarios. The highest algal biomass peaks, 1.07 (± 0.10) and 1.04 (± 0.06) × 10^8 cells L^{-1}, were observed in the microcosms with intermediate CO_2 levels (pH 8.10 and 7.90 respectively). Intra- and extracellular phenolic compounds were identified and quantified by Reverse Phase-High Performance Liquid Chromatography (RP-HPLC). The highest concentrations of total exuded phenolics were found in cultures with lower cell densities, at pH 8.25 (43±3 nM) and 7.75 (18.0±0.9 nM). Accumulation of intracellular phenolic compounds was observed in cells with decreasing pH, reaching the maximum level (9.24±0.19 attomol $cell^{-1}$) at the lowest pH (7.75). The total carbohydrate content inside the cells increased with decreasing pH from 8.25 to 8.10, remaining constant at pH 7.90, and decreasing at lower pH. The presence of antioxidants was determined by 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical inhibition and ferric-reducing antioxidant power (FRAP) assays. The highest activity in both tests was exhibited by cells grown at pH 7.75.

Keywords

Emiliania huxley, phenolic compounds, carbohydrates, antioxidants, ocean acidification, RP-HPLC.

Introduction

Microalgae play a key role in marine ecosystems, forming the basis of the marine food chain as they are responsible for almost half of the total primary production (Usher et al., 2014; Dedman et al., 2023). They constitute a potential feedstock because of their valuable metabolites such as pigments, vitamins, proteins, carbohydrates, and lipids with valued fatty acids. Marine phytoplankton, primarily diatoms and coccolithophorids groups, drive the oceanic carbon cycle by sequestering inorganic carbon from the atmosphere during photosynthesis (Marinov et al., 2010). Coccolithophores are the most abundant calcifying phytoplankton and form gigantic blooms throughout the oceans, especially in mid-latitudes. The coccolithophore *Emiliania huxleyi* is the main contributor to calcareous sea sediments, making it particularly sensitive to ocean acidification and playing a crucial ecological role (Arundhathy et al., 2021; Westbroek, 1992). When exposed to elevated CO_2 and low pH, it reduces its growth rate and level of calcification, resulting in thinner coccosphaeres (Kholssi et al., 2023; Mackey et al., 2015; Meier et al., 2014).

Global environmental changes, in particular those related to increasing temperature and decreasing pH, profoundly affect ocean ecosystems at many levels, as these are the two main variables controlling all chemical and biological cycles, with a major impact on the growth and metabolic functions of microalgae (Berge et al., 2010; Dedman et al., 2023; Kholssi et al., 2023; Lu et al., 2013). The absorption of anthropogenic CO_2 into seawater lowers its pH with adverse consequences for marine ecosystems and human societies (Gruber et al., 2023; Jiang et al., 2023; Lida et al., 2021). In fact, pre-industrial seawater pH (8.25) has already dropped to 8.10 and is expected to reach a pH of 7.85 in this century (Jacobson, 2005). For instance, pH homeostasis, which regulates the pH inside and outside the cell, is critical for the growth and metabolism of most microorganisms,

including microalgae (Barakat et al., 2021; Guan and Liu, 2020; Lund et al., 2020). Different algal species show different optimal pH ranges for maximum growth (Hoppe et al. 2011; Kholssi et al., 2023). Changes in environmental pH could have consequences on the competitiveness of both sensitive and tolerant microalgae in mixed phytoplankton communities, modifying their structure, composition, and distribution, which are crucial in mitigating global environmental change by fixing and transporting carbon from the upper to the deep ocean in the major global carbon sink (Eltanahy and Torky, 2021; Kholssi et al., 2023, Marinov et al., 2010). Such changes could also affect the species at a higher trophic level, resulting in a potential shift in biodiversity (Jin and Kirk, 2018; Vasconcelos et al., 2002). Spisla et al. (2021) reported that extreme CO_2 events modify the composition of particulate organic matter, which leads to a substantial reorganization of the planktonic community, affecting multiple trophic levels from phytoplankton to primary and secondary consumers (Trombetta et al., 2019). Nelson et al. (2020) found modifications of planktonic and benthic communities in response to reduced seawater pH (from pH 8.1 to 7.8 and 7.4), concluding that a re-arrangement of the biofilm microbial communities occurred through a potential shift from autotrophic to heterotrophic dominated biofilms. In addition, microbial biofilms obtained under reduced pH altered settlement rates in invertebrate larvae of *Galeolaria hystrix*. Barcelos e Ramos et al. 2022 showed that coexistence with other microorganisms modifies the response of *E. huxleyi* to increased CO_2, markedly decreasing its growth rate at elevated CO_2 concentrations with bacteria *Idiomarina abyssalis* (I. abyssalis) and *Brachybacterium* sp. Moreover, elevated CO_2 concentrations increased organic carbon and decreased inorganic carbon content of *E. huxleyi* cells in the presence of *I. abyssalis*, but not *Brachybacterium* sp.

Changes in phytoplankton communities due to variation in seawater acidity alter the composition of the organic ligands that these communities release into the surrounding environment (Samperio-Ramos et al., 2017). These ligands are crucial in the formation of metal complexes to acquire micronutrients, sequester toxic metals, and to establish electrochemical gradients resulting in changes in the speciation, the bioavailability, and the toxicity of trace metals (Harmesa et al., 2022; Santana-Casiano et al., 2014). Iron is an essential micronutrient for phytoplankton involved in fundamental cellular processes, including respiration, photosynthesis, nitrogen uptake, and nitrogen fixation (Raven et al., 1999; Hogle et al., 2014). It controls the productivity, species composition, and trophic structure of microbial communities over large regions of the ocean (González et al., 2019; Hunter and Boyd, 2007). Iron concentrations in ocean waters are very low due to its low solubility and effective removal from the ocean surface by phytoplankton (Liu and Millero, 2002). Complexation with organic compounds is one of the mechanisms for maintaining dissolved iron concentrations above its inorganic solubility, while potentially reducing the concentrations of soluble and bioavailable inorganic species (Hunter and Boyd, 2007; Shaked and Lis, 2012). A decrease in seawater pH from 8.1 to 7.4 will increase Fe(III) solubility by approximately 40%, which could have a large impact on biogeochemical cycles (Morel and Price, 2003; Millero et al., 2009). Organic matter exuded by marine microorganisms can form Fe(III) complexes that modify Fe(II) oxidation rates and promote the reduction of Fe(III) to Fe(II) in seawater. Under acidifying conditions, some research work has shown that the residence time of the reduced form of essential trace metals increases as their oxidation rate decreases (Pérez-Almeida et al., 2022; Santana-Casiano et al., 2014).

Carbohydrates are one of the major components of the dissolved organic carbon (DOC) pool in marine environments, representing 3-50% of dissolved and colloidal organic

matter (Giljan et al., 2023; Hassler et al. 2011; Wang et al., 2006). Phenolic compounds are secondary metabolites synthesized as a defense mechanism of organisms exposed to abiotic stresses (salinity, metal toxicity, heat, acidification, cold, extreme light, nutrient deficiency, UV radiation) (López et al. 2015; Parvin et al., 2022). Their antioxidant nature enhances tolerance of organisms to adverse climatic conditions that induce an increase in reactive oxygen species (ROS) (Gauthier et al. 2020; Sachdev et al. 2021; Vázquez et al., 2022). Both types of compounds can influence the iron chemistry and bioavailability (Perez-Almeida et al., 2022; Santana-Casiano et al., 2014; Santschi et al., 2003). Catechin, sinapic acid and gallic acid were found to be weak Fe-binding ligands that increased the persistence of dissolved Fe, regenerating Fe(II) in seawater from 0.05% to 11.92% (González et al. 2019). Hassler et al. (2011) reported that the addition of glucuronic acid to natural planktonic assemblages increased iron bioavailability for eukaryotic phytoplankton. Furthermore, iron bioavailability also increased when three different saccharides were used in cultured and natural eukaryotic phytoplankton populations, suggesting that this is a generalizable phenomenon.

The effect of pH changes on the speciation of metal-organic complexes and on the redox kinetics of metals in the marine environment is not as well characterized as that of inorganic ligands due to the heterogeneous composition of dissolved organic matter and their unknown structure. Therefore, studying the nature of these organic ligands will allow a comprehensive understanding of the consequences of acidification on ocean biogeochemical processes.

This work aimed to determine how marine acidification may affect the composition of cells and exudates from *Emiliania huxleyi*. Therefore, cultures of *E. huxleyi* were grown at four different pH (7.75, 7.90, 8.10, and 8.25) reached by bubbling CO_2 in the culture seawater (Samperio-Ramos et al., 2017). The four experimental scenarios represent

interglacial, close to contemporary, and two future ocean acidification conditions based on the Intergovernmental Panel on Climate Change projections (IPCC, 2014). Intra- and extracellular phenolic compounds (gallic acid (GAL), protocatechuic acid (PCA), p-coumaric acid (COU), ferulic acid (FA), catechin (CAT), vanillic acid (VAN), epicatechin (ECAT), syringic acid (SYR), rutin (RU) and gentisic acid (GA)) were identified and quantified by RP-HPLC. The total carbohydrate content of cells was quantified using the phenol/ sulfuric acid (PSA) assay (Dubois et al., 1956). The presence of antioxidants was assessed by the antioxidant activity of cells and exudates determined by DPPH radical scavenging activity test (RSA) and FRAP assay (Sethi et al., 2020).

2. Materials and methods

2.1 Chemicals

Methanol (HPLC gradient grade) was purchased from Scharlab (Barcelona, Spain). Formic acid (synthesis grade), $Fe_3Cl \cdot 6H_2O$, and $FeSO_4 \cdot 7H_2O$ were supplied by Panreac (Barcelona, Spain), D-Glucose (Glc), phenol, DPPH and 2,4,6-tri(2-pyridyl)-triazine (TPTZ) by Sigma-Aldrich (St. Louis, MO, USA). Polyphenol standards were supplied as follows: GAL, PCA, COU, FA, CAT, VAN, ECAT, and SYR by Sigma–Aldrich Chemie (Steinheim, Germany); RU and GA by Merck (Darmstadt, Germany. Ultrapure water was obtained from a Milli-Q system from Millipore (Bedford, MA, USA).

2.2 Cultures

Axenic cultures of *E. huxleyi* (strain RCC1238) were supplied by the Spanish Bank of Algae (BEA) in f/2 medium. *E. huxleyi* coccolithophore was cultured with an initial cell density of 10^6 cells L^{-1} under different pCO_2-controlled seawater pH conditions (7.75,

7.90, 8.10, and 8.25), measured on the free hydrogen ion scale pH_F=-log[H+] with a Ross Combination glass body electrode calibrated daily with TRIS buffer solutions. For this purpose, a gaseous mixture of CO_2-free air and pure CO_2 (up to CO_2 levels 900, 600, 350, and 225 µatm, respectively) was bubbled in the culture medium with an equipment that modulates the CO_2 flow once the desired pH is reached, keeping it constant (±0.02). To maintain suspended cells and homogeneity of CO_2 in solution, the cultures were shaken at 60 rpm with a Teflon-coated magnetic stirrer. All materials were cleaned following a standard protocol (Achterberg et al., 2001) and subsequently autoclaved at 121 °C for 30 min prior to use. Once the seawater reached the desired pCO_2/pH value, coccolithophorides were inoculated into the batch cultures. Stock cultures were maintained in the experimental conditions for 48 h before starting each experiment, which allowed acclimation to the CO_2 concentration. Five experimental batch cultures were carried out at each pH treatment. Seawater enriched with exudates was filtered using acid-cleaned and combusted polycarbonate (Nucleopore) syringe-filters (0.45 µm) to prevent cell breakage. A more detailed description is given by Samperio-Ramos et al. (2017).

2.3. Cells and exudates extract preparation prior to HPLC, carbohydrates, and antioxidant activities quantifications

Cells cultured for 8 days in 700 mL of seawater were freeze-dried and suspended in 5 mL of acetone. The samples were sonicated for 2 min, stirred for 5 min, and centrifuged at 7000 rpm for 15 min. The supernatant was separated, and the residue was extracted with 15 mL of acetone for 30 min and centrifuged again. All supernatants were collected and evaporated to dryness, and the resulting residue was dissolved in 5 mL of methanol. These samples were used for phenolic compounds and carbohydrates quantifications and DPPH inhibition and FRAP values determinations.

Seawater samples (700 mL) enriched with exudates were previously subjected to solid phase extraction (SPE) at a flow rate of 2 mL min^{-1} using Macherey-Nagel Chromabond Easy cartridges (500 mg). The retained analytes were eluted with 12 mL of methanol, which was subsequently evaporated on a rotary evaporator. The residue was dissolved in 300 µl of methanol and filtered through a 0.22 µm filter to be injected into the HPLC equipment. These samples were used to quantify phenolic compounds and DPPH inhibition values.

2.4. Phenolic profile of E. huxleyi cells and exudates

Methanol extract of cells (2 mL) prepared as described in section 2.3 was evaporated, the residue was dissolved in methanol (200 µL) and filtered with a syringe filter (0.2 µm). Chromatographic analysis of cells and exudates was made according to Santiago-Díaz et al. (2023), with a Jasco LC-4000 HPLC equipment provided with a PU-4180 quaternary pump, an AS-4150 autosampler, an MD-4015 photodiode array detector, LC-Net ll interface, a Phenomenex C18 column (250 mm x 4.6 mm, 5 µm) and a Phenomenex guard column maintained at 30ºC. The elution was performed with water containing 0.1% formic acid (phase A) and methanol (phase B), with a flow rate of 1 mL min^{-1} and injection volume of 10 µL. The gradient elution method for A was 0 min, 75%; 30 min, 40%; 40 min, 40%; and finally, the column was washed and reconditioned. For quantification, simultaneous monitoring was set at 270 nm (GAL, PCA, CAT, VAN, RU, ECAT, and SYR) and 324 nm (GA, COU, and FA). Samples were analysed by triplicate, and the results were expressed as attomol $cell^{-1}$ (amol $cell^{-1}$).

2.5 Determination of carbohydrate content

The total carbohydrates contents in *E. huxleyi* cells were determined following the phenol sulfuric acid method (Dubois et al., 1956) with some modifications. The cells extract (1.5 mL) was concentrated, and the residue was dissolved in 0.8 mL of methanol. The reaction was carried out by mixing 80 □L of sample or standard with 150 □L of phenol (5%) and 1 mL of concentrated sulfuric acid. The resulting solution was heated at 100ºC for 5 min, and the absorbance was recorded at 490 nm in a UV-VIS spectrophotometer. The results were expressed as femtomole of glucose equivalent per cell (fmol Glc eq $cell^{-1}$) calculated from the calibration curve of 0.14 to 2.78 mM glucose, y=0.0032x + 0.0863; R^2=0.9971.

2.6. Antioxidant activities of algae and exudates

The antioxidant properties of the cell extracts were evaluated according to Sethi et al. (2020) with modifications. The ability to reduce complex TPTZ-Fe(III) to TPTZ-Fe(II) by donating an electron was measured as follows: The FRAP reagent was prepared with acetate buffer solution 0.3 M (pH 3.6), 10 mM of TPTZ in HCl (40 mM), and 2.5 mL of $FeCl_3 \cdot 6H_2O$ solution (20 mM) (in the ratio of 10:1:1). Methanol extracts (50 □L) were mixed with 1 mL of freshly prepared and pre-warmed FRAP reagent (37ºC) for 13 min. The mixture was introduced in an ice bath, and the absorbance was measured at 593 nm. Results were expressed as fmol of reduced Fe(III) per cell and calculated from a calibration curve constructed with $FeSO_4 \cdot 7H_2O$ concentrations ranging from 0.15 to 2.20 mM (regression line equation y = 0.3942x + 0.1497, R^2 =0.996). The estimation was carried out in triplicate, and the results were averaged.

The capacity of cell extracts and exuded compounds of inhibiting DPPH radical was determined by mixing 0.8 mL of DPPH solution (0.053 mM) with 30 □L of cell extracts, and with 50 □L for each sample of exuded compounds. The absorbance was recorded after 20 min at 515 nm using a UV-visible spectrophotometer (Shimadzu Pharmaspec

1800). The total amount of inhibited DPPH radical per cell was calculated from a calibration curve of 0.03 to 0.1 mM DPPH, y = 11.987x - 0.1352; R^2=0.9996.

3. Results and discussion

3.1. Cell growth

The acidification conditions did not significantly affect the final cell densities, which increased during 8 days from an initial value of 10^6 cells L^{-1} to 9.98 (± 0.53) × 10^7 (pH 8.25), 1.07 (± 0.13) × 10^8 (pH 8.10), 1.04 (± 0.07) × 10^8 (pH 7.90) and 9.15 (± 0.41) × 10^7 (pH 7.75) (Fig. 1). The highest peaks of algae biomass, 1.07 (± 0.10) and 1.04 (± 0.07) × 10^8 cells L^{-1}, were recorded in the microcosms with intermediate CO_2 levels 350 µat and 600 µat (pH 8.10 and 7.90 respectively) (Samperio-Ramos et al., 2017). After 8 days, growth rates remained fairly constant in all experimental acidification conditions, indicating that the stationary phase had been reached (Fig.1). Heidenreich et al. (2019) also concluded that growth rates of both haploid and diploid *E. huxleyi* cells were unaffected by ocean acidification (culture medium adjusted to pCO_2 400 vs. 1000 µatm). Fukuda et al. (2014) reported similar results for *E. huxleyi* harvested in CO_2-enriched seawater, whose growth was unaffected. However, the authors observed different responses depending on the acidification method, i.e. *E. huxleyi* showed severe growth damage in experiments carried out by acidification by HCl, which also changes the seawater alkalinity that does not happen when bubbling CO_2 is used, concluding that coccolithophore *E. huxleyi* has the ability to respond positively to CO_2 acidification. In contrast to these results, Vázquez et al. (2022) found that CO_2 enrichment aeration up to lower pH than those here (1200 µatm, pH 7.62) induced growth rates of the coccolithophore more negatively affected concerning the control (400 µatm, pH 8.10) than low pH reached without CO_2 enrichment.

Discrepancies in experimental results may be due to different environmental factors, culture, light conditions, etc. (Tong et al. 2017; Gafar et al. 2019). Langer et al. (2009) observed different responses to acidification among four strains of *E. huxleyi* in all parameters tested, and no strain showed a previously described response pattern for this species, i.e. growth rate exhibited a marked increase in one strain and slight changes in the remaining strains, decreasing in two of them and increasing in the other.

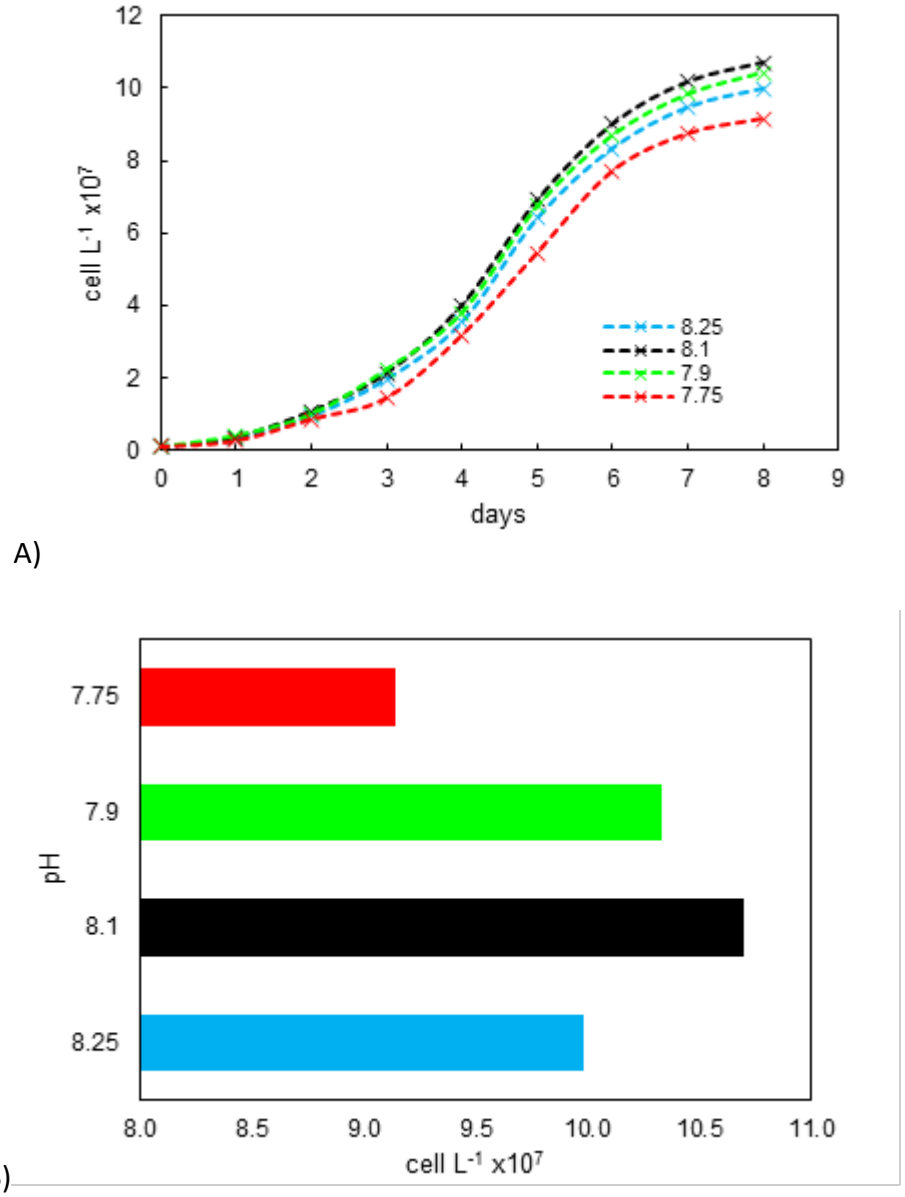


Figure 1. Growth curves (A) and cell densities (B) of coccolithophore *Emiliania huxleyi* cultivated under four different pH conditions indicated in different colors

3.2. Phenolic contents of cells and exudates

The phenolic profile of cell extracts and seawater samples enriched with *E. huxleyi* exudates are summarized in Table 1. GAL, PA, ECAT, and RU were detected in both cells and exudates, whereas VAN and COU were only detected in cells. The other polyphenols were below the limit of quantification (LOQ). At all pH conditions, PA, ECAT, and RU were detected in the seawater enriched with exudates and VAN in cells.

Table 1. Phenolic compounds in cells and exudates of *E. huxleyi* under different pH conditions.

Phenolic compound[a]	pH 7.75 (pCO_2 900µat)		pH 7.90 (pCO_2 600µat)		pH 8.10 (pCO_2 350µat)		pH 8.25 (pCO_2 225µat)	
	Cell	Exuded	Cell	Exuded	Cell	Exuded	Cell	Exuded
GAL	0.13±0.03	-	-	-	0.25±0.07	-	-	45±0
PCA	-	74±9	0.16±0.01	34±2	-	26.3±0.2	-	298±22
ECAT	1.2±0.1	34±1	-	40±5	-	70±2	-	77±6
VAN	6.44±0.06	-	2.5±0.1	-	2.30±0.05	-	2.5±0.2	-
COU	-	-	-	-	1.4±0.2	-	-	-
RU	1.47±0	12.1±0.4		20±2		13.2±0.3		11.2±0.8
Sum	9.24±0.19	120.1±10.4	2.66±0.11	94±9	3.95±0.32	109.5±2.5	2.5±0.2	431.2±28.8
Levels[b] (nM)		18.0±0.9		9.6±0.8		11.7±0.3		43±3

[a]Results are expressed as attomol $cell^{-1}$ (means ± standard deviation of three measurements).

[b]Results are expressed as nanomol L^{-1} (means ± standard deviation of three measurements).

Abbreviations: GAL: gallic acid; PCA: protocatechuic acid; ECAT: epicatechin; VAN: vanillic acid; COU: p-coumaric acid; RU: rutin.

The highest concentrations of total exuded polyphenols were found in cultures at pH 8.25 (43±3 nM) and 7.75 (18.0±0.9 nM), where cell densities were lower (9.98×10^7 and 9.15×10^7 respectively). Under these pH conditions, each cell exuded a higher amount of

phenolic compounds than those exuded under pH 7.90 and 8.10, where a greater number of cells (1.04×10^8 and 1.07×10^8 respectively) exuded less polyphenols (Fig. 2).

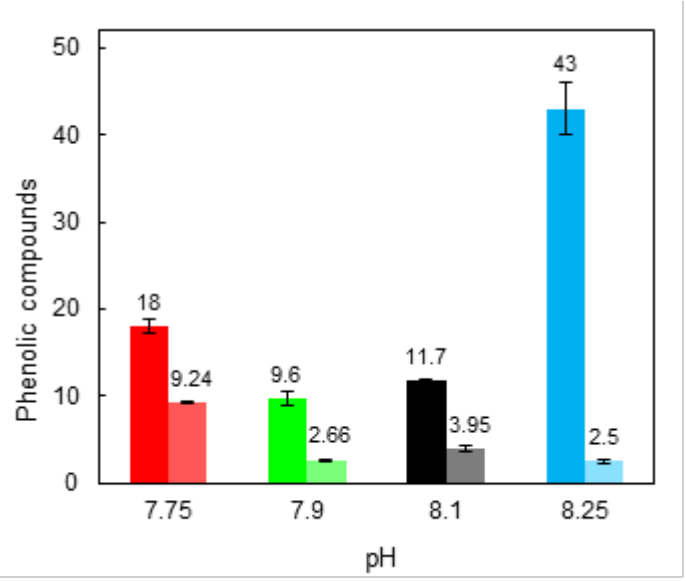


Figure 2. Total sum of the identified intracellular polyphenols expressed as attomol $cell^{-1}$ (in light color) and concentration (nM) of exuded polyphenols (in dark color) by diatoms grown under reduced pH conditions.

The contents of phenolic compounds inside the cells increased with the decline of pH up to pH 7.75, reaching the maximum level (9.24±0.19 attomol $cell^{-1}$). These results agree with those reported by Jin et al. (2015), who evidenced that phytoplankton grown under the CO_2 levels predicted for the end of this century showed accumulation of phenolic compounds, increased by 46–212% compared with that obtained at the current CO_2 level. Subsequently, zooplankton fed with phytoplankton grown in acidified seawater showed 28% to 48% higher phenolic content. This transfer of accumulated phenolic compounds to higher trophic levels could have serious consequences for the marine ecosystem and seafood quality. Polyphenol accumulation has also been observed in terrestrial plants (Bai

et al., 2019; Kaur et al., 2022; Lwalaba et al., 2020). However, Arnold et al. (2012) reported a loss of phenolics in the seagrasses *Cymodocea nodosa, Ruppia maritima*, and *Potamogeton perfoliatus* grown in acidified seawater, where the pH decreased up to 7.3, and the CO_2 level increased ten-fold.

Vázquez et al. (2022) showed that CO_2 enrichment aeration (1200 µatm, pH 7.62) induced metabolic stress and accumulation of reactive oxygen species (ROS) in the *E. huxleyi* strain used in their experiments. This could explain the accumulation of phenolic compounds at pH 7.75 (9.24±0.19 amol $cell^{-1}$), considered to play a significant role as a defense mechanism due to their antioxidant and ROS scavenging properties (Salam et al., 2023).

No correlation was found between phenolic compounds inside and outside the cell. The extracellular phenolic compound's behavior was opposite to that described above for intracellular. The exuded compounds decreased from 43 ± 3 nM at pH 8.25 to 9.6±0.8 nM at pH 7.90 (Table 1). These results partially agree with those reported by López et al. (2015) for *Dunaliella tertiolecta* growing under high levels of copper, where the concentration of phenolic compounds declined from 9.4±0.6 nM in seawater cultures without Cu addition to 8.4±0.4 and 8.6±0.4 nM in the copper enriched seawater and increased 1.4 times concerning the control into the cells grown under the highest Cu level. However, at the lowest pH of 7.75, the level of phenolic compounds was enhanced to 18.0±0.9 nM, higher than that at pH 7.90 (9.6 ± 0.8 nM) but lower than the initial value at pH 8.25 (43 ±3). At this pH, the intracellular content experienced the maximum increase with respect to the initial one (3.7 times higher). This increase may be explained by their potential to scavenge harmful ROS, whose enhanced production has been linked to the pH decrease by Vázquez et al. (2022). The phenolic profile differences found inside and outside the cells could be explained by the different mechanisms to counter pH

acidification inside (intrinsic buffers such as ionizable groups on amino acids, phosphates and other molecules; Na^+–H^+ exchangers and bicarbonate transporters, membrane permeability, among others) and the changes of metabolic pathways involved (Barcelos e Ramos et al., 2010; Casey et al., 2010) and outside, limited to membrane permeability and exuded material.

At pH 7.90, an exception was found in this study. The content of intracellular phenolic compounds decreased compared to that found at pH 8.10 but increases with respect to the initial pH 8.25 (1.06 times higher). The decreases in pH in this study were 0.15, 0.35 and 0.50 units with respect to the initial pH 8.25 (pH 8.1, 7.90 and 7.75, respectively). Suffrian et al. (2011) reported measurements of intracellular pH in *E: huxleyi* showing the presence of mechanisms that counteract cell acidification despite elevated membrane proton permeability and that an external pH shift of 1 was required to acidify the cell by 0.56 pH units. The impact of these changes does not necessarily have to be proportional to the pH change, taking into account the wide range of biological processes dependent on the acid-base properties of the molecules involved, and the different physiological state of cells grown under different conditions, as well as the impact of these changes on the structure, and functions of organelles, as well as on the overall organization of the cell (Antosiewicz and Kane, 2022).

Polyphenols modify Fe(II) oxidation rates by promoting the reduction of Fe(III) to Fe(II) in seawater. The effect of the polyphenol gallic acid on Fe oxidation and reduction was studied by Pérez-Almeida et al. (2022), concluding that gallic acid reduces Fe(III) to Fe(II) in seawater, with a more pronounced effect as pH decreases, allowing Fe(II) to be present for longer periods and improving its bioavailability. The authors found that 69.3% of the initial Fe(II) was oxidized after 10 min at pH = 8.0 in the absence of gallic acid, while only 37.5% was oxidized with 100 nM gallic acid and after 30 min. The reduction

of Fe(III) to Fe(II) by gallic acid was faster as pH decreased. The same results were observed for catechin and sinapic acid, which also favoured the regeneration of Fe(II) in seawater, increasing the amount of regenerated Fe(II) as pH decreased, concluding that acidification may contribute to an increase in the level of reduced iron in the environment (Santana_Casiano et al., 2014). This could be the reason for the decrease of gallic acid and the remaining phenolic compounds in the extracellular medium, and the increase of their presence inside the cell.

3.3. Total carbohydrate content of cells

Total carbohydrates in cells and exudates of *E. huxleyi* are shown in Table 2. No correlation was found between intra- and extracellular contents. The amount of total intracellular carbohydrates remained almost constant between pH 8.25 and pH 7.9 and decreased slightly at pH 7.75. Similar results were observed for exuded carbohydrates, which did not undergo any change as the pH dropped to pH 7.75. Our results agree with those of Araujo and Tabano (2005), who reported that CO_2 addition to the culture seawater lowered the carbohydrates content of marine diatom *Chaetoceros* cf. *wighamii.* Thornton (2009) reported that the planktonic diatom *Chaetoceros muelleri* grown at pH 6.8, 7.4, 7.9, and 8.2 showed a decreased proportion of total carbohydrate within the cells and increased levels of dissolved exuded carbohydrates into the surrounding medium with the decrease in pH. Thornton used a different method to maintain pH, a biological buffer (25 mM HEPES (Sigma-Aldrich, St. Louis, Mo, USA)) and daily titration with the addition of a small amount of HCl, which changes the seawater alkalinity. Fukuda et al. (2014) observed increased production and storage of polysaccharides by the coccolithophore *E. huxleyi* stimulated by acidification with CO_2 enrichment. Jones et al. (2013) compared the response of the coccolithophore species *E. huxleyi* cultured at two

pH conditions reached by bubbling CO_2 (pH 7.94 and 7.47 at the time of the harvesting), when as little as 5% of dissolved inorganic carbon (DIC) was consumed, indicating low chemical shift throughout the experiments. The higher CO_2 level evidenced cellular responses to stress such as decreased growth rates, but proteins associated with many key metabolic processes remained unaltered, thus maintaining many biological functions. Diverse metabolic responses of the coccolithophore *E. huxleyi* to ocean acidification and to combinations of ocean acidification with other environmental factors have been described (Tong et al. 2017; Gafar et al. 2019). Xie et al. (2021) studied the effects of high and low DIC concentration (from 900 to 4,930 μmol kg^{-1}) and reduced pH value (from 8.04 to 7.70) on physiological rhythms, element contents and macromolecules of the coccolithophore *Emiliania huxleyi*, concluding that its response is highly dependent on the DIC. Compared to high pH conditions, low pH and DIC concentration led to increases in particulate organic carbon (POC) and particulate organic nitrogen (PON) contents with less impact on protein and carbohydrate contents; however, high DIC and low pH reduced POC, PON, protein, and carbohydrate contents. Grosse et al. (2020) investigated the effects of seawater acidification on dissolved and particulate amino acids and carbohydrates in arctic and sub-arctic planktonic communities in two large-scale experiments in a pH range similar to ours here (control mesocosm: pCO_2 185 μatm /pH 8.32; mesocosm pCO_2 between 270 and 1420 μatm/pH 8.18–7.51). The authors concluded that the relative composition of amino acids and carbohydrates did not change as a direct consequence of increased pCO_2, and the observed changes depended mainly on the composition of the phytoplankton community.

Table 2. Intracellular and extracellular carbohydrates of *Emiliania huxleyi* harvested under different pH conditions

pH	Intracellular carbohydrates[a]	Extracellular carbohydrates[b]
8.25 (pCO_2 225µat)	27.0 ± 2.2	59.24 ± 3.97
8.10 (pCO_2 350µat)	30.4 ± 3.2	60.99 ± 6.57
7.90 (pCO_2 600µat)	30.3 ± 3.7	63.19 ± 9.81
7.75 (pCO_2 900µat)	23.8 ± 1.9	62.60 ± 5.23

[a]The results are expressed as femtomole glucose equivalent cell^{-1}

[b]Extracellular released carbohydrates (femtomole of glucose equivalent cell^{-1}).

The results are expressed as means ± standard deviation of three measurements.

Similar effects to those observed here have been found in the response of macroalgae to elevating ocean acidification. Gao et al. (2017) reported that pCO_2 does not affect the carbohydrate content of algae *Ulva rigida* investigated under pH 7.95 and 7.55. Barakat et al. (2021) studied the effect of the acid stress on the green alga *Ulva fasciata* subjected to four levels of pCO_2, 280, 550, 750, and 1050 µatm (pH values 7.2, 7.6, 7.86, 8.1 (control)), and found similar production of carbohydrates at the three lowest pH (46.13, 46.96 and 46.04% of dry weight respectively), while the control (pH 8.1) showed 42.37 % of dry weight.

3.4. Antioxidant activities

Table 3 shows the antioxidant activities of extracts of cells *E. huxleyi* grown in seawater enriched with several levels of CO_2 and those of the compounds exuded by these cells. Cells with the highest phenolic content (pH 7.75) gave the most increased scavenging activity (8.1±0.1 fmol cell^{-1}) and iron-reducing capacity (10.2±0.5 fmol cell^{-1}), indicating

that these cells grown in the most acidified media produce relevant amounts of antioxidants. At higher pH, the ability to reduce iron remains almost constant. At the same time, the free RSA increases as pH decreases, following the same tendency as the content of analysed polyphenols. However, the exudates do not exhibit the same tendency. As the pH decreases, the total content of exuded polyphenols also decreases and the exuded compounds' RSA increases up to pH 7.90, stating that polyphenols are not responsible for this activity. By lowering from pH 7.90 to pH 7.75, polyphenols increase and DPPH inhibition decreases.

Significant correlation was found between FRAP and total intracellular phenolic content ($r=0.928$, $p<0.05$) suggesting that the reducing power in cells is associated with these compounds, which reduce Fe(III) to Fe(II). This could also corroborate that the polyphenols exuded at lower pH decrease as their reducing power is no longer necessary because under these conditions the persistence of essential metals such as Fe(II) increases, and their concentration and reducing power inside the cell increases to compensate for oxidative stress. The FRAP test is based on the transfer of an electron, whereas the DPPH test includes the transfer of both a hydrogen atom and an electron. These different chemical reactions mechanisms and their kinetics could explain the differences between FRAP and DPPH results and may be the reason for the lack of correlation between the phenolic content and RSA.

Table 3. Antioxidant activities of exudates and extracts of *E. huxleyi* cells grown in seawater enriched with CO_2.

pH	FRAP[a]	RSA	
		Cells[b]	Exudates[c]
8.25 (pCO_2 225µat)	9.5±0.6	3.4±0.2	26±1
8.10 (pCO_2 350µat)	9.3±0.3	2.3±0.3	37±2
7.90 (pCO_2 600µat)	9.3±0.7	6.0±0.2	40±2
7.75 (pCO_2 900µat)	10.2±0.5	8.1±0.1	26.1±0.4

[a]Results are expressed as fmol of reduced Fe(III) per cell.

[b]The results are expressed as fmol of inhibited DPPH per cell

[c]The results are expressed as nmol of inhibited DPPH L^{-1}

All results are expressed as means ± standard deviation of three measurements

Abbreviations: FRAP: ferric-reducing antioxidant power; RSA: radical scavenging activity

The results found in the literature on the effect of acid stress on the biochemical composition of microorganisms and their exudates are apparently contradictory. However, a large number of factors (initial cell density, grown phase, culture media composition, light photoperiod and light intensity applied during culture, pH, among others) strongly influence the toxicity of pollutants in algal bioassay stimulating and/or inhibiting the production of different metabolites through the regulation of metabolic pathways (Barcelos e Ramos et al., 2010; Santiago-Díaz et al., 2023; Singh and Shrivastava, 2016; Tandon et al., 2017; Zhang et al., 2019). In addition, the antioxidant activity of cell extracts and exudates depends on their complex mixture of compounds and the interactions between them, as well as the assay mechanism/kinetic (Šamec et al., 2021; Sethi et al., 2020). We have detected different carbohydrate contents that showed

significant negative correlation with the FRAP values, which may indicate that these compounds are not responsible for this reducing activity. In addition, we only identified a small sample of phenolics.

On the other hand, the pH range for optimal growth varies depending on the microalgae species, and even the sensitivity of *E. huxleyi* to acidification appears to be strain-specific (Langer et al., 2009). Borchard and Engel (2015) reported that *E. huxleyi* ability to acclimate to different CO_2 concentrations during the stationary phase of growth was responsible for the absence of a CO_2 effect found on primary production and exudation in their study.

The responses of marine organisms to ocean acidification are very complex and appear to depend on many factors (Grosse et al., 2020). Changes in seawater pH influence the protonation of biological molecules and could modify their charge and negatively affect metabolic processes. Several mechanisms have been described to maintain the pH inside cells, such as changing the lipid composition of the cytoplasmic membrane or increasing the production of cyclopropane fatty acids to reduce proton permeability (Lund et al., 2020). Ocean acidification will directly affect marine organisms, altering the structure and functions of marine ecosystems. The accumulation of phenolic compounds leads to functional consequences in primary and secondary producers, with the possibility that fishery industries could be influenced as a result of progressive ocean change (Gattuso et al., 2015; Jin et al., 2015; Trombetta et al., 2019).

Conclusion

Global environmental change influences the growth and metabolic functions of microalgae affecting their communities' structure and compositions depending on the sensitivity of different taxonomic groups, with implications for higher trophic levels and

biodiversity loss. Limited research has been focused on the ecological consequences of joint action of seawater pH decrease, warming, and changes in salinity, among others. In this study, the phenolic profiles of *E. huxleyi* cells grown under four marine acidification scenarios, as well as their exudates, have been determined for the first time. Different phenolic profiles of cells and exudates were found. Intra- and extracellular carbohydrate levels also showed slight modifications with a pH decrease. These changes in metabolites increased the intracellular reducing ability (FRAP) and the capacity to inhibit radicals, whose accumulation is associated with enhanced oxidative stress. The functional consequences of these variations observed here in response to reduced pH are a potential factor leading to readjustments in phytoplankton community structure and diversity and possible alteration in marine ecosystems.

References

Achterberg EP, Holland TW, Bowie AR, Mantoura, R. F. C., and Worsfold, P. J.: Determination of iron in seawater, Anal. Chim. Acta, 442(1), 1–14. https://doi.org/10.1016/j.talanta.2018.05.071, 2001.

Antosiewicz, J. M., and Kane, P. M.: Editorial: Intracellular Molecular Processes Affected by pH. Front. Mol. Biosci. Sec Biophysics 9, 891533, https://doi: 10.3389/fmolb.2022.891533, 2022.

Araujo, Sd.C., and García, V.M.T.: Growth and biochemical composition of the diatom Chaetoceros cf. wighamii brightwell under different temperature, salinity and carbon dioxide levels. I. Protein, carbohydrates and lipids. Aquaculture 246(1-4), 405–412. https://doi.org/10.1016/j.aquaculture.2005.02.051, 2005.

Arnold, T., Mealey, C., Leahey, H., Miller, A.W., Hall-Spencer J.M., Milazzo, M., and Maers, K.: Ocean Acidification and the Loss of Phenolic Substances in Marine Plants, PLoS One, 7(4), e35107. https://doi.org/10.1371/journal.pone.0035107, 2012.

Arundhathy, M., Jyothibabu, R., and Santhikrishnan, S.: (2021). Coccolithophores: an environmentally significant and understudied phytoplankton group in the Indian Ocean. Environ. Monit. Assess., 193, 144, https://doi.org/10.1007/s10661-020-08794-1, 2021.

Bai, Y., Wang, G., Cheng, Y., Shi, P., Yang, C., Yang, H., and Xu, Z.: Soil acidification in continuously cropped tobacco alters bacterial community structure and diversity via the accumulation of phenolic acids, Sci. Rep., 9, 12499, https://doi.org/10.1038/s41598-019-48611-5, 2019.

Barakat, K. M., El-Sayed, H. S., Khairy, H. M., El-Sheikh, M. A., Al-Rashed, S. A., Arif, I. A., and Elshobary, M. E.: Effects of ocean acidification on the growth and biochemical composition of a green alga (*Ulva fasciata*) and its associated microbiota, Saudi J. Biol. Sci., 28(9), 5106–5114. https://doi.org/10.1016/j.sjbs.2021.05.029, 2021.

Barcelos e Ramos, J., Müller, M.N., and Riebesell, U.T.: Short-term response of the coccolithophore Emiliania huxleyi to an abrupt change in seawater carbon dioxide concentrations, Biogeoscience, 7(1), 177–186, https://bg.copernicus.org/articles/7/177/2010/, 2010.

Barcelos e Ramos, J., Ribeiro, S. C., Schulz, K. G., Coelho, F. J. R. D. C., Oliveira, V., Cunha, A., Gomes, N. C. M., Brownlee, C., Passow, U., de Azevedo, E.B.: *Emiliania huxleyi*-Bacteria Interactions under Increasing CO_2 Concentrations. Microorganisms. 13, 10(12), 2461, https://doi.org/10.3390/microorganisms10122461, 2022.

Berge, T., Daugbjerg, N., Andersen, B., and Hansen, P. J.: Effect of lowered pH on marine phytoplankton growth rates, Mar. Ecol. Prog. Ser., 416, 79–91. https://doi.org/10.3354/meps08780, 2010.

Borchard, C., and Engel, A.: Size-fractionated dissolved primary production and carbohydrate composition of the coccolithophore *Emiliania huxleyi*, Biogeoscience, 12, 1271–1284. https://doi.org/10.5194/bg-12-1271-2015, 2015.

Casey, J., Grinstein, S., and Orlowski, J.: Sensors and regulators of intracellular pH, Nat. Rev. Mol. Cell. Biol. 11, 50–61. https://doi.org/10.1038/nrm2820, 2010.

Dedman, C. J., Barton, S., Fournier, M., and Rickaby, R. E. M.: The cellular response to ocean warming in Emiliania huxleyi, Front. Microbiol., 14, 1177349. https://doi.org/10.3389/fmicb.2023.1177349, 2023.

Dubois, K. M., Gilles, K. A., Hamilton, G. J. K., Rebers, P. A., and Smith, F.: Colorimetric method for determination of sugars and related substances, Analyt. Chem., 28, 350–356, https://doi.org/10.1021/ac60111a017, 1956.

Eltanahy, E., and Torky, A.: Chapter 1 microalgae as cell factories: food and feed-grade high-value metabolites, Microalgal Biotechnol., 1–35, https://doi.org/10.1039/9781839162473-00001, 2021.

Fukuda, S-y., Suzuki, Y., and Shiraiwa, Y.: Difference in physiological responses of growth, photosynthesis and calcification of the coccolithophore *Emiliania huxleyi* to acidification by acid and CO_2 enrichment, Photosynth. Res., 121, 299–309. https://doi.org/10.1007/s11120-014-9976-9, 2014.

Gafar, N. A., Eyre, B. D.,l and Schulz, K.G.: A comparison of species specific sensitivities to changing light and carbonate chemistry in calcifying marine phytoplankton, Sci Rep., **9**, 2486, https://doi.org/10.1038/s41598-019-38661-0, 2019.

Gao, G., Clare, A. S., Rose, C., and Caldwell G. C.: Eutrophication and warming-driven green tides (Ulva rigida) are predicted to increase under future climate change scenarios, Mar. Pollut. Bull., 114(1), 439–447, https://doi.org/10.1016/j.marpolbul.2016.10.003, 2017.

Gattuso, J. P., Magnan, A., Billé, R., Cheung, W., Howes, E., Joos, F., Allemand, D., Bopp, L., Cooley, S. R., Eakin, C. M., Hoegh-Guldberg, O., Kelly, R. P., Pörtner, H.-O., Rogers, A. D., Baxter, J. M., Laffoley, D., Osborn, D., Rankovic, A., Rochette, J., Sumalia, U. R., Treyer, S., and Turley, C.: Contrasting futures for ocean and society from different anthropogenic CO_2 emissions scenarios, Science, 349 (6243), doi: 10.1126/science.aac472, 2015.

Gauthier, M. R., Senhorinho, G. N. A. and Scott, J. A.: Microalgae under environmental stress as a source of antioxidants, Algal Res., 52, 102104. https://doi.org/10.1016/j.algal.2020.102104, 2020.

Giljan, G., Brown, S., and Lloyd, C.C., Ghobrial, S., Amann, R., Arnosti, C.: Selfish bacteria are active throughout the water column of the ocean, ISME Commun., 3(1), 11, https://doi.org/10.1038/s43705-023-00219-7, 2023.

González, A. G, Cadena-Aizaga, M. I., Sarthou, G., González-Dávila, M., and Santana-Casiano, J. M.: Iron complexation by phenolic ligands in seawater, Chem. Geol., 511, 380–388, https://doi.org/10.1016/j.chemgeo.2018.10.017, 2019.

Grosse, J., Endres, S., and Engel, A.: Ocean acidification modifies biomolecule composition in organic matter through complex interactions, Sci. Rep., 10, 20599, https://doi.org/10.1038/s41598-020-77645-3, 2020.

Gruber, N., Bakker, D. C. E., DeVries, T., Gregor, L., Hauck, J., Landschützer, P., McKinley G. A.: Trends and variability in the ocean carbon sink, Nat. Rev. Earth Environ., 4, 119–134, https://doi.org/10.1038/s43017-022-00381-x, 2023.

Guan, N., and Liu, L.: Microbial response to acid stress: mechanisms and applications, Appl. Microbiol. Biotechnol., 104, 51–65, https://doi.org/10.1007/s00253-019-10226-1, 2020.

IPCC Climate Change. Synthesis Report, 2014. Contribution of Working Groups I, II and III to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change. In: R.K. Pachauri and L.A. Meyer, Ed. IPCC, Geneva, Switzerland.

Harmesa, Wahyudi, A. J., Lestari, and Taufiqurrahman, E.: Variability of trace metals in coastal and estuary: Distribution, profile, and drivers, Mar. Pollut. Bull., 174, 113173, https://doi.org/10.1016/j.marpolbul.2021.113173, 2022.

Hassler, C. S., Schoemann, V., Nichols, C. M., Butler, E. C. V., and Boyd, P. W.: Saccharides enhance iron bioavailability to Southern Ocean phytoplankton, Proc. Natl. Acad. Sci., 108(3), 1076–1081, https://doi.org/10.1073/pnas.101096310, 2011.

Heidenreich, E., Wördenweber, R., Kirschhöfer, F., Nusser, M., Friedrich, F., Fahl, K., Kruse, O., Rost, B., Franzreb, M., Brenner-Weiss, G., Rokitta, S.: Ocean acidification has little effect on the biochemical composition of the coccolithophore Emiliania huxleyi, PLoS One, 14(7), e0218564, https://doi.org/10.1371/journal.pone.0218564, 2019.

Hogle, S. L., Barbeau, K. A., and Gledhill, M.: Heme in the marine environment: from cells to the iron cycle. Metallomics 6, 1107–1120, https://doi.org/10.1039/c4mt00031e, 2014.

Hoppe, C. J. M., Langer, G., and Rost, B.: Emiliania huxleyi shows identical responses to elevated pCO2 in TA and DIC manipulations, J. Experimental Mar. Biol. Ecol., 406 (1–2), 54-62, https://doi.org/10.1016/j.jembe.2011.06.008, 2011.

Hunter, K. A., and Boyd, P. W.: Iron-binding ligands and their role in the ocean biogeochemistry of iron, Environ. Chem., 4, 221–232, doi:10.1071/EN07012, 2007.

Jacobson, M.Z.: Studying ocean acidification with conservative, stable numerical schemes for nonequilibrium air-ocean exchange and ocean equilibrium chemistry, J. Geophys. Res., 110, D07302, https://doi.org/10.1029/2004JD005220, 2005.

Jiang, J., Cao, L., Jin, X., Yu, Z., Zhang, H., Fu, J., and Jiang, G.: Response of ocean acidification to atmospheric carbon dioxide removal. J. Environ. Sci., https://doi.org/10.1016/j.jes.2023.04.029, 2023.

Jin, P., Wang, T., Liu, N., Dupont, S., Beardall, J., Boyd, P. W., Riebesell, U., and Gao, K.: Ocean acidification increases the accumulation of toxic phenolic compounds across trophic levels, Nat. Commun., 6, 8714, https://doi.org/10.1038/ncomms9714, 2015.

Jin, Q., and Kirk, M. F.: pH as a primary control in environmental microbiology: 1. Thermodynamic Perspective, Front. Environ. Sci., 6, 21, https://doi.org/10.3389/fenvs.2018.00021, 2018.

Jones, B. M., Iglesias-Rodriguez, M. D., Skipp, P. J., Edwards, R. J., Greaves, M. J., Young, J. R., Elderfield, H., and O'Connor, C. D.: Responses of the *Emiliania huxleyi* Proteome to Ocean Acidification, PLoS One, 8(4), e61868, https://doi.org/10.1371/journal.pone.0061868, 2013.

Kaur, S., and Samota, M. K.: Choudhary, M. Choudhary, M., Pandey, A. K., Sharma, A., and Thakur, J.: How do plants defend themselves against pathogens-Biochemical mechanisms and genetic interventions, Physiol. Mol. Biol. Plants, 28, 485–504, https://doi.org/10.1007/s12298-022-01146-y, 2022.

Kholssi, R., Lougraimzi, H., and Moreno-Garrido, I.: Effects of global environmental change on microalgal photosynthesis, growth and their distribution, Mar. Environ. Res., 184, 105877, https://doi.org/10.1016/j.marenvres.2023.105877, 2023:

Langer, G., Nehrke, G., Probert, I., Ly, J., and Ziveri, P.: Strain-specific responses of *Emiliania huxleyi* to changing seawater carbonate chemistry, Biogeosciences, 6(11), 2637–2646, https://doi.org/10.5194/bg-6-2637, 2009.

Lida, Y., Takatani, Y., Kojima, A., and Ishij, M.: Global trends of ocean CO_2 sink and ocean acidification: an observation-based reconstruction of surface ocean inorganic carbon variables, J. Oceanogr., 77, 323–358, https://doi.org/10.1007/s10872-020-00571-5, 2021.

Liu, X. W., and Millero, F. J.: The solubility of iron in seawater. Mar. Chem., 77, 43–54, https://doi.org/10.1016/S0304-4203(01)00074-3, 2002.

López, A., Rico, M., Santana-Casiano, J.M., and González, A.G.: Phenolic profile of Dunaliella tertiolecta growing under high levels of copper and iron, Environ. Sci. Pollut. Res., 22, 14820–14828, https://doi.org/10.1007/s11356-015-4717-y, 2015.

Lund, P. A., De Biase, D., Liran, O., Scheler, O., Mira, N. P., Cetecioglu, Z., Fernández, E. N., Bover-Cid, S., Hall, R., Sauer, M., and O'Byrne, C.: Understanding How Microorganisms Respond to Acid pH Is Central to Their Control and Successful Exploitation, Front. Microbiol. Sec. Microbial Physiol. Metabolism, 11, https://doi.org/10.3389/fmicb.2020.556140, 2020.

Lwalaba, J. L. W., Zvobgo, G., Mwamba, T. M., Louis, L. T., Fu, L., Kirika, B. A., Tshibangu, A. K., Adil, M. F., Sehar, S., Mukobo, R. P., and Zhang, G.: High accumulation of phenolics and amino acids confers tolerance to the combined stress of cobalt and copper in barley (Hordeum vulagare), Plant Physiol. Biochem., 155, 927–937, https://doi.org/10.1016/j.plaphy.2020.08.038, 2020.

Lu, X., Tian, C., Pei, H., Hu, W., and Xie, J.: Environmental factors influencing cyanobacteria community structure in Dongping Lake, China, J. Environ. Sci., 25(1), 2196-2206, https://doi.org/10.1016/S1001-0742(12)60297-6, 2013.

Mackey, K. R. M., Morris, J. J., Morel, F. M. M., and Kranz, S. A.: Response of Photosynthesis to Ocean Acidification, Oceanography, 28(2), 74–91, http://www.jstor.org/stable/24861872, 2015.

Marinov, I., Doney, S. C., and Lima, I. D.: Response of ocean phytoplankton community structure to climate change over the 21st century: partitioning the effects of nutrients, temperature and light, Biogeoscience, 7, 3941–3959, https://doi.org/10.5194/bg-7-3941-2010, 2010.

Meier, K. J. S., Beaufort, L., Heussner, S., and Ziveri, P.: The role of ocean acidification in *Emiliania huxleyi* coccolith thinning in the Mediterranean Sea, Biogeoscience, 11, 2857–2869, https://doi.org/10.5194/bg-11-2857-2014, 2014.

Millero, F. J., Woosley, R., Ditrolio, B., and Waters, J.: Effect of ocean acidification on the speciation of metals in seawater. Oceanography 22, 72–85, doi: 10.5670/oceanog.2009.98, 2009.

Morel, F. M. M., and Price, N. M.: The biogeochemical cycles of trace metals in the oceans. Science 300, 944–947, doi: 10.1126/science.1083545, 2003.

Nelson, K. S., Baltar, F., Lamare, M. D., and Morales, S. E.: Ocean acidification affects microbial community and invertebrate settlement on biofilms, Sci. Rep., 10, 3274, https://doi.org/10.1038/s41598-020-60023-4, 2020.

Parvin, K., Nahar, K., Mohsin, S.M., Al Mahmud, J., Fujita, M., and Hasanuzzaman, M.: Plant Phenolic Compounds for Abiotic Stress Tolerance. In: Hasanuzzaman, M., Ahammed, G.J., Nahar, K. (eds) Managing Plant Production Under Changing Environment. Springer, Singapore, https://doi.org/10.1007/978-981-16-5059-8_8, 2022.

Pérez-Almeida, N., González, A. G., Santana-Casiano, J. M., and González-Dávila, M.: Ocean Acidification Effect on the Iron-Gallic Acid Redox Interaction in Seawater, Front. Mar. Sci. Sec. Mar. Biogeochem., 9, https://doi.org/10.3389/fmars.2022.837363, 2022.

Raven, J. A., Evans, M. C. W., and Korb, R. E.: The role of trace metals in photosynthetic electron transport in O_2-evolving organisms. Photosyn. Res., 60, 111–149, https://doi.org/10.1023/A:1006282714942, 1999.

Sachdev, S., Ansari, S. A., Ansari, M.I., Fujita, M., and Hasanuzzaman, M.: Abiotic Stress and Reactive Oxygen Species: Generation, Signaling, and Defense Mechanisms, Antioxidants, 10(2), 277, https://doi.org/10.3390/antiox10020277, 2021.

Salam, U., Ullah, S., Tang, Z.-H., Elateeq, A.A., Khan, Y., Khan, J., Khan, A., and Ali, S.: Plant Metabolomics: An Overview of the Role of Primary and Secondary Metabolites against Different Environmental Stress Factors, Life, 13, 706, https://doi.org/10.3390/life13030706, 2023.

Šamec, D., Karalija, E., Šola, I., Bok, V. V., and Salopek-Sond, B.: The role of polyphenols in abiotic stress response: the influence of molecular structure, Plants, 10(1), 118, https://doi.org/10.3390/plants10010118, 2021.

Samperio-Ramos, G., Santana-Casiano, J. M., González-Dávila, M., Ferreira, S., and Coimbra, M. A.: Variability in the organic ligands released by *Emiliania huxleyi* under simulated ocean acidification conditions, AIMS Environ. Sci., 4(6), https://doi.org/10.3934/environsci.2017.6.788, 2017.

Santana-Casiano, J. M., González-Dávila, M., González, A.G., Rico, M., López, A., and Martel, A.: Characterization of phenolic exudates from Phaeodactylum tricornutum and their effects on the chemistry of Fe(II)–Fe(III), Mar. Chem., 158, 10–16, https://doi.org/10.1016/j.marchem.2013.11.001, 2014.

Santiago-Díaz, P., Rivero, A., Rico, M., González-González, A., González-Dávila, M., and Santana-Casiano, M.: Copper toxicity leads to accumulation of free amino acids and polyphenols in Phaeodactylum tricornutum diatoms, Environ. Sci. Pollut. Res., 30, 51261–51270, https://doi.org/10.1007/s11356-023-25939-0, 2023.

Santschi, P. H., Hung, C.-C., Schultz, G., Alvarado-Quiroz, N., Guo, L., Pinckney, J., and Walsh, I.: Control of acid polysaccharide production and ^{234}Th and POC export fluxes by

marine organisms, Geophys. Res. Lett., 30, 1044, https://doi.org/10.1029/2002GL016046, 2003.

Sethi, S., Joshi, A., Arora, B., Bhowmik, A., Sharma, R.R., and Kumar, P.: Significance of FRAP, DPPH, and CUPRAC assays for antioxidant activity determination in apple fruit extracts, Eur. Food Res. Technol., 246, 591–598, https://doi.org/10.1007/s00217-020-03432-z, 2020.

Shaked, Y., and Lis, H.: Disassembling iron availability to phytoplankton. Front. Microbiol., 3, 123, https://doi.org/10.3389/fmicb.2012.00123, 2012.

Singh, P. K., and Shrivastava, A. K.: Role of initial cell density of algal bioassay of toxic chemicals, J. Basic Microbiol., 56, 812–819, https://doi.org/https://doi.org/10.1002/jobm.201500597, 2016.

Spisla, C., Taucher, J., Bach, L. T., Haunost, M., Boxhammer, T., King, A. L., Jenkins, B. D., Wallace, J. R., Ludwig, A., Meyer, J., Stange, P., Minutolo, F., Lohbeck, K. T., Nauendorf, A., Kalter, V., Lischka, S., Sswat, M., Dörner, I., Ismar-Rebitz, S. M. H., Aberle, N., Yong, J. C., Bouquet, J.-M., Lechtenbörger, A. K., Kohnert, P., Krudewig, M., and Riebesell, U.: Extreme Levels of Ocean Acidification Restructure the Plankton Community and Biogeochemistry of a Temperate Coastal Ecosystem: A Mesocosm Study, Front. Mar. Sci., 7, https://www.frontiersin.org/articles/10.3389/fmars.2020.611157, 2021.

Suffrian, K., Schulz, K.G., Gutowska, M.A., Riebesell, U. and Bleich, M.: Cellular pH measurements in *Emiliania huxleyi* reveal pronounced membrane proton permeability, New Phytologist., 190, 595-608, https://doi.org/10.1111/j.1469-8137.2010.03633.x, 2011.

Tandon, P., Jin, Q., and Huang, L.: A promising approach to enhance microalgae productivity by exogenous supply of vitamins, Microb. Cell Factories, 16, 1-13, https://doi.org/10.1186/s12934-017-0834-2, 2017, 2011.

Thornton, D. C. O.: Effect of low pH on carbohydrate production by a marine planktonic diatom (*Chaetoceros muelleri*), Int. J. Ecol., https://doi.org/10.1155/2009/105901, 2009.

Trombetta, T., Vidussi, F., Mas, S., Parin, D., Simier, M., and Mostajir, B.: Water temperature drives phytoplankton blooms in coastal waters. PloS One, 14, 1–28, https://doi.org/10.1371/journal.pone.0214933, 2019.

Tong, S., Hutchins, D. A., and Gao, K.: Physiological and biochemical responses of Emiliania huxleyi to ocean acidification and warming are modulated by UV radiation, Biogeosciences, 16, 561-572, https://doi.org/10.5194/bg-16-561-2019, 2019.

Usher, P. K., Ross, A. B., Camargo-Valero, M. A., Tomlin, A. S., and Gale, W. F.: An overview of the potential environmental impacts of large-scale microalgae cultivation, Biofuels 5, 331–349, https://doi.org/10.1080/17597269.2014.913925, 2014.

Vasconcelos, M. T. S. D., Leal, M.F.C., and van den Berg, C.M.G.: Influence of the nature of the exudates released by different marine algae on the growth, trace metal uptake and exudation of Emiliania huxleyi in natural seawater, Mar. Chem., 77(2–3), 187–210, https://doi.org/10.1016/S0304-4203(01)00087-1, 2002.

Vázquez, V., León, P., Gordillo, F. J. L., Jiménez, C., Concepción, I., Mackenzie, K., Bresnan, E., and Segovia, M.: High-CO_2 Levels Rather than Acidification Restrict Emiliania huxleyi Growth and Performance, Microb. Ecol., 86, 127–143, https://doi.org/10.1007/s00248-022-02035-3, 2022.

Xie, E., Xu, K., Li, Z., Li, W., Yi, X., Li, H., Han, Y., Zhang, H., Zhang, Y.: Disentangling the Effects of Ocean Carbonation and Acidification on Elemental Contents and

Macromolecules of the Coccolithophore Emiliania Huxley, Front. Microbiol., 20 Sec. Aquatic. Microbiol., 12, https://doi.org/10.3389/fmicb.2021.737454, 2021.

Zhang, Y., Wu, H., Yuan, C., Li, T., and Li, A.: Growth, biochemical composition, and photosynthetic performance of Scenedesmus acuminatus during nitrogen starvation and resupply, J. Appl. Phycol., 31, 2797–2809, https://doi.org/10.1007/s10811-019-01783-z, 2019.

Wang, D. L., Henrichs, S. M., and Guo, L.D.: Distributions of nutrients, dissolved organic carbon and carbohydrates in the western Arctic Ocean, Cont. Shelf Res., 26(14), 1654-1667, https://doi.org/10.1016/j.csr.2006.05.001, 2006.

Westbroek, P.: The coccolithophore Emiliania huxleyi and global climate, The Paleontological Society Special Publications, 6, 309-309, doi:10.1017/S2475262200008698, 1992.

4.3. Aplicación de la metodología analítica desarrollada y optimizada a diferentes materiales vegetales

Los trabajos correspondientes a los apartados 4.3.1 y 4.3.2 se han realizado en colaboración con el BEA, que ha identificado y suministrado las especies de microalgas investigadas. Estas reaccionan a cambios externos, sintetizando metabolitos secundarios y cambiando su entorno intracelular y extracelular, siendo de especial interés por su amplio potencial.

4.3.1. Metabolitos bioactivos de microalgas procedentes de las Islas Canarias para aplicaciones en alimentación humana y animal

Las Islas Canarias son de origen volcánico y subtropical, y se encuentran sometidas a altos niveles de radiación solar durante todo el año. Estas condiciones ambientales generan diversos hábitats y ecosistemas, forzando la adaptación de microorganismos mediante la acumulación de metabolitos que podrían resultar de gran interés biotecnológico. Las microalgas son una fuente rica en compuestos bioactivos, capaces de reducir los niveles de CO_2 y con una producción de bajo coste. Estas características las convierten en una alternativa sostenible y ecológica para la nutrición y la salud en el futuro.

En este estudio, se desarrollaron y optimizaron metodologías de análisis de metabolitos orgánicos en tres cepas de microalgas de agua dulce recolectadas en diferentes localizaciones de las Islas Canarias. Las microalgas analizadas fueron: *Spirogyra* sp., *Cosmarium* sp. y *Cosmarium blytii*. Estos microorganismos se seleccionaron por sus actividades biológicas y por su elevada tasa de crecimiento y productividad de biomasa.

Se llevó a cabo un análisis de carbohidratos totales por reacción con el reactivo de antrona, y un análisis de tres aminoácidos (ácido glutámico, ácido aspártico y prolina) por RP-HPLC-DAD. Además, se evaluó la capacidad antioxidante de los extractos en metanol de las tres microalgas mediante el ensayo de RSA.

Para el análisis de carbohidratos totales se compararon dos métodos de extracción: (i) con HCl 3M durante 5 horas a 100ºC, (ii) con agua a temperatura ambiente durante 1 hora. Los extractos preparados con el primer método proporcionan una mayor cantidad de carbohidratos, ya que en condiciones ácidas a elevadas temperaturas se produce la

hidrólisis de los enlaces glucosídicos que mantienen los monosacáridos unidos como disacáridos, oligosacáridos y polisacáridos. La concentración del ácido, la temperatura y el tiempo son parámetros determinantes en la hidrólisis de estos compuestos. Además, se debe tener en cuenta la robustez de la pared celular del alga, por lo que el empleo de métodos mecánicos de extracción favorecería la liberación de los metabolitos de las células.

La microalga con mayor contenido en carbohidratos totales fue *Cosmarium* sp. seguida de *Cosmarium blytii* y *Spirogyra* sp. (43,2, 41,8 y 35,9%, respectivamente). Los elevados contenidos en carbohidratos de estas algas las convierten en potenciales fuentes de estos compuestos con numerosas propiedades (antioxidantes, antifúngicas, prebióticos...).

La determinación de aminoácidos se llevó a cabo mediante una extracción con metanol diluido en agua y una posterior derivatización con PITC. Este procedimiento de extracción sin hidrólisis permite la cuantificación de aminoácidos libres. El alga *Cosmarium blytii* presentó hasta 5 veces más aminoácidos totales analizados (24,02 mg g^{-1}) que Cosmari*um* sp. y *Spirogyra* sp., siendo el ácido glutámico el mayoritario. El contenido en ácido glutámico y aspártico de *Cosmarium blytii* es mayor que el de algas ampliamente conocidas y analizadas como *Spirulina platensis*, *Dunaliella salina* o *Tetraselmis suecica*. Tanto el ácido glutámico como el aspártico son los aminoácidos más abundantes en microalgas y poseen numerosos beneficios en la salud.

En cuanto a la capacidad antioxidante de los extractos celulares, *Spirogyra* sp. y *Cosmarium blytii* mostraron una mayor actividad antioxidante que el aditivo sintético BHT (por sus siglas en inglés “butylated hydroxytoluene”). Ambas microalgas podrían ser una fuente de antioxidantes con suficiente potencial para sustituir a antioxidantes sintéticos como el BHT (clasificado como promotor de cáncer).

Los extractos de las microalgas verdes *Cosmarium blytii*, *Cosmarium* sp. y *Syporiga* sp. son ricos en carbohidratos y contienen ácido glutámico, ácido aspártico y prolina. Estos compuestos junto con su actividad antioxidante podrían mejorar el valor nutricional de las dietas humanas y animales, lo que podría beneficiar la economía local de las Islas Canarias mediante nuevas actividades industriales y comerciales de aprovechamiento de esta materia en diferentes áreas.

Los resultados de este trabajo fueron publicados la revista *Chemistry and Biodiversity* en 2022.

doi.org/10.1002/cbdv.202200230 RESEARCH ARTICLE CHEMISTRY & BIODIVERSITY

Bioactive Metabolites of Microalgae from Canary Islands for Functional Food and Feed Uses

Paula Santiago-Díaz,[a] Milagros Rico,*[a] Argimiro Rivero,[a] and Magdalena Santana-Casiano[a]

[a] Departamento de Química, Instituto Universitario de Oceanografía y Cambio Global, Universidad de Las Palmas de Gran Canaria, Campus de Tafira, Las Palmas de Gran Canaria, Canary Islands, Spain, e-mail: milagros.ricosantos@ulpgc.es

Three freshwater microalgae (*Spirogyra* sp., *Cosmarium* sp., and *Cosmarium blytii*) collected from several locations in Gran Canaria have been studied to explore their potential as a novel source of bioactive compounds for biotechnological applications. Soluble carbohydrates were quantified after extraction with 3 M HCl at 100 °C, ranging from 35.8 to 43.3 %, and with water at room temperature, ranging from 19 to 22.8 %. Amino acids glutamic acid, proline and aspartic acid were quantified by RP-HPLC. Glutamic acid was the most abundant, ranging from 12.2 to 3.63 mg g ^{1} of dry biomass. *Cosmarium blytii* was the richest sample in amino acids (24.02 mg g ^{1} of dry weight). In addition, *Cosmarium blytii* and *Spyrogira* sp. exhibited higher radical scavenging activity (RSA) against 1,1-diphenyl-2-picrylhydrazyl (DPPH) than that of the synthetic antioxidant butylhydroxytoluene (BHT), commonly used as food additive. These results show a great potential of these microalgae for exploitation in the food, feed and pharmaceutical industries.

Keywords: microalgae, amino acids, carbohydrates, radical scavenging activity, RP-HPLC.

Introduction

The 2019 Revision of World Population Prospects of United Nations estimates that world's population will exceed 9.8 billion and could peak at nearly 11 billion around 2100. This growing population will require 60 % more food by 2050 and therefore, brings additional challenges to eradicate poverty, combat hunger and malnutrition, and strengthen the coverage and quality of health. In addition, high consumption of processed food is associated with higher environmental costs and higher greenhouse gas emissions and has negative impact on health.[1] Changes in human and animal nutrition are essential to achieving several of the Sustainable Development Goals (https://sdgs.un.org/es/goals).

The ability of microalgae in mitigating CO_2 levels together with their production methods at a low cost,[2] make them a novel environmentally friendly source of bioactive compounds with potential use in human and animal healthy diets and in pharmaceutical industries.[2–3] Microalgae can play a very important role in improving nutrition and health in the future, as well as combating hunger and undernutrition due to their high levels of multifunctional compounds.[3] Several health benefits have been attributed to some macro and microalgae components such as phenolic compounds, polysaccharides, proteins, lipids, polyunsaturated fatty acids (PUFAs), and other secondary metabolites. Algal polysaccharides have been described as critically important for industrial and nutritional purposes[4] as their activities include antioxidant, antifungal, antibacterial, antiviral, antitumoral, anti-inflammatory and immunomodulatory properties, among others.[5–6] These polysaccharides derived from microalgae have been used in the food industry as fat substitute for texturizing, thickeners, stabilizers, and emulsifiers[7] and as nutraceuticals and low-sweetness humectants.[8] In addition, balanced diets in amino acids of natural origin and safe sources are strongly recommended as several diseases are linked with their lack or excess.[9] Proteins are one of the main components of microalgae, reaching up to 70 % of dry biomass and containing up

to 50% of essential amino acids, with higher antioxidant capacities compared to common proteins in human diet.[10–11] They also exhibit anti-hypertensive, immune-modulatory, anti-cancer, hepatoprotective, anti-atherosclerotic, anticoagulant, anti-UV radiation, anti-osteoporosis and anti-microbial activities.[12]

As food ingredients, amino acids glutamic and aspartic acids contribute to enhance food flavour of meat, soy sauce, seafood and some processed food.[13–14] Besides, supplementation with glutamic acid could be advantageous due to its beneficial effects in: (i) protecting intestinal function from the oxidative stress, (ii) regulating the intestinal damage cause by mycotoxins, (iii) lowering blood pressure, (iv) reducing cardiovascular disease risk, (v) increasing the efficacy and decreasing the toxicity of anticancer drugs.[15–17] Meanwhile, aspartic acid in human diet is involved in: (i) regulating the secretion of important hormones, (ii) alleviating intestinal damage, (iii) improving liver energy status, (iv) reducing the oxidative stress in the kidneys.[18–19] Dietary supplementation of proline may also be advantageous under certain physiological and pathological conditions as an important regulator of cell metabolism and physiology, affecting fetal survival, growth, and development.[20–21]

Food products incorporating microalgae improve their nutritional and textural properties.[22–25] In fact, evidences of health benefits and enhanced nutritional properties of different food formulations incorporating microalgae have been reported.[22] Gouveia et al.[23] observed an improved resistance to oxidation of food emulsions enriched with microalgal biomass of *Chlorella vulgaris* and *Haematococcus pluvialis* (up to 2.00% w/w). Recently, microalgae have also been introduced as protein resource to produce meat analogs, which are considered healthier than red meat.[24] Žugčić et al.[25] reported that beef patties prepared with microalgal proteins (with 1% *Chlorella* or with 1% *Spirulina* of 60 and 70% of purity, respectively) showed increased concentrations of all amino acids, especially aspartic and glutamic acids, which were the predominant amino acids, concluding that microalgae proteins could be useful candidates for new meat products in the food and feed industries.

On the other hand, microalgae are more nutritious than the traditional sources of animal and aquatic feed.[26] The increasing limitations in wild fish harvest and the benefits of microalgae components described above as well as the economically advantageous production are increasing the demand for microalgae-based feed in aquaculture[27] Besides, inclusion of microalgae in feed could improve animal growth and meat quality. Broiler chickens fed with the same dose (1 g/kg diet) of microalgae *Chlorella vulgaris*, *Spirulina platensis*, and *Amphora coffeaformis* for 36 days, showed a positive effect on performance and improved antioxidant status and meat quality of breast muscle.[28] Kirchgessner et al.[29] found that proline supplementation (0.35, 0.7, 1.05, 1.4, and 2.1%) of young pigs improved their daily growth rate and feed efficiency and reduced their concentrations of urea in plasma.

Microalgae components levels are strongly dependent on several factors such as culture media composition and available nitrogen[30] light photoperiod and light intensity applied during culture[31] and microalgae growth phase of harvesting, among others.[32] Therefore, manipulation of microalgae culture medium and/or conditions can increase the production of bioactive metabolites.[33] Sui et al.[31] studied the impact of two light:dark illumination cycles (12:12 and 24:0 h) on *Dunaliella salina* protein composition, concluding that continuous illumination led to higher suspended protein content, with enhanced proportion of all individual essential amino acids (up to 44% of the total protein content), while cells cultured under 12:12 h light:dark cycle showed 30% of essential amino acids. Permanent illumination stimulated the production of several essential amino acids affecting the free and total amino acid profiles of cells.

The Canary Islands are mountainous with a subtropical volcanic origin, supporting high levels of solar radiation all year round. These environmental conditions generate highly diverse habitats and ecosystems, forcing microorganisms to adapt and accumulate metabolites that might be interesting from a biotechnological approach. The three microalgae strains analysed in the present study were collected from different locations and environments and studied to investigate their potential in developing food products with high nutritional and therapeutic values. Microalgae *Spirogyra* sp., *Cosmarium* sp. and *Cosmarium blytii* were provided by the Spanish Bank of Algae. They were selected because of the wide variety of reported activities for these strains collected from other regions.[34–35] Besides, they showed high values for growth and biomass productivities in previous studies performed by the Spanish Bank of Algae in order to select new species with possibilities from the biotechnological point of view. For this purpose, microalgae extracts were screened for their antioxidant activities by inhibition of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical and compared with those of

synthetic pure compounds with known antioxidant activities and widely used as food additives (butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT)).[36] The total carbohydrates content was determined by the anthrone method, and amino acids proline, glutamic and aspartic acids were identified and quantified using RP-HPLC. The present study contributes to consider this microalgae collection as a potential source of bioactive components for future developments in the food, feed, cosmetic and pharmaceutical industries.

Results and Discussion

The previous treatments for effective mechanical extraction of metabolites from cells and their storage[37–39] have an important impact in the quantification of the cell's components. Cell wall disruption is a necessary preliminary step to quantify the total content of each metabolite and to prevent wrong measurements. It depends on the species being investigated (including cell wall type) and their physiological state.

Visca et al.[37] compared two previous drying methods of cells for hydrolysing and extracting carbohydrates from *Scenedesmus* sp. and *Chlorella* sp. biomass: (i) cells were dried at 105 °C for 12 h; (ii) cells were freeze-dried. Under the first drying conditions, *Scenedesmus* sp. and *Chlorella* sp. showed 30.5 and 17.7 % carbohydrates, respectively. The authors found that *Scenedesmus* sp. reached much higher content of carbohydrates (58.7 and 51.8 %, depending on the extracting solvent) when freeze drying pretreatment was used and lipids were previously removed. However, the carbohydrate content of *Chlorella* sp. was not affected because a simple freeze-drying step was not enough to break the robust cell wall, which requires methods combining freeze-drying with sonication or ball-milling.[40] Kröger et al.[38] observed that freeze-drying produced cell wall damage of microalgae *Scenedesmus rubescens*, improving the extraction yields. Besides, several drying methods have also been studied by de Farias Neves et al.,[39] who confirmed that freeze-drying is the most suitable microalgae drying method without bioactive compounds loss.

In this study, cells were freeze-dried and the cell wall rupture was confirmed by making observations with microscope to ensure accurate quantification of metabolites.[41]

RSA of Microalgae Extracts

DPPH free radical scavenging assay was used for screening the antioxidant activity of samples (microalgae extracts and pure compounds). Methanol was selected as extracting solvent because previous studies performed in our laboratory showed the highest levels of natural antioxidants such as phenolic compounds associated to this solvent in the extraction of algal material.[42] According to the currently food additive regulations, synthetic antioxidants BHA and BHT are lawful for using individually or in combination as preservatives in many food at a maximum level of 0.02 %, or 200 ppm.[43] Therefore, this concentration was used to test their antioxidant activity. In the last decades, there has been a great interest to replace these synthetic antioxidants with additives of natural origin to prolong the shelf life of foodstuffs because BHA and BHT are classified as cancer promoters.[44] In the present study, microalgae *Cosmarium blytii* and *Spyrogira* sp. extracts exerted higher RSA than BHT (21.2, 21.1 and 14.4 % inhibition rate, respectively) but lower than BHA (33.4 %) at the permitted level (0.02 %). Results are summarized in *Figure 1*.

The antiradical activity observed in the extracts of microalgae is very important due to the deleterious role of free radicals in food and in biological systems. Antioxidants present in the extracts react with free radicals by donating an electron or hydrogen radical to reduce them to a stable molecule or a less reactive radical, helping to mitigate the oxidative stress.[3]

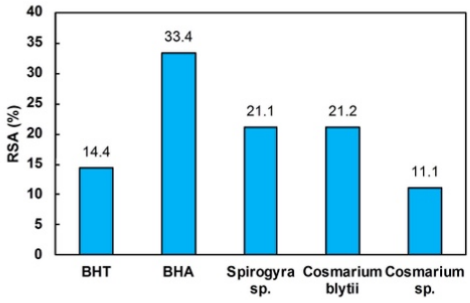


Figure 1. Radical scavenging activities (RSA) of microalgal samples and synthetic antioxidants BHT and BHA calculated as follows: RSA = 100× (1 − absorbance in the presence of sample/absorbance in the absence of sample) and expressed as percentage inhibition.

Carbohydrates Quantification

Acid hydrolysis conditions have an important effect in the quantification of carbohydrates, alongside the pretreatment and storage of microalgae cells. Carbohydrates were quantified using the anthrone assay.[45] They were heated with concentrated sulfuric acid producing furfural derivatives, which reacted with the anthrone reagent to yield a blue-green complex. The concentrations of carbohydrates were calculated from a calibration curve prepared with glucose as described below, and the results are shown in *Table 1*.

The extracts prepared by heating with 3 M HCl at 100 °C for 5 h yielded the highest amounts of carbohydrates, confirming that the yield of hydrolysis of microalgae biomass is highly dependent on temperature, time and acid concentration. In these conditions, the glycosidic bonds that maintain monosaccharides joined as polysaccharides are broken and the concentration of carbohydrates increases. These results are consistent with previous finding,[46] where drastic hydrolysis conditions (3 M H_2SO_4, 4 h, 100 °C) were required to quantify total monosaccharide content of flaxseed polysaccharides, and milder conditions (0.2 M H_2SO_4, 28 h, 80 °C) gave much lower rate of polysaccharides degradation.

Cosmarium sp. yielded the highest content of carbohydrates followed by *Cosmarium blytii* and *Spirogyra* sp. (43.2, 41.8 and 35.9%, respectively). Our results also align well with those reported by Mutripah et al.,[47] who hydrolysed the samples in an autoclave at 121 °C for only 15 min with lower concentration of sulfuric acid (0.2 M), reaching up to 46.9% carbohydrates.

In this work, microalgae samples extracted with water for 1 h at room temperature showed between 19 and 22% carbohydrates. Our results agree partially with those reported by Kostas et al.,[48] who used an optimized hydrolysis protocol (11 M concentrated sulfuric acid at 37 °C for 60 min) for extracting carbohydrates from seaweed *Laminaria digitata* and *Chondrus crispus* biomass previously dried in a fan oven at 80 °C and milled (21 and 40% carbohydrates, respectively). However, Militão et al.[49] found lower amounts of carbohydrates than those found in this study, in microalgae *Pseudopediastrum boryanum* and *Scenedesmus obliquus* cultivated at three different temperatures, oven dried at 65 °C, and not subjected to acid hydrolysis. *Pseudopediastrum boryanum* cultured at 30 °C gave the highest content of total carbohydrates followed by *Scenedesmus obliquus* cultivated at 20 °C (6.17 and 3.33 mg g^{-1} of biomass, respectively).

Algal polysaccharides have shown numerous health benefits cited above to be used as functional food or nutraceuticals.[5–6,8] The high content of polysaccharides quantified in this study in samples of novel microalgae confirms their potential possibilities to be considered as novel sources of bioactive components in food, feed and biomedical applications.

Free Amino Acids Analysis

Amino acids require derivatization prior to HPLC analysis to introduce chromophores in their chemical structures in order to improve their sensitivity to UV absorption and fluorescence detection. Therefore, the reagent phenyl isothiocyanate (PITC) was used to prepare amino acids derivatives in this work. A simple, rapid, accurate and economical RP-HPLC method was developed to determine the content of glutamic and aspartic acids, and proline in freshwater microalgae extracts. Good linearity was observed in the range of concentration 20 to 800 μg mL^{-1} with regression coefficients not lower than 0.9964. Recoveries were found in the range between 101 ± 9 and 108 ± 2%, and relative standard deviations (RSD) between 0.73 and 2.31%. The amounts determined for each amino acid are given in *Table 2*.

Cosmarium blytii showed the highest content of each amino acid, being glutamic acid the most abundant (12 mg g^{-1} of dry weight). As compared to

Table 1. Total carbohydrate content expressed as mg of glucose equivalent per g of dry biomass of three freshwater microalgae.

Algae sample	Kaiser method (%)* 3 M HCl at 100 °C for 5 h	Jansen method (%)* Water for 1 h at room temperature
Spirogyra sp.	359±1 (35.9)	227.97±0.02 (22.8)
Cosmarium blytii	417.72±0.07 (41.8)	210.96±0.03 (21.1)
Cosmarium sp.	432.9±0.1 (43.3)	190.9±0.1 (19)

Results are expressed as mean ± standard deviation of three measurements. *In parentheses, results mean gram of glucose equivalent per 100 g of dry biomass.

Table 2. Free amino acids contents, expressed as mg g^{-1} of dry weight, of three freshwater microalgae extracted with aqueous methanol.

Amino acid	*Spyrogyra* sp.	*Cosmarium blytii*	*Cosmarium* sp.
Glutamic acid	1.10±0.03	12±1	2.9±0.1
Aspartic acid	2.47±0.03	8.4±0.6	0.74±0.02
Proline	0.63±0.06	3.62±0.05	0.8±0.1
Total	4.20	24.02	4.44

Results are expressed as mean ± standard deviation of three measurements.

Cosmarium sp., *Spyrogyra* sp. yielded lower amount of proline and glutamic acid but higher level of aspartic acid.

Numerous previous studies focused on the determination of total and free amino acid composition of seaweeds and microalgae showed that aspartic and glutamic acids were the most abundant (up to 26% of the free amino acid fraction).[11,50] Kolmakova and Kolmakov[51] reviewed the results available in the literature on the experimental determinations of the total content of essential and nonessential amino acids of green and diatom microalgae and cyanobacteria, concluding that the percentage composition of both types of amino acids is stable for all these microorganisms under consideration. Derrien et al.[52] studied the amino acid profile of five microalgae (*Tetraselmis suecica*, *Skeletonema costatum*, *Chaetoceros calcitrans*, *Thalassiosira* sp. and *Isochrysis galbana*) showing that arginine, tyrosine, aspartic and glutamic acids were responsible for more than 60% of the total amino acid concentration in all five species. These authors reported higher amounts of aspartic acid in microalgae *Tetraselmis suecica*, *Skeletonema costatum* and *Chaetoceros costatum* (20, 11.8 and 11.4 mg g^{-1} of dry weight, respectively) than those found here, and lower amounts in microalgae *Isochrysis galbana* and *Thalassiosira* sp. (0.3 and 1.5 mg g^{-1} of dry weight, respectively). However, *Cosmarium blytii* in this study showed lower content of glutamic acid than those reported by Derrien et al.[52] for *Thalassiosira* sp., but higher content than those in the other four microalgae. Dewi et al.[53] tested six different methods for extracting glutamic acid from *Spirulina* sp., reaching contents (in mg g^{-1} of biomass) between 83.7 in fresh biomass dried at temperature below 60 °C for 10 h, and 23.5 in dried biomass macerated with 50% ethanol (1:20, w/v) at room temperature for 4 days. However, five different samples of three microalgae species, *Spirulina platensis*, *Dunaliella salina*, and *Tetraselmis suecica*, yielded lower contents of glutamic and aspartic acids than *Cosmarium blytii* here and lower content of aspartic acid than those found in this study in *Cosmarium* sp. and *Syporiga* sp.[54] Tibbets et al.[55] studied the amino acid profile of *Nannochloropsis granulate* subjected to supercritical CO_2 extraction at 70 and 90 °C. These authors found lower amounts of aspartic acid (from 0.052 to 0.093 mg g^{-1} of dry weight) and glutamic acid (from 0.812 to 1.093 mg g^{-1} of dry weight) but higher content of proline (from 11.97 to 12.25 mg g^{-1} of dry weight) than those found here.

The present investigation shows the potential of *Cosmarium blytii*, *Cosmarium* sp. and *Syporiga* sp. for application in the food and feed industry.

Conclusions

The high contents of carbohydrates and the presence of glutamic and aspartic acids, and proline in the extracts derived from green microalgae *Cosmarium blytii*, *Cosmarium* sp. and *Syporiga* sp. confirm their potential possibilities as food and feed sources in future human and animal diets. In addition, *Cosmarium blytii* and *Syporiga* sp. exhibited higher RSA than BHT, and their inclusion in feed and human food could improve their antioxidant activity and nutritional values. Our results provide sufficient evidence for further research about the viability of these microalgae in the healthy food, feed and pharmaceutical industries. This could become a revulsive for the growth of the local economy by developing new commercial and industrial activities aimed at feed production for aquaculture companies in the Canary Islands.

Experimental Section

Chemicals

Methanol and acetonitrile (HPLC grade), hydrochloric acid (37%), sodium carbonate and sodium bicarbonate (analytical grade) were purchased from Scharlab

(Barcelona, Spain). PITC (reagent grade), DPPH, BHA, BHT, by Sigma–Aldrich (St. Louis, MO, USA). Amino acids standards of analytical grade (aspartic acid, glutamic acid and proline) were provided by Merck (Darmstadt, Germany). Ultrapure water was obtained from a Milli-Q system from Millipore (Bedford, MA, USA).

Algal Material

Microalgae *Cosmarium blytii* BEA0204B, *Cosmarium* sp. BEA0208B and *Spyrogyra* sp. BEA0666B were isolated and supplied by the Spanish Bank of Algae (located in Taliarte, SE coast of Gran Canaria), where the identification by microscopy techniques and molecular methods was carried out according to their stablished procedures. This microalgae Collection is recognized by the World Intellectual Property Organization (WIPO) as one of the twelve accredited international authorities for identifying and deposit of microorganisms (algae) through the Budapest Treat. The samples were collected from several locations in Gran Canaria (Canary Islands) in March 2016: *Cosmarium blytii* (road GC 323 to El Hornillo); *Cosmarium* sp. (thirst in Lugarejos Dam) and *Spyrogyra* sp. (Barranco de Azuaje) and were grown to 2 L Erlenmeyer flasks under controlled conditions (temperature: 23 ± 2 °C; light irradiance: < 100 micromols photons $m^{-2}s^{-1}$ and photoperiod 16:8 h light:dark in Waris-H media. All screened microalgae were collected in the exponential phase of growth. Then, they were centrifuged (15 min, 8000 rpm), freeze dried and reserved in darkness until analysis.

RSA of Microalgae Extracts

RSA was determined by measuring the loss of color of radical DPPH after reaction with the extracts derived from microalgae.[56] Freeze-dried microalgae (10 mg) were mixed with methanol (1 mL) using a vortex (Vortex Ika Genius 3) for 20 min. The mixture was heated at 40 °C for 10 min and sonicated for 10 min (this step was performed twice). Then, the extracts were centrifuged for 10 min at 9000 rpm in a microcentrifuge (Thermo Scientific, Heraeus fresco 17) and filtered. The supernatant was evaporated to dryness in a rotary vacuum evaporator, the residue was resolved in methanol (100 μL) and 25 μL of this solution were mixed with DPPH solution (975 μL 0.1 mM). After 20 min incubation, absorbance was measured at 515 nm using a Shimadzu 1800 UV/VIS spectrophotometer. The inhibition percentage was calculated by application of the equation: RSA = 100 (1 – absorbance in the presence of sample/absorbance in the absence of sample). The concentration 0.2 g L^{-1} was used to test the antioxidant activity of pure compounds BHA and BHT following the same procedure described above. All assays were carried out in triplicate and the results are expressed as average values.

Carbohydrates Quantification

Freeze-dried algal material (30 mg) was extracted: (i) with 12 mL of 3 M HCl for 5 h (100 °C) for the determination of total carbohydrates according to Kaiser and Benner;[57] (ii) with water at room temperature during 1 h according to Jansen[58] with modifications. After centrifugation of each extract at 3500 rpm for 10 min, the supernatant was collected and stored at 4 °C.

The amounts of carbohydrates in the extracts were determined using the colorimetric method described by Brooks et al.[45] with modifications. Anthrone reagent was prepared fresh daily by dissolving anthrone (200 mg) in concentrated sulfuric acid (100 mL). This reagent (2 mL) was mixed with 1 mL of each sample (microalgae extracts and standard solutions), vortexed for 30 seconds and heated for 10 min in a boiling water bath. The test tubes were cooled in an ice bath for 10 min and the absorbance was recorded at 625 nm on a Shimadzu UV-1800 spectrophotometer. A standard calibration curve was prepared with solutions of glucose in the range of concentrations from 100 to 180 μg mL^{-1} Three replicates were used for the determination of carbohydrates concentrations from the regression line equation ($y = 0.0103x + 0.7353$; $R^2 = 0.9954$). The results are expressed as mg of glucose equivalent g^{-1} of dry algal biomass and as percentage of glucose per 100 g of dry biomass.

Amino Acids Determination

Free amino acids were extracted according to Sommaruga and Tartarotti[59] with modifications. Freeze-dried microalgae (400 mg) were mixed with 8 mL of aqueous methanol (20%) in a test tube and incubated in a water bath at 45 °C for 2 h. Then, the test tubes were sonicated in an ultrasonic bath (J.P. Selecta) for 10 min and centrifuged at 3500 rpm for 15 min at room temperature. The supernatant was collected and evaporated in a rotary vacuum evaporator (Eppendorf,

Concentrator plus) at 30 °C (6800 rpm). The residues were resolved in 200 μL of ultra-pure water.

Phenyl isothiocyanate (PITC) derivatization according to Vemuri[60] was performed as follows: the samples (200 μL) were mixed with 100 μL of a solution of PITC in acetonitrile (4% v/v) and 100 μL of solution of sodium bicarbonate (5% w/v) using a vortex for 1 min. Then, the mixture was heated at 40 °C in a water bath for 10 min. Later, 100 μL of 1% w/v sodium carbonate solution were added and the resulting mixture was heated again at 40 °C for 5 min. The samples were allowed to reach room temperature and finally, 400 μL of aqueous methanol (30%) were added, and the solution was filtered through a 45 μm nylon syringe to be injected into the HPLC system.

The content of glutamic and aspartic acids, and proline in freshwater microalgae cells was determined by RP-HPLC according to Shi et al.[61] with modifications. Therefore, it was used a Jasco LC-4000 HPLC equipment connected to a ChromNav software for data acquisition and provided with a PU-4180 quaternary pump, a vacuum degasser, an AS-4150 autosampler, a MD-4015 photodiode array detector and a LC-NetII interface. A reverse phase Luna-C18 (250× 4.6 mm, 5 μm) column was used with a guard column (10×4.6 mm, 5 μm) from Phenomenex and a gradient system involving two mobile phases (eluent A was ultra-pure water with 0.1 M sodium acetate buffer and eluent B was methanol) at 30 °C. The samples were injected in triplicate with a flow rate of 1.0 mL min^{-1}. The injection volume was 20 μL and the elution conditions were as follows: 0–4 min, isocratic 100% A; 4–8 min, linear gradient from 0% to 2% B; 8–17 min, linear gradient from 2% to 30% B; 17–27 min, linear gradient from 30% to 50% B; 27–37 min, linear gradient from 50% to 100% B and, finally, washing and reconditioning of the column. The detection was carried out at 254 nm. All the analyses were performed in triplicate and the results were expressed as average value ± standard deviation. Each standard was individually tested to determine its retention times (RT). The calibration curves were prepared with standard solutions of proline, and aspartic and glutamic acids at five different concentrations (from 20 to 800 μg mL^{-1}). The linearity was assessed by linear regression analysis, which was calculated by the least square method. Reproducibility was expressed as RSD of six replicate samples of each standard and the accuracy was expressed as the recovery of the standards.

Acknowledgements

This study received financial support from the ATOPFe project (CTM2017-83476-P) given by the Ministerio de Economía y Competitividad from Spain, and from the Universidad de Las Palmas de Gran Canaria (PIFULPGC-2019). The participation of Paula Santiago was funded through a PhD scholarship from the Universidad de Las Palmas de Gran Canaria (PIFULPGC-2019) to join the Ph.D. Program in Oceanography and Global Change (DOYCAG), promoted by the Institute of Oceanography and Global Change (IOCAG). The authors would like to thank the Plataforma Oceánica de Canarias – Consorcio público – ICTS (plocan.eu) for allowing us to use the chromatographic equipment.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Author Contribution Statement

Paula Santiago-Díaz has performed the acquisition and analysis of data and participated in writing the article; Milagros Rico and Argimiro Rivero have made conception and design, analysis of data and written the manuscript; Magdalena Santana-Casiano has contributed with project administration and funding acquisition, provision of resources; review and editing. All the authors have critically revised and approved the submitted manuscript.

References

[1] Food and Agricultural Organization of the United Nations (FAO), Rome, 'The Future of food and agriculture-trends and challenges', **2017**.

[2] R. C. Vieira, J. A. Medeiros, M. A. A. do Nascimento, A. K. de Souza Abud, A. Raymundo, C. E. de Farias Silva, in ' *New and Future Developments in Microbial Biotechnology and Bioengineering*', (Eds.: A. A. Rastegari, A. N. Yadav, N. Yadav), Elsevier **2020**, pp. 19–30.

[3] A. K. Koyande, K. W. Chew, K. Rambabu, Y. Tao, D. T. Chu, P. L. Show, 'Microalgae: A potential alternative to health supplementation for humans', *Food Sci. Hum Wellness* **2019**, *8*, 16–24.

[4] A. Usman, S. Khalid, A. Usman, Z. Hussain, Y. Wang, in ' *Algae Based Polymers, Blends, and Composites*', (Eds.: K. M. Zia, M. Zuber, M. Ali), Elsevier **2017**, pp. 115–153.

[5] M. F. de Jesus Raposo, A. M. M. Bernardo De Morais, R. M. S. Costa De Morais, in ' *Polysaccharides: Bioactivity and Biotechnology*', **2015**, pp. 1683–1727.
[6] S. Amna Kashif, Y. J. Hwang, J. K. Park, 'Potent biomedical applications of isolated polysaccharides from marine microalgae Tetraselmis species', *Bioprocess Biosyst. Eng.* **2018**, *41*, 1611–1620.
[7] M. Koller, A. Muhr, G. Braunegg, 'Microalgae as versatile cellular factories for valued products', *Algal Res.* **2014**, *6*, 52–63.
[8] S. Patel, A. Goyal, 'Functional oligosaccharides: Production, properties and applications', *World J. Microbiol. Biotechnol.* **2011**, *27*, 1119–1128.
[9] G. Wu, 'Amino acids: metabolism: functions, and nutrition', *Amino Acids* **2009**, *37*, 1–17.
[10] E. W. Becker, 'Micro-algae as a source of protein', *Biotechnol. Adv.* **2007**, *25*, 207–210.
[11] E. F. Vieira, C. Soares, S. Machado, M. Correia, M. J. Ramalhosa, M. T. Oliva-teles, A. Paula Carvalho, V. F. Domingues, F. Antunes, T. A. C. Oliveira, S. Morais, C. Delerue-Matos, 'Seaweeds from the Portuguese coast as a source of proteinaceous material: Total and free amino acid composition profile', *Food Chem.* **2018**, *269*, 264–275.
[12] R. Sathya, D. Mubarakali, J. Mohamedsaalis, J.-W. Kim, 'A systemic review on microalgal peptides: Bioprocess and sustainable applications', *Sustainability* **2021**, *13*, 3262–3277.
[13] B. P. Halpern, 'Glutamate and the flavor of foods', *J. Nutr.* **2000**, *130*, 910S–914S.
[14] X.-Y. You, Y. Xu, Z.-B. Huang, Y.-P. Li, 'Nonvolatile taste compounds of jiangluobo (A traditional Chinese fermented food)', *J. Food Qual.* **2010**, *33*, 477–489.
[15] A. Jennings, A. MacGregor, A. Welch, P. Chowienczyk, T. Spector, A. Cassidy, 'Amino acid intakes are inversely associated with arterial stiffness and central blood pressure in women', *J. Nutr.* **2015**, *145*, 2130–2138.
[16] S. Dutta, S. Ray, K. Nagarajan, 'Glutamic acid as anticancer agent: An overview', *Saudi Pharm. J.* **2013**, *21*, 337–343.
[17] B. Mohanty, A. Mahanty, S. Ganguly, T. V. Sankar, K. Chakraborty, A. Rangasamy, B. Paul, D. Sarma, S. Mathew, K. K. Asha, B. Behera, M. Aftabuddin, D. Debnath, P. Vijayagopal, N. Sridhar, M. S. Akhtar, N. Sahi, T. Mitra, S. Banerjee, P. Paria, D. Das, P. Das, K. K. Vijayan, P. T. Laxmanan, A. P. Sharma, 'Amino Acid Compositions of 27 Food Fishes and Their Importance in Clinical Nutrition', *J. Amino Acids* **2014**, *2014*, 1–7.
[18] P. Kang, Y. Liu, H. Zhu, S. Li, H. Shi, F. Chen, W. Leng, D. Pi, Y. Hou, D. Yi, 'The effect of aspartate on the energy metabolism in the liver of weanling pigs challenged with lipopolysaccharide', *Eur. J. Nutr.* **2015**, *54*, 581–588.
[19] S. Ichikawa, T. Gohda, M. Murakoshi, Z. Li, E. Adachi, T. Koshida, Y. Suzuki, 'Aspartic acid supplementation ameliorates symptoms of diabetic kidney disease in mice', *FEBS Open Bio* **2020**, *10*, 1122–1134.
[20] G. Wu, F. W. Bazer, S. Datta, G. A. Johnson, P. Li, M. C. Satterfield, T. E. Spencer, 'Proline metabolism in the conceptus: implications for fetal growth and development', *Amino Acids* **2008**, *35*, 691–702.
[21] G. Wu, F. W. Bazer, R. C. Burghardt, G. A. Johnson, S. W. Kim, D. A. Knabe, P. Li, X. Li, J. R. McKnight, M. C. Satterfield, T. E. Spencer, 'Proline and hydroxyproline metabolism: Implications for animal and human nutrition', *Amino Acids* **2011**, *40*, 1053–1063.
[22] M. P. Caporgno, A. Mathys, 'Trends in Microalgae Incorporation Into Innovative Food Products With Potential Health Benefits', *Front. Nutr. Sci.* **2018**, *5*, article 58 (10 pages).
[23] L. Gouveia, A. Raymundo, A. P. Batista, I. Sousa, J. Empis, 'Chlorella vulgaris and Haematococcus pluvialis biomass as coloring and antioxidant in food emulsions', *Eur. Food Res. Technol.* **2006**, *222*, 362–367.
[24] Y. Fu, T. Chen, S. H. Y. Chen, B. Liu, P. Sun, H. Sun, F. Chen, 'The potentials and challenges of using microalgae as an ingredient to produce meat analogs', *Trends Food Sci. Technol.* **2021**, *112*, 188–200.
[25] T. Žugčić, R. Abdelkebir, F. J. Barba, A. Rezek-Jambrak, F. Gálvez, S. Zamuz, D. Granato, J. M. Lorenzo, 'Effects of pulses and microalgal proteins on quality traits of beef patties', *Journal of Food Sci. Technol.* **2018**, *55*, 4544–4553.
[26] G. Dineshbabu, G. Goswami, R. Kumar, A. Sinha, D. Das, 'Microalgae-nutritious, sustainable aqua- and animal feed source', *J. Funct. Foods* **2019**, *62*, 103545–103559.
[27] S. Nagappan, P. Das, M. AbdulQuadir, M. Thaher, S. Khan, C. Mahata, H. Al-Jabri, A. K. Vatland, G. Kumar, 'Potential of microalgae as a sustainable feed ingredient for aquaculture', *J. Biotechnol.* **2021**, *341*, 1–20.
[28] S. El-Bahr, S. Shousha, A. Shehab, W. Khattab, O. Ahmed-Farid, I. Sabike, O. El-Garhy, I. Albokhadaim, K. Albosadah, 'Effect of Dietary Microalgae on Growth Performance, Profiles of Amino and Fatty Acids, Antioxidant Status, and Meat Quality of Broiler Chickens', *Animal* **2020**, *10*, 761–775.
[29] V. M. Kirchgessner, J. Fickler, F. X. Roth, 'Effect of dietary proline supply on N-balance of piglets, 3: The importance of non-essential amino acids for protein retention', *J. Anim. Physiol. Anim. Nutr.* **1995**, *73*, 57–75.
[30] Y. Zhang, H. Wu, C. Yuan, T. Li, A. Li, 'Growth, biochemical composition, and photosynthetic performance of Scenedesmus acuminatus during nitrogen starvation and resupply', *J. App. Phycol.* **2019**, *31*, 2797–2809.
[31] Y. Sui, M. Muys, P. Vermeir, S. D'Adamo, S. E. Vlaeminck, 'Light regime and growth phase affect the microalgal production of protein quantity and quality with Dunaliella salina', *Bioresour. Technol.* **2019**, *275*, 145–152.
[32] E. Granum, S. Kirkvold, S. M. Myklestad, 'Cellular and extracellular production of carbohydrates and amino acids by the marine diatom Skeletonema costatum: Diel variations and effects of N depletion', *Mar. Ecol. Prog. Ser.* **2002**, *242*, 83–94.
[33] E. Curcuraci, S. Manuguerra, C. M. Messina, R. Arena, G. Renda, T. Ioannou, V. Amato, C. Hellio, F. J. Barba, A. Santulli, 'Culture Conditions Affect Antioxidant Production, Metabolism and Related Biomarkers of the Microalgae Phaeodactylum tricornutum', *Antioxidants* **2022**, *11*, 411–427.
[34] R. Challouff, R. B. Dhieb, H. Omrane, K. G. And, H. B. Ouda, 'Antibacterial, antioxidant and cytotoxic activities of extracts from the thermophilic green alga, Cosmarium sp', *Afr. J. Biotechnol.* **2012**, *11*, 14844–14849.
[35] J. Kumar, P. Dhar, A. B. Tayade, D. Gupta, O. P. Chaurasia, D. K. Upreti, K. Toppo, R. Arora, M. R. Suseela, R. B. Srivastava, 'Chemical composition and biological activities

of *trans*-Himalayan alga Spirogyra porticalis (Muell.) Cleve', *PLoS One* **2015**, *10*, e0118255–e0118278.
[36] A. Augustyniak, G. Bartosz, A. Čipak, G. Duburs, L. Horáková, W. Łuczaj, M. Majekova, A. D. Odysseos, L. Rackova, E. Skrzydlewska, M. Stefek, M. Štrosová, G. Tirzitis, P. R. Venskutonis, J. Viskupicova, P. S. Vraka, N. Žarković, 'Natural and synthetic antioxidants: An updated overview', *Free Radical Res.* **2010**, *44*, 1216–1262.
[37] A. Visca, F. di Caprio, R. Spinelli, P. Altimari, A. Cicci, G. Iaquaniello, L. Toro, F. Pagnanelli, 'Microalgae cultivation for lipids and carbohydrates production', *Chem. Eng. Trans.* **2017**, *57*, 127–132.
[38] M. Kröger, M. Klemm, M. Nelles, 'Extraction behavior of different conditioned S. Rubescens', *Energies* **2019**, *12*, 1336–1342.
[39] F. de Farias Neves, M. Demarco, G. Tribuzi, in ' *Microalgae: From Physiology to Application*', **2019**.
[40] W. A. Stirk, P. Bálint, M. Vambe, C. Lovász, Z. Molnár, J. van Staden, V. Ördög, 'Effect of cell disruption methods on the extraction of bioactive metabolites from microalgal biomass', *J. Biotechnol.* **2020**, *307*, 35–43.
[41] C. Safi, M. Charton, A. V. Ursu, C. Laroche, B. Zebib, P. Y. Pontalier, C. Vaca-Garcia, 'Release of hydro-soluble microalgal proteins using mechanical and chemical treatments', *Algal Res.* **2014**, *3*, 55–60.
[42] I. Jerez-Martel, S. García-Poza, G. Rodríguez-Martel, M. Rico, C. Afonso-Olivares, J. L. Gómez-Pinchetti, 'Phenolic profile and antioxidant activity of crude extracts from microalgae and cyanobacteria strains', *J. Food Qual.* **2017**, Article ID 2924508 (8 pages).
[43] A. Pop, B. Kiss, F. Loghin, 'Endocrine disrupting effects of butylated hydroxyanisole (BHA – E320)', *Clujul Med.* **2013**, *86*, 16–20.
[44] A. A. M. Botterweck, H. Verhagen, R. A. Goldbohm, J. Kleinjans, P. A. van den Brandt, 'Intake of butylated hydroxyanisole and butylated hydroxytoluene and stomach cancer risk: results from analyses in the Netherlands Cohort Study', *Food Chem. Toxicol.* **2000**, *38*, 599–605.
[45] J. R. Brooks, V. K. Griffin, M. W. Kattan, 'A modified method for total carbohydrate analysis of glucose syrups, maltodextrins, and other starch hydrolysis products', *Cereal Chem.* **1986**, *63*, 465–466.
[46] T. H. Emaga, N. Rabetafka, C. S. Blecker, M. Paquot, 'Kinetics of the hydrolysis of polysaccharide galacturonic acid and neutral sugars chains from flaxseed mucilage [Cinétique d'hydrolyse des chaines de sucres neutres et d'acide galacturonique des polysaccharides du mucilage de graines de lin]', *Biotechnol. Agron. Soc. Environ.* **2012**, *16*, 139–147.
[47] S. Mutripah, M. D. N. Meinita, J.-Y. Kang, G.-T. Jeong, A. B. Susanto, R. E. Prabowo, Y.-K. Hong, 'Bioethanol production from the hydrolysate of Palmaria palmata using sulfuric acid and fermentation with brewer's yeast', *J. App. Phycol.* **2014**, *26*, 687–693.
[48] E. T. Kostas, S. J. Wilkinson, D. A. White, D. J. Cook, 'Optimization of a total acid hydrolysis based protocol for the quantification of carbohydrate in macroalgae', *J. Algal Biomass Util.* **2016**, *7*, 21–36.
[49] F. P. Militão, V. O. Fernandes, K. v Bastos, A. P. Martins, P. Colepicolo, L. P. Machado, 'Nutritional value changes in response to temperature, microalgae mono and mixed cultures [Alterações no valor nutricional de microalgas em resposta a variação de temperatura, cultivo unialgal e misto]', *Acta Limnol. Bras.* **2019**, *31*, e17–e28.
[50] I. Peinado, J. Girón, G. Koutsidis, J. M. Ames, 'Chemical composition, antioxidant activity and sensory evaluation of five different species of brown edible seaweeds', *Food Res. Int.* **2014**, *66*, 36–44.
[51] A. A. Kolmakova, V. I. Kolmakov, 'Amino Acid Composition of Green Microalgae and Diatoms, Cyanobacteria, and Zooplankton (Review)', *Ecol. Physiol. Biochem. Hydrobionts* **2019**, *12*, 452–461.
[52] A. Derrien, L. J. M. Coiffard, C. Coiffard, Y. de Roeck-Holtzhauer, 'Free amino acid analysis of five microalgae', *J. App. Phycol.* **1998**, *10*, 131–134.
[53] E. N. Dewi, U. Amalia, M. Mel, 'The Effect of Different Treatments to the Amino Acid Contents of Micro Algae Spirulina sp.', *Aquat. Procedia* **2016**, *7*, 59–65.
[54] M. Herrero, E. Ibáñez, S. Fanali, A. Cifuentes, 'Quantitation of chiral amino acids from microalgae by MEKC and LIF detection', *Electrophoresis* **2007**, *28*, 2701–2709.
[55] S. M. Tibbetts, W. J. Bjornsson, P. J. McGinn, 'Biochemical composition and amino acid profiles of Nannochloropsis granulata algal biomass before and after supercritical fluid CO2 extraction at two processing temperatures', *Anim. Feed Sci. Technol.* **2015**, *204*, 62–71.
[56] V. Bondet, W. Brand-Williams, C. Berset, 'Kinetics and mechanisms of antioxidant activity using the DPPH• free radical method', *LWT Food Sci. Technol.* **1997**, *30*, 609–615.
[57] K. Kaiser, R. Benner, 'Determination of amino sugars in environmental samples with high salt content by high-performance anion-exchange chromatography and pulsed amperometric detection', *Anal. Chem.* **2000**, *72*, 2566–2572.
[58] E. Jansen, 'Simple Determination of Sugars in Cigarettes', *Journal of Analytical & Bioanalytical Techniques* **2014**, *5*, 5–7.
[59] R. Sommaruga, B. Tartarotti, 'A Note of Caution on Using Absolute Methanol Concentrations and Low Temperatures to Quantify Mycosporine-Like Amino Acids in Algae', *J. Phycol.* **2002**, *38*, 33–33.
[60] S. Vemuri, 'Analysis of Amantadine Hydrochloride-Phenyl Isothiocyanate Complex in Bulk and Pharmaceutical Dosage Forms by RP HPLC-PDA Method', *Br. J. Pharm. Res.* **2014**, *4*, 278–288.
[61] Z. Shi, H. Li, Z. Li, J. Hu, H. Zhang, 'Pre-column Derivatization RP-HPLC Determination of Amino Acids in Asparagi Radix before and after Heating Process', *IERI Procedia* **2013**, *5*, 351–356.

Received March 14, 2022
Accepted August 15, 2022

Bioactive Metabolites of Microalgae from Canary Islands for Functional Food and Feed Uses

Citation Data: Chemistry and Biodiversity, ISSN: 1612-1880, Vol: 19, Issue: 9, Page: e202200230
Publication Year: 2022

Scopus metrics

7 80th percentile
Citations in Scopus

1,68
Field-Weighted citation impact

Views count
Last updated on 19 January 2023

13
Views count 2022

13
Views count 2014-2023

PlumX metrics

Captures

21
Readers

Citations

4
Citation Indexes

Journal Citation Reports™

CHEMISTRY & BIODIVERSITY

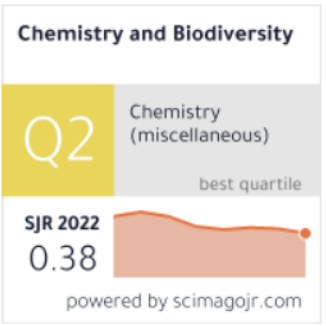
Publisher name: WILEY-V C H VERLAG GMBH

Journal Impact Factor ™

2.9
2022

2.8
Five Year

JCR Category	Category Rank	Category Quartile
BIOCHEMISTRY & MOLECULAR BIOLOGY *in SCIE edition*	192/285	Q3
CHEMISTRY, MULTIDISCIPLINARY *in SCIE edition*	97/178	Q3



4.3.2. Caracterización de nuevas especies de microalgas seleccionadas por su actividad antioxidante y contenido en polifenoles, aminoácidos y carbohidratos

En este estudio se analizaron aminoácidos y polifenoles mediante RP-HPLC-DAD en tres microalgas de agua dulce del Phylum *Chlorophyta*. Las microalgas analizadas pertenecen a las especies *Chloromonas cf. Reticulata, Pseudopediastrum boryanum* y *Chlorodium saccharophilum,* y no habían sido estudiadas anteriormente por su composición bioquímica. La metodología optimizada, junto con el análisis espectrofotométrico UV-VIS de carbohidratos totales y el ensayo de capacidad antioxidante (RSA), permitió una caracterización más completa de estas tres especies evaluando sus potencial nutricional, funcional y biomédico.

Se determinaron 10 aminoácidos tanto en su forma libre como aminoácidos totales. Para la cuantificación de los aminoácidos libres, se llevó a cabo una ruptura celular de las algas mediante shock osmótico con agua destilada seguido de un baño termostático a 70ºC y posterior baño de ultrasonidos. Con ayuda de un microscopio se aseguró la ruptura de las células. Para la determinación de los aminoácidos totales se realizó una hidrólisis ácida (HCl 6 M, 110ºC, 24h) con una neutralización posterior.

El contenido y el perfil de aminoácidos libres de las microalgas pueden cambiar drásticamente en función de las condiciones de cultivo (nitrógeno, fotoperíodo, intensidad lumínica…) e incluso en función del estado de crecimiento en el que el alga es recolectada. Las tres especies mostraron diferencias en cuanto a la cantidad de aminoácidos libres, siendo el ácido glutámico el aminoácido encontrado en mayor cantidad.

De los aminoácidos totales, sólo un 10% representan aminoácidos libres, por lo que, aunque el alga *C. saccharophilum* mostró el mayor contenido en aminoácidos libres entre las analizadas, fue la que mostró menor contenido de aminoácidos totales. Esto dependerá de los aminoácidos analizados y del método de extracción e hidrólisis. De los 10 aminoácidos totales evaluados, se encontraron grandes cantidades de aminoácidos esenciales, especialmente en *P. boryanum* y *C. saccharophilum* (72,6 y 61,2 %, respectivamente). Estos porcentajes y su equivalente en mg por gramo de material algal son comparables con el contenido en aminoácidos esenciales de productos comerciales para nutrición humana y animal.

El contenido en carbohidratos totales se encontró entre un 45,1 y un 52,7%, coincidiendo con los hallados en otras microalgas sometidas al mismo procedimiento de hidrólisis. El contenido en carbohidratos puede depender del tratamiento de extracción previo a la hidrólisis, como la liofilización o el secado. Incluso, la naturaleza del alga puede ser determinante, ya que aquellas con paredes celulares robustas precisan de métodos de ruptura celular mecánicos adicionales posteriores a la liofilización.

De los 10 compuestos fenólicos analizados, sólo en *C. saccharophilum* se detectaron todos, mientras que, en el resto, determinados polifenoles no pudieron ser detectados. De la misma forma, esta microalga obtuvo el mayor contenido de polifenoles totales (55,83 μg g^{-1} en peso seco) seguida por *C. reticulata* y *P. boryanum* (27,10 y 26,40 μg g^{-1} en peso seco, respectivamente). En este estudio, la especie de microalga con mayor contenido en polifenoles no coincide con la de mayor capacidad antioxidante (*P. boryanum*). Debe considerarse, que sólo se analizaron 10 polifenoles, obviando la presencia y acción de otros polifenoles y compuestos que también poseen capacidad antioxidante y podrían contribuir a este resultado. Por otro lado, todos los extractos de las microalgas presentaron una considerable capacidad antioxidante, siendo mayor que el hallado para el aditivo sintético BHT. Como se ha indicado anteriormente, este antioxidante sintético comúnmente empleado como antioxidante en la industria alimentaria es un posible promotor del cáncer capaz de inducir citotoxicidad y apoptosis. Por lo que, el empleo de estas especies como fuente de antioxidantes podría proponerse como un sustituto de origen natural, seguro y con un valor nutricional añadido por su contenido en aminoácidos y carbohidratos.

Además de la aplicación de dos nuevos métodos de RP-HPLC-DAD simples, sensibles y reproducibles para el análisis de aminoácidos y polifenoles, en este trabajo se ha conseguido la caracterización bioquímica de tres especies de microalgas diferentes. Las actividades antioxidantes de los extractos derivados de estas, así como sus contenidos de aminoácidos, compuestos fenólicos y carbohidratos, confirman el potencial de estas especies de microalgas como una nueva fuente de compuestos bioactivos para aplicaciones en alimentación humana y animal, así como en biomedicina.

Los resultados de esta investigación fueron publicados en la revista *Marine Drugs* en 2022.

marine drugs

MDPI

Article

Characterization of Novel Selected Microalgae for Antioxidant Activity and Polyphenols, Amino Acids, and Carbohydrates

Paula Santiago-Díaz [1,2], Argimiro Rivero [1,2], Milagros Rico [1,2,*] and Juan Luis Gómez-Pinchetti [2,3]

[1] Departamento de Química, Universidad de Las Palmas de Gran Canaria, Campus de Tafira, 35017 Las Palmas de Gran Canaria, Spain; paula.santiago@ulpgc.es (P.S.-D.); argimiro.rivero@ulpgc.es (A.R.)
[2] Instituto de Oceanografía y Cambio Global (IOCAG), Campus de Taliarte, Universidad de Las Palmas de Gran Canaria, Unidad Asociada ULPGC-CSIC, 35214 Telde, Spain; juan.gomez@ulpgc.es
[3] Banco Español de Algas (BEA), Universidad de Las Palmas de Gran Canaria, Muelle de Taliarte s/n, 35214 Telde, Spain
* Correspondence: milagros.ricosantos@ulpgc.es; Tel.: +34-928-454418

Abstract: The biochemical composition of three novel selected microalgae strains (Chlorophyta) was evaluated to confirm their potential possibilities as new sustainably produced biomass with nutritional, functional, and/or biomedical properties. Extracts from cultured *Pseudopediastrum boryanum*, *Chloromonas cf. reticulata*, and *Chloroidium saccharophilum* exhibited higher radical scavenging activity of DPPH (1,1-diphenyl-2-picrylhydrazyl) when compared to butylated hydroxytoluene (BHT), but lower than butylated hydroxyanisole (BHA). Total phenolic compounds and amino acids were determined by newly developed RP-HPLC methods. Total phenolic contents, as $\mu g\ g^{-1}$ of dry biomass, reached 27.1 for *C. cf. reticulata*, 26.4 for *P. boryanum*, and 55.8 for *C. saccharophilum*. Percentages of total analysed amino acids were 24.3, 32.1, and 18.5% of dry biomass, respectively, presenting high values for essential amino acids reaching 54.1, 72.6, and 61.2%, respectively. Glutamic acid was the most abundant free amino acid in all microalgae samples, followed by proline and lysine in *C. saccharophilum* and *P. boryanum*, and methionine and lysine in *C. reticulata*. Soluble carbohydrates in aqueous extracts ranged from 39.6 for *C. saccharophilum* to 49.3% for *C. reticulata*, increasing values to 45.1 for *C. saccharophilum* and 52.7% for *P. boryanum* in acid hydrolysates of dried biomass. Results confirmed the potential possibilities of these microalgae strains.

Keywords: amino acids; carbohydrates; microalgae; phenolic compounds; radical scavenging activity (RSA); RP-HPLC

Citation: Santiago-Díaz, P.; Rivero, A.; Rico, M.; Gómez-Pinchetti, J.L. Characterization of Novel Selected Microalgae for Antioxidant Activity and Polyphenols, Amino Acids, and Carbohydrates. *Mar. Drugs* **2022**, *20*, 40. https://doi.org/10.3390/md20010040

Academic Editor: Carlos Almeida

Received: 17 December 2021
Accepted: 29 December 2021
Published: 30 December 2021

1. Introduction

Changes in human and animal nutrition are essential, among other actions, to achieve several of the UN Sustainable Development Goals [1]. Diets rich in meat and processed foods are detrimental to health and are also associated with higher environmental costs and greenhouse gas emissions. Therefore, it is a priority to reduce their consumption in order to mitigate their negative impact on health [2].

Algae in general and microalgae in particular are described as a novel rich source of nutrients and contain natural products with several properties and applications in many industrial fields, including food, feed, cosmetics, pharmaceuticals, and biofuel production [3–5].

Polysaccharides from macro- and microalgae are considered a source of dietary fibre with bioactive properties improving the levels of blood glucose and cholesterol [6,7]. These algal polysaccharides also show other potent biological activities such as antioxidant, antifungal, antiviral, antibacterial, and antitumoral properties; tyrosinase inhibitory activity; and anti-inflammatory and immunomodulatory characteristics [6,8].

Balanced diets in amino acids of natural origin and safe sources are strongly recommended [9]. Proteins are one of the main components of microalgae, reaching up to 70%

of dry biomass in some species [10,11] and containing up to 50% essential amino acids and higher antioxidant activities than those of common proteins in the human diet [12,13]. Several microalgal peptides also exhibit antihypertensive, immunomodulatory, anticancer, hepatoprotective, antiatherosclerosis, anticoagulant, anti-UV radiation, antiosteoporosis, and antimicrobial activities [14]. Studies focused on drugs combined with glutamic acid (glutamate) confirmed an increase in their efficacy [15]. In addition, glutamic and aspartic acids contribute to enhancing (i) the flavour of meat, soy sauce, seafood, and some processed foods [16] and to (ii) protein solubility for pharmaceutical uses [17]. Dietary supplementation with proline may also be advantageous under certain physiological and pathological conditions [18].

In addition, different types of antioxidants such as phenolic compounds from seaweeds and microalgae have also been reported [19–21]. Food enrichment with microalgae is a simple and well-known method for improving the physicochemical, nutritional, and sensory properties [22]. The antioxidant capacity and phenolic content of broccoli soup increased when freeze-dried *Spirulina* sp., *Chlorella* sp., or *Tetraselmis* sp. was added at concentrations ranging from 0.5 to 2.0% (*w/v*) [23]. According to Žugčić et al. [24], beef patties prepared with microalgal proteins (1% *Chlorella* or 1% *Spirulina* of 60 and 70% purity, respectively) increased the concentrations of all amino acids, especially aspartic and glutamic acids, concluding that microalgae proteins could be useful candidates for new meat products in the food and feed industries [25].

Considering all this application potential and despite the rich biodiversity of microalgae, only a few species are exploited from a biotechnological point of view, and only 18 species of the phylum Chlorophyta are being produced in Europe [26]. The objective of this study was to evaluate the biochemical composition of three selected, not previously studied, freshwater microalgae strains *Chloromonas cf. reticulata*, *Pseudopediastrum boryanum*, and *Chloroidium saccharophilum* for their potential in developing food and feed products with high nutritional and therapeutic/functional values. For this purpose, methanol extracts obtained from laboratory-controlled cultured biomass were screened for their capacity to scavenge the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical and compared with food additives (butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT)) with known antioxidant activities [27]. Ten different phenolic compounds and ten selected amino acids were identified and quantified by newly developed RP-HPLC methods, and total soluble carbohydrate contents obtained by two different extraction protocols were also determined.

2. Results

2.1. Radical Scavenging Activity

As it is observed in Figure 1, extracts from *Pseudopediastrum boryanum* exhibited the highest capacity to scavenge free radical DPPH (30.19%), followed by *Chloroidium saccharophilum* (26.95%) and *Chloromonas cf. reticulata* (19.33%). All microalgae samples showed a higher RSA than BHT (17.37%) and lower activity than BHA (48.69%).

2.2. Identification and Quantification of Phenolic Compounds

The identification and quantification of 10 polyphenols were achieved by an updated RP-HPLC method in less than 30 min (retention times ranged from 4.69 to 25.87 min). Linearity was evaluated using the method of least squares of a plot of integrated peak area versus mean concentration from three area measurements. The correlation coefficients were not less than 0.9995. Precision was assessed using six determinations at 1 μg mL^{-1} and expressed as relative standard deviation (RSD), which ranged from 1.80 to 3.85%. The limits of detection (LOD) and the limits of quantification (LOQ) were calculated assuming a minimum detectable signal-to-noise level of 3 and 10, respectively. LOD were found to be in the range of 0.0221–0.2003 μg mL^{-1}, and the LOQ were observed in the range of 0.0736–0.6676 μg mL^{-1}. The recoveries were found in the range from 91.8 to 109.2%.

All ten analysed phenolic compounds were identified for the strain *Chloroidium saccharophilum* (Table 1). Rutin and protocatechuic, coumaric, ferulic, and gentisic acids were not detected in the extracts obtained from *Chloromonas reticulata*. *Pseudopediastrum boryanum* showed a lack of coumaric and gentisic acids. *C. saccharophilum* exhibited the highest content of phenolic compounds (55.83 μg g^{-1} of dry weight) followed by *C. reticulata* and *P. boryanum* (27.10 and 26.40 μg g^{-1} of dry weight), respectively.

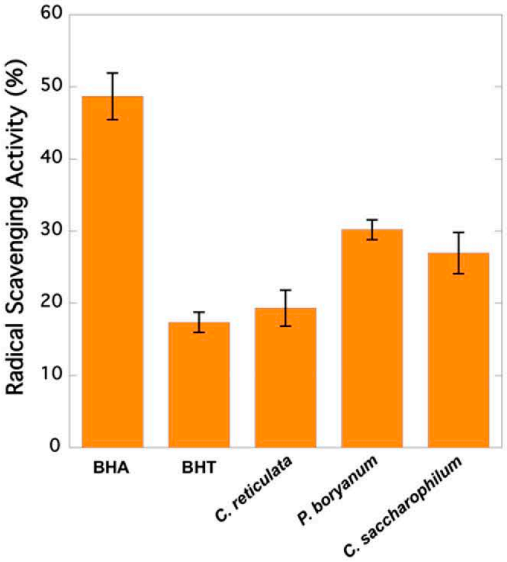


Figure 1. Radical scavenging activities (RSA) of synthetic compounds (BHA and BHT) and microalgae extracts expressed as DPPH inhibition percentage: 100 × (1 − Abs in the presence of sample/Abs in the absence of sample).

Table 1. Total polyphenol contents (μg g^{-1} of dry biomass) of the analysed microalgae strains.

Polyphenol	*Chloromonas cf. reticulata*	*Pseudopediastrum boryanum*	*Chloroidium saccharophilum*
Gallic acid	5.08 ± 0.25	1.44 ± 0.99	2.88 ± 1.22
Protocatechuic acid	n.d. *	1.23 ± 0.11	3.08 ± 1.34
Catechin	5.68 ± 0.74	4.14 ± 0.13	12.34 ± 1.13
Vanillic acid	1.61 ± 0.21	2.63 ± 0.23	14.15 ± 2.84
Epicatechin	10.43 ± 0.21	5.27 ± 0.84	1.90 ± 0.33
Syringic acid	3.12 ± 0.32	1.12 ± 0.10	5.84 ± 0.66
Rutin	n.d. *	6.40 ± 1.03	6.87 ± 3.42
Gentisic acid	n.d. *	n.d. *	2.42 ± 2.21
Coumaric acid	n.d. *	n.d. *	3.95 ± 2.09
Ferulic acid	n.d. *	2.44 ± 0.34	2.40 ± 1.67
Total	27.10 ± 2.03	26.40 ± 4.02	55.83 ± 16.90

* n.d.: not detected.

2.3. Identification and Quantification of Free and Total Analysed Amino Acids

A new RP-HPLC method was developed for detecting and quantifying 10 amino acids in microalgae extracts in less than 35 min (the retention times ranged from 6.19 to 34.87 min). Correlation coefficients were not lower than 0.9976. Precision expressed as RSD, the LODs, and the LOQs were calculated as above. RSD ranged from 1.73 to 3.88%. The percentage recoveries were from 92.4 to 101.8%. The LOD ranged from 0.0006 to 0.01 μg mL^{-1}, and

the LOQ from 0.0015 to 0.0335 μg mL^{-1}. This methodology was fast, precise, and accurate and allowed the simultaneous quantification of 10 amino acids with a high sensitivity and reproducibility.

All 10 free amino acids studied were present in all the microalgae strains (Table 2). Glutamic acid was the most abundant in all microalgae samples, ranging from 461.82 to 5630.37 μg g^{-1} of dry weight. *C. saccharophilum* exhibited a remarkably higher amount of each single amino acid compared to the other microalgae strains, reaching 20.46 mg of total free amino acids per gram of dry biomass.

Table 2. Free analysed amino acid contents (μg g^{-1} of dry weight) of the three microalgae strains.

Amino Acid	*Chloromonas cf. reticulata*	*Pseudopediastrum boryanum*	*Chloroidium saccharophilum*
Arginine	107.88 ± 1.67	448.00 ± 90.00	668.34 ± 48.16
Glutamic acid	461.82 ± 1.70	1937.00 ± 28.00	5630.37 ± 135.89
Aspartic acid	89.31 ± 0.55	157.57 ± 16.49	805.95 ± 18.82
Proline	112.99 ± 0.51	1282.00 ± 38.00	5546.42 ± 141.14
Methionine	146.00 ± 6.30	201.00 ± 77.00	805.95 ± 90.52
Valine	130.00 ± 0.38	285.00 ± 6.14	1379.65 ± 159.98
Lysine	196.84 ± 0.87	697.65 ± 52.14	2847.93 ± 157.67
Isoleucine	65.34 ± 0.08	86.03 ± 4.00	866.81 ± 15.26
Phenylalanine	107.42 ± 0.19	108.00 ± 2.00	1379.65 ± 30.57
Histidine	116.67 ± 1.22	154.00 ± 21.00	530.92 ± 38.35
Sum of amino acids in μg g^{-1} of dry weight			
∑NEEA	772.0 ± 4.43	3824.6 ± 172.5	12,651.1 ± 344.0
∑EEA	762.3 ± 9.04	1531.7 ± 162.3	7810.9 ± 492.5
∑FAA	1534.33 ± 13.5	5356.3 ± 334.8	20,462.0 ± 836.5

∑NEEA, sum of non-essential amino acids; ∑EEA, sum of essential amino acids; ∑FAA, sum of free amino acids.

Apart from values for glutamic acid, Table 2 shows that the most abundant free amino acids in *C. saccharophilum* were proline, lysine, valine, and phenylalanine; in *P. boryanum* were proline, lysine, and arginine; and in *C. reticulata* were lysine and methionine. The percentages of essential amino acids were 49.7% in *C. reticulata*, 38.2% in *C. saccharophilum*, and 28.6% in *P. boryanum* (Table 2).

Table 3 shows the total content of the ten amino acids analysed in the acid-hydrolysed microalgae extracts. Total contents of these amino acids in *P. boryanum*, *C. reticulata*, and *C. saccharophilum* were 32.06, 24.27, and 18.45% on a dry weight basis, with high percentages of the analysed essential amino acids: 72.6, 54.1, and 61.2%, respectively. Lysine was the most abundant amino acid in *C. saccharophilum* (36.49 mg g^{-1} of dry weight), proline in *C. reticulata* (51.69 mg g^{-1} of dry weight), and methionine in *P. boryanum* (137.20 mg g^{-1} of dry weight).

2.4. Carbohydrate Contents

Aqueous extraction of *C. saccharophilum, P. boryanum, and C. reticulata* biomass showed 39.64, 43.24, and 49.25% in soluble carbohydrates, respectively (Figure 2). The highest carbohydrate contents reaching 45.06, 52.67, and 51.24% were obtained when extractions were carried out under acid hydrolysis conditions.

Table 3. Contents of 10 analysed amino acids (mg g^{-1} of dry weight) of three microalgae strains.

Amino Acid	*Chloromonas cf. reticulata*	*Pseudopediastrum boryanum*	*Chloroidium saccharophilum*
Arginine	21.29 ± 3.47	7.12 ± 0.75	5.98 ± 0.66
Glutamic acid	23.84 ± 6.25	19.48 ± 1.41	30.50 ± 1.28
Aspartic acid	14.67 ± 3.59	11.38 ± 0.84	3.44 ± 0.21
Proline	51.69 ± 1.75	49.90 ± 4.25	31.68 ± 1.42
Methionine	40.81 ± 9.78	137.20 ± 24.41	29.89 ± 8.96
Valine	19.72 ± 1.79	20.21 ± 1.59	18.48 ± 0.45
Lysine	31.56 ± 1.66	36.20 ± 2.32	36.49 ± 1.99
Isoleucine	13.28 ± 1.35	13.43 ± 1.32	11.36 ± 0.71
Phenylalanine	19.76 ± 1.37	20.01 ± 1.20	14.92 ± 0.21
Histidine	6.07 ± 0.13	5.63 ± 0.69	1.81 ± 0.01
Sum of amino acids in mg g^{-1} of dry weight (%)			
∑NEEA	111.5 ± 15.1 (45.9)	87.9 ± 7.25 (27.4)	71.6 ± 3.57 (38.8)
∑EEA	131.2 ± 16.1 (54.1)	232.7 ± 30 (72.6)	112.9 ± 11.3 (61.2)
∑TAA	242.7 ± 31.1	320. 6 ± 38.1	184.5 ± 14.9

∑NEEA, sum of non-essential amino acids; ∑EEA, sum of essential amino acids; ∑TAA, sum of total amino acids.

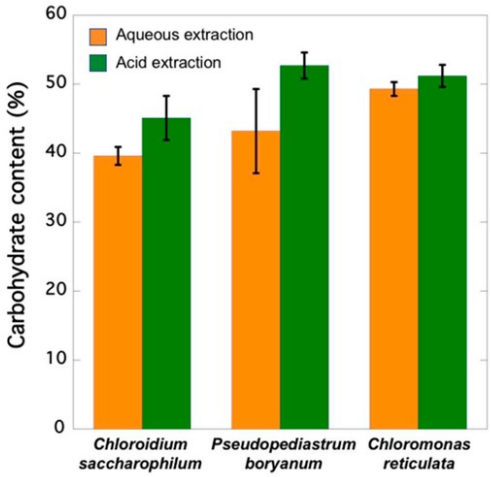


Figure 2. Carbohydrate content (as percent of glucose equivalents per dry weight) of the analysed microalgae strains.

3. Discussion

3.1. Algal Material and Extraction Procedures

The Canary Islands are mountainous with a sub-tropical volcanic origin, supporting high levels of solar radiation all year round. These environmental conditions generate highly diverse habitats and ecosystems, forcing microorganisms to adapt and accumulate metabolites that might be interesting from a biotechnological approach [28].

The selected microalgae strains analysed in the present study were bioprospected from different locations and environments and, after clonal isolation, adapted to laboratory growth conditions and BG-11 culture media before scale-up for the evaluation of growth characteristics and biomass production.

Several factors should be considered in the extraction and quantification of metabolites, such as the previous treatment of microalgae cells and storage, the hydrolysis conditions (time, temperature, and acid concentration), and the mechanical/chemical extraction methods. Cell wall disruption is a necessary preliminary step to make the cell contents

accessible and digestible and prevent incorrect measurements. The efficiency of cell disruption methods depends on the species being investigated (including cell wall type) and their physiological state. Therefore, the total rupture of the cell membrane should be confirmed by making observations with a microscope to prevent underestimation of the metabolite contents [11].

Kröger et al. [29] compared several methods for effective extraction from the microalgae *Scenedesmus rubescens*, concluding that freeze-drying produces cell wall damage and therefore improves the extraction yields. Moreover, several drying methods have also been studied by de Farias Neves et al. [30], who confirmed that freeze-drying is the most suitable microalgae drying method without bioactive compound loss. For all these reasons, in our work, biomass was freeze-dried and cells examined under a microscope to check complete cell wall breaking.

3.2. Antioxidant (RSA) Activity and Phenolic Contents

The synthetic compounds BHA and BHT are widely used as antioxidant food additives at a maximum level of 0.02% [31]. However, these compounds are classified as cancer promoters capable of inducing cytotoxicity and apoptosis [32]. Therefore, many studies have extensively studied their replacement by safer and inexpensive natural antioxidants. Phenolic compounds of natural origin exhibit antioxidant properties capable of extending food shelf life by preventing rancidity due to oxidation. They also exhibit protective effects against oxidative stress in biological systems [19].

Previous studies showed that gentisic acid, gallic acid, catequin, epicatechin, protocatechuic, and syringic acids exhibited higher antioxidant activity than BHT and BHA [33]. Catechin, epicatechin, gallic acid, and vanillic acid, among others, are better radical scavengers than many monomeric flavones and even flavonols because they act particularly well as H-atom donors [34]. The presence of these compounds in microalgae increases their potential health benefits. The microalgae species *C. saccharophilum*, *P. boryanum*, and *C. reticulata* showed a higher RSA than that of BHT (Figure 1). Gentisic and coumaric acids were not detected in the extracts derived from *P. boryanum* (Table 1), which showed the highest RSA (30.19%) and the lowest phenolic content (26.40 $\mu g\ g^{-1}$ of dry biomass). All ten phenolic compounds, gallic acid, protocatechuic acid, catechin, vanillic acid, rutin, epicatechin, syringic acid, gentisic acid, coumaric acid, and ferulic acid, were detected in *C. saccharophilum*, which exhibited the highest content of phenolic compounds, followed by *C. reticulata* and *P. boryanum* (55.83, 27.10, and 26.40 $\mu g\ g^{-1}$ of dry biomass, respectively). The lack of five analysed phenolics (rutin and coumaric, ferulic, protocatechuic, and gentisic acids) was observed in the extracts prepared with *C. reticulata*, which gave the lowest relative RSA (19.33%), but a phenolic content similar to that of *P. boryanum* (Table 1). Similar findings were reported by Corrêa da Silva et al. [35] who studied the total phenolic content and antioxidant activity of microalgae *P. boryanum* grown in different culture media. Their results also showed extracts with higher phenolic content (measured through the Folin–Ciocalteu assay) but lower antioxidant activity (assay ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)). No correlation between DPPH inhibition and phenolic contents was found by Blagojević et al. [36], who evaluated the phenolic profiles and antioxidant activities of 10 cyanobacterial strains cultured in BG11 medium in the presence and absence of nitrogen. In our study, a small group of phenolic compounds was quantified. The antioxidant response of these compounds varies remarkably depending on their chemical structure [34], and other possible antioxidants present in the mixture have not been considered, as phenolics are not the only contributors to the antioxidant activities in algae. In addition, the extracts are complex mixtures including active components at low levels, and their activities depend on the relative concentrations of these components and the interfering compounds as well as the synergistic, additive, or antagonistic effects between them [37].

Samples in this study showed a higher content of phenolic compounds than five different microalgae and cyanobacterial species evaluated by Klejdus et al. [38], who

identified eight phenolic compounds and quantified the highest content in the green microalgae *Spongiochloris spongiosa* (6.656 μg g^{-1} of dry biomass). Blagojević et al. [36] only detected 8 of 45 polyphenols investigated in several cyanobacteria species. These authors observed that cyanobacteria *Arthrospira* S1 and *Anabaena* C2 showed a lower total phenolic content (24.05 and 18.72 μg g^{-1} of biomass, respectively) than those obtained in our samples. Onofrejová et al. [39] identified 12 phenolic compounds in the freshwater microalgae *Spongiochloris spongiosa* and cyanobacterium *Anabela doliolum*, also quantifying lower contents (5.1 and 3.6 μg g^{-1}, respectively) than those determined in the present study.

3.3. Free Amino Acid Contents

In this study, 10 amino acids were selected because of their antioxidant properties and their important role in cellular metabolism as key precursors for synthesis of several metabolites [9,40–43]. Therefore, a well-balanced diet can ensure the intake needs of essential and non-essential amino acids of the body to function properly.

All microalgal samples presented the 10 free amino acids evaluated—histidine, methionine, valine, lysine, isoleucine, phenylalanine, arginine, proline, and glutamic and aspartic acids—with quantitative differences for individual compounds between the three different strains (Table 2). Glutamic acid was the most abundant, ranging from 461.8 to 5630.4 μg g^{-1} of dry biomass. These results are in accordance with those reported by Vendruscolo et al. [44], who quantified 15 amino acids in two Chlorophyceae (*Chlorella vulgaris* and *Scenedesmus obliquus*) and two cyanobacteria (*Aphanothece microscopica* and *Phormidium autumnale*), concluding that glutamic acid was the most abundant detected amino acid in three of them (*Scenedesmus obliquus* showed a higher content of alanine). Our findings also agree partially with previous studies focused on the free amino acid profile determination of seaweeds and microalgae, which confirmed that glutamic and aspartic acids were the most abundant free amino acids (up to 26% of the free amino acid fraction) [45,46].

C. saccharophilum showed the highest amount of total analysed free amino acids, reaching 20.5 mg g^{-1} of dry biomass (13.34 and 3.82 times higher than those of *C. reticulata* and *P. boryanum*, respectively). Vendruscolo et al. [44] reported lower amounts of the total sum of 15 amino acids quantified in two Chlorophyceae and two cyanobacteria (ranging from 0.371 to 1.525 mg g^{-1} of dry biomass). Machado et al. [47] determined the total sum of 20 free amino acids in four seaweed species (*Porphyra dioica*, *Porphyra umbilicalis*, *Gracilaria vermiculophylla*, and *Ulva rigida)*, which ranged from 3.36 to 16.17 mg g^{-1} of dry biomass. Their values were higher than those for *C. reticulata,* but lower than those found for *C. saccharophilum* in this study (1.53 mg and 20.46 mg g^{-1} of dry biomass, respectively).

Apart from glutamic acid, Table 2 shows that the most abundant free amino acids were proline, lysine, valine, and phenylalanine in *C. saccharophilum*; proline, lysine, and arginine in *P. boryanum*; and lysine and methionine in *C. reticulata*. On the contrary, Vendruscolo et al. [44] only detected methionine and lysine in one of the four analysed microalgae (30.55 and 8.05 μg g^{-1} of dry biomass, respectively, in *Scenedesmus obliquus*), and Machado et al. [47] only identified methionine in one of the above cited red seaweed (160 μg g^{-1} of dry biomass in *Porphyra dioica*).

Under natural conditions, the composition of free amino acids in microalgae can vary dramatically during the growing season. Kolmakova et al. [45] concluded that the composition of free amino acids of diatoms and green microalgae and cyanobacteria is extremely sensitive to external factors such as available nitrogen and light intensity and photoperiod and also depends on the growth phase of the microalgae culture [48]. According to Granum et al. [49], extracellular free amino acid contents exuded by the marine diatom *Skeletonema costatum* changed drastically from the exponential to stationary growth phase, and the cellular free amino acid levels reached values between 8% (end of scotophase) and 22% (end of photophase) of cellular organic N and decreased by 90% within 24 h of N depletion. In fact, intracellular amino acids have been proposed to be used as an index of the physiological status of the diatom *Rhizosolenia delicatula* [50].

3.4. Total Contents of Analysed Amino Acids

Kolmakova et al. [45] reported that the percentages of total essential and non-essential amino acids in diatoms and green microalgae and cyanobacteria are stable and show a common profile, with leucine as the most abundant essential amino acid, methionine and histidine as the least abundant, and glutamic and aspartic acids as the most abundant non-essential amino acids (up to 20% of the sum of amino acids). However, methionine was the most abundant amino acid in *P. boryanum* in the present study (137.2 mg g^{-1} of dry weight). Several authors have confirmed that non-essential glutamic and aspartic acids in the cell hydrolysates constituted 22–44% of the total amino acids in algae [51]. Cobos et al. [52] reported relatively similar amino acid profiles in four freshwater Chlorophyta microalgae, *Ankistrodesmus* sp., *Haematococcus* sp., *Scenedesmus* sp., and *Chlorella* sp., where aspartic acid ranged from 20.94 to 38.21 mg g^{-1} of dry weight and leucine from 20.08 to 40.99 mg g^{-1} of dry weight, and the least abundant amino acid was histidine, ranging from 4.10 to 7.24 mg g^{-1} of dry weight. The percentages of glutamic and aspartic acids in this work were 9.6, 15.9, and 18.4% in *P. boryanum*, *C. reticulata*, and *C. saccharophilum*, respectively, which would presumably decrease if a larger number of amino acids were analysed. In accordance with Cobos et al. [52] and Kolmakova et al. [45], the least abundant amino acid was histidine, ranging from 1.81 to 6.07 mg g^{-1} of dry weight (Table 3). However, methionine, proline, and lysine were the most abundant amino acids in *C. reticulata* and *P. boryanum*, while *C. saccharophilum* gave a higher content of lysine, proline, and glutamic acid. Lysine was the most abundant amino acid in *C. saccharophilum* (36.49 mg g^{-1} of dry weight) and proline in *C. reticulata* (51.69 mg g^{-1} of dry weight). Several studies have also exhibited relevant amounts of lysine and proline [11,53]. *C. saccharophilum*, which showed the highest free amino acid content, presented the lowest total content of analysed amino acids after acid hydrolysis, followed by *C. reticulata* and *P. boryanum* (184.5, 242.7, and 320.6 mg g^{-1} of dry weight, respectively). The analysis of total amino acids does not distinguish between free amino acids, which represent less than 10% of the total amino acids, and those which are bound in proteins [54]. This fact can lead to significant differences between free and total amino acid profiles. In addition, it is important to note that high total analysed amino acid values were obtained, considering that only 10 amino acids were quantified in the present study. Machado et al. [47] evaluated the presence of 20 amino acids in four macroalgae (*Porphyra dioica*, *Porphyra umbilicalis*, *Gracilaria vermiculophylla*, and *Ulva rigida*) and reported total contents of amino acids ranging from 96.22 to 286.56 mg g^{-1} of dry weight, and from 57.63 to 173.08 mg g^{-1} of dry weight if only the 10 amino acids analysed in our study were considered. These authors found fractions of free amino acids ranging from 3.15 to 7.18 g per 100 g of total amino acids and from 3.56 to 5.57% considering only our 10 amino acids. Vieira et al. [13] reported higher fractions of free amino acids (grams per 100 g of total amino acids) ranging from 6.47 to 24.0% in brown seaweed species and from 3.40 to 14.0% in red and green seaweeds. These differences may be due to the fact that the extraction of free amino acids was carried out with 0.2M perchloric acid, which could have hydrolysed peptides and increased the amount of free amino acids versus the aqueous extraction performed by Machado et al. [47]. Our results showed lower fractions of the 10 analysed free amino acids in *C. reticulata* and *P. boryanum* (0.63 and 1.67%, respectively) and a higher fraction in *C. saccharophilum* (11.1%) than those reported by Machado et al. [47].

Higher percentages of the analysed essential amino acids were found in *P. boryanum* and *C. saccharophilum* (72.6 and 61.2%, respectively) than those found by Machado et al. [46] when only the 10 amino acids evaluated in this study were considered (between 54% in *Gracilaria vermiculophylla* and 57.87% in *Ulva rigida*). *C. reticulata* showed a similar percentage of the analysed essential amino acids (54.1%). In accordance with Sui et al. [55], these high percentages could be due to the illumination cycle applied during the culture (18:6 h L:D) and the late-exponential growth phase for harvesting cells. Araya et al. [56] quantified the content of seventeen amino acids in five species of microalgae (*Haematococcus pluvialis*, *Skeletonema costatum*, *Arthrospira* sp., *Acutodesmus acuminatus*, and *Botryococcus*

braunii). Their results showed that the highest amount of amino acids (267.6 mg g^{-1}) was found in *Arthrospira* sp., and the other four species contained lower amounts than those found in this study (below 141.3 mg g^{-1} found in *Botryococcus braunii*).

Lourenço et al. [54] found different amino acid contents (as percentage of dry matter) in *Chlorella minutissima* and *Prorocentrum minimum* at the following growth phases: mid-exponential (24.79 and 24.18%, respectively), late-exponential (36.96 and 30.40%, respectively), early stationary (36.12 and 27.44%, respectively), and late-stationary (22.46 and 26.25, respectively).

On the other hand, different microalgae species show specific needs of L:D cycles and light intensity for productive photosynthesis [57]. Long illumination periods have been previously reported as a stress condition to induce the accumulation of lipids and carotenoids such as astaxanthin [58]. Sui et al. [55] studied the impact of two L:D cycles (12:12 and 24:0 h) on *Dunaliella salina* protein production, concluding that continuous illumination led to higher protein content (0.62 g L^{-1} on day 16 in the exponential phase), which decreased in the stationary phase (0.49 g L^{-1} on day 28). On the contrary, microalgae cultured under a 12:12 h L:D cycle gave a constant accumulation of proteins that reached 0.43 g L^{-1} in the stationary phase. The contents of all individual essential amino acids increased between 5% and 58% in cells cultured under an L:D cycle, reaching 30% of the total protein, and increased dramatically by 17-125% in cells cultured under continuous illumination, reaching 44% of the total protein content. Seyfabadi et al. [59] studied the behaviour of *Chorella vulgaris* incubated at 37.5, 62.5, and 100 µmol photons m^{-2} s^{-1} irradiance and 8:16, 12:12, and 16:8 h L:D photoperiods. It was confirmed that a longer illumination period increases protein contents. In fact, the cycle 16:8 h L:D yielded the highest protein accumulation under each irradiance assayed, reaching the maximum at 100 µmol photons $m^{-2}s^{-1}$ irradiance and a 16:8 h L:D cycle. The photoperiod 16:8 h L:D used in the present study also might stimulate the production of several essential amino acids.

Gorissen et al. [60] analysed the amino acid contents after the acid hydrolysis of 35 protein samples commercially available as isolated protein powder suitable for application in human nutrition or animal feeds. *P. boryanum* showed a higher total amount of six essential amino acids than several of these dietary protein samples, whose content of eight essential amino acids was quantified (oat (137), lupin (131), wheat (180), hemp (116), microalgae (157), soy (199), brown rice (221), corn (210), and egg (165), where values in parentheses mean mg g^{-1} of raw material). *C. saccharophilum* and *C. reticulata* also showed comparable contents to several protein sources analysed by Gorissen et al. [60]. The high levels of the analysed amino acids with high percentages of essential amino acids make microalgae strains in our study a novel source of essential amino acids with potential use in food and functional products.

3.5. Carbohydrate Contents

C. saccharophilum, *C. reticulata*, and *P. boryanum* showed 45.1, 51.2, and 52.7% in carbohydrates, respectively (Figure 2). These results partially agree with those reported by Schulze et al. [61] who analysed freeze-dried biomass from 46 microalgae after hydrolysis with 2N HCl for 1 h and revealed carbohydrate contents ranging from 16.5% (*Mychonastes* sp.) to 71.6% (*Porphyridium purpureum*). Templenton et al. [62] published lower amounts of carbohydrates in strains of *Phaeodactylum tricornutum*, *Nannochloropsis* sp., and *Chlorella vulgaris* (19.6, 8.6, and 20.5% of dry weight, respectively) than those observed in our study by applying the same hydrolysis conditions. This might be due to the fact that biomass was air dried before the acid hydrolysis process in their study, and we used freeze-dried biomass. Our results align well with those of Visca et al. [63], who compared two drying methods before extracting *Scenedesmus* sp. and *Chlorella* sp. biomass: (1) cells were dried at 105ºC for 12 h, and (2) cells were freeze-dried, and then both were subjected to similar hydrolysis conditions as those described in the present work. *Scenedesmus* sp. yielded 30.5% carbohydrates and reached a maximum carbohydrate purity when freeze-drying

pretreatment was used and lipids were removed (58.7% and 51.8%, depending on the extracting solvent). The authors concluded that *Chlorella* sp. carbohydrate content (17.7%) was not affected by the freeze-drying process because its cell wall is weaker. However, Safi et al. [11] reported that *Chlorella* sp. has a robust cell wall, and its disruption is a necessary preliminary step to quantify the total/maximum content of each metabolite. These findings agree with those reported by Stirk et al. [64], who observed that a simple freeze-drying step is not enough to break the tough cell wall of *Chlorella* sp., which requires methods combining freeze-drying with sonication or ball-milling.

Carbohydrate contents evaluated without methods involving previous acid hydrolysis in 12 species of seaweeds washed with tap water and air dried on blotting paper to remove excess water ranged from 20.47 to 23.9% carbohydrates [65]. Our results obtained without acid pretreatment showed higher contents than those described by Manivannan et al. [65]: *C. saccharophilum*, *P. boryanum*, and *C. reticulata* yielded 39.6, 43.2, and 49.3% carbohydrates, respectively. These results agree with the total carbohydrate content quantified in microalgae *Neochloris oleoabundans* (~40%) by Suarez Garcia et al. [66].

Further analytical studies of oligosaccharide structure and composition in the samples of novel microalgae, including the strains analysed in the present study, are necessary to unlock the potential applications of these microalgae and their components. In particular, algal polysaccharides have shown numerous industrial applications including antioxidant and antitumor effects, immunostimulating functions, cosmetics and cosmeceuticals, or prebiotic properties as functional foods or nutraceuticals [67,68].

4. Materials and Methods

4.1. Chemicals

Methanol (HPLC gradient grade) and tetrachloroethylene (synthesis grade) were purchased from Scharlab (Barcelona, Spain). Triethylamine (analysis quality) and phenylisothiocyanate (PITC) of reagent grade were supplied by Panreac (Barcelona, Spain); 1,1-diphenyl-2-picrylhydrazyl (DPPH), butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and anthrone (reagent grade) were supplied by Sigma-Aldrich (St. Louis, MO, USA). Formic acid (synthesis grade) and amino acids (aspartic acid, glutamic acid, histidine, arginine, proline, valine, lysine, methionine, isoleucine, and phenylalanine) were provided by Merck (Darmstadt, Germany). Phenolic compounds were supplied as follows: gallic acid, protocatechuic acid, (−) epicatechin, ferulic acid, *p*-coumaric acid, vanillic acid, syringic acid, and (+) catechin by Sigma–Aldrich Chemie (Steinheim, Germany), and rutin and gentisic acid by Merck (Darmstadt, Germany). Ultrapure water was obtained from a Milli-Q system from Millipore (Bedford, MA, USA).

4.2. Algal Material

Three microalgae clonal strains of the Phylum Chlorophyta were selected and provided by the Culture Collection at the Spanish Bank of Algae (located in Taliarte; east coast of Gran Canaria): *Chloromonas. cf. reticulata* (BEA0990B; Order Chlamydomonadales), *Pseudopediastrum boryanum* (BEA0190B; Order Sphaeropleales), and *Chloroidium saccharophilum* (BEA0031B; Order Watanabeales). Strains were isolated from samples bioprospected in Gran Canaria (Canary Islands), except *C. saccharophilum*, which was collected in the Chihuahua region (Mexico). *C. reticulata* was obtained from a terrestrial habitat in the central countryside area of the island, and *P. boryanum* was collected from a moist rock curtain in the northwest. Cultures were scaled up to 2 L Erlenmeyer flasks under controlled conditions (temperature: 23 ± 2 °C; irradiance: <100 µmol photons m^{-2} s^{-1}; and photoperiod 16:8 light:dark (L:D)) in BG-11 culture medium (pH adjusted to 7.4), and continuous aeration was supplied with CO_2 pulses at a rate of 1 min per hour. Growth curves were followed, and samples were harvested at the final exponential growth phase. After centrifugation at 8000 rpm for 15 min, biomass samples were freeze dried (6.5 L Labconco, Kansas City, MO, USA) and kept in sealed vials in darkness before analysis.

4.3. Radical Scavenging Activity (RSA) Measurements

Freeze-dried microalgae biomass (25 mg) was mixed with methanol (1.5 mL) using a vortex (Vortex Ika Genius 3) for 20 min. The mixture was heated at 40 °C for 10 min and sonicated for 10 min (this step was performed twice). Then, the algal material was centrifuged for 10 min at 9000 rpm in a microcentrifuge (Thermo Scientific, Heraeus fresco 17) and removed by filtration. The filtrate was evaporated to dryness in a rotary vacuum evaporator, and the residue was dissolved in methanol (100 µL).

The RSA of samples was evaluated using the DPPH free radical assay described by Bondet et al. [69] with some modifications. Briefly, 25 µL of the samples and standards BHA and BHT (0.2 g L^{-1}) was mixed with 975 µL of DPPH solution (0.1 mM). The obtained mixture was vortexed and incubated for 20 min at room temperature in darkness. The neutralization of DPPH radical leads a decrease in absorbance monitored against a methanol blank at 515 nm using a Shimadzu 1800 UV-Vis spectrophotometer. The radical inhibition percentage was calculated by application of the equation

$$\text{RSA} = 100 \times (1 - \text{Abs in the presence of sample/Abs in the absence of sample}) \quad (1)$$

Measurements were taken in triplicate, and the results were averaged.

4.4. Phenolic Compounds Determination

The extraction of phenolic compounds was carried out as described in the above section (RSA measurements) by mixing 200 mg of biomass with 10 mL of methanol. Once the filtrate was evaporated, the dry residue was suspended in 5 mL of acidified water (pH 1.5). Then, solid phase extraction (SPE) was used following the procedure of Dvořáková et al. [70] with modifications. The cartridges (Chromabon Easy, Macherey-Nagel, 500 mg, particle size 93 µm) were conditioned by successive elution with 2 mL of water, 6 mL of methanol, and 2 mL of water. The suspension was passed through the cartridge, and the retained phenolics were eluted with acetone (6 mL) and evaporated to dryness in a rotary vacuum evaporator. Finally, the residue was dissolved in 200 µL of mobile phase, filtered through a 0.20 µm nylon syringe, and transferred to a vial. Three replicates were used for the quantification.

Chromatographic analysis was performed with a Jasco LC-4000 HPLC instrument equipped with a quaternary pump (PU-4180), an autosampler (AS-4150), photodiode array detector (MD-4015), and an LC-Net interface II. Data acquisition was carried out with ChromNav software. The phenolics were separated with a Varian C18 column (250 mm × 4.6 mm, 5 µm) and a guard column maintained at 30 °C. The gradient elution was performed using water with 0.1% formic acid as mobile phase A and methanol as mobile phase B with the following elution programme for eluent A: 0–5 min, 80% isocratic; 5–30 min, linear gradient from 80% to 40%. Finally, column was washed and reconditioned. Simultaneous monitoring was set at 270 nm (gallic acid, protocatechuic acid, catechin, vanillic acid, rutin, epicatechin, and syringic acid) and 324 nm (gentisic acid, coumaric acid, and ferulic acid) for quantification (Figure 3). Five different concentrations of each compound in the range of 1 to 50 mg L^{-1} were injected in triplicate. The presence of polyphenols in the extracts was confirmed by comparison of their retention times and overlaying of UV spectra with those of individual standard compounds.

4.5. Amino Acid Composition Determination

Extractions of amino acids were performed according to Machado et al. [47] with modifications. A total of 10 mg of freeze-dried biomass was mixed with 5 mL of deionized water for 2 min. The mixture was heated in a water bath at 70 °C for 30 min and was sonicated in an ultrasonic bath (Selecta, Spain) for 5 min followed by centrifugation for 10 min at room temperature (3000 rpm). The supernatant was collected and stored at −20 °C until analysis. Samples were examined under a microscope (Olympus BX40 model) to check complete cell wall lysis.

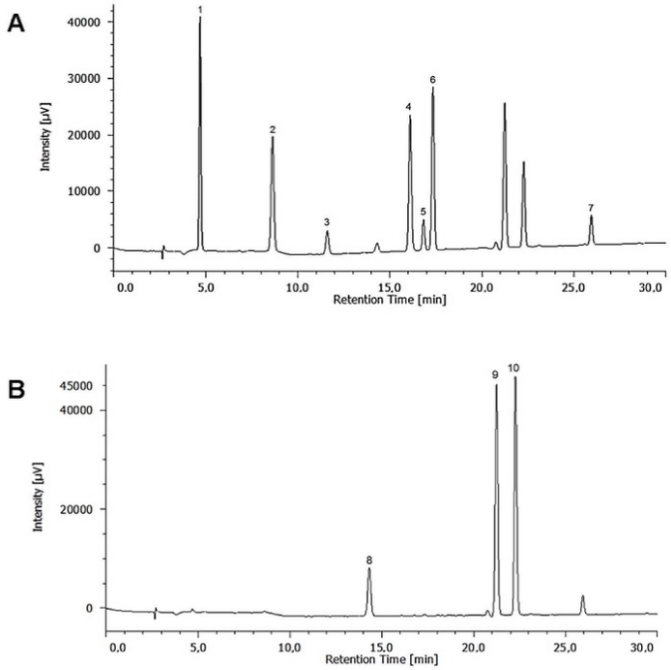


Figure 3. HPLC chromatograms of standard polyphenols: (**A**) 270 nm: 1, gallic acid; 2, protocatechuic acid; 3, catechin; 4, vanillic acid; 5, epicatechin; 6, syringic acid; 7, rutin. (**B**) 324 nm: 8, gentisic acid; 9, coumaric acid; 10, ferulic acid.

Protein hydrolysis for the total amino acid extraction was carried out as follows: HCl (6M, 2 mL) was added to 100 mg of freeze-dried microalgae in test tubes which were flushed under a N_2 stream and heated in an oven at 110 °C for 24 h. Then, the mixture was neutralised by adding NaOH (6M), and deionized water was added up to 5 mL. The extracts were stored at −20 °C until analysis.

The amino acid derivatization procedure of Shi et al. [71] was modified by adding 5 mL of sample solution to 2.5 mL of PITC (1M in acetonitrile) and 2.5 mL of triethylamine (1M in acetonitrile). The resulting solution was stirred for 1 h at room temperature. Subsequently, 5 mL of tetrachloroethylene was added, the mixture was vigorously shaken, and the upper layer was collected. This step was performed twice, and the final solution was filtered through a 0.22 µm nylon syringe filter. Three replicates were used for the analysis.

Chromatographic analysis of six essential amino acids (histidine, methionine, valine, lysine, isoleucine, and phenylalanine) and four non-essential (arginine, proline and glutamic, and aspartic acids) was carried out with a Jasco LC-4000 HPLC instrument, as described above. The amino acid derivatives were separated with a Phenomenex C18 column (250 mm × 4.6 mm, 5 µm) and a Phenomenex guard column maintained at 30 °C. The gradient elution was performed using water with 0.1% formic acid as mobile phase A and methanol as mobile phase B. The elution programme applied for eluent A was 0 min, 75%; 30 min, 40%; 40 min, 40%. Finally, column was washed and reconditioned. The flow rate was 1 mL min^{-1}, and the injection volume was 10 µL. The calibration curves were made by plotting the integrated peak areas of the samples versus the concentration. Five different concentrations of each amino acid in the range of 1 to 40 mg L^{-1} were injected in triplicate. The presence of amino acids was confirmed by comparing their retention times with those of standard compounds (Figure 4).

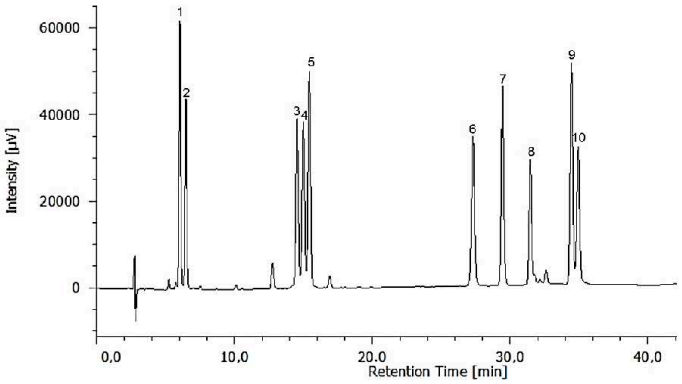


Figure 4. HPLC chromatogram of standard amino acids at 254 nm: 1, histidine; 2, arginine; 3, glutamic acid; 4, aspartic acid; 5, proline; 6, methionine; 7, valine; 8, lysine; 9, isoleucine; 10, phenylalanine.

4.6. Carbohydrates Quantification

Freeze-dried algal material was subjected to different pretreatments: (i) acid hydrolysis according to Templenton et al. [62], where the algal biomass (25 mg) was mixed with 250 µL of sulfuric acid (72 wt %) in a water bath at 30 °C for 1 h and then was heated at 121 °C in sulfuric acid (4 wt %) in an autoclave (Micro 8, JP Selecta SA); (ii) aqueous extraction was carried out by stirring the biomass (25 mg) with 2.5 mL of ultrapure water during 1 h at room temperature according to Jansen [72]. Both extracts were centrifuged (3500 rpm for 10 min) and recovered by filtration.

Carbohydrate contents were determined using the colorimetric method described by Brooks et al. [73] with modifications. Anthrone reagent was prepared fresh daily by dissolving anthrone (200 mg) in 72% sulfuric acid (100 mL). This reagent (2 mL) was mixed with 1 mL of each sample (microalgae extracts and standard solutions), vortexed for 30 s, and heated for 10 min at 100 °C in a water bath. The test tubes were cooled in an ice bath for 5 min, and the absorbance was recorded at 505 nm on a Shimadzu UV-1800 spectrophotometer. A standard calibration curve was prepared with solutions of glucose in the range of concentrations from 20 to 200 µg mL^{-1}. The results were expressed as grams of glucose equivalent (percentage of dry biomass). Three replicates were used for the determination of carbohydrate concentrations.

5. Conclusions

Two new simple, sensitive, accurate, and reproducible RP-HPLC methods were developed for detecting and quantifying 10 amino acids and 10 phenolic compounds in three novel selected microalgae strains. The antioxidant activities of extracts derived from *Chloromonas cf. reticulata*, *Psudopediastrum boryanum*, and *Chloroidium saccharophilum* determined in this study, as well as their amino acid, phenolic, and carbohydrate contents, confirm the potential possibilities of these microalgae species to be considered as novel source of bioactives for food, feed, and biomedical applications. Further research is needed to determine the impact of the growth conditions, illumination cycles during cell culture, their ability to accumulate metabolites, and, therefore, their specific potential.

Author Contributions: Conceptualization, A.R. and M.R.; Data curation, P.S.-D.; Formal analysis, P.S.-D., A.R. and M.R.; Funding acquisition, J.L.G.-P.; Investigation, P.S.-D., A.R., M.R. and J.L.G.-P.; Methodology, A.R., M.R. and J.L.G.-P.; Resources: J.L.G.-P.; Software, P.S.-D. and A.R.; Supervision, A.R.; Validation, P.S.-D. and A.R.; Visualization, P.S.-D., M.R. and J.L.G.-P.; Writing—original draft, M.R.; Writing—review and editing, P.S.-D., A.R., M.R. and J.L.G.-P. All authors have read and agreed to the published version of the manuscript.

Funding: The participation of Paula Santiago was funded through a PhD scholarship from the Universidad de Las Palmas de Gran Canaria (PIFULPGC-2019) to join the PhD Program in Oceanography and Global Change (DOYCAG), promoted by the Institute of Oceanography and Global Change (IOCAG). J.L. Gomez Pinchetti thanks the financial support of the European Territorial Cooperation Program PCT-MAC 2014-2020 through the Project REBECA-CCT (MAC/1.1.B/269) and the H2020-INFRADEV-03-2019 Project IS_MIRRI21 (No. 871129).

Data Availability Statement: The data used to support the findings of this study are included in the article.

Acknowledgments: The authors thank the Plataforma Oceánica de Canarias—Consorcio público—ICTS (plocan.eu) for allowing us to use the chromatographic equipment.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. UN General Assembly. *Transforming Our World: The 2030 Agenda for Sustainable Development*; United Nations: New York, NY, USA, 2015.
2. FAO. *The Future of Food and Agriculture-Trends and Challenges*; FAO: Rome, Italy, 2017.
3. Ördög, V.; Stirk, W.A.; Lenobel, R.; Bancířová, M.; Strnad, M.; Van Staden, J.; Szigeti, J.; Németh, L. Screening Microalgae for Some Potentially Useful Agricultural and Pharmaceutical Secondary Metabolites. *J. Appl. Phycol.* **2004**, *16*, 309–314. [CrossRef]
4. Nethravathy, M.U.; Mehar, J.G.; Mudliar, S.N.; Shekh, A.Y. Recent Advances in Microalgal Bioactives for Food, Feed, and Healthcare Products: Commercial Potential, Market Space, and Sustainability. *Compr. Rev. Food Sci. Food Saf.* **2019**, *18*, 1882–1897. [CrossRef]
5. Martínez Andrade, K.A.; Lauritano, C.; Romano, G.; Ianora, A. Marine Microalgae with Anti-Cancer Properties. *Mar. Drugs* **2018**, *16*, 165. [CrossRef] [PubMed]
6. Bauer, S.; Jin, W.; Zhang, F.; Linhardt, R.J. The Application of Seaweed Polysaccharides and Their Derived Products with Potential for the Treatment of Alzheimer's Disease. *Mar. Drugs* **2021**, *19*, 89. [CrossRef]
7. de Jesus Raposo, M.F.; de Morais, A.M.M.B.; de Morais, R.M.S.C. Emergent Sources of Prebiotics: Seaweeds and Microalgae. *Mar. Drugs* **2016**, *14*, 27. [CrossRef]
8. Amna Kashif, S.; Hwang, Y.J.; Park, J.K. Potent Biomedical Applications of Isolated Polysaccharides from Marine Microalgae *Tetraselmis* Species. *Bioprocess. Biosyst. Eng.* **2018**, *41*, 1611–1620. [CrossRef] [PubMed]
9. Wu, G. Amino Acids: Metabolism: Functions, and Nutrition. *Amino Acids* **2009**, *37*, 1–17. [CrossRef] [PubMed]
10. Koyande, A.K.; Chew, K.W.; Rambabu, K.; Tao, Y.; Chu, D.-T.; Show, P.-L. Microalgae: A Potential Alternative to Health Supplementation for Humans. *Food Sci. Hum. Wellness* **2019**, *8*, 16–24. [CrossRef]
11. Safi, C.; Charton, M.; Ursu, A.V.; Laroche, C.; Zebib, B.; Pontalier, P.Y.; Vaca-Garcia, C. Release of Hydro-Soluble Microalgal Proteins Using Mechanical and Chemical Treatments. *Algal Res.* **2014**, *3*, 55–60. [CrossRef]
12. Kazir, M.; Abuhassira, Y.; Robin, A.; Nahor, O.; Luo, J.; Israel, A.; Golberg, A.; Livney, Y.D. Extraction of Proteins from Two Marine Macroalgae, *Ulva* sp. and *Gracilaria* sp., for Food Application, and Evaluating Digestibility, Amino Acid Composition and Antioxidant Properties of the Protein Concentrates. *Food Hydrocoll.* **2019**, *87*, 194–203. [CrossRef]
13. Vieira, E.F.; Soares, C.; Machado, S.; Correia, M.; Ramalhosa, M.J.; Oliva-Teles, M.T.; Paula Carvalho, A.; Domingues, V.F.; Antunes, F.; Oliveira, T.A.C.; et al. Seaweeds from the Portuguese Coast as a Source of Proteinaceous Material: Total and Free Amino Acid Composition Profile. *Food Chem.* **2018**, *269*, 264–275. [CrossRef] [PubMed]
14. Vitali, A. Proline-Rich Peptides: Multifunctional Bioactive Molecules as New Potential Therapeutic Drugs. *Curr. Protein Pept. Sci.* **2015**, *16*, 147–162. [CrossRef]
15. Zou, C.; Brewer, M.; Cao, X.; Zang, R.; Lin, J.; Deng, Y.; Li, C. Antitumor Activity of 4-(N-Hydroxyphenyl)Retinamide Conjugated with Poly(l-Glutamic Acid) against Ovarian Cancer Xenografts. *Gynecol. Oncol.* **2007**, *107*, 441–449. [CrossRef] [PubMed]
16. You, X.-Y.; Xu, Y.; Huang, Z.-B.; Li, Y.-P. Nonvolatile Taste Compounds of Jiangluobo (A Traditional Chinese Fermented Food). *J. Food Qual.* **2010**, *33*, 477–489. [CrossRef]
17. Trevino, S.R.; Scholtz, J.M.; Pace, C.N. Amino Acid Contribution to Protein Solubility: Asp, Glu, and Ser Contribute More Favorably than the Other Hydrophilic Amino Acids in RNase Sa. *J. Mol. Biol.* **2007**, *366*, 449–460. [CrossRef] [PubMed]
18. Wu, G.; Bazer, F.W.; Burghardt, R.C.; Johnson, G.A.; Kim, S.W.; Knabe, D.A.; Li, P.; Li, X.; McKnight, J.R.; Satterfield, M.C.; et al. Proline and Hydroxyproline Metabolism: Implications for Animal and Human Nutrition. *Amino Acids* **2011**, *40*, 1053–1063. [CrossRef]
19. Sansone, C.; Brunet, C. Promises and Challenges of Microalgal Antioxidant Production. *Antioxidants* **2019**, *8*, 199. [CrossRef]
20. Rico, M.; López, A.; Santana-Casiano, J.M.; González, A.G.; González-Dávila, M. Variability of the Phenolic Profile in the Diatom *Phaeodactylum tricornutum* Growing under Copper and Iron Stress. *Limnol. Oceanogr.* **2013**, *58*, 144–152. [CrossRef]
21. Coulombier, N.; Jauffrais, T.; Lebouvier, N. Antioxidant Compounds from Microalgae: A Review. *Mar. Drugs* **2021**, *19*, 549. [CrossRef]

22. Nova, P.; Martins, A.P.; Teixeira, C.; Abreu, H.; Silva, J.G.; Silva, A.M.; Freitas, A.C.; Gomes, A.M. Foods with Microalgae and Seaweeds Fostering Consumers Health: A Review on Scientific and Market Innovations. *J. Appl. Phycol.* **2020**, *32*, 1789–1802. [CrossRef]
23. Lafarga, T.; Acién-Fernández, F.G.; Castellari, M.; Villaró, S.; Bobo, G.; Aguiló-Aguayo, I. Effect of Microalgae Incorporation on the Physicochemical, Nutritional, and Sensorial Properties of an Innovative Broccoli Soup. *LWT* **2019**, *111*, 167–174. [CrossRef]
24. Žugčić, T.; Abdelkebir, R.; Barba, F.J.; Rezek-Jambrak, A.; Gálvez, F.; Zamuz, S.; Granato, D.; Lorenzo, J.M. Effects of Pulses and Microalgal Proteins on Quality Traits of Beef Patties. *J. Food Sci. Technol.* **2018**, *55*, 4544–4553. [CrossRef] [PubMed]
25. Fu, Y.; Chen, T.; Chen, S.H.Y.; Liu, B.; Sun, P.; Sun, H.; Chen, F. The Potentials and Challenges of Using Microalgae as an Ingredient to Produce Meat Analogues. *Trends Food Sci. Technol.* **2021**, *112*, 188–200. [CrossRef]
26. Fernandes, T.; Cordeiro, N. Microalgae as Sustainable Biofactories to Produce High-Value Lipids: Biodiversity, Exploitation, and Biotechnological Applications. *Mar. Drugs* **2021**, *19*, 573. [CrossRef] [PubMed]
27. Augustyniak, A.; Bartosz, G.; Čipak, A.; Duburs, G.; Horáková, L.; Łuczaj, W.; Majekova, M.; Odysseos, A.D.; Rackova, L.; Skrzydlewska, E.; et al. Natural and Synthetic Antioxidants: An Updated Overview. *Free Radic. Res.* **2010**, *44*, 1216–1262. [CrossRef]
28. Malavasi, V.; Soru, S.; Cao, G. Extremophile Microalgae: The Potential for Biotechnological Application. *J. Phycol.* **2020**, *56*, 559–573. [CrossRef] [PubMed]
29. Kröger, M.; Klemm, M.; Nelles, M. Extraction Behavior of Different Conditioned, *S. Rubescens*. *Energies* **2019**, *12*, 1336. [CrossRef]
30. De Farias Neves, F.; Demarco, M.; Tribuzi, G. Drying and Quality of Microalgal Powders for Human Alimentation. In *Microalgae: From Physiology to Application*; InTech Open: London, UK, 2019; ISBN 978-1-83880-035-2.
31. Pop, A.; Kiss, B.; Loghin, F. Endocrine Disrupting Effects of Butylated Hydroxyanisole (BHA–E320). *Clujul Med.* **2013**, *86*, 16–20. [PubMed]
32. Saito, M.; Sakagami, H.; Fujisawa, S. Cytotoxicity and Apoptosis Induction by Butylated Hydroxyanisole (BHA) and Butylated Hydroxytoluene (BHT). *Anticancer Res.* **2003**, *23*, 4693–4701. [PubMed]
33. Jerez-Martel, I.; García-Poza, S.; Rodríguez-Martel, G.; Rico, M.; Afonso-Olivares, C.; Gómez-Pinchetti, J.L. Phenolic Profile and Antioxidant Activity of Crude Extracts from Microalgae and Cyanobacteria Strains. *J. Food Qual.* **2017**, *2017*, 2924508. [CrossRef]
34. Quideau, S.; Deffieux, D.; Douat-Casassus, C.; Pouységu, L. Plant Polyphenols: Chemical Properties, Biological Activities, and Synthesis. *Angew. Chem. Int. Ed.* **2011**, *50*, 586–621. [CrossRef] [PubMed]
35. Corrêa da Silva, M.G.; Pires Ferreira, S.; Dora, C.L.; Hort, M.A.; Giroldo, D.; Prates, D.F.; Radmann, E.M.; Bemvenuti, R.H.; Costa, J.A.V.; Badiale-Furlong, E.; et al. Phenolic Compounds and Antioxidant Capacity of *Pediastrum boryanum* (Chlorococcales) Biomass. *Int. J. Environ. Health Res.* **2020**, 1–13. [CrossRef]
36. Blagojević, D.; Babić, O.; Rašeta, M.; Šibul, F.; Janjušević, L.; Simeunović, J. Antioxidant Activity and Phenolic Profile in Filamentous Cyanobacteria: The Impact of Nitrogen. *J. Appl. Phycol.* **2018**, *30*, 2337–2346. [CrossRef]
37. Jacobo-Velázquez, D.A.; Cisneros-Zevallos, L. Correlations of Antioxidant Activity against Phenolic Content Revisited: A New Approach in Data Analysis for Food and Medicinal Plants. *J. Food Sci.* **2009**, *74*, R107–R113. [CrossRef] [PubMed]
38. Klejdus, B.; Kopecký, J.; Benešová, L.; Vacek, J. Solid-Phase/Supercritical-Fluid Extraction for Liquid Chromatography of Phenolic Compounds in Freshwater Microalgae and Selected Cyanobacterial Species. *J. Chromatogr. A* **2009**, *1216*, 763–771. [CrossRef]
39. Onofrejová, L.; Vašíčková, J.; Klejdus, B.; Stratil, P.; Mišurcová, L.; Kráčmar, S.; Kopecký, J.; Vacek, J. Bioactive Phenols in Algae: The Application of Pressurized-Liquid and Solid-Phase Extraction Techniques. *J. Pharm. Biomed. Anal.* **2010**, *51*, 464–470. [CrossRef]
40. Hwang, H.-S.; Winkler-Moser, J.K.; Liu, S.X. Study on Antioxidant Activity of Amino Acids at Frying Temperatures and Their Interaction with Rosemary Extract, Green Tea Extract, and Ascorbic Acid. *J. Food Sci.* **2019**, *84*, 3614–3623. [CrossRef]
41. Marquis, V.; Smirnova, E.; Poirier, L.; Zumsteg, J.; Schweizer, F.; Reymond, P.; Heitz, T. Stress- and Pathway-Specific Impacts of Impaired Jasmonoyl-Isoleucine (JA-Ile) Catabolism on Defense Signalling and Biotic Stress Resistance. *Plant Cell Environ.* **2020**, *43*, 1558–1570. [CrossRef]
42. Rubino, J.T.; Franz, K.J. Coordination Chemistry of Copper Proteins: How Nature Handles a Toxic Cargo for Essential Function. *J. Inorg. Biochem.* **2012**, *107*, 129–143. [CrossRef]
43. Dietzen, D. Amino Acids, Peptides, and Proteins. In *Principles and Applications of Molecular Diagnostics*; Elsevier: Amsterdam, The Netherlands, 2018; pp. 345–380. ISBN 9780128160619.
44. Vendruscolo, R.G.; Facchi, M.M.X.; Maroneze, M.M.; Fagundes, M.B.; Cichoski, A.J.; Zepka, L.Q.; Barin, J.S.; Jacob-Lopes, E.; Wagner, R. Polar and Non-Polar Intracellular Compounds from Microalgae: Methods of Simultaneous Extraction, Gas Chromatography Determination and Comparative Analysis. *Food Res. Int.* **2018**, *109*, 204–212. [CrossRef]
45. Kolmakova, A.A.; Kolmakov, V.I. Amino Acid Composition of Green Microalgae and Diatoms, Cyanobacteria, and Zooplankton (Review). *Ecol. Physiol. Biochem. Hydrobionts* **2019**, *12*, 452–461. [CrossRef]
46. Peinado, I.; Girón, J.; Koutsidis, G.; Ames, J.M. Chemical Composition, Antioxidant Activity and Sensory Evaluation of Five Different Species of Brown Edible Seaweeds. *Food Res. Int.* **2014**, *66*, 36–44. [CrossRef]
47. Machado, M.; Machado, S.; Pimentel, F.B.; Freitas, V.; Alves, R.C.; Oliveira, M.B.P.P. Amino Acid Profile and Protein Quality Assessment of Macroalgae Produced in an Integrated Multi-Trophic Aquaculture System. *Foods* **2020**, *9*, 1382. [CrossRef]
48. Paliwal, C.; Mitra, M.; Bhayani, K.; Bharadwaj, S.V.V.; Ghosh, T.; Dubey, S.; Mishra, S. Abiotic Stresses as Tools for Metabolites in Microalgae. *Bioresour. Technol.* **2017**, *244*, 1216–1226. [CrossRef]

49. Granum, E.; Kirkvold, S.; Myklestad, S.M. Cellular and Extracellular Production of Carbohydrates and Amino Acids by the Marine Diatom *Skeletonema costatum*: Diel Variations and Effects of N Depletion. *Mar. Ecol. Prog. Ser.* **2002**, *242*, 83–94. [CrossRef]
50. Martin-Jézéquel, V.; Sournia, A.; Birrien, J.-L. A Daily Study of the Diatom Spring Bloom at Roscoff (France) in 1985. III. Free Amino Acids Composition Studied by HPLC Analysis. *J. Plankton Res.* **1992**, *14*, 409–421. [CrossRef]
51. Fleurence, J. Seaweed Proteins: Biochemical, Nutritional Aspects and Potential Uses. *Trends Food Sci. Technol.* **1999**, *10*, 25–28. [CrossRef]
52. Cobos, M.; Pérez, S.; Braga, J.; Vargas-Arana, G.; Flores, L.; Paredes, J.D.; Maddox, J.D.; Marapara, J.L.; Castro, J.C. Nutritional Evaluation and Human Health-Promoting Potential of Compounds Biosynthesized by Native Microalgae from the Peruvian Amazon. *World J. Microbiol. Biotechnol.* **2020**, *36*, 121. [CrossRef] [PubMed]
53. Biancarosa, I.; Espe, M.; Bruckner, C.G.; Heesch, S.; Liland, N.; Waagbø, R.; Torstensen, B.; Lock, E.J. Amino Acid Composition, Protein Content, and Nitrogen-to-Protein Conversion Factors of 21 Seaweed Species from Norwegian Waters. *J. Appl. Phycol.* **2017**, *29*, 1001–1009. [CrossRef]
54. Lourenço, S.O.; Barbarino, E.; Lavín, P.L.; Lanfer Marquez, U.M.; Aidar, E. Distribution of Intracellular Nitrogen in Marine Microalgae: Calculation of New Nitrogen-to-Protein Conversion Factors. *Eur. J. Phycol.* **2004**, *39*, 17–32. [CrossRef]
55. Sui, Y.; Muys, M.; Vermeir, P.; D'Adamo, S.; Vlaeminck, S.E. Light Regime and Growth Phase Affect the Microalgal Production of Protein Quantity and Quality with *Dunaliella salina*. *Bioresour. Technol.* **2019**, *275*, 145–152. [CrossRef]
56. Araya, M.; García, S.; Rengel, J.; Pizarro, S.; Álvarez, G. Determination of Free and Protein Amino Acid Content in Microalgae by HPLC-DAD with Pre-Column Derivatization and Pressure Hydrolysis. *Mar. Chem.* **2021**, *234*, 103999. [CrossRef]
57. Cheirsilp, B.; Torpee, S. Enhanced Growth and Lipid Production of Microalgae under Mixotrophic Culture Condition: Effect of Light Intensity, Glucose Concentration and Fed-Batch Cultivation. *Bioresour. Technol.* **2012**, *110*, 510–516. [CrossRef]
58. Wong, Y. Effects of Light Intensity, Illumination Cycles on Microalgae *Haematococcus pluvialis* for Production of Astaxanthin. *J. Mar. Biol. Aquac.* **2016**, *2*, 1–6. [CrossRef]
59. Seyfabadi, J.; Ramezanpour, Z.; Khoeyi, Z.A. Protein, Fatty Acid, and Pigment Content of Chlorella Vulgaris under Different Light Regimes. *J. Appl. Phycol.* **2011**, *23*, 721–726. [CrossRef]
60. Gorissen, S.H.M.; Crombag, J.J.R.; Senden, J.M.G.; Waterval, W.A.H.; Bierau, J.; Verdijk, L.B.; van Loon, L.J.C. Protein Content and Amino Acid Composition of Commercially Available Plant-Based Protein Isolates. *Amino Acids* **2018**, *50*, 1685–1695. [CrossRef]
61. Schulze, C.; Strehle, A.; Merdivan, S.; Mundt, S. Carbohydrates in Microalgae: Comparative Determination by TLC, LC-MS without Derivatization, and the Photometric Thymol-Sulfuric Acid Method. *Algal Res.* **2017**, *25*, 372–380. [CrossRef]
62. Templeton, D.W.; Quinn, M.; Van Wychen, S.; Hyman, D.; Laurens, L.M.L. Separation and Quantification of Microalgal Carbohydrates. *J. Chromatogr. A* **2012**, *1270*, 225–234. [CrossRef] [PubMed]
63. Visca, A.; Di Caprio, F.; Spinelli, R.; Altimari, P.; Cicci, A.; Iaquaniello, G.; Toro, L.; Pagnanelli, F. Microalgae Cultivation for Lipids and Carbohydrates Production. *Chem. Eng. Trans.* **2017**, *57*, 127–132. [CrossRef]
64. Stirk, W.A.; Bálint, P.; Vambe, M.; Lovász, C.; Molnár, Z.; van Staden, J.; Ördög, V. Effect of Cell Disruption Methods on the Extraction of Bioactive Metabolites from Microalgal Biomass. *J. Biotechnol.* **2020**, *307*, 35–43. [CrossRef] [PubMed]
65. Manivannan, K.; Thirumaran, G.; Devi, G.K.; Hemalatha, A.; Anantharaman, P. Biochemical Composition of Seaweeds from Mandapam Coastal Regions along Southeast Coast of India. *Am. Eurasian J. Bot.* **2008**, *1*, 32–37.
66. Suarez Garcia, E.; Suarez Ruiz, C.A.; Tilaye, T.; Eppink, M.H.M.; Wijffels, R.H.; van den Berg, C. Fractionation of Proteins and Carbohydrates from Crude Microalgae Extracts Using an Ionic Liquid Based-Aqueous Two Phase System. *Sep. Purif. Technol.* **2018**, *204*, 56–65. [CrossRef]
67. Jutur, P.P.; Nesamma, A.A.; Shaikh, K.M. Algae-Derived Marine Oligosaccharides and Their Biological Applications. *Front. Mar. Sci.* **2016**, *3*, 83. [CrossRef]
68. Costa, J.A.V.; Lucas, B.F.; Alvarenga, A.G.P.; Moreira, J.B.; de Morais, M.G. Microalgae Polysaccharides: An Overview of Production, Characterization, and Potential Applications. *Polysaccharides* **2021**, *2*, 759–772. [CrossRef]
69. Bondet, V.; Brand-Williams, W.; Berset, C. Kinetics and Mechanisms of Antioxidant Activity Using the DPPH·Free Radical Method. *LWT Food Sci. Technol.* **1997**, *30*, 609–615. [CrossRef]
70. Dvořáková, M.; Hulín, P.; Karabín, M.; Dostálek, P. Determination of Polyphenols in Beer by an Effective Method Based on Solid-Phase Extraction and High Performance Liquid Chromatography with Diode-Array Detection. *Czech J. Food Sci.* **2007**, *25*, 182–188. [CrossRef]
71. Shi, Z.; Li, H.; Li, Z.; Hu, J.; Zhang, H. Pre-Column Derivatization RP-HPLC Determination of Amino Acids in Asparagi Radix before and after Heating Process. *IERI Procedia* **2013**, *5*, 351–356. [CrossRef]
72. Jansen, E. Simple Determination of Sugars in Cigarettes. *J. Anal. Bioanal. Tech.* **2014**, *5*, 5–7. [CrossRef]
73. Brooks, J.R.; Griffin, V.K.; Kattan, M.W. A Modified Method for Total Carbohydrate Analysis of Glucose Syrups, Maltodextrins, and Other Starch Hydrolysis Products. *Cereal Chem.* **1986**, *63*, 465–466. [CrossRef]

4.3.3. Estudio del potencial nutricional y farmacéutico de *Artemisia thuscula*

El crecimiento demográfico previsto en las próximas décadas junto con el estilo de vida poco saludable que se han impuesto en los últimos años y los factores ambientales que pueden afectar negativamente a la salud, exigen nuevas fuentes nutricionales, así como nuevos enfoques preventivos. En este contexto, los compuestos bioactivos que poseen las plantas podrían emplearse tanto en nutrición como en medicina para abordar esta compleja problemática.

Artemisia thuscula comúnmente conocida como "incienso canario" es una planta endémica de las Islas Canarias empleada tradicionalmente como remedio natural diurético, hipoglucémico, antiespasmolítico, anticatarral, entre otros. La información etnofarmacológica documentada del uso tradicional de plantas medicinales puede ayudar al descubrimiento de compuestos terapéuticamente efectivos para la salud humana. El género *Artemisia* ha sido objeto de estudio y la caracterización de compuestos específicos ha dado lugar al desarrollo de nuevos fármacos. Sin embargo, su composición bioquímica no ha recibido tanta atención. Por ello, este trabajo se centra en la caracterización de metabolitos orgánicos de la especie junto con la evaluación de su capacidad antioxidante. Para una completa evaluación del potencial de la planta se analizan por separado las hojas, los tallos y las raíces.

Se lleva a cabo un análisis proximal de humedad, fibra, grasas, minerales y carbohidratos. Tanto las hojas, como los tallos y las raíces presentaron un elevado contenido en humedad y fibra. Además, el contenido en minerales (expresado como porcentaje de ceniza) se encuentra dentro del rango esperado en alimentos. La cantidad de carbohidratos hallada en todas las secciones de la planta fue menor que la determinada en el mismo género y en otras muestras vegetales. Estas características junto con su bajo contenido en grasas la convierten en una opción saludable para dietas bajas en calorías, colesterol, azúcar y sal.

Con el método optimizado para la identificación y cuantificación de 15 aminoácidos, se consigue realizar un perfil bastante completo de aminoácidos esenciales y no esenciales que componen la planta. Se lleva a cabo una cuantificación de aminoácidos totales después de una hidrólisis de las proteínas y péptidos del material vegetal. Se detectaron y cuantificaron los 15 aminoácidos totales (8 esenciales y 7 no esenciales) en todas las muestras, a excepción de la glutamina. En las hojas se observó el

mayor contenido en aminoácidos, tanto esenciales como no esenciales, seguido de los tallos y finalmente las raíces. La ratio de aminoácidos esenciales con respecto al total se encuentra dentro de los estándares que definen una proteína de alta calidad por la Organización Mundial de la Salud. Entre los aminoácidos no esenciales, los más abundantes son el ácido glutámico y el aspártico, coincidiendo con los datos observados en la bibliografía para muchas especies vegetales. Entre los aminoácidos esenciales destaca la metionina en el tallo de *A. thuscula* (12,08 mg g^{-1}), aminoácido que suele ser deficitario en dietas basadas en cereales y posee diversos beneficios para la salud. Por ello, la suplementación con aminoácidos derivados de esta especie, tanto de sus hojas, tallos y raíces, podría mejorar la calidad nutricional en alimentación humana y animal.

Se identificaron y cuantificaron 10 compuestos fenólicos y se determinó espectrofotométricamente la cantidad total de flavonoides de cada parte de la planta. Las raíces presentaron un mayor contenido en flavonoides totales en comparación con los tallos y las hojas, lo que coincide con algunas plantas herbáceas como *Justicia montana* y *Scutellaria baicalensis*. Sin embargo, en la mayoría de los estudios consultados, el contenido en flavonoides predomina en hojas, flores o semillas y no en las raíces. Los flavonoides están relacionados con los mecanismos de adaptación y tolerancia a condiciones de estrés, y con el crecimiento y desarrollo de las plantas, por lo que su acumulación en las diferentes partes dependerá de estos factores y en gran medida de las condiciones ambientales. En cuanto al perfil fenólico, este difiere entre hojas, tallos y raíces siendo la epicatequina el compuesto predominante en tallos y raíces, mientras que en las hojas es el ácido ferúlico. Tanto la epicatequina como el ácido ferúlico se encuentran relacionados con la protección de las plantas, cuyas cantidades encontradas en *A. thuscula* son comparables con las de frutas y verduras.

Los compuestos fenólicos derivados de plantas, y en concreto los encontrados en los extractos de hojas, tallo y raíz de *A. thuscula* poseen diversas propiedades bioactivas como antioxidantes, antiinflamatorios, antimicrobianos, anticancerígenos, entre otros. Los extractos fenólicos de esta planta podrían tener potencial como agentes farmacéuticos o sustitutos de aditivos sintéticos alimentarios o cosméticos.

Para la determinación de pigmentos, se empleó únicamente hojas frescas y se compararon dos métodos diferentes de extracción que permitían cuantificar diferentes tipos de pigmentos. En el análisis de clorofila a y b, la mezcla acetona:agua resultó en un

mayor rendimiento de extracción, ya que en material vegetal con gran contenido en agua como *A. thuscula* (61% humedad en hojas) la clorofila precisa de disolventes polares. En el caso de la mezcla acetona:hexano, de menor polaridad, se extraen solo parcialmente compuestos polares, por lo que su rendimiento es menor.

La ratio de clorofila a: clorofila b coincide con el hallado para el mismo género *Artemisia*, además de encontrarse licopeno en los extractos de acetona:hexano, que, junto con la presencia de β-caroteno hacen de las hojas de *A. thuscula* una fuente de pigmentos con actividades biológicas y potenciales aplicaciones para la industria textil, cosmética y de alimentación.

En este estudio se llevaron a cabo cinco ensayos *in-vitro* de análisis de capacidad antioxidante de los extractos de las diferentes partes de la planta. Los extractos de las raíces presentaron una mayor capacidad antioxidante en los ensayos RSA, FRAP y CUPRAC, y menor actividad en los ensayos de capacidad complejante de Cu(II) y Fe(II) en comparación con los tallos y las raíces. Para poder estudiar la relación entre los diferentes metabolitos analizados y la capacidad antioxidante se realizó una matriz de correlación. De estos resultados y su correlación se obtuvo que:

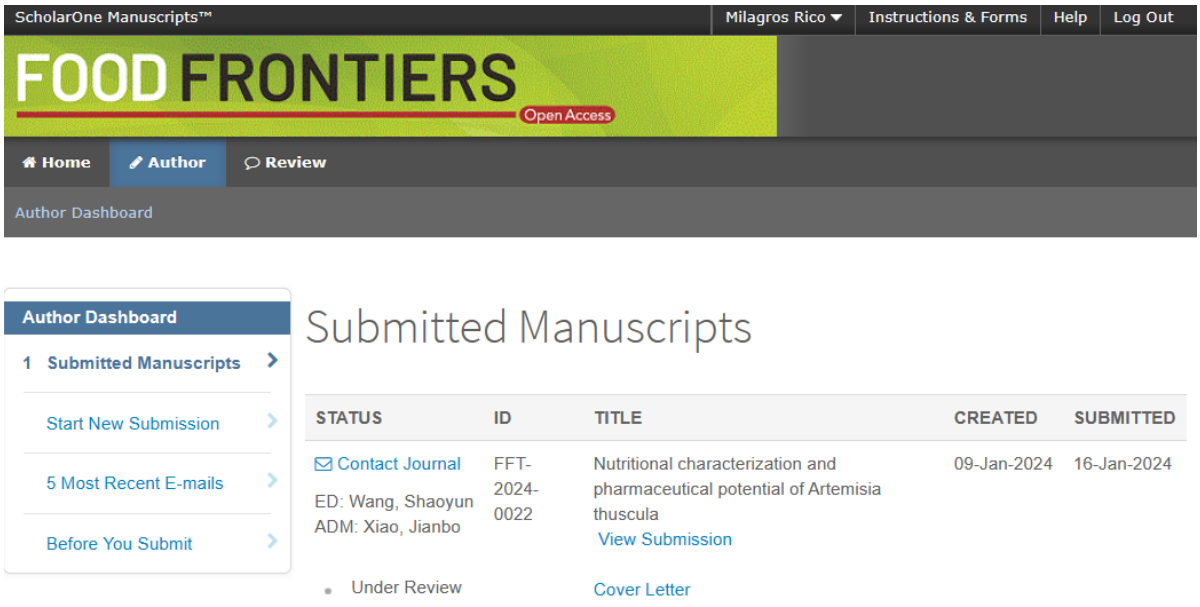
(i) Los extractos de las raíces, hojas y tallos presentan una mayor capacidad antioxidante (RSA) que el aditivo sintético BHT en todas las muestras, mientras que el extracto de las raíces fue el único con mayor capacidad que el BHA.

(ii) La actividad antioxidante evaluada por medio del ensayo RSA correlaciona significativamente ($r=0,992$ y $p<0,05$) con los flavonoides, encontrados en cantidades elevadas en las raíces de la planta.

(iii) La capacidad complejante de Fe(II) correlaciona significativamente con los polifenoles y los aminoácidos totales ($p<0,05$, $r=0,995$ y $0,994$ respectivamente).

Debido a su alto contenido en fibra y bajo en grasas y carbohidratos, la incorporación de *A. thuscula* en alimentos podría mejorar sus propiedades, como textura, consistencia y retención de agua. Además, es una fuente rica en polifenoles y aminoácidos esenciales, beneficiosos contra diversas enfermedades. Sus actividades antioxidantes en hojas, tallos y raíces muestran un gran potencial como suplementos dietéticos, así como en el desarrollo de fármacos más efectivos y seguros.

Este trabajo ha sido enviado a la revista *Food Frontiers* y se encuentra bajo revisión.


ScholarOne Manuscripts™
Milagros Rico
Instructions & Forms
Help
Log Out
FOOD FRONTIERS
Open Access
Home
Author
Review
Author Dashboard
Author Dashboard
1 Submitted Manuscripts
Start New Submission
5 Most Recent E-mails
Before You Submit
Submitted Manuscripts
STATUS
ID
TITLE
CREATED
SUBMITTED
Contact Journal
ED: Wang, Shaoyun
ADM: Xiao, Jianbo
FFT-2024-0022
Nutritional characterization and pharmaceutical potential of Artemisia thuscula
View Submission
09-Jan-2024
16-Jan-2024
Under Review
Cover Letter

Nutritional characterization and pharmaceutical potential of *Artemisia thuscula*

Composition of *Artemisia thuscula*

Milagros Rico[1,2,*], Paula Santiago-Díaz[1,2]

[1]Departamento de Química, Universidad de Las Palmas de Gran Canaria, Campus de Tafira, 35017 Las Palmas de Gran Canaria, Spain.

[2]Instituto de Oceanografía y Cambio Global (IOCAG), Universidad de Las Palmas de Gran Canaria, Unidad Asociada ULPGC-CSIC, Las Palmas de Gran Canaria, Spain.

*Corresponding author at: Departamento de Química, Facultad de Ciencias del Mar, Universidad de Las Palmas de Gran Canaria, Campus de Tafira, 35017 Las Palmas de Gran Canaria, Canary Islands, Spain.

*Corresponding author: milagros.ricosantos@ulpgc.es

Abstract

As the world's population grows, the demand for alternative nutritional and medicinal sources increases, and interest in those of plant origin is growing. The biochemical composition and antioxidant activity of leaves, stems and roots of *Artemisia thuscula* were studied. For this purpose, their proximate composition, total flavonoids and pigments were determined. In addition, the identification and quantification of 15 essential and non-essential amino acids and 10 phenolic compounds was performed by reverse-phase high performance liquid chromatography with diode array detector (RP-HPLC). The antioxidant potential was evaluated by different tests: radical scavenging activity (RSA), cupric ion reducing antioxidant capacity (CUPRAC), ferric reducing antioxidant power (FRAP), Cu^{2+} chelating activity (CuCA) and Fe^{2+} chelating activity

(FeCA). The plant material showed a high fibre and mineral content, a low fat content, and a profile rich in essential amino acids, fulfilling the requirements to be part of a plant-based nutrition. Roots were the main source of identified phenolic compounds (2.59 mg g^{-1} of dry weight), and total flavonoids quantified with $AlCl_3$ spectrophotometric method (8.47 mg g^{-1} of dry weight). All plant parts showed antioxidant potential. A significant correlation was found between RSA and flavonoid contents, while FeCA correlated with amino acids and phenolic compounds. This study provides new insights into the utilisation of different parts of *A. thuscula* plant as an accessible and inexpensive source of bioactive compounds.

KEYWORDS

Artemisia thuscula, antioxidant capacity, proximate composition, phenolics, flavonoids, pigments, amino acids

1 INTRODUCTION

As the world's population continues to grow (projected to increase from 7.6 billion to 9.7 billion by 2050), the urgency to discover and employ alternative nutritional sources becomes an imperative. Furthermore, the World Health Organization (WHO) stresses that vascular and respiratory diseases, diabetes, cancer, depression, and anxiety stem from unhealthy lifestyle, pollution, and reactive rather than preventive medicine (WHO, 2022). For this reason, plant-based nutrition guidelines have gained interest, as they are characterized by their high content of vitamins, minerals, fiber and antioxidants (Neufingerl & Eilander, 2022). Documented ethnopharmacological information on the traditional use of medicinal plants can provide information on therapeutically effective compounds for human health (Heinrich & Gibbons, 2001).

Incorporating plants with considerable water, mineral and fibre content into the human diet could help maintain adequate hydration, promote digestive health, and help regulate blood sugar and cholesterol levels (Cummings & Engineer, 2018; Soetan et al., 2010). A balanced diet rich in amino acids from natural and safe sources is also strongly recommended as they provide several health benefits: (i) improve protein synthesis, muscle growth and athletic performance; (ii) improve health problems at different life stages, such as fetal growth restriction, infertility and infections; (iii) prevent excessive fat accumulation, obesity, diabetes and cardiovascular diseases (Wu, 2009).

Phenolic compounds, including flavonoids, are secondary plant metabolites with diverse physiological functions in reproduction, growth, and nutrition (Harborne & Williams, 2000). They also have a protective effect against abiotic and biotic stresses (Naikoo et al., 2019), microbial pathogens or insects (Tak & Kumar, 2020) and a wide range of human health benefits (anti-inflammatory, antimicrobial, anticancer, reduction of cardiovascular and atherosclerosis risk, glucose regulators and neuroprotective effect) (Abdallah & Esmat, 2017; Lutz et al., 2019; Ullah et al., 2020) mainly due to their antioxidant properties. Phenolic composition and antioxidant capacity depend on the *Artemisia* species (Bogdanova et al., 2017; Carvalho et al., 2011) and have potential from a dietary and nutritional perspective. In addition, natural pigments are also powerful antioxidants with multiple health benefits (Lu et al., 2021).

Artemisia comprises a large and diverse genus of small herbs and shrubs with more than 500 species distributed worldwide. It has been studied for its wide variety of compounds with biological activities with effects on the central nervous and cardiovascular systems (Bora & Sharma, 2011). Characterization of phytochemicals specific to this genus had led to the discovery of new drugs such as the compound artemisinin with antimalarial properties (Pellicer et al., 2018). *Artemisia thuscula* (A. canariensis) is an endemic species

of the Canary Island characterized by its strong aroma, silver-grey leaves and yellow flowers. It is locally called as incienso canario and has been traditionally used as diuretic, hypoglycemic, antidiarrhoeic, uricosuric, stomachic spasmolytic, carminative, vermifuge, tranquillizer, pectoral, and anticatarrhal (Benjumea et al., 2005; Perfumi et al., 1995). However, little is known about the biochemical characterization and composition of this species and its antioxidant capacity for potential applications in medicine, nutrition, and cosmetic.

The complete characterization of the plant *A. thuscula,* leaves, stems and roots, was carried out by analyzing its proximate composition (moisture, ash, crude fibre, crude fat and total carbohydrate content) and pigments, the identification and quantification of 15 essential and non-essential amino acids and 10 phenolic compounds by reverse-phase high performance liquid chromatography with diode array detector (RP-HPLC-DAD), the determination of total flavonoid content and the evaluation of the antioxidant capacity through the application of various assays (DPPH, FRAP, CUPRAC, Cu^{2+}-chelating capacity and Fe^{2+}-chelating capacity). Finally, a correlation analysis was conducted to explore the relationship between the analyzed metabolites and antioxidant activities.

The aim of this study is to evaluate the potential of bioactive compounds and composition of different parts of *A. thuscula,* as well as its antioxidant capacity, for applications in nutrition, pharmacology, agriculture, among others. This is the first report in a full characterization of *A. thuscula* with a leaves, stems, and roots profiling of phenolics and amino acids, proximate composition analysis and antioxidant capacity determination.

2 MATERIALS AND METHODS

2.1 Chemicals

Sodium hydroxide, D-glucose, aluminium chloride, iron (II) sulphate heptahydrate, iron (III) chloride hexahydrate, triethylamine (TEA), butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) were supplied by Panreac (Barcelona, Spain). Sulfuric acid (97%), hydrochloric acid (37%) and acetone were provided by Honeywell (Charlotte, USA). Phenylisothiocyanate (PITC), anthrone, sodium nitrite, pyrocatechol violet (PV), ferrozine, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,9-dimethyl-1,10-phenanthroline (neocuproine), 2,4,6-tri(2-pyridyl)-triazine (TPTZ), 6-hidroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (Trolox), and amino acids standards were purchased from Sigma-Aldrich Chemie (Steinheim, Germany). Tetrachloroethylene (TClE) was supplied by Labkem (Barcelona, Spain), hexane by VWR (Radnor, USA) and methanol gradient grade by Scharlab (Barcelona, Spain). Ammonium acetate, copper (II) chloride were provided by Merck (Hohenbrunn, Germany).

Polyphenol standards were supplied as follows: quercetin, gallic acid, protocatechuic acid, p-coumaric acid, ferulic acid, catechin, vanillic acid, epicatechin, syringic acid, and by Sigma-Aldrich Chemie (Steinheim, Germany); rutin and gentisic acid by Merck (Darmstadt, Germany). Ultrapure water was obtained from a Milli-Q system from Millipore (Bedford, MA, USA).

2.2 Plant material

Artemisia thuscula was collected at Barranquillo Frío, 745 m altitude, Santa María de Guia, Gran Canaria, Canary Islands in February 2023. Two voucher specimens were deposited at the Herbarium of the Viera y Clavijo Botanical Garden in Las Palmas de Gran Canaria (LPA: 41174 and LPA: 41175).

2.3 Preparation of leaves, roots and stem samples

Sections of *A. thuscula* (leaves, roots and stem) were separated, air-dried, weighed and frozen. Samples were freeze-dried, ground with a blender (Moulinex, 600 W, Ecully Cedex, France) and stored in the dark at 8ºC until analysis.

2.4 Proximate composition

The determination of proximate composition of leaves, stems and roots of *A. thuscula* was carried out using the methods described by Ilodibia et al. (2016) with modifications. The results were calculated on a dry weight (dw) basis, as a weight percentage of the freeze-dried weight.

2.4.1 Determination of moisture content

The moisture content was calculated as $[W_0\text{-}W_1/W_0] \times 100$, where W_0 was the initial air-dried sample mass before freeze-drying and W_1 the final mass of dry residue after freeze-drying.

2.4.2 Determination of total ash content

The total ash content was determined by burning 100 mg of freeze-dried sample (W_0) in a muffle furnace at 550ºC in a previously weighed porcelain crucible (W_1). Once completely converted to ash, the crucible containing the sample was cooled in a desiccator and then weighed (W_2). The percentage of ash was calculated as $[W_2\text{-}W_1/W_0] \times 100$.

2.4.3 Determination of crude fibre

The crude fibre was determined by boiling 100 mg of freeze-dried sample (W_0) in 3 mL of 1.25% H_2SO_4 at reflux for 30 min, keeping the volume constant by adding hot distilled

water. The boiled mixture was centrifuged, and the supernatant was discarded. The residue was rinsed with portions of hot distilled water and boiled with 1.25% of NaOH (3 mL) at reflux for 30 min. The centrifugation and washing steps were repeated as above, and the resulting precipitate was transferred to a previously weighed crucible and dried at 105ºC in an oven until a constant weight (W_1). Then, the crucible was taken to a muffle furnace until the sample was converted to ashes (W_2). The crude fibre content was calculated according to the following formula: Crude fibre (%) = [W_1-W_2/W_0] x 100.

2.4.4 Determination of crude fat

The crude fat content was evaluated by mixing the freeze-dried sample (250 mg = W_0) with 10 mL of hexane in a flask of known mass (W_1). The resulting solution was stirred at reflux for 3 h and evaporated to dryness using a rotary evaporator. The flask with the residue was cooled in a desiccator and weighed (W_2). The percentage of crude fat was determined as follows: Fat (%) = [W_2-W_1/W_0] x 100.

2.4.5 Determination of total carbohydrates

Carbohydrates were hydrolyzed according to Templeton et al. (2012). Freeze-dried samples (25 mg) were mixed with 250 µL of sulfuric acid (72%) in a water bath at 30ºC for 1 h. Distilled water was added to a final concentration of 4 % sulfuric acid and the resulting solutions were heated for 1 h in an autoclave (120ºC). Total carbohydrate content was quantified by the anthrone method (Brooks et al., 1986) in which 1 mL of anthrone reagent (200 mg of anthrone in 100 mL of 96% H_2SO_4) reacts with 0.5 mL of each sample in a boiling water bath for 10 min. The absorbance was measured at 505 nm and a calibration curve was made from 12.5 to 150 mg L^{-1} of glucose. The results were expressed as percentage of glucose.

2.5 Quantification of amino acids

The amino acid extraction, derivatization and chromatographic analysis were performed according to Santiago-Díaz et al. (2022) as briefly described here: Freeze-dried samples (25 mg) were mixed with 2 mL of 6M HCl for 24h in an oven at 110ºC. The hydrolysates were separated by centrifugation (3000 rpm, 10 min) and filtered (0.45 um syringe filter). The resulting solution was neutralized with 6 M NaOH, derivatized with PITC (0.1 M) and TEA (1M) and kept for 1h at room temperature. Amino acid derivatives were extracted with TClE and hexane and filtered through 0.2 μm syringe filters.

The chromatographic determination was carried out with a Jasco LC-4000 HPLC equipment provided with a PU-4180 quaternary pump, an AS-4150 autosampler, an MD-4015 photodiode array detector, LC-Net ll interface, a Phenomenex C18 column (250 mm x 4.6 mm, 5 μm) and a Phenomenex guard column maintained at 30ºC.

Calibration curves were made for 15 L-amino acids (histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, valine, arginine, asparagine, aspartic acid, glutamic acid, glutamine, proline and serine) from 1 to 100 mg L^{-1}. Results were expressed as mg g^{-1} dw.

2.6 Quantification of phenolic compounds

Phenolic compounds were extracted twice from 200 mg of plant material with 10 mL of methanol by stirring for 30 min and then sonicating for 10 min. The mixture was centrifuged (3000 rpm, 15 min), the supernatants were collected, evaporated and the residue was dissolved in 2 mL of methanol and filtered through 0.2 μm syringe filters.

The quantification of 10 phenolic compounds (gallic acid, protocatechuic acid, catechin, vanillic acid, rutin, epicatechin, syringic acid, gentisic acid, coumaric acid and ferulic acid) was performed following the RP-HPLC-DAD procedure developed by Santiago-

Díaz et al. (2022) with the chromatographic equipment described above. The calibration curves ranged from 1 to 50 mg L^{-1} and the results were expressed as μg g^{-1} dw.

2.7 Quantification of flavonoids

The extraction of flavonoids was made by mixing the samples (100 mg) with methanol (3 mL). Quantification was performed following the colorimetric method reported by Chang and Kim (2018). In brief, 250 μL of the extract was mixed with distilled water (1.25 mL) and 5% $NaNO_2$ (100 μL), and stirred for 6 min. Then, 15 μL of 10% $AlCl_3$ (w/v) was added and after 5 min, 500 μL of 1 mM NaOH and distilled water (2.75 mL) were added. Quercetin was used for the calibration curve in the range of 10-1000 mg L^{-1}. The results were expressed as mg of quercetin equivalent per gram of dry weight.

2.8 Quantification of pigments

Pigments from fresh leaves of *A. thuscula* (500 mg) were extracted by sonication for 3 min in an ice-water bath using 5 mL of acetone:hexane mixture (2:3) for chlorophyll a (Chl a) and b (Chl b), β-carotene, and lycopene, or acetone-water mixture (4:1) for Chl a and b and total carotenoids. Homogenates were centrifuged at 7500 rpm for 5 minutes. The absorbance of each supernatant was measured at 453, 505, 645 and 663 nm for the first mixture, and at 663.6 nm for Chl a, 646.6 nm for Chl b and 470.0 nm for carotenoids for the second. The pigment contents were calculated from equations taken from Branisa et al. (2014) and expressed as μg per gram of fresh weight.

2.9 Evaluation of antioxidant activities

2.9.1 Radical Scavenging Activity.

Free radical scavenging activity of plant extracts was measured by radical DPPH inhibition assay (Sethi et al., 2020). Briefly, 50 µL of methanolic extracts or BHA and BHT solutions (0.2 µg L^{-1}) were mixed with 1 mL of 2.5 mM DPPH reagent. After 10 min in the dark at room temperature, the absorbance was recorded at 515 nm against a blank. The percentage of inhibition was calculated as $[A_0\text{-}A_1/A_0]$ x 100, where A_0 was the absorbance of DPPH reagent and A_1 the absorbance of the sample.

2.9.2 Ferric Reducing Antioxidant Power (FRAP).

The ferric reducing antioxidant power was determined according to Sethi et al. (2020) with modifications. Briefly, 1.5 mL of FRAP reagent (50 mL of 300 mM sodium acetate buffer at pH 3.6, 5 mL of 10 mM TPTZ and 5 mL of 20 mM $FeCl_3$) was mixed with 50 µL of sample and incubated at 37ºC for 30 min. The absorbance was recorded at 593 nm and results were expressed as µmol of reduced Fe^{3+} per gram of dry weight calculated from a calibration curve of $FeSO_4$ ranging between 0.04 and 0.80 mM.

2.9.3 Cupric Ion Reducing Antioxidant Capacity (CUPRAC).

The reducing capacity of cupric ion was evaluated following Apak et al. (2007) with modifications. CUPRAC reagent was prepared with 10 mL of 10 mM $CuCl_2$, 10 mL of 7.5 mM neocuproine and 10 mL of ammonium acetate buffer at pH 7.0. The mixture was diluted with 48.4 mL of distilled water. The assay was carried out by mixing 20 µL of sample with 980 µL of diluted CUPRAC reagent, the absorbance measured at 450 nm and the results were expressed as µmol of Trolox equivalents per gram of dry weight from a calibration curve made from 0.2 to 0.6 mM.

2.9.4 Iron chelating activity

The Fe^{2+} chelating activity was determined by adding 50 µL of 5 mM ferrozine to a mixture of sample (200 µL) and 0.1 M sodium acetate buffer (pH 4.9, 1 mL) previously left for 30 min at room temperature (Sánchez-Vioque et al., 2013). The resulting solution was incubated in dark at room temperature for 30 min and the absorbance was measure at 562 nm. The percentage of inhibition of ferrozine-Fe^{2+} complex formation was calculated as $[A_0\text{-}A_1/A_0]$ x 100, where A_0 was the absorbance of the control reaction and A_1 the absorbance of the sample.

2.9.5 Copper chelating activity.

The Cu^{2+} chelating activity was evaluated by adding 20 µL of 4 mM PV to a mixture of 50 µL of sample, 700 µL of 50 mM sodium acetate buffer pH 6.0 and 20 µL of 5 mM $CuCl_2$ (previously incubated for 30 min at room temperature) (Sánchez-Vioque et al., 2013). The resulting solution was kept in dark at room temperature for 30 min and absorbance was measured at 632 nm. The percentage of inhibition of PV-Cu^{2+} complex formation was calculated as $[A_0\text{-}A_1/A_0]$ x 100, where A_0 was the absorbance of the control reaction and A_1 the absorbance of the sample.

2.10 Statistical analysis

A Pearson's correlation test was performed using the Jamovi program (2022) to determine the degree of relationship between pairs of variables. Each plant part was analysed in triplicate for every analytical method and the results were presented as mean ± standard deviation. Pearson's correlation was conducted using averaged values of each variable, and *p*-values of <0.05 were considered statistically significant.

3 RESULTS AND DISCUSSION

3.1 Proximate composition

The proximate composition and total carbohydrate content of the different parts of *A. thuscula* are presented in Table 1. The results reveal that all plant parts are rich in water, with a moisture content above 39%. These amounts agree with the moisture content in *Gomphrena celosioides* with a stem, leaf and root percentage of 64.20, 58.60 and 50.35 %, respectively (Ilodibia et al., 2016). The high moisture content in leaves makes them more susceptible to degradation, however, the concept of moisture alone is not sufficient to determine chemical stability of foodstuffs (Bell, 2020).

Table 1 near here

The ash percentage, which measures mineral content, was higher in leaves (8.69%) than in stem (2.26%) and roots (4.07%). Tripathi et al. (2015) and Zaman et al. (2022) reported lower ash content in leaves of *Artemisia nilagirica* (7.83%) and *Artemisia maritima* (6.32%), respectively. Considering that ash content in food can vary from 0 to 12% (Harris & Marshall, 2017), *A. thuscula* leaves could be a good source of inorganic compounds essential in the nutrition of humans and animals (Soetan et al., 2010).

The highest amount of crude fibre was found in the roots (50.48%) and is comparable to that quantified by Asuk et al. (2015) in roots of *Jatropha curcas* shrub (43.33%). The content in stems (39.59 %) was similar to that in the stem of *Manihot esculenta Crantz* (39.51%), a plant commonly used as a food source in developing countries (Idris et al., 2020; Li et al., 2017). Leaves showed lower fibre content (15.85%) than that reported by Zaman et al. (2022) for *Artemisia marina* (21.06%). The amount of crude fat was low, as expected in *Artemisia* species (Zaman et al., 2022). It was higher in leaves (0.072%), followed by stems (0.035%) and roots (0.014%) (Table 1). The incorporation of *A.*

thuscula into food products could improve their properties (texture, consistency, water and oil retention and emulsion) (Elleuch et al., 2011).

Carbohydrates are the main source of energy in the human diet and have various physiological and health benefits. Their levels remained relatively constant in leaves, stems and roots, within a range of 19.40-21.84 %. These results agree with the carbohydrate range reported by Caidan et al. (2014) for leaves, stems, and roots of *R. Amabilis* (20.2-21.7%). However, this range is below the amount found in *Artemisia marina* leaves (49.63%) (Zaman et al., 2022) and in other plant species, where the percentage of carbohydrates can be up to 70.8% for roots, 49.9% for leaves and 72% for stems (Idris et al., 2020; Nkafamiya et al., 2016). Most studies of proximate composition in plant samples determine the carbohydrate content subtracting the moisture, protein, fat and ash content. This determination can lead to an overestimation of carbohydrate amounts, as it includes non-carbohydrate components and combines all analytical errors from the other analysis (Cummings & Stephen, 2007).

The proximate composition of *A. thuscula* meets the requirements as part of a plant-based diet, defined as relatively low in fat, cholesterol, salt, animal products, and sugar, while minimizing processed foods, added sugars, oils, and foods of animal origin (Neufingerl & Eilander, 2022).

Its high fibre amount, combined with its low-fat content (less than 0.072%) and the carbohydrate content, lower than that of bread (49.9%) and rice (29.2%) (Roe et al., 2015), make it suitable for low-calorie diets (Mallillin et al., 2008).

3.2 Quantification of amino acids

The amino acid content depends on several factors, e.g., the total free amino acid concentrations of *Artemisia abrotanum L* in pre-blooming and blooming periods were

4.53 and 6.06 mg g^{-1}, respectively (Cansever & Söğüt, 2022). All amino acids analysed here were detected and quantified, except for glutamine, which was not detected in any plant sample (Table 2). The highest content of amino acids, both essential and non-essential, was found in leaves of *A. thuscula,* followed by stems and roots. Protein quality is determined by its content of essential amino acids, as these cannot be produced by the human body and must be obtained from the diet. The ratios of EAA/TAA and EAA/NEAA for considering a protein to be of high quality are 0.4 and 0.6, respectively, according to the WHO definition (Zhang et al., 2023). These ratios in leaves, stems, and root of *A. thuscula* ranged from 0.4 to 0.67 (EAA/TAA) and from 0.68 to 2.03 (EAA/NEAA), fulfilling this requirement. Ochkur et al. (2013) reported EAA/NEAA ratios ranging between 0.64 and 0.84 and EAA/TAA rations ranging between 0.38 and 0.45 in the herbs of *A. vulgaris L., A. abrotanum L., A. annua L., A. absinthium L., and A. austriaca Jacq.* collected during flowering in summer in Ukraine.

Table 2 near here

The most abundant non-essential amino acids (NEAA) in leaves, stems, and roots were glutamic acid and aspartic acid. Among the essential amino acids (EAA), leucine and lysine were the most predominant. These results are comparable with those presented by Nkafamiya et al. (2016), where glutamic and aspartic acids were the major NEAA (110.9 mg/g and 109.7 mg/g, respectively) and leucine and lysine were the predominant EAA (129.7 mg/g and 128.5 mg/g, respectively) in leaves of *Azanza garckeana*. Similar results were found in the tree leaves of *Moringa Oleifera* (Cao et al., 2023).

The amount of methionine quantified in the stems of *A. thuscula* (12.08 mg g^{-1}) was more than 9.58 times higher than that of the other essential amino acids, and that of lysine was 4.43 mg g^{-1} in the leaves. These results agree with those reported by Caidan et al. (2014), where methionine was the predominant amino acid in *Rubus amabilis* stems. Moreover,

the contents of both methionine and lysine were higher than those found in whole wheat (5.27 and 3.77 mg g^{-1}, respectively), brown rice (0.89 and 0.98 mg g^{-1}, respectively) and yellow maize (0.97 and 1.42 mg g^{-1}, respectively) (Galili & Amir, 2013). It is important to highlight that both compounds are limiting amino acids in legumes, cereals, fruits and seeds (staple foods in developing countries), which decreases their nutritional value (Wenefrida et al., 2009).

All plant parts studied here are rich in essential amino acids, suggesting that they are a good source of amino acids for food and health applications. Therefore, supplementation with amino acids derived from leaves, stems and roots of *A. thuscula* at a safe dose could improve the nutritional quality of foods and forages (Navik et al., 2021).

3.3 Phenolic compounds

Phenolic compounds derived from plant sources exhibit a wide array of bioactive properties including antioxidant, anti-inflammatory, antimicrobial, anticancer and neuroprotective effects (Abdallah & Esmat, 2017; Lutz et al., 2019). Flavonoids are the most ubiquitous and widely distributed group of phenolic compounds in plants, occurring in almost all plant components (Shen et al., 2022). The total flavonoid content quantified in leaves, stems, and roots of *A. thuscula* are summarized in Table 3. In addition, five hydroxybenzoic acid derivatives (gallic, syringic, vanillic, gentisic and protocatechuic acids) and two hydroxycinnamic acid derivatives (coumaric and ferulic acids) were also quantified.

Table 3 near here

The total flavonoid content evaluated here in the aerial part of *A. thuscula* (8.47 and 6.65 mg quercetin g^{-1} dw in leaves and stems, respectively) is over the content reported by Carvalho et al. (2011) in six different *Artemisia* species (between 0.3 and 1.9 µg quercetin

g^{-1} dw). John et al. (2013) found lower quantities of flavonoids (between 12.5 and 28.6 μg quercetin g^{-1} dw) in all plant parts of six different species of the genus *Justicia*. Five of these species showed lower flavonoid content in roots than in leaves, and only *Justicia montana* exhibited higher content in roots than in stems and leaves. This last result agrees with that obtained in this study, in which a higher concentration was found in roots of *A. thuscula* (13.30 mg quercetin g^{-1} dw). However, the highest flavonoid content usually occurs in leaves, flowers and seeds, and their distribution is affected by several factors such as variation and intensity of light (Asuk et al., 2015; Shen et al., 2022). In addition, flavonoids play a crucial role in the mechanisms of adaptation and tolerance to stress conditions, and in plant growth and development (Naikoo et al. 2029; Shen et al., 2022), so their accumulation in the roots or elsewhere in the plant depends on those factors and, to a large extent, it depends on environmental conditions (Taylor & Grotewold, 2005). For example, Ginkgo trees accumulate flavonoids to improve *G. biloba* tolerance to soil salt and heavy metal stress (Shen et al., 2022; Xu et al., 2020).

The phenolic profile differs among leaves, stems, and roots of *A. thuscula* (Table 3). Leaves were the richest in phenolic compounds, with ferulic acid being the most predominant, followed by stems and roots. Caffeic acid and ferulic acid conjugates were found to be the most prevalent hydroxycinnamic acids in all examined leaves of the various *Artemisia* species (Carvalho et al., 2011). Similar findings were observed by Liu et al. (2022), who found that ferulic acid presented the highest percentage (0.289%) in leaves of *Sinopodophyllum hexandrum*, a medicinal plant. Ferulic acid is covalently bound to lignin and polysaccharides in plant cell walls, forming physical and chemical barriers that protect cell wall carbohydrates from microbial attack and enzymatic hydrolysis (Wang et al., 2022).

Vanillic acid was found exclusively in *A. thuscula* roots (101.01 μg g^{-1} dw), while epicatechin was the most abundant phenolic compound in stems and roots. These results are totally consistent with previous analyses of *Vaccinium angustifolium* reported by Harris et al. (2007), who quantified remarkable contents of epicatechin in stem and roots, and identified vanillic acid only in roots. The main role of epicatechin in plant protection is related to its antioxidant and antimicrobial effect, and preventing the attack of herbivores (Li et al., 2022). Its molecular structure is a key factor for its radical proliferation-inhibiting activity with important protective functions that promote human health and longevity (antioxidant, antidiabetic, anti-inflammatory, cardio and brain protection) (Prakash et al., 2019). Therefore, the high level of epicatechin found in different parts of *A. thuscula* in this study (227-503 μg g^{-1}) compared to that in commonly consumed food, beverages, fruits and vegetables (703.6 μg in dark chocolate, 114.8 μg in blackberry, 79.7 μg in peach, 79.3 μg in green tea infusion and 52.4 μg in black grapes per 1 g fw) is especially noteworthy in the context of human nutrition (Prakash et al., 2019).

A. thuscula extracts rich in phenolic compounds can be considered potential pharmaceutical agents, promising substitutes of food additives and flavouring agents, and bioactive ingredients in cosmeceutical products, among others.

3.4 Pigments

The pigment contents of fresh leaves of *A. thuscula* are presented in Table 4. Acetone-water extraction gave a higher yield than acetone-hexane for Chl a and b. Plant material with considerable water content, such as *A. thuscula* (61%) requires polar solvents (acetone, methanol or ethanol) for chlorophyll extraction. For this purpose, hexane is less

suitable because it partially extracts polar pigments such as Chl b or xanthopylls (Lichtenthaler & Buschmann, 2001).

Table 4 near here

In this study, Chl a was predominates over Chl b with remarkable contents (564.6 and 314.1 μg g^{-1} fw, respectively). The ratio Chl a: Chl b was 1.8 in both extraction solvents, which agrees with the ratio of 1.79 found by Todaria (1986) in plants of the same genus collected at 550 m altitude (*Artemisia scoparia*). This ratio is generally described to be about 3:1 in higher plants. However, it varies with pollution and environmental stress conditions, including seasonal and regional climatic changes (Carvalho et al., 2011). In addition, the content of pigments of sun and shade leaves also varies, being higher in sun leaves (Lichtenthaler & Babani, 2022). Chl a:Chl b ratio varied between 3.39 and 0.27 in *Artemisia priceps* (*Artemisia scoparia*) and in *Artemisia arborescens* leaves, respectively, when five *Artemisia* species were examined by Carvalho et al. (2011). Doddavarapu et al. (2021) studied forty commonly plants belonging to four different families (*Apocyanaceae, Asteraceae, Euphorbiaceae and Fabaceae*) to compare their chlorophyll concentration. Twenty-six of them showed a higher of Chl a content and the other 14 exhibited higher Chl b level. The maximum and minimum Chl a:Chl b ratios were 7.4:1 in *Cajanus cajan, Fabaceae*, and 0.399:1 in *Senna siamea, Fabaceae,* respectively.

A regular intake of chlorophyll increases the efficacy of magnesium, helps the blood in carrying the much-needed oxygen to all cells and tissues, neutralizes free radicals, keeps the circulatory and digestive systems much healthier and protects against cancer (Doddavarapu et al., 2021). Moreover, lycopene, whose content in leaves of *A. thuscula* was 25.09 μg g^{-1} fw, may have a protective role against certain types of cancer, the development of Type 2 diabetes and cardiovascular disease (Zheng et al., 2004). *Artemisia thuscula* is an inexpensive and very easily available source of pigments with a

high number of biological activities and could be used in the textile, cosmetic, food and feed industries (Brudzyńska et al., 2021).

3.5 Antioxidant activities

Currently, there is no widely accepted method to test the antioxidant capacity of various compounds in different matrices. The application of several antioxidant assays allows the comparison of different antioxidant mechanisms. Therefore, in this study five different in vitro assays were carried out to evaluate the antioxidant potential of the leaves, stems, and roots in *A. thuscula* (Table 5). In addition, to clarify the contribution of each group of analysed bioactive compounds to antioxidant activity, correlations among antioxidant activity and metabolites were determined (Table 6).

Table 5 near here

Radical scavenging activity, which determines the ability of a compound/extract to neutralize free radicals associated with the development of various diseases (Apak et al., 2007), was higher in *A. thuscula* root extracts. This result aligns well with those reported by El Diwani et al. (2009), where *Jatropha curcas* root extracts exhibited higher RSA than stem and leaves. In addition, all extracts studied here showed a greater RSA than that of the synthetic food additive BHT, and the root extract also gave higher activity than that of BHA. Both compounds are categorised as carcinogens with the ability to induce cytotoxicity and apoptotic cell death (Saito et al., 2003). Therefore, antioxidants from *A. thuscula* could be a natural substitute for BHA and BHT (Halliwell et al., 1995). Furthermore, the RSA assay showed a high significant correlation with flavonoids ($r=0.992$) (Table 6). Quantification of total flavonoid content in the presence of $NaNO_2$ has been shown to be specific for rutin, luteolin, catechins and phenolic acids (Pękal and

Pyrzynska, 2014). The strong positive correlations found between flavonoids and RSA could indicate the presence of these molecules containing catecholic moieties.

The reducing power evaluated by CUPRAC and FRAP methods refers to the capacity of a molecule to transfer electrons in order to counteract harmful free radicals or reactive species (Apak et al., 2007). All plant sections of *A. thuscula* exhibited a greater capacity to reduce Cu^{2+} to Cu^{1+} than to reduce Fe^{3+} to Fe^{2+} with predominant activity in roots followed by leaves and stems. These results partially agree with those of Saunoriūtė et al. (2023), who found that methanolic extracts of two different *Artemisia* species (*Artemisia abrotanum L.* and *Artemisia absinthium* L) showed better results in the CUPRAC assay than in the FRAP assay, with higher CUPRAC activity in the *Artemisia vulgaris* aerial parts.

Table 6 near here

Leaves of *A. thuscula* exhibited the greatest Fe^{2+}-chelating activity, up to 2.87 and 3.51 times higher than stems and roots, respectively. Iron chelating capacity is significantly correlated with the total phenolic and total amino acid contents ($p<0.05$, $r=0.995$ and 0.994, respectively), which are also correlated with each other (Table 6). Reciprocal interactions between phenolic compounds and proteins lead to various nutritional, functional and structural changes, with antioxidant activities being of great importance in terms of organic chemistry as well as health and nutrition (Chu et al., 2018; Hilal et al., 2022). Protein/phenolic ratio is an important factor that could affect the interactions between phenolic compounds and proteins with synergistic or antagonistic actions. Protein surface structure, total charges, and secondary structures, among other, also exhibit varying degrees of correlation with the non-covalent binding proteins to specific phenolic substances (Hilal et al., 2022; Lin et al., 2023).

Extensive research has confirmed that chelating agents effectively stabilize transition metals, limiting their role as catalysts and thus inhibiting the generation of initial free radicals. Consequently, this process helps to suppress lipid peroxidation in both biological and food systems (Yu et al., 2002). Recently, iron and copper chelators have been investigated for cancer drug design and development (Gaur et al., 2018; Kontoghiorghes & Kontoghiorghe, 2020).

Artemisia thuscula plant is demonstrated to be a rich source of antioxidant compounds. Each part contains unique bioactive compounds that contribute to their antioxidant effects. The leaves, stems and roots offer different antioxidant profiles, presenting alternative paths for obtaining specific antioxidant advantages. Trendafilova et al. (2020) evidenced the beneficial effect on human and animal health of consuming edible *Artemisia* species, as well as their food applications and therapeutic effects. For example, *A. annua* leaves, capable of inhibiting inflammation, repairing the skin barrier, improving damaged skin, and reducing redness and other symptoms of sensitive skin, are consumed in salads in some Asian countries and in the United States, and are marketed as dietary supplements. *Artemisia thuscula* has been used as an expectorant and anti-catarrhal, as well as in the treatment of gastrointestinal diseases in traditional medicine. This herb has sedative properties and controls epileptic seizures, tension and anxiety (Benjumea et al., 2005; Darias et al., 1986; Perfumi et al., 1995). Its composition, with the presence of several eudesmanolides and sesquiterpene lactones as heliangolide (Breton et al., 1985; Gonzalez et al., 1983), and the properties determined in this work promise great benefits for human health through its use in the food and pharmaceutical industries.

4 CONCLUSION

Due to the high fibre content and low fat and carbohydrate content of *A. thuscula*, its incorporation into food products could improve their properties, such as texture, consistency, water and oil retention and emulsification. In addition, *A. thuscula* is a rich source of phenolics, flavonoids and essential amino acids with known favourable biochemical effects on multiple diseases. The antioxidant activities of leaves, stems and roots of *A. thuscula* show great potential as dietary supplements, functional foods and as a source of new, more effective and safer drugs.

REFERENCES

Abdallah, H. M., & Esmat, A. (2017). Antioxidant and anti-inflammatory activities of the major phenolics from Zygophyllum simplex L. J. Ethnopharmacol. 205, 51–56. https://doi.org/10.1016/j.jep.2017.04.022

Apak, R., Güçlü, K., Demirata, B., Özyürek, M., Çelik, S., Bektaşoğlu, B., … Özyurt, D. (2007). Comparative Evaluation of Various Total Antioxidant Capacity Assays Applied to Phenolic Compounds with the CUPRAC Assay. Molecules, 12(7), 1496–1547. http://dx.doi.org/10.3390/12071496

Asuk, A. A., Agiang, M. A., Dasofunjo, K., & Willie, A. J. (2015). The biomedical significance of the phytochemical, proximate and mineral compositions of the leaf, stem bark and root of Jatropha curcas. Asian Pacific Journal of Tropical Biomedicine 5(8), 650–657. https://doi.org/10.1016/j.apjtb.2015.05.015

Bell, L. N. (2020). Moisture effects on food's chemical stability. In Water activity in foods: fundamentals and applications, 227-253. https://doi.org/10.1002/9781118765982.ch9

Benjumea, D., Abdala, S., Hernandez-Luis, F., Pérez-Paz, P., & Martin-Herrera, D.

(2005). Diuretic activity of Artemisia thuscula, an endemic canary species. Journal of Ethnopharmacology 100(1-2), 205–209. https://doi.org/https://doi.org/10.1016/j.jep.2005.03.005

Bogdanova, E. S., Zakhozhiy, I. G., Tabalenkova, G. N., & Rozentsvet, V. A. (2017). Bioaccumulation of nutrients , lipids , pigments , antioxidants and essential oils in plants of genus Artemisia growing in lake Elton region (South- East of the European part of Russia). Journal of Medicinal Plants Studies 5(2), 270–275.

Bora, K. S., & Sharma, A. (2011). The genus Artemisia: A comprehensive review. Pharmaceutical Biology 49(1), 101–109. https://doi.org/10.3109/13880209.2010.497815

Branisa, J., Jenisova, Z., Porubska, M., Jomova, K., & Valko, M. (2014). Spectrophotometric determination of chlorophylls and carotenoids. An effect of sonication and sample processing. Microbiology, Biotechnology and Food Sciences, 3(2), 61–64.

Breton, J. L., Camps, F., Coll, J., Eguren, L., Gavin, J. A., Gonzalez, A. G., … Torramilans, J. (1985). Isolation and structural elucidation of heliangolidin, a new sesquiterpene lactone from artemisia canariensis. Tetrahedron, 41(15), 3141–3146. https://doi.org/https://doi.org/10.1016/S0040-4020(01)96668-2

Brooks, J. R., Griffin, V. K., & Kattan, M. W. (1986). A modified method for total carbohydrate analysis of glucose syrups, maltodextrins, and other starch hydrolysis products. Cereal Chemistry, 63(5), 465–466. http://www.aaccnet.org/publications/cc/backissues/1986/Documents/chem63_465.pdf

Brudzyńska, P., Sionkowska, A., & Grisel, M. (2021). Plant-derived colorants for food, cosmetic and textile industries: A review. Materials, 1413), 1–18.

https://doi.org/10.3390/ma14133484

Caidan, R., Cairang, L., Liu, B., & Yourui, S. (2014). Amino acid, fatty acid, and mineral compositions of fruit, stem, leaf and root of Rubus amabilis from the Qinghai-Tibetan Plateau. Journal of Food Composition and Analysis, 33(1), 26–31. https://doi.org/10.1016/j.jfca.2013.09.009

Cao, J., Shi, T., Wang, H., Zhu, F., Wang, J., Wang, Y., … Su, E. (2023). Moringa oleifera leaf protein: Extraction, characteristics and applications, Journal of Food Composition and Analysis, 119, 105234. https://doi.org/10.1016/j.jfca.2023.105234

Carvalho, I. S., Cavaco, T., & Brodelius, M. (2011). Phenolic composition and antioxidant capacity of six artemisia species. Industrial Crops and Products, 33(2), 382–388. https://doi.org/10.1016/j.indcrop.2010.11.005

Cansever, I. & Söğüt, Ö. (2022). Determination of Total Protein and Free Amino Acid Content of Artemisia abrotanum L. in the Blooming and Pre-Blooming Period. Bezmialem Science 10(5), 623-627. https://api.semanticscholar.org/CorpusID:247730221

Chang, E., & Kim, C. Y. (2018). Lipid Peroxidation and Antioxidant Activities of the Aqueous Rhizome Extract of Rheum officinale Baillon. Journal of Food Quality, 2018. https://doi.org/10.1155/2018/5258276

Chu, Q., Bao, B., & Wu, W. (2018). Mechanism of interaction between phenolic compounds and proteins based on non-covalent and covalent interactions. Medicine Ressearch 2:180014. doi: 10.21127/yaoyimr20180014

Cummings, J. H., & Stephen, A. M. (2007). Carbohydrate terminology and classification. European Journal of Clinical Nutrition, 61, S5–S18. https://doi.org/10.1038/sj.ejcn.1602936

Cummings, J. H., & Engineer, A. (2018). Denis Burkitt and the origins of the dietary fibre

hypothesis. Nutrition Research Reviews, 31(1), 1–15. https://doi.org/10.1017/S0954422417000117

Darias, V., Bravo, L., Barquin, E., Herrera, D. M., & Fraile, C. (1986). Contribution to the ethnopharmacological study of the Canary Islands. Journal of Ethnopharmacology, 15(2), 169–193. https://doi.org/https://doi.org/10.1016/0378-8741(86)90154-6

Doddavarapu, B., Crasta, G. L., & Shankar, M. (2021). Comparative studies on chlorophyll concentration in some important plant families. Journal of Pharmacognosy and Phytochemistry 10(3), 214-220. https://doi.org/10.22271/phyto.2021.v10.i3c.14074

El Diwani, G., El Rafie, S., & Hawash, S. (2009). Antioxidant activity of extracts obtained from residues of nodes leaves stem and root of Egyptian Jatropha curcas. Journal of Pharmacy and Pharmacology, 3, 521–530

Elleuch, M., Bedigian, D., Roiseux, O., Besbes, S., Blecker, C., & Attia, H. (2011). Dietary fibre and fibre-rich by-products of food processing: Characterisation, technological functionality and commercial applications: A review. Food Chemistry, 124(2), 411–421. https://doi.org/10.1016/j.foodchem.2010.06.077

Galili, G., & Amir, R. (2013). Fortifying plants with the essential amino acids lysine and methionine to improve nutritional quality. Plant Biotechnology Journal, 11(2), 211–222. https://doi.org/10.1111/pbi.12025

Gaur, K.; Vázquez-Salgado, A. M.; Duran-Camacho, G.; Dominguez-Martinez, I.; Benjamín-Rivera, J. A.; Fernández-Vega, L.; …, Tinoco, A. D. (2018). Iron and Copper Intracellular Chelation as an Anticancer Drug Strategy. Inorganics, 6(4), 126. https://doi.org/10.3390/inorganics6040126

Gonzalez, A. G., Bermejo, J., & Zaragoza, T. (1983). Eudesmanolides from Artemisia

canariensis. Phytochemistry, 22(6), 1509–1510.

Halliwell, B., Murcia, M. A., Chirico, S., & Aruoma, O. I. (1995). Free Radicals and Antioxidants in Food and in vivo: What they do and how they work. Critical Reviews in Food Science and Nutrition, 35(1-2), 7–20. https://doi.org/10.1080/10408399509527682

Harborne, J. B., & Williams, C. A. (2000). Advances in flavonoid research since 1992. Phytochemistry, 55(6), 481–504. https://doi.org/https://doi.org/10.1016/S0031-9422(00)00235-1

Harris, C. S., Burt, A. J., Saleem, A., Le, P. M., Martineau, L. C., Haddad, P. S., … Arnason, J. T. (2007). A single HPLC-PAD-APCI/MS method for the quantitative comparison of phenolic compounds found in leaf, stem, root and fruit extracts of Vaccinium angustifolium. Phytochemical Analysis, 18(2), 161–169. https://doi.org/https://doi.org/10.1002/pca.970

Harris, G. K., & Marshall, M. R. (2017). Ash Analysis. Food Analysis (287–297). https://doi.org/10.1007/978-3-319-45776-5_16

Heinrich, M., & Gibbons, S. (2001). Ethnopharmacology in drug discovery: an analysis of its role and potential contribution. Journal of Pharmacy and Pharmacology, 53(4), 425–432. https://doi.org/10.1211/0022357011775712

Hilal, Y., Busra, G. S., Ufuk, C. H., Tugba, O., & Esra, C. (2022). Chemistry of Protein-Phenolic Interactions Toward the Microbiota and Microbial Infections. Frontiers in Nutrition, 9, https://www.frontiersin.org/articles/10.3389/fnut.2022.914118

Idris, S., Rosnah, S., Nor, M. Z. M., Mokhtar, M. N., & Abdul Gani, S. S. (2020). Physicochemical composition of different parts of cassava (Manihot esculenta crantz) plant. Food Research, 4, 78–84. https://doi.org/10.26656/fr.2017.4(S1).S33

Ilodibia, C. V., Ewere, F. U., Akachukwu, E. E., Adimonyemma, R. N., Igboabuchi, N.

A., & Okeke, N. F. (2016). Proximate composition, vitamin and anatomical studies on Gomphrena celosioides. Annual Research and Review in Biology, 10(3), 1–6. https://doi.org/10.9734/ARRB/2016/25546

John, B., Reddy, V. R. K., Reddy, V., & Ct, S. (2013). Total Phenolics and Flavonoids in Selected Justicia Species. Journal of Pharmacognosy and Phytochemistry, 2(4), 72–73. https://api.semanticscholar.org/CorpusID:185475118

Kontoghiorghes, G. J., & Kontoghiorghe, C. N. (2020). Iron and Chelation in Biochemistry and Medicine: New Approaches to Controlling Iron Metabolism and Treating Related Diseases. Cells, 9 (6), 1456. https://doi.org/10.3390/cells9061456

Li S, Cui Y, Zhou Y, Luo Z, Liu J, & Zhao M. (2017). The industrial applications of cassava: current status, opportunities and prospects. Journal of the Science of Food and Agriculture, 97(8), 2282–2290. doi: 10.1002/jsfa.8287

Li, X., Zhang, J., Lin, S., Xing, Y., Zhang, X., Ye, M., … Sun X. (2022). (+)-Catechin, epicatechin and epigallocatechin gallate are important inducible defensive compounds against Ectropis grisescens in tea plants. Plant, Cell & Environment, 45, 496–511. https://doi.org/10.1111/pce.14216

Lichtenthaler, H., & Buschmann, C. (2001). Extraction of Photosynthetic Tissues: Chlorophylls and Carotenoids. Current Protocols in Food Analytical Chemistry, 1(1), F4.2.1-F4.2.6.

Lichtenthaler, H.K., & Banani, F. (2022). Contents of photosynthetic pigments and ratios of chlorophyll a/b and chlorophylls to carotenoids (a+b)/(x+c) in C4 plants as compared to C3 plants. Photosynthetica, 60, 3-9. https://doi.org/10.32615/ps.2021.041

Lin, D., Sun, L.-C., Huo, W.-S., Zhang, L.-J., Chen, Y.-L. Miao, S., & Cao, M.-J. (2023). Improved functionality and safety of peptides by the formation of peptide-

polyphenol complexes, Trends in Food Science & Technology, 141, 104193. https://doi.org/10.1016/j.tifs.2023.104193

Liu, W., Zhang, Z., Zhang, T., Qiao, Q., & Hou, X. (2022). Phenolic profiles and antioxidant activity in different organs of Sinopodophyllum hexandrum. Frontiers in Plant Science, 13, 1–11. https://doi.org/10.3389/fpls.2022.1037582

Lu, W., Shi, Y., Wang, R., Su, D., Tang, M., Liu, Y., & Li, Z. (2021). Antioxidant activity and healthy benefits of natural pigments in fruits: A review. International Journal of Molecular Sciences, 22(9). https://doi.org/10.3390/ijms22094945

Lutz, M., Fuentes, E., Ávila, F., Alarcón, M., & Palomo, I. (2019). Roles of phenolic compounds in the reduction of risk factors of cardiovascular diseases. Molecules, 24(2), 1–15. https://doi.org/10.3390/molecules24020366

Mallillin, A. C., Trinidad, T. P., Raterta, R., Dagbay, K., & Loyola, A. S. (2008). Dietary fibre and fermentability characteristics of root crops and legumes. British Journal of Nutrition, 100(3), 485–488. https://doi.org/10.1017/S000711450891151X

Naikoo, M. I., Dar, M. I., Raghib, F., Jaleel, H., Ahmad, B., Raina, A., … Naushin, F. (2019). Role and regulation of plants phenolics in abiotic stress tolerance: An overview. Plant Signaling Molecules: Role and Regulation under Stressful Environments, 157–168. https://doi.org/10.1016/B978-0-12-816451-8.00009-5

Navik, U., Sheth, V. G., Khurana, A., Jawalekar, S. S., Allawadhi, P., Gaddam, R. R., … Tikoo, K. (2021). Methionine as a double-edged sword in health and disease: Current perspective and future challenges. Ageing Research Reviews, 72, 101500. https://doi.org/10.1016/j.arr.2021.101500

Neufingerl, N., & Eilander, A. (2022). Nutrient intake and status in adults consuming plant-based diets compared to meat-eaters: A systematic review. Nutrients, 14(1). https://doi.org/10.3390/nu14010029

Nkafamiya, I., Ardo, B., Osemeahon, S., & Akinterinwa, A. (2016). Evaluation of Nutritional, Non-nutritional, Elemental Content and Amino Acid Profile of Azanza garckeana (Goron Tula). British Journal of Applied Science & Technology, 12(6), 1–10. https://doi.org/10.9734/bjast/2016/19811

Ochkur, A.V., Kovaleva, A.M., & Kolesnik, Y.S. (2013). Amino-Acid Composition of Subgenus Artemisia Herbs. Chemistry of Natural Compounds 49, 589–591. https://doi.org/10.1007/s10600-013-0684-z

Pękal, A., & Pyrzynska, K. Evaluation of Aluminium Complexation Reaction for Flavonoid Content Assay. Food Analytical Methods 7, 1776–1782 (2014). https://doi.org/10.1007/s12161-014-9814-x

Pellicer, J., Saslis-Lagoudakis, C. H., Carrió, E., Ernst, M., Garnatje, T., Grace, O. M., … Rønsted, N. (2018). A phylogenetic road map to antimalarial Artemisia species. Journal of Ethnopharmacology, 225, 1–9. https://doi.org/10.1016/j.jep.2018.06.030

Perfumi, M., Paparelli, F., & Cingolani, M. L. (1995). Spasmolytic Activity of Essential Oil of Artemisia thuscula Cav. from the Canary Islands. Journal of Essential Oil Research, 7(4), 387–392. https://doi.org/10.1080/10412905.1995.9698545

Prakash, M., Basavaraj, B.V., & Chidambara-Murthy, K.N. (2019). Biological functions of epicatechin: Plant cell to human cell health, Journal of Functional Foods, 52, 14-24, 1756-4646. https://doi.org/10.1016/j.jff.2018.10.021

Roe, M., Pinchen, H., Church, S., & Finglas, P. (2015). McCance and Widdowson's The Composition of Foods Seventh Summary Edition and updated Composition of Foods Integrated Dataset. Nutrition Bulletin, 40(1), 36–39. https://doi.org/10.1111/nbu.12124

Saito, M., Sakagami, H., & Fujisawa, S. (2003). Cytotoxicity and apoptosis induction by butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT). Anticancer

Research, 23(6C), 4693–4701.

Sánchez-Vioque, R., Polissiou, M., Astraka, K., Mozos-Pascual, M. de los, Tarantilis, P., Herraiz-Peñalver, D., & Santana-Méridas, O. (2013). Polyphenol composition and antioxidant and metal chelating activities of the solid residues from the essential oil industry. Industrial Crops and Products, 49, 150–159. https://doi.org/10.1016/j.indcrop.2013.04.053

Santiago-Díaz, P., Rivero, A., Rico, M., & Gómez-Pinchetti, J. L. (2022). Characterization of Novel Selected Microalgae for Antioxidant Activity and Polyphenols, Amino Acids, and Carbohydrates. Marine Drugs, 20(1). https://doi.org/10.3390/md20010040

Saunoriūtė, S., Ragažinskienė, O., Ivanauskas, L., Marksa, M., Laužikė, K., & Raudonė, L. (2023). Phenolic Diversity and Antioxidant Activity of Artemisia abrotanum L. and Artemisia absinthium L. during Vegetation Stages. Separations, 10(10), 545. http://dx.doi.org/10.3390/separations10100545

Sethi, S., Joshi, A., Arora, B., Bhowmik, A., Sharma, R.R., & Kumar, P. (2020). Significance of FRAP, DPPH, and CUPRAC assays for antioxidant activity determination in apple fruit extracts. European Food Research Technology 246, 591–598. https://doi.org/10.1007/s00217-020-03432-z

Shen, N., Wang, T., Gan, Q., Liu, S., Wang, L., & Jin, B. (2022). Plant flavonoids: Classification, distribution, biosynthesis, and antioxidant activity. Food Chemistry, 383, 132531. https://doi.org/10.1016/j.foodchem.2022.132531

Soetan, K. O., Olaiya, C. O., & Oyewole, O. E. (2010). The importance of mineral elements for humans , domestic animals and plants : A review. African Journal of Food Science, 4, 200–222.

Tak, Y., & Kumar, M. (2020). Phenolics: A Key Defence Secondary Metabolite to

Counter Biotic Stress. Plant Phenolics in Sustainable Agriculture, 309–329. https://doi.org/10.1007/978-981-15-4890-1_13

Taylor, L. P., & Grotewold, E. (2005). Flavonoids as developmental regulators. Current Opinion in Plant Biology, 8(3), 317–323. https://doi.org/10.1016/j.pbi.2005.03.005

Templeton, D. W., Quinn, M., Van Wychen, S., Hyman, D., & Laurens, L. M. L. (2012). Separation and quantification of microalgal carbohydrates. Journal of Chromatography A, 1270, 225–234. https://doi.org/10.1016/j.chroma.2012.10.034

Todaria, N. P. (1986). Changes in Pigments and Total Phenolics in Artemisia Species Grown at Different Altitudes in the Garhwal Himalaya. Biologia Plantarum 28(4), 307–309. https://doi.org/10.1007/BF02902300

Trendafilova, A., Moujir, L.M., Sousa, P.M.C., & Seca, A.M.L. (2020). Research Advances on Health Effects of Edible Artemisia Species and Some Sesquiterpene Lactones Constituents. Foods, 10(1), 65. https://doi.org/10.3390/foods10010065

Tripathi, Y., C., Bisht, V., & Anjum, N. (2015). Compositional Analysis and In-Vitro Antioxidant Activity of Essential Oil of Artemisia nilagirica Leaves. World Journal of Pharmaceutical Research 4(9):1663-1679. https://api.semanticscholar.org/CorpusID:212510425

Ullah, A., Munir, S., Badshah, S. L., Khan, N., Ghani, L., Poulson, B. G., … Jaremko, M. (2020). Important Flavonoids and Their Role as a Therapeutic Agent. Molecules, 25(22), 5243. https://doi.org/10.3390/molecules25225243

Wang, Y.-L., Wang, W.-K., Wu, Q.-C., & Yang, H.-J. (2022). The release and catabolism of ferulic acid in plant cell wall by rumen microbes: A review. Animal Nutrition, 9, 335-344. https://doi.org/10.1016/j.aninu.2022.02.003

Wenefrida, I., Utomo, S. H., Blanche, B. S., & Linscombe, D. S. (2009). Enhancing Essential Amino Acids and Health Benefit Components in Grain Crops for Improved

Nutritional Values. Recent Patents on DNA & Gene Sequences, 3(3), 219–225. https://doi.org/http://dx.doi.org/10.2174/187221509789318405

World Health Organization. (2022). Bending the Trends To Promote Health and Well-being: a strategic foresight on the future of health promotion. World Health Organization. https://iris.who.int/handle/10665/363391. Licencia: CC BY-NC-SA 3.0 IGO.

Wu, G. (2009). Amino acids: metabolism: functions, and nutrition. Amino Acids, 37, 1–17. https://doi.org/10.1007/s00726-009-0269-0

Xu, N., Liu, S., Lu, Z., Pang, S., Wang, L., Wang, L., & Li, W. (2020). Gene Expression Profiles and Flavonoid Accumulation during Salt Stress in Ginkgo biloba Seedlings. Plants, 9(9), 1162. http://dx.doi.org/10.3390/plants9091162

Yu, L., Perret, J., Davy, B., Wilson, J., & Melby, C. L. (2002). Antioxidant properties of cereal products. Journal of Food Science, 67(7), 2600–2603. https://doi.org/10.1111/j.1365-2621.2002.tb08784.x

Zaman, S., Barkatulllah, Zahoor, M., Shah, S. W. A., Ullah, Z., Ullah, R., & Alotaibi, A. (2022). Pharmacognostic evaluation of Artemisia maritima L. a highly medicinal specie of genus Artemisia. Saudi Journal of Biological Sciences, 29(10), 103419. https://doi.org/10.1016/j.sjbs.2022.103419

Zhang, Y., Yang, B., Yang, l., Jiang, S., Lu, J., & Lin, L. (2023). Comparison of the nutritional qualities of the pond, rice-field and wild crayfish (Procambarus clarkii) meat. Food Chemistry Advances, 2, 100272, https://doi.org/10.1016/j.focha.2023.100272

Zheng, Z., Yin, Y., Lu, R., & Jiang, Z. (2019). Lycopene Ameliorated Oxidative Stress and Inflammation in Type 2 Diabetic Rats. *Journal of Food Science, 84,* 1194-1200. https://doi.org/10.1111/1750-3841.14505

TABLE 1. Proximate composition of leaves, stems, and roots of *Artemisia thuscula*.

	Leaves	Stem	Roots
Moisture†	61.48 ± 2.95	39.51 ± 7.77	45.20 ± 2.02
Ash†	8.69 ± 0.08	2.26 ± 0.43	4.07 ± 0.59
Crude fibre†	15.85 ± 0.76	39.59 ± 6.37	50.48 ± 9.45
Crude fat†	0.072 ± 0.002	0.035 ± 0.007	0.014 ± 0.004
Carbohydrates‡	17.40 ± 2.21	21.84 ± 0.45	18.66 ± 1.36

†Percentage on a dry weight basis.

‡Percentage of glucose equivalents per gram of dry weight.

All results are expressed as mean ± standard deviation (n=3).

TABLE 2. Amino acid composition of leaves, stems, and roots of *Artemisia thuscula.*

Amino acid	Leaves	Stem	Roots
		Essential amino acids	
Histidine	2.44 ± 0.01	0.66 ± 0.02	0.74 ± 0.00
Isoleucine	1.65 ± 0.03	0.41 ± 0.01	0.69 ± 0.01
Leucine	4.71 ± 0.10	1.13 ± 0.00	1.74 ± 0.01
Lysine	4.43 ± 0.01	1.26 ± 0.01	1.50 ± 0.02
Methionine	1.13 ± 0.02	12.08 ± 0.39	0.58 ± 0.04
Phenylalanine	2.43 ± 0.02	0.58 ± 0.01	0.90 ± 0.01
Threonine	2.84 ± 0.01	0.92 ± 0.00	1.35 ± 0.05
Valine	2.84 ± 0.01	0.75 ± 0.02	1.09 ± 0.01
		Non-essential amino acids	
Arginine	0.52 ± 0.01	0.23 ± 0.00	< LOQ
Asparagine	1.70 ± 0.02	1.05 ± 0.03	1.20 ± 0.02
Aspartic acid	12.11 ± 0.04	1.98 ± 0.02	2.83 ± 0.06
Glutamine	n.d.	n.d.	n.d.
Glutamic acid	9.37 ± 0.05	2.48 ± 0.03	3.07 ± 0.19
Proline	6.78 ± 0.02	2.10 ± 0.01	1.72 ± 0.01
Serine	3.89 ± 0.20	1.28 ± 0.01	2.01 ± 0.08
		Sum of amino acids	
∑EAA	22.98 ± 0.22 (40.4)	18.00 ± 0.47 (67.0)	8.59 ± 0.16 (44.2)
∑NEAA	33.85 ± 0.33 (59.6)	8.88 ± 0.11 (33.0)	10.83 ± 0.36 (55.8)
∑TAA	56.83 ± 0.55	26.88 ± 0.58	19.42 ± 0.51
EAA/TAA	0.40	0.67	0.44
EAA/NEAA	0.68	2.03	0.79

Results are expressed as mg g^{-1} of dry weight (mean ± standard deviation (n=2)).

Abbreviations: LOQ.: limit of quantification; n.d.: not detected; ∑EAA: sum of essential amino acids; ∑NEAA: sum of non-essential amino acids; ∑TAA: total content of amino acids.

TABLE 3. Individual phenolic compounds and flavonoid contents of leaves, stems, and roots of *Artemisia thuscula.*

Phenolic compound†	Leaves	Stem	Roots
Gallic acid	15.01 ± 3.53	<LOQ	<LOQ
Protocatechuic acid	43.27 ± 3.98	103.53 ± 10.67	n.d.
Catechin	180.33 ± 39.24	109.84 ± 26.47	n.d.
Vanillic acid	n.d.	n.d.	101.01 ± 7.653
Epicatechin	227.61 ± 61.17	503.12 ± 87.22	449.58 ± 27.93
Syringic acid	22.09 ± 5.69	16.71 ± 2.39	55.43 ± 7.80
Rutin	144.87 ± 0.65	156.19 ± 20.93	81.22 ± 7.53
Gentisic acid	n.d.	n.d.	n.d.
Coumaric acid	n.d.	308.20 ± 58.28	320.43 ± 41.26
Ferulic acid	1965.60 ± 236.55	178.51 ± 30.67	79.77 ± 9.53
Total	2598.79 ± 350.81	1376.09 ± 236.63	1087.45 ± 101.69
Flavonoids ‡	8.47 ± 1.15	5.65 ± 0.36	13.30 ± 1.14

†Results are expressed as μg per gram of dry weight.

‡Results are expressed as mg of quercetin equivalent per gram of dry weight.

All results are expressed as mean ± standard deviation (n=2).

Abbreviations: LOQ.: limit of quantification; n.d.: not detected.

TABLE 4. Pigment composition of *Artemisia thuscula* leaves extracts in two organic solvent mixtures.

	Solvent	
Pigment	Acetone:hexane (2:3)	Acetone:water (4:1)
Chlorophyll a	304.50 ± 60.52	564.6 ± 54.5
Chlorophyll b	168.77 ± 28.24	314.1 ± 18.6
Lycopene	25.09 ± 2.12	-
β-carotene	2.68 ± 0.90	-
Total carotenoids	-	72.6 ± 8.5

All results are expressed as μg per gram of fresh weight (mean ± standard deviation (n=3)).

Abbreviations: - not determined with the method.

TABLE 5. Antioxidant activities of leaves, stems, and roots of *Artemisia thuscula* extracts.

	RSA[†]	FRAP[‡]	CUPRAC[§]	FeCA[¶]	CuCA[¶¶]
Leaves	76.4 ± 2.2	11.25 ± 0.27	58.10 ± 2.88	33.4 ± 0.5	19.0 ± 0.1
Stem	65.9 ± 3.7	2.49 ± 0.19	42.44 ± 12.83	11.6 ± 1.4	14.1 ± 1.0
Roots	87.7 ± 0.9	77.28 ± 1.62	156.46 ± 11.69	9.5 ± 0.5	n.d.
BHA	78.9 ± 3.23				
BHT	32.7 ± 1.4				

[†] Inhibited DPPH (%).

[‡] μmol of Fe^{3+}reduced to Fe^{2+} per gram of dry weight.

[§] μmol of Trolox equivalents per gram of dry weight.

[¶] Formation of ferrozine-Fe^{2+} complex inhibited (%).

[¶¶]Formation of PV-Cu^{2+} complex inhibited (%).

All results are expressed as mean ± standard deviation (n=3).

Abbreviations: RSA: radical scavenging activity; FRAP: ferric reducing antioxidant power; CUPRAC: cupric ion reducing antioxidant capacity; CuCA: Cu^{2+} chelating activity; FeCA: Fe^{2+} chelating activity; BHA: butylated hydroxyanisole; BHT; butylated hydroxytoluene; n.d.: no detected.

TABLE 6. Statistical relationship between metabolite contents and antioxidant activities of leaves, stems, and roots of *Artemisia thuscula* using Pearson's correlation test.

	Pearson coeficients	TCH	∑TAA	∑Polyphenols	∑Flavonoids
TCH		1			
∑TAA	R	-0.575	1		
	p value	0.695	-		
∑Polyphenols	R	-0.582	1.000**	1	
	p value	0.698	0.003	-	
∑Flavonoids	R	-0.579	-0.334	-0.325	1
	p value	0.697	0.608	0.605	-
RSA	R	-0.679	-0.209	-0.201	0.992*
	p value	0.738	0.567	0.564	0.041
FRAP	R	-0.345	-0.569	-0.562	0.965
	p value	0.612	0.693	0.690	0.085
CUPRAC	R de Pearson	-0.363	-0.553	-0.546	0.970
	p value	0.618	0.687	0.684	0.078
CuCA	R de Pearson	-0.006	0.822	0.817	-0.812
	p value	0.502	0.193	0.196	0.801
FeCA	R de Pearson	-0.662	0.994*	0.995*	-0.228
	p value	0.730	0.035	0.032	0.573

*p < 0.05, **p < 0.01

Abbreviations: TCH: Total carbohydrate content; ∑TAA: total content of amino acids; RSA: radical scavenging activity; FRAP: ferric reducing antioxidant power; CUPRAC: cupric ion reducing antioxidant capacity; CuCA: Cu^{2+} chelating activity; FeCA: Fe^{2+} chelating activity; BHA: butylated hydroxyanisole; BHT; butylated hydroxytoluene.

4.4. Resultados de las estancias de investigación en centros internacionales

4.4.1. Estancia en el Departamento de Química de la Universidad de Aveiro

Este apartado recoge los resultados obtenidos de la investigación llevada a cabo durante la estancia en el Departamento de Química de la Universidad de Aveiro.

Mediante la metodología descrita en el Capítulo 3 (apartados 3.4 y 3.5 respectivamente) se llevó a cabo la determinación de carbohidratos totales disueltos y ácidos urónicos totales disueltos en:

(i) Aguas de cultivo enriquecidas con exudados del alga *Phaeodactylum tricornutum* expuestas a diferentes niveles de Cu (0,31, 0,79 y 1,57 µM) y a un cultivo control sin adición de este metal.

(ii) Aguas de cultivo enriquecidas con exudados del alga *Emiliania huxleyi* sometidas a diferentes condiciones de pH del medio (7,75, 7,9, 8,1, 8,25).

4.4.1.1. Determinación de carbohidratos y ácidos urónicos en exudados de *Phaeodactylum tricornutum* expuesta a Cu

Tanto la determinación de carbohidratos como la de ácidos urónicos en estas muestras no precisó de una etapa previa de concentración. La cantidad exudada por la diatomea en las condiciones de cultivo del control, subletal y letal fue suficientemente elevadas para permitir su análisis directo.

Los carbohidratos totales en exudados de la diatomea *P. tricornutum* se analizaron a los 12 y 18 días de cultivo, y los resultados (expresados en pmol de equivalentes de glucosa exudados por célula) se muestran en la tabla 11. La cantidad de carbohidratos aumenta a medida que lo hace la concentración de Cu en el medio. La mayor cantidad se alcanza a 1,57 µM de Cu(II) a los 12 y 18 días de cultivo, siendo hasta 13 y 39 veces mayor que el control respectivamente.

Tabla 11. Contenido en carbohidratos totales disueltos (pmol Glc eq cel^{-1}) en agua de mar enriquecida con exudados de *P. tricornutum* bajo diferentes concentraciones de Cu y ciclos de cultivo

Ciclo (días)	Control	Cu(II) 0,31 µM	Cu(II) 0,79 µM	Cu(II) 1,57 µM
12	0,830 ± 0,051	1,182 ± 0,247	2,22 ± 0,68	11,5 ± 2,6
18	0,357 ± 0,047	0,680 ± 0,056	10,3 ± 1,0	34,2 ± 6,0

Estos resultados son similares a los observados por Pistocchi et al. (2000) en cinco especies de diatomeas y dinoflageladas expuestas a diferentes concentraciones de Cu (desde 0,16 hasta 0,79 μM). La cantidad de carbohidratos liberados fue mayor en presencia de niveles más elevados de Cu en comparación con el control, siendo este incremento más pronunciado durante la fase estacionaria de crecimiento de las algas.

Los grupos funcionales de los carbohidratos y de los ácidos urónicos, como el carboxilo (-COOH), el hidroxilo (-OH) y la amida (N-H) están relacionados con la formación de complejos con iones metálicos, lo que les confiere una alta afinidad por metales como el Cu (Hou et al., 2013). Tonietto et al. (2014) concluyeron que existe una fuerte correlación (r=0,997) entre los carbohidratos y la concentración de ligandos de Cu exudados por la cianobacteria *Cylindrospermopsis raciborskii.* Por tanto, cabe esperar que la exudación de carbohidratos al medio por la diatomea *P. tricornutum* represente un mecanismo de protección ante la toxicidad del Cu.

Para la determinación de ácidos urónicos se realizó una optimización para adaptar la reacción a los volúmenes y concentraciones de las muestras. Además, se aplicó la separación de estos compuestos por peso molecular empleando la metodología descrita en el capítulo 3 (apartado 3.6) del presente documento. Los resultados se muestran en la tabla 12 y se expresan en pmol de equivalentes de ácido galacturónico exudados por célula. Para cada tratamiento con Cu y para el control se realizó la separación de los exudados por peso molecular, analizando, por un lado, el total de los ácidos urónicos presentes y, por otro, el material de menor de 1kDa.

Los resultados muestran que la liberación de ácidos urónicos por la diatomea también está condicionada por la concentración de Cu en el medio. Un aumento en la concentración de este metal da lugar a un incremento en los niveles de ácidos urónicos extracelulares, alcanzando el máximo de concentración al nivel más letal de Cu (hasta 12 y 94 veces mayor que el control a los 12 y 18 días, respectivamente).

Tabla 12. Contenido en ácidos urónicos totales disueltos (pmol Gal eq cel^{-1}) en agua de mar enriquecida con exudados de *P. tricornutum* bajo diferentes concentraciones de Cu y ciclos de cultivo

	12 días		18 días	
	< 1kDa	Totales	< 1kDa	Totales
Control	0,735±0,080	0,830 ± 0,064	0,294±0,000	0,335 ± 0,016
Cu(II) 0,31 µM	0,105±0,010	1,30 ± 0,11	0,498±0,061	0,683 ± 0,052
Cu(II) 0,79 µM	4,12±0,93	4,78 ± 0,96	10,7±0,7	7,32 ± 0,42
Cu(II) 1,57 µM	11,3±0,5	10,3 ± 1,8	44,4±2,3	31,5 ± 4,8

La composición de ácidos urónicos en el material polimérico exudado por las microalgas en presencia de Cu ha sido ampliamente estudiada. Especies como la diatomea *Aulacoseira granulata* libera compuestos de gran peso molecular (>10 kDa) que presentan ácido glucurónico en su estructura. Las cargas negativas de los ácidos urónicos presentes en los polímeros exudados pueden interactuar con los metales disueltos en el medio siendo los grupos carboxílicos de estos, un sitio de unión para los metales. De hecho, a mayor contenido de ácidos urónicos en el material extracelular polimérico de *A. granulata*, mayor capacidad de complejación de Cu (Gouvêa et al., 2005). Estos resultados coinciden con los reportados por Lombardi et al. (2005) que concluyen que la capacidad de formar complejos con Cu de los exopolisacáridos del alga *Scenedesmus acuminatus* está dominada por la presencia de ácido galacturónico y glucurónico. La correlación entre los ácidos urónicos disueltos y el Cu en aguas naturales podría indicar que el estrés por metales da lugar a la liberación de ácidos urónicos por parte del fitoplancton como medida de tolerancia y adaptación a ese estrés oxidativo (Hung y Santschi, 2001).

Sin embargo, en los resultados obtenidos en el estudio de *P. tricornutum* debe tenerse en cuenta ciertas desventajas que conlleva el método de análisis empleado, ya que puede dar lugar a sobreestimaciones de la cantidad de ácidos urónicos. Esto se debe a la interferencia de los azúcares neutros en la reacción que genera una coloración marrón al añadir el ácido sulfúrico a temperaturas de 100ºC produciendo una mayor cuantificación de la cantidad de ácidos urónicos. Aunque el empleo del m-fenilfenol reduce este efecto, no llega a eliminar por completo la interferencia de los azúcares neutros. Esto explicaría

por qué la cantidad de ácidos urónicos obtenidos en el análisis de los exudados es similar a la de carbohidratos analizados en las mismas muestras de *P. tricornutum*, cuando otros autores han encontrado que la composición de ácidos urónicos en los exopolisacáridos de las microalgas varía entre un 3 y 19% de ácidos urónicos (Lombardi et al., 2005; Lombardi y Vieira, 1999). Filisetti-Cozzi y Carpita (1991) consiguen corregir la interferencia de la coloración marrón con la adición de pequeñas cantidades de sulfamato.

En cuanto a la separación por peso molecular, esta clasificación es relevante ya que experimentos llevados a cabo por Lombardi et al. (2005) concluyeron que la capacidad de complejación de Cu era mayor en ligandos de menor peso molecular (<12 kDa) en exudados del alga *Scenedesmus acuminatus*. Los resultados del presente estudio en la diatomea *P. tricornutum* muestran como la mayoría de los ácidos urónicos exudados pertenecían a la fracción de menor peso molecular (<1kDa) que aumentaba su concentración en el medio a medida que aumentaban los niveles de Cu en el cultivo. Esta respuesta puede atribuirse a un mecanismo para sobrellevar la toxicidad de este metal, disminuyendo así su disponibilidad en el medio e inhibiendo su actividad por medio de la complejación de los ácidos urónicos disueltos. Sin embargo, autores como De Ruiter et al. (1992) establecen que el empleo de ácido sulfúrico para el análisis de ácidos urónicos no es suficiente para romper por completo los enlaces β(1-4) de los polímeros que contienen estas moléculas, por lo que en el análisis de ácidos urónicos totales sólo se estarían cuantificando los ácidos urónicos como monómeros y los parcialmente hidrolizados. Para hidrolizar por completo los polímeros que contienen ácidos urónicos es necesario una hidrólisis prolongada y, una vez hidrolizados, los ácidos urónicos pueden degradarse formando lactonas en cantidades irreproducibles (Blake y Richards, 1968). Por otro lado, Yapo (2012) recoge que, en el caso de muestras solubles en agua, el paso previo de pre-hidrólisis no es necesario ya que los polímeros complejos se hidrolizan con el ácido sulfúrico concentrado a elevadas temperaturas. Por tanto, dada las contradicciones encontradas en la literatura, para poder evaluar la capacidad de hidrólisis, la efectividad de este método y su posible aplicación es necesario realizar más experimentos.

4.4.1.2. Determinación de carbohidratos y ácidos urónicos en exudados de *Emiliania huxleyi* expuesta a diferentes pH

Para el análisis de los exudados de las aguas de cultivo del alga *Emiliania huxleyi* se precisó de una etapa previa de concentración, ya que tanto los carbohidratos como los ácidos urónicos no se encontraban en una concentración lo suficientemente elevada para su determinación.

El procedimiento de concentración para el análisis de carbohidratos consistió en tomar 1 mL de la muestra en un tubo de ensayo apropiado para su empleo en el equipo de concentración de vacío. Una vez evaporado completamente, el residuo se disolvió en 160 □L de agua destilada.

La concentración para la determinación de ácidos urónicos se llevó a cabo partiendo de 500 □L de muestra de agua de mar enriquecida con los exudados del alga y llevados a sequedad en tubos de ensayo adecuados para el equipo de concentración por vacío. Una vez evaporado por completo, el residuo se disolvió en 125 □L de agua destilada.

Ambos procedimientos se realizaron por triplicado y cada réplica se sometió al análisis correspondiente. Para la separación por peso molecular de los ácidos urónicos se empleó el mismo método empleado para la separación de carbohidratos Una vez separado, los 20 mL se liofilizan y el residuo se disuelve en 2 mL de agua destilada y se toman los 125 □L necesarios para su determinación.

Tabla 13. Carbohidratos y ácidos urónicos totales en agua de mar enriquecida con exudados de *Emiliania huxleyi* cultivada bajo diferentes pH

	pH			
	7,75	7,9	8,1	8,25
Carbohidratos totales[a]	0,166 ± 0,071	1,45 ± 0,53	0,139 ± 0,007	0,143 ± 0,009
Ácidos urónicos[b]				
Totales	0,030 ± 0,024	0,630 ± 0,067	< LOQ	0,025 ± 0,031
<1kDa	0,015±0,004	0,690±0,085	0,078 ± 0,006	0,025 ± 0,001

[a]nmol de equivalentes de glucosa exudado por célula

[b]pmol de equivalentes de ácido galacturónico exudado por célula

La máxima cantidad de carbohidratos totales exudados por célula se observa a pH 7,9, seguido por los cultivos a pH 7,75 y 8,25, mientras que en el medio de cultivo obtenido simulando el pH actual (8,1), la liberación de carbohidratos es menor. Se observa una tendencia parecida a la de los estudios llevados a cabo por Samperio-Ramos et al. (2017), donde la cantidad de carbohidratos se determina con el reactivo TPTZ, y se expresa en fmol de C por célula y por día.

En cuanto a los ácidos urónicos, las concentraciones obtenidas son menores que la de los carbohidratos liberados al medio. A esto, junto con las desventajas ya mencionadas en el apartado anterior, se le suma la baja sensibilidad del método en este rango de concentraciones. Tal y como se puede observar, las desviaciones estándar en algunos casos son de la misma magnitud que la media. Por otro lado, cabe destacar que las muestras se han sometido a un procedimiento de concentración, lo que aumenta la cantidad de cloruro sódico (NaCl) presente. La presencia de NaCl puede disminuir la intensidad del color del derivado del ácido urónico formado, en función de la concentración de esta sal, así como de la cantidad de ácidos urónicos presentes en la muestra. Además, concentraciones elevadas de NaCl (más de 0,8 N) producen un burbujeo que puede afectar a la medida, por lo que en estos casos se recomienda la dilución de la muestra por debajo de 0,4 N (Yapo, 2012).

4.4.2. Estancia en el Departamento de Ciencias Animales y Ecología Acuática de la Universidad de Gante

Este apartado recoge los resultados obtenidos de la investigación llevada a cabo durante la estancia en Departamento de Ciencias Animales y Ecología Acuática de la Universidad de Gante.

El objetivo del trabajo realizado durante la estancia se centró en el estudio de los efectos de toxicidad de las combinaciones binarias de metales Cd-Cr, Cd-Ni, Cr-Ni, y la combinación ternaria de Cr-Cd-Ni en la microalga de agua dulce *Pseudokirchneriella subcapitata.*

La evaluación de los posibles efectos tóxicos y los riesgos asociados a la exposición de mezclas de productos químicos en los ecosistemas es un desafío complejo de gran importancia en la actualidad (Spurgeon et al., 2010). Estas combinaciones de sustancias tóxicas pueden dar lugar a sinergismos o antagonismos de sus efectos en el

medio, es decir, desviaciones de los efectos de la mezcla predichos bajo la suposición de que los componentes de esta producen toxicidad sin interactuar entre sí (suposición de no interacción o adición). Por tanto, cuando se observa sinergismo, las sustancias están interactuando de manera cooperativa produciendo un efecto conjunto mayor de lo esperado, mientras que, en el antagonismo la combinación de sustancias reduce los efectos de la toxicidad de la mezcla (Martin et al., 2021).

Reconocer los límites de toxicidad de estos compuestos en los ecosistemas acuáticos es de vital importancia para poder establecer los estándares de seguridad de determinadas sustancias. La presencia de metales como el Cd, el Ni y el Cr pueden producir efectos dañinos para los organismos de los ecosistemas acuáticos (Kahlon et al., 2018). A pesar de que el efecto de los metales de manera individual está ampliamente estudiado, estos se encuentran en la naturaleza como combinaciones, y su impacto como mezcla debe ser considerado.

Para evaluar estos efectos se emplean comúnmente dos modelos predictivos de toxicidad de mezclas: la adición de concentraciones y la acción independiente (CA por sus siglas en inglés "Concentration Adition" e IA por "Independent Action", respectivamente). El primer modelo asume que las sustancias de la mezcla tienen el mismo modo de acción, mientras que el segundo supone que los compuestos actúan de manera independiente. La suposición fundamental es que las sustancias no interactúan entre sí. Sin embargo, las interacciones sinérgicas pueden ocurrir si los efectos conjuntos observados son significativamente mayores que los predichos por estos modelos basados en la toxicidad individual de los componentes de la mezcla. En contraste, las interacciones antagonistas ocurren si los efectos conjuntos observados son significativamente menores que los predichos por estos modelos.

Para llevar a cabo el estudio, el alga *Pseudokirchneriella subcapitata* fue cultivada en el medio OECD enriquecido con 0,4 □g L^{-1} de Mn con una densidad inicial de 10^4 célula mL^{-1}. La densidad celular se determinó tras 24, 48 y 72 horas de cultivo mediante un contador coulter (*Coulter counter*). El pH fue regulado durante todo el test con 3,6 mM del buffer MOPs (por sus siglas en inglés "3-(N-morpholino)propanesulfonic acid") y la temperatura se mantuvo a 21ºC. La iluminación fue constante durante la duración del test con un rango de entre 60-120 □mol $m^{-2}s^{-1}$. Las medidas se realizan por triplicado y se le aplica una corrección de partículas con agua de mar artificial.

Las combinaciones binarias (Cd-Cr, Cd-Ni y Cr-Ni), la combinación ternaria (Cd-Cr-Ni) y los experimentos de cada metal individual (Cd, Cr y Ni) se llevaron a cabo por separado de manera simultánea. Para los test de toxicidad tanto de las combinaciones binarias, la ternaria y las individuales se ensayaron 7 combinaciones de concentraciones diferentes, además de un control sin la adición de metales. Estas concentraciones se encuentran resumidas en las tablas 14, 15 y 16 para los metales individuales, las mezclas binarias y la mezcla ternaria individualmente.

Para la respuesta individual de cada metal, así como para la evaluación de la toxicidad de la mezcla ternaria se empleó el *equitoxic ray design,* un diseño basado en la contribución igualitaria de cada metal a las unidades de toxicidad (TU, *toxic units*). Las TUs se definen como la concentración del metal *i* (C_{Mei}) dividido por el 50% de la concentración efectiva del metal *i* ($EC50_{Mei}$) (Ecuación [1]) y se seleccionan de manera que, en la curva de respuesta relativa (RR) medida en % (Ecuación [2]) para el modelo de IA, se encuentren: un punto con un 0-10% de efecto parcial de toxicidad, otros dos con un 90-100% y al menos 3 puntos que se encuentren entre el 10-90% de efecto parcial de toxicidad.

$$TU_i = \frac{C_{Mei}}{EC50_{Mei}} \quad [1]$$

$$RR_{i,IA}(\%) = 100/\left(1+\left(\frac{C_{Mei}}{EC50_{Mei}}\right)^{\beta_{Mei}}\right) \quad [2]$$

Donde β es el parámetro de pendiente de la curva. Los valores de EC50 de cada metal empleados, así como los valores de β para las diferentes combinaciones se obtuvieron de los experimentos de cada metal individual.

La RR se relaciona con el efecto de la toxicidad en ambos modelos mediante la ecuación [3]:

$$E(\%) = 100 - RR(\%) \quad [3]$$

Donde E es el efecto, en este caso, la inhibición del crecimiento de *P. subcapitata.*

El rango de concentraciones de cada metal en cada uno de los experimentos se encuentra en las tablas 14, 15 y 16.

Con los rangos de concentración definidos para cada experimento, se aplica el método de IA y de CA para la predicción de la RR del test de toxicidad. El procedimiento

de cálculo de estos parámetros viene detallado en el estudio de Nys et al. (2016). Los resultados para cada combinación de metales, así como para los metales individuales se muestran a continuación:

Tabla 14. Concentraciones, TUs y respuesta relativa de los test de toxicidad de Cd, Cr y Ni

Concentración (□g L^{-1})	TU	RR (%) modelo IA
Cd		
0	0,01	100
6,5	0,07	98,87
12,9	0,15	96,39
25,9	0,29	89,05
51,8	0,59	71,24
103,5	1,18	43,02
Ni		
0	0,01	100
27,3	0,07	99,27
54,7	0,15	97,38
109,4	0,29	91,02
218,8	0,58	73,46
437,5	1,16	43,03
875,0	2,32	17,09
1750	4,65	5,33
Cr		
0	0,01	100
32,3	0,07	99,98
64,7	0,15	99,78
129,4	0,29	98,04
258,7	0,59	84,57
517,5	1,17	37,58
1034,9	2,35	6,20
2069,8	4,69	0,72

A continuación, se muestran las curvas de RR aplicando el modelo de acción independiente de los metales individuales.

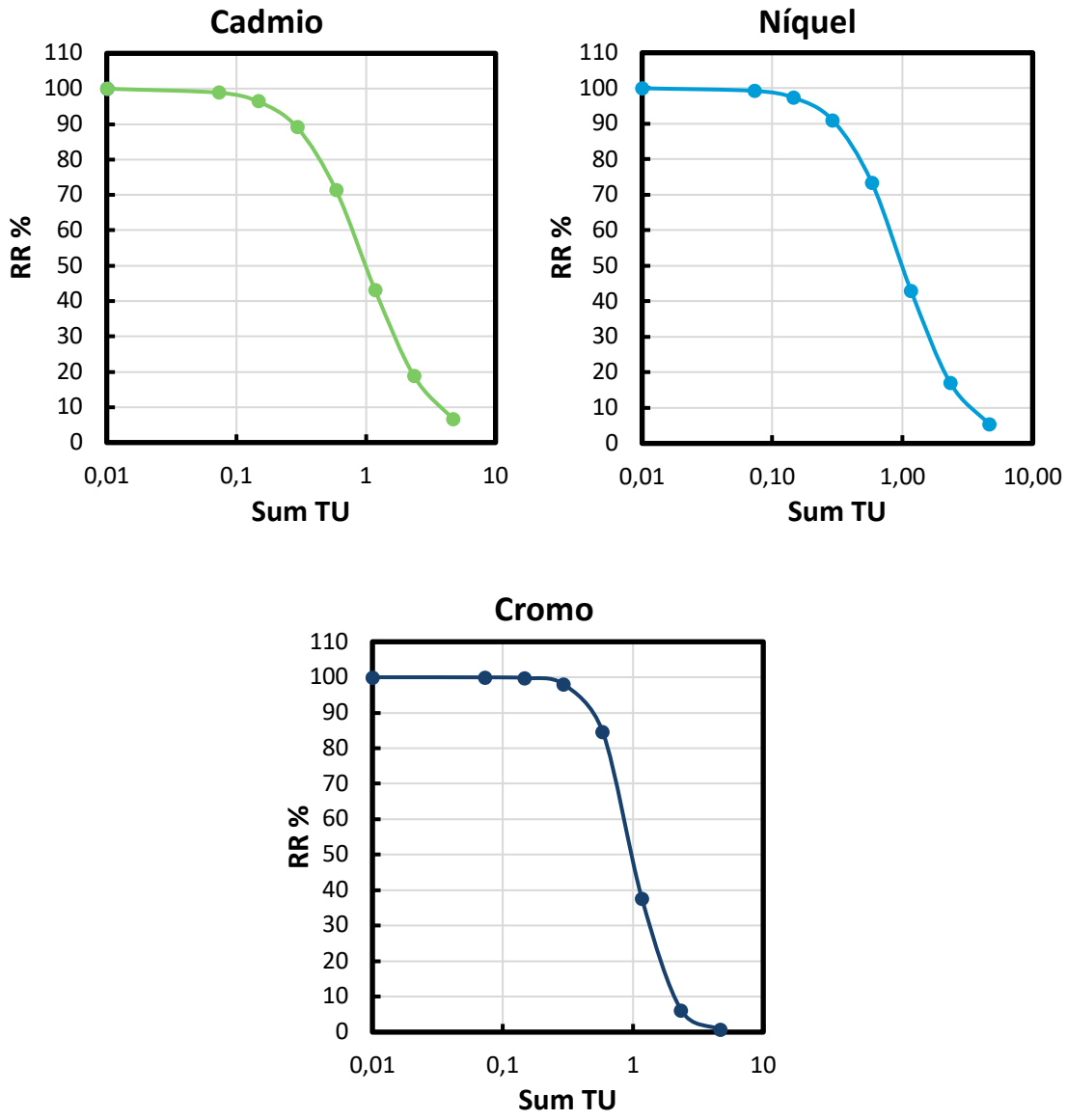


Figura 14. Curvas de respuesta RR (%) aplicando el modelo de acción independiente (IA) para cada uno de los metales (Cd, Ni y Cr) individualmente

Una vez seleccionadas las concentraciones de los metales individuales, se procede a simular la RR(%) para cada una de las mezclas binarias y para la mezcla ternaria. En cada caso, se aplicará la RR al modelo de acción independiente (Ecuación [4]) y al modelo de adición de concentraciones (Ecuación [5]).

$$RR_{mix}=100 \text{ x } \prod_{i=1}^{n}\left(\frac{1}{1+\left(\frac{C_{Me_i}}{EC50_{Me_i}}\right)^{\beta_{Me_i}}}\right) \qquad [4]$$

$$\sum_{i=1}^{n} \frac{C_{Me}}{EC_{50_{Me_i}} x \left(\frac{100-RR_{mix}}{RR_{mix}}\right)^{\frac{1}{\beta_{Me_i}}}} = 1 \qquad [5]$$

Donde RR_{mix} es la RR estimada de la mezcla, y *n* es el número de componentes de la muestra. Los resultados de las respuestas estimadas para cada modelo y mezclas binarias y terciarias se muestran en las tablas 15 y 16 respectivamente. Las concentraciones de los metales se expresan en □g L^{-1}.

Tabla 15. Concentraciones y respuesta relativa de los modelos IA y CA de los test de toxicidad de mezclas binarias de Cd, Cr y Ni

Mezcla binaria Cd-Cr			
Cd	Cr	RR(%) modelo IA	RR(%) modelo CA
0	0	100	100
6,5	32,3	98,84	98,13
11,6	58,2	96,83	94,34
21	104,8	91,15	83,20
37,7	188,6	75,95	58,48
67,9	339,5	42,45	27,19
122,2	611,1	9,48	8,41
220	1100,0	0,89	2,03
Mezcla binaria Cd-Ni			
Cd	Ni	RR(%) modelo IA	RR(%) modelo CA
0	0	100	100
6,5	27,3	98,14	96,86
11,6	49,2	94,89	91,60
21	88,6	86,34	79,15
37,7	159,5	67,51	57,09
67,9	287,0	38,01	31,70
122,2	516,7	12,90	13,93
220	930	2,67	5,34
Mezcla binaria Cr-Ni			
Cr	Ni	RR(%) modelo IA	RR(%) modelo CA
0	0	100	100
32,3	27,3	99,25	98,65
58,2	49,2	97,68	95,39
104,8	88,6	92,82	84,92
188,6	159,5	78,14	59,38
339,5	287,0	43,57	26,47
611,1	516,7	9,32	7,71
1100,0	930	0,80	1,79

Las representaciones de las curvas de las mezclas binarias con ambos modelos aplicados se muestran en la siguiente figura:

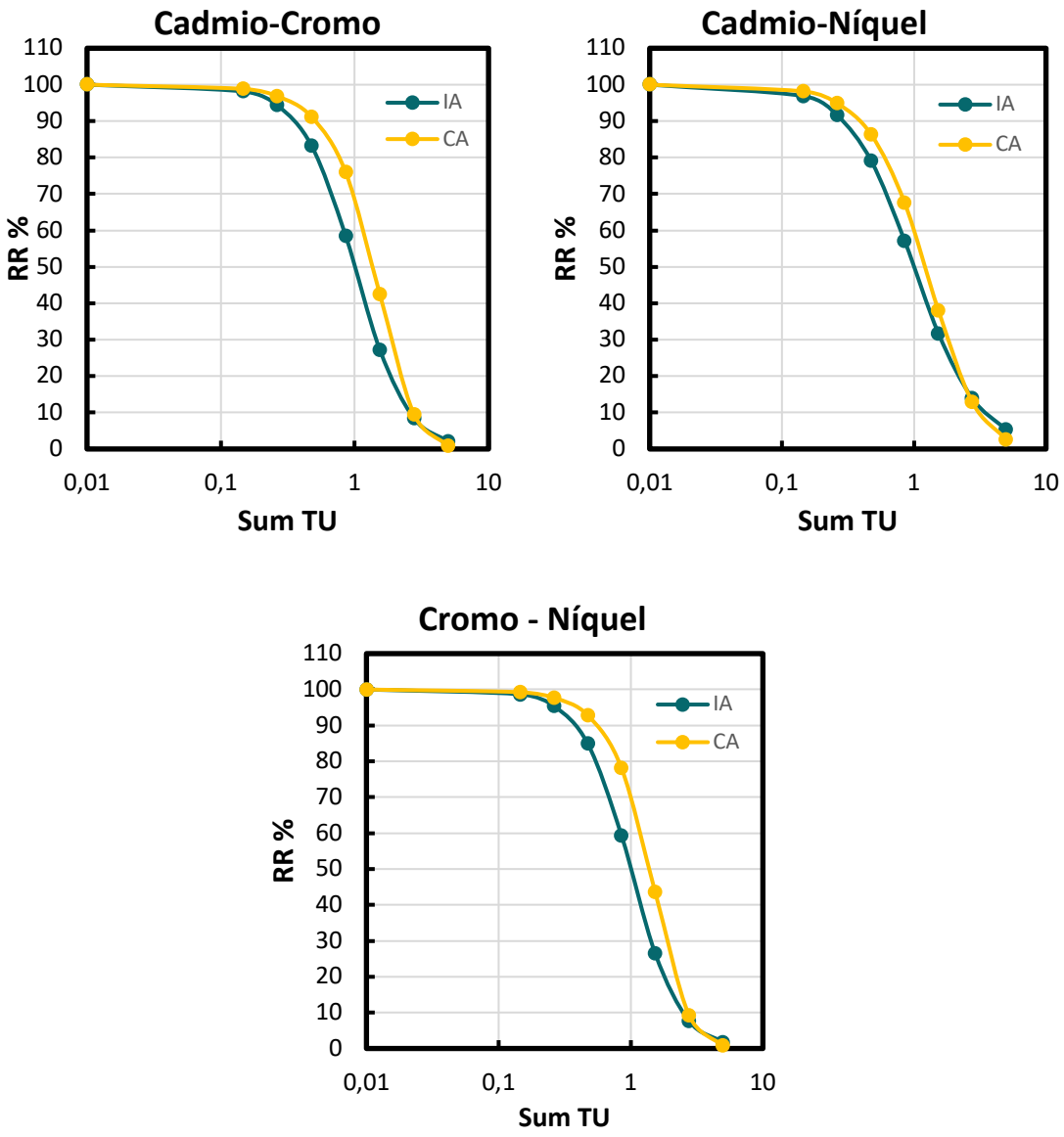


Figura 15. Curvas de respuesta RR (%) aplicando el modelo IA y CA para cada una de las mezclas binarias

Tal y como se puede ver en la Figura 15, el modelo CA es más conservador que el IA. El modelo CA predice una RR menor en todas las combinaciones de metales, es decir, un efecto de inhibición del crecimiento mayor.

Tabla 16. Concentraciones, TUs y respuesta relativa de los modelos IA y CA de los test de toxicidad de la combinación ternaria de Cd, Cr y Ni

Mezcla ternaria Cd-Cr-Ni					
Cd	Cr	Ni	Sum TU	RR(%) modelo IA	RR(%) modelo CA
0	0	0	0,01	100	100
3,6	18,0	15,2	0,12	99,34	98,86
6,5	32,3	27,3	0,22	98,13	96,78
11,6	58,2	49,2	0,40	94,72	91,12
21,0	104,8	88,6	0,71	85,50	77,28
37,7	188,6	159,5	1,28	63,29	37,24
67,9	339,5	287,1	2,30	26,52	14,20
122,2	611,1	516,7	4,15	3,37	4,18

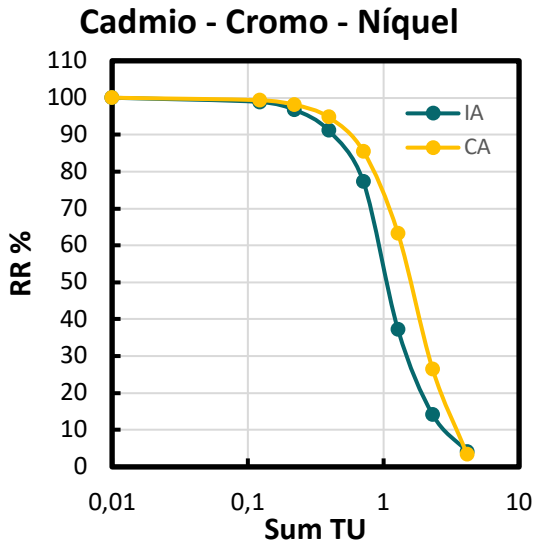


Figura 16. Curva de respuesta RR (%) aplicando el modelo IA y CA para la mezcla ternaria

Una vez diseñados los diferentes test de toxicidad, se llevan a cabo todos los experimentos simultáneamente, en las mismas condiciones. Mediante la medida de la densidad celular se calcula la velocidad de crecimiento normalizada con respecto al control en % para cada mezcla, así como para los metales individuales. De esta manera obtendríamos la respuesta observada en porcentaje.

A partir de las curvas de crecimiento de los tres metales individuales se obtienen los nuevos parámetros experimentales de β y de EC50, y se vuelve a aplicar los modelos

IA y CA a las mezclas binarias y a la mezcla ternaria de Cd, Cr y Ni. Así, se tienen las respuestas estimadas (%) de cada uno de los modelos a partir de las condiciones experimentales.

Para poder determinar el efecto sinérgico o antagónico de las mezclas de metales, se representa la RR(%) estimada frente a la RR(%) observada experimentalmente. El resultado se muestra a continuación:

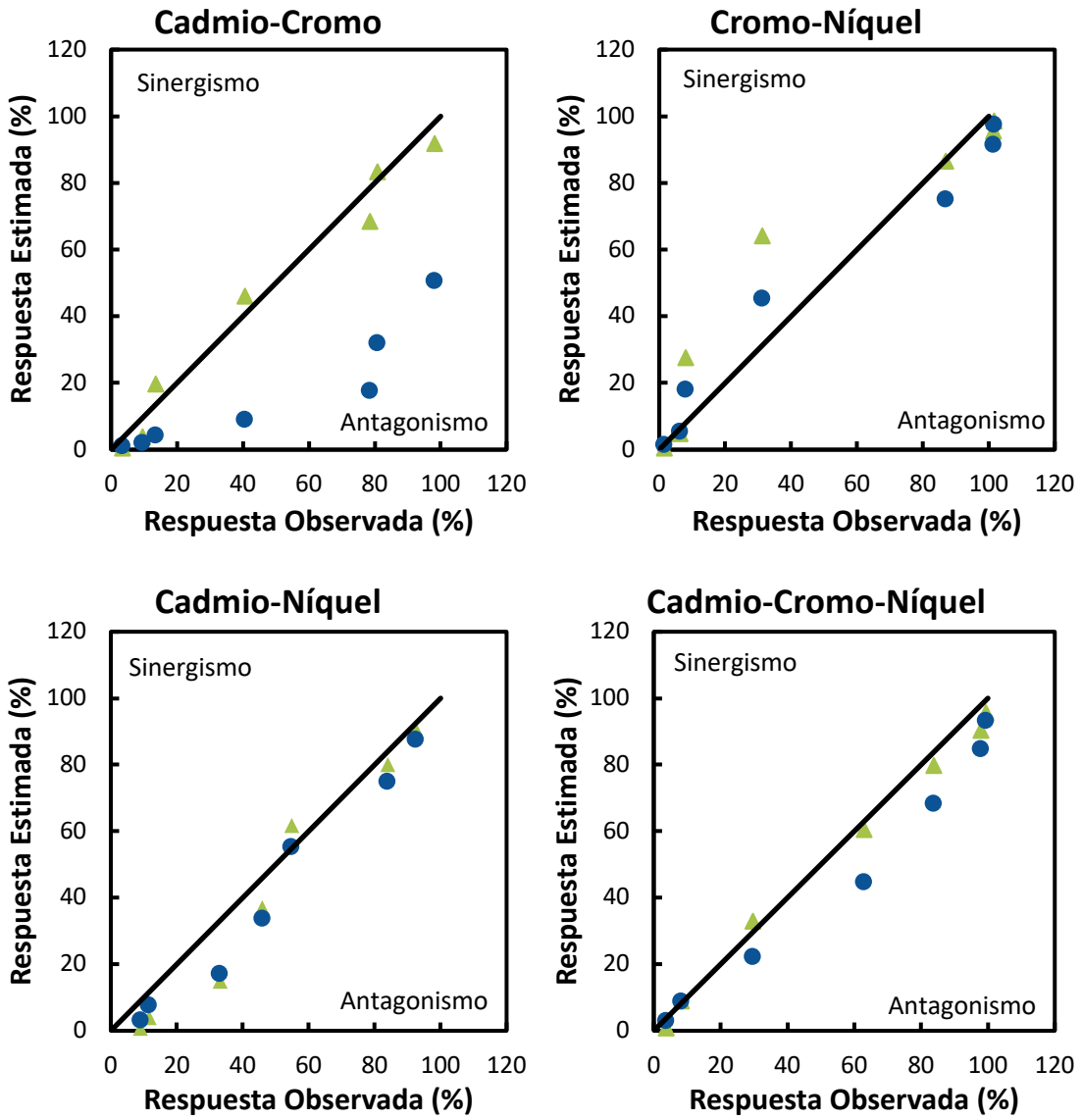


Figura 17. Curvas de respuesta estimada frente a la respuesta observada para los modelos IA (triángulos) y CA (círculos), en cada una de las mezclas de los test de toxicidad

Mediante estas representaciones (Figura 17) se puede concluir el tipo de efecto interactivo observado en las mezclas de metales dependiendo del modelo utilizado para este análisis.

Las predicciones del modelo IA se encuentran relativamente cercanas a las respuestas observadas en todos los experimentos. Por tanto, se detecta un efecto de no interacción relativo al este modelo de IA. Es decir, la combinación de estos metales tanto binaria como ternaria no mostraron una influencia unos sobre otros, sino que se comportaron de manera independiente. Sin embargo, para el modelo CA se observa que la respuesta estimada en la mayoría de los casos fue menor que la observada. Esto sugiere que el modelo CA tiende a una interacción antagónica, es decir, los metales interfieren entre sí en sus acciones dando lugar a un efecto menor que el esperado si tuvieran un efecto aditivo. Para poder confirmar que los efectos antagonistas relativos al modelo CA son significativos se debe realizar un análisis estadístico, empleando un parámetro de desviación (a) que indica la desviación de la no interacción.

Estos resultados constituyen un estudio preliminar de los efectos de todas las combinaciones posibles de los tres metales tóxicos Cd, Cr y Zn. El estudio permite comprobar cómo se desvían dos modelos ampliamente aplicados en la evaluación de riesgos de sustancias tóxicas de la respuesta real observada, y de esta manera poder seleccionar el modelo que mejor se ajuste para la predicción de la toxicidad.

CAPÍTULO 5. CONCLUSIONES

La metodología desarrollada y optimizada para la identificación y cuantificación de aminoácidos, polifenoles y carbohidratos es sensible y reproducible para su aplicación en células de microalgas y en el medio de cultivo enriquecido con sus exudados, así como en diferentes materiales vegetales.

Los resultados ponen de manifiesto la implicación de los metabolitos orgánicos analizados en la adaptación y tolerancia de las microalgas marina *Phaeodactylum tricornutum* y *Emiliania huxleyi* frente a condiciones de estrés ambiental como la toxicidad por la presencia de metales pesados y la acidificación oceánica.

De los estudios de *Phaeodactylum tricornutum* se concluye lo siguiente:

(i) Concentraciones de 0,31 μM de Cu(II) en el medio de cultivos resulta subletal reduciendo la densidad celular inicial en un 37%, mientras que con 0,79 y 1,31 μM de Cu(II) se reduce en 82 y 91% respectivamente, siendo letales para el crecimiento de la diatomea.

(ii) La acumulación de aminoácidos y polifenoles intracelulares, así como la liberación de carbohidratos al medio de cultivo por la diatomea marina se encuentran considerablemente afectadas por la presencia de Cu, experimentando un gran incremento a medida que aumentan los niveles de este metal.

(iii) El compuesto fenólico cuya concentración en el interior de la célula aumenta en mayor medida es el ácido gálico, el más activo inhibiendo radicales DDPH.

(iv) Entre los aminoácidos, la metionina, la histidina y la prolina son los que mayor acumulación experimentan en las células, posiblemente por su capacidad de complejar y transportar iones metálicos a través de la pared celular, así como de inhibir la formación de ROS.

(v) La naturaleza y la cantidad de polifenoles extracelulares varía en función de la concentración de Cu en el medio, siendo su comportamiento diferente a la de los polifenoles intracelulares.

(vi) La capacidad antioxidante de los extractos de las células de *Phaeodactylum tricornutum* y de sus exudados, también se ve intensificada en respuesta al estrés oxidativo, corroborado por las elevadas cantidades de MDA intracelulares.

(vii) La correlación entre el MDA y los polifenoles producidos por la diatomea demuestran la relación de estos metabolitos secundarios en el mecanismo de defensa del alga frente al estrés por Cu.

(viii) La capacidad complejante de Cu mostró la mayor actividad antioxidante, cuya correlación significativa con polifenoles y carbohidratos extracelulares podría indicar la relevancia de ambos metabolitos en la reducción de la toxicidad del Cu.

Del estudio de *Emiliania huxleyi* se extraen las siguientes conclusiones:

(i) La variación en el pH del medio de cultivo no afecta a su crecimiento con un ligero aumento de la densidad celular a valores intermedios, debido a su capacidad de adaptación.

(ii) La acidificación oceánica modifica la composición bioquímica intracelular y extracelular del alga.

(iii) Es la primera caracterización hasta la fecha de los compuestos fenólicos exudados y acumulados en esta especie de microalga marina, cultivada bajo diferentes condiciones de acidificación del medio.

(iv) No existe correlación entre los polifenoles intra- y extracelulares: el contenido de polifenoles intracelulares es mayor en los cultivos de menor pH mientras que los liberados al medio obtienen su máximo en las condiciones de mayor y menor acidificación.

(v) No existe correlación entre los carbohidratos intra- y extracelulares: los carbohidratos acumulados en las células y liberadas al medio se comportan de manera diferente según las necesidades de la célula en cada una de sus condiciones.

(vi) El perfil polifenólico intracelular y extracelular difiere en función de las condiciones del medio.

(vii) El cambio en la producción y exudación de estos metabolitos orgánicos en el alga se relacionan con las diferentes estrategias de adaptación y tolerancia a la acidificación del medio. Esto se puede observar también en la variación de la capacidad antioxidante de las células que es mayor en el pH más bajo, mientras que en los exudados es mayor a pH intermedios.

De la aplicación de la metodología desarrollada se concluye:

(i) Las microalgas de agua dulce *Cosmarium blytii, Cosmarium* sp., *Spyrogira* sp., *Chloromonas cf. Reticulata, Pseudopediastrum boryanum* y *Chlorodium saccharophilum*, presentan cantidades elevadas de carbohidratos, aminoácidos esenciales y polifenoles, que junto con la actividad antioxidante de sus extractos celulares las convierten en una potencial fuente de compuestos bioactivos para su empleo en nutrición y biomedicina.

(ii) La caracterización bioquímica de las hojas, tallos y raíces de la planta terrestre *Artemisia thuscula* revela su valor nutricional, con potenciales aplicaciones en la medicina, cosmética y nutrición.

CHAPTER 5. CONCLUSIONS

The methodology developed and optimized for the identification and quantification of amino acids, polyphenols, and carbohydrates is sensitive and reproducible for its application in microalgal cells and in culture medium enriched with their exudates, as well as in different plant materials.

The results highlight the involvement of organic metabolites in the adaptation and tolerance of the marine microalgae *Phaeodactylum tricornutum* and *Emiliania huxleyi* to environmental stress conditions such as toxicity due to the presence of heavy metals and ocean acidification.

From the study of *Phaeodactylum tricornutum,* the following conclusions are drawn:

(i) Concentrations of 0.31 μM Cu(II) in the culture medium are sublethal, leading to a 37% reduction in initial cell density. In contrast, concentrations of 0.79 and 1.31 μM Cu(II) cause a decrease of 82% and 91%, respectively, and are lethal, inhibiting the growth of the diatom.

(ii) The accumulation of intracellular amino acids and polyphenols, as well as the release of carbohydrates into the culture medium by the marine diatom, are affected by the presence of Cu, increasing as levels rise.

(iii) The phenolic compound whose concentration increases the most inside the cell is gallic acid, the most active in inhibiting DDPH radicals.

(iv) Among the amino acids, methionine, histidine, and proline show the greatest accumulation in the cells, possibly due to their ability to complex and transport metal ions, as well as to inhibit ROS formation.

(v) The nature and quantity of extracellular polyphenols vary depending on the concentration of Cu in the medium, differing from the accumulation of intracellular polyphenols.

(vi) The antioxidant capacity of *Phaeodactylum tricornutum* cells extracts and its exudates is also intensified in response to oxidative stress, corroborated by the high amounts of intracellular MDA. The correlation between MDA and the polyphenols demonstrates the role of these secondary metabolites in the defense mechanism against Cu stress.

(vii) The Cu chelating capacity exhibited the highest antioxidant activity, and its significant correlation with extracellular polyphenols and

carbohydrates suggests the importance of these metabolites in mitigating Cu toxicity.

From the study of *Emiliania huxleyi,* the following conclusions are drawn:

(i) Variations in the pH of the culture medium do not affect its growth, with a slight increase in cell density at intermediate values, attributable to its adaptive capacity.

(ii) Ocean acidification modifies the intracellular and extracellular biochemical composition of the alga.

(iii) This is the first characterization to date of the phenolic compounds exuded and accumulated in this species, cultivated under different conditions of medium acidification.

(iv) There is no correlation between intra- and extracellular polyphenols: the content of intracellular polyphenols is higher in cultures with lower pH, while those released into the medium obtain its maximum under conditions of both higher and lower acidification.

(v) There is no correlation between intra- and extracellular carbohydrates: the carbohydrates accumulated within the cells and released into the medium behave differently depending on the cell's needs under each condition.

(vi) The intracellular and extracellular polyphenolic profiles vary depending on the culture conditions.

(vii) The changes in the production and exudation of these organic metabolites in the alga are associated with different strategies for adaptation and tolerance to medium acidification. This is also observed in the variation of the antioxidant capacity of cell extracts, which is higher at the lowest pH, while in the exudates, it is greater at intermediate pH levels.

From the application of the developed methodology, the following conclusions are drawn:

(i) The freshwater microalgae *Cosmarium blytii, Cosmarium* sp., *Spyrogira* sp., *Chloromonas* cf. *reticulata*, *Pseudopediastrum boryanum*, and *Chlorodium saccharophilum*, contain high amounts of carbohydrates, essential amino acids and polyphenols. This compounds in combination

with the antioxidant capacity of their cell extracts, make them a potential source of bioactive molecules for use in nutrition and biomedicine.

(ii) The biochemical characterization of the leaves, stems, and roots of the terrestrial plant *Artemisia thuscula* reveals its nutritional value with potential applications in medicine, cosmetics, and nutrition.

CAPÍTULO 6. ACTIVIDADES COMPLEMENTARIAS A LA ACTIVIDAD INVESTIGADORA

Durante el período de doctorado, además de las actividades de investigación que permitieron el desarrollo del presente documento de Tesis Doctoral, se han realizado diversas actividades complementarias que han sido fundamentales tanto para el desarrollo profesional como investigador y docente, como para alcanzar los objetivos específicos establecidos. Las actividades más destacadas se describen a continuación:

ACTIVIDADES FORMATIVAS

PROGRAMA DE DOCTORADO

- Introducción a Matlab y R. 60 horas. Notable.
- Búsqueda de referencias bibliográficas sobre resultados de investigación. 10 horas. Sobresaliente.
- Presentación pública de trabajo de investigación desarrollado. 10 horas. Sobresaliente.

COMPLEMENTARIAS

- Indicios de calidad en las publicaciones científicas. 8 horas. ULPGC.
- Redacción de artículos científicos en ciencias. 6 horas. ULPGC.
- Empleabilidad en los estudios de doctorado: especial referencia al autoempleo. 10 horas. ULPGC.
- Inglés para la comunicación científica: características y herramientas. 15 horas. ULPGC.
- Transferencia de resultados de investigación, gestión de propiedad intelectual. 6 horas. ULPGC.
- Seminario online Troubleshooting series: Reproducibilidad en áreas. Parte II. 2 horas. Waters Cromatografía.

MOVILIDAD A CENTROS DE INVESTIGACIÓN INTERNACIONALES

- Universidade de Aveiro (Portugal). Del 01/02/2022 al 03/05/2022. Estancia en el Departamento de Química bajo la supervisión del Prof. Dr. Manuel António Coimbra Rodrigues da Silva para la adquisición de conocimientos sobre diferentes técnicas analíticas de determinación de carbohidratos.
- Universiteit Gent (Bélgica). Del 15/09/2022 al 15/12/2022. Estancia en el laboratorio GhenToxLab (Ghent University Environmental Toxicology Unit) bajo

la supervisión del Prof. Dr. Karel De Schamphelaere para la adquisición de conocimientos sobre el cultivo y desarrollo de test de toxicología en microalgas relacionadas con metales.

DIVULGACIÓN CIENTÍFICA

PARTICIPACIÓN EN CONGRESOS INTERNACIONALES

- *25th International Symposium on Advances in Extraction Technologies.* Arona, julio 2023. Presentación del póster "Characterization of leaves, stem and roots of *Artemisia thuscula* for potential food and pharmaceutical applications".
- *25th International Symposium on Advances in Extraction Technologies.* Arona, julio 2023. Presentación del póster "Extraction and analysis of phenolic compounds in *Phaeodactylum tricornutum* cells in Cu polluted cultures".
- *VII International Symposium on Marine Sciences*. Las Palmas de Gran Canaria, julio 2022. Presentación de la comunicación oral "Behaviour of free amino acids in *Phaeodactylum tricornutum* cells under copper stress".
- *VII International Symposium on Marine Sciences*. Las Palmas de Gran Canaria, julio 2022. Participación en el póster "Quantification of carotenoids and chlorophylls in algae".
- *VII International Symposium on Marine Sciences*. Las Palmas de Gran Canaria, julio 2022. Participación en el póster "Emerging challenges: Microalgae, waste or healthy food?".
- *VII International Symposium on Marine Sciences*. Las Palmas de Gran Canaria, julio 2022. Participación en el póster "Identification of phenolic compounds exuded by *Emiliania huxleyi* under acidification conditions".
- *VII International Symposium on Marine Sciences.* Las Palmas de Gran Canaria, julio 2022. Participación en el póster "Seasonal variability of the phenolic profiles of *Cymopolia Barbata* and *Lobophora variegata*".
- *VI International Symposium on Marine Sciences*. Barcelona, julio 2020. Presentación del póster "The amino acid profile on the marine diatom *Phaeodactylum tricornutum* under different copper levels".

OTRAS ACTIVIDADES DE DIVULGACIÓN CIENTÍFICA

- Artículo en la revista OKEANOS. Revista de la Sociedad Atlántica de Oceanógrafos titulado "¿Química orgánica en oceanografía?" en 2022.
- Artículo en la revista OKEANOS. Revista de la Sociedad Atlántica de Oceanógrafos titulado "Microalgas marinas como fuente de aminoácidos" en 2021.
- Participación en la sección "Preséntame tu tesis" del programa *El Laboratorio* de RTVC.
- Coautoría del artículo "Microalgas: Una alternativa frente a los ultraprocesados", publicado en *The Conversation* (España) en diciembre de 2021.
- Participación en *Macaronight - La noche de los investigadores*. Septiembre 2021. Presentación de la ponencia "Envenenamiento por metales: ¿cómo se defienden las microalgas en el medio marino?".

INNOVACIÓN EDUCATIVA

- Coautoría del artículo "Comparación de estrategias docentes en prácticas de laboratorio de química" enviado a la *Revista de Docencia Universitaria (REDU)*. 2024.
- Presentación del póster "Aplicación de la metodología basada en resolución de problemas en prácticas de laboratorio de química en enseñanza superior" en el *V Congreso Internacional de Innovación Docente e Investigación en Educación Superior: Nuevas tendencias para el cambio en la enseñanza superior de las Áreas de Conocimiento.* Sevilla, noviembre 2023.
- Coautoría del artículo "Implementation of the inverted classroom in organic chemistry laboratory practices" en la revista *International Journal of Human Sciences Research.* 2023. Indicio de calidad: Arts and Humanities Citation Index (AyHCI).
- Participación en el grupo de innovación educativa de la ULPGC: GIEMAR-Grupo de Innovación Educativa en Ciencias Marinas. Cursos 2022/2023 y 2023/2024.
- Coautoría de la comunicación oral "Un enfoque seguro, ecológico y sostenible para el estudio de la aditividad de absorbancias en espectrofotometría UV-VIS

en prácticas de laboratorio de Química" presentada en el *IV Congreso Internacional de Didáctica de la Química.* Galicia, mayo 2023.

- Coautoría de la comunicación oral "Implantación del aula invertida en prácticas de laboratorio de Química Orgánica" presentada en las *IX Jornadas Iberoamericanas de Innovación Educativa en el ámbito de las TIC y las TAC (INNOEDUCATIC 2022).* Las Palmas de Gran Canaria, noviembre 2022.
- Realización del curso *Iniciación a la Educación Educativa*. ULPGC. 2 ECTS. Curso 2020/2021.

PARTICIPACIÓN EN PROYECTOS

- Miembro del equipo de trabajo en el proyecto "Respuesta del Fe en un océano acidificado (FeRIA)" (PID2021-123997NB-I00) financiado por el Ministerio de Ciencia e Innovación.
- Miembro del equipo de trabajo en el proyecto "Análisis multidisciplinar de sumideros de carbono azul en aguas costeras (MultiCoast)" (TED2021-130892B-I00) financiado por el Ministerio de Ciencia e Innovación.
- Participación en el proyecto de Innovación Educativa "Metodologías activas y gamificación para la mejora del autoaprendizaje y la motivación del alumnado" (PIE 2022-66) financiado por la Unión Europea, NextGenerationEU, UNIDIGITAL - PIEFI - Línea 3. Contenidos y programas de formación.
- Participación en el proyecto de Innovación Educativa "Mejora del autoaprendizaje a través de la motivación del alumnado. Proyección hacia potenciales futuros estudiantes" (PIE 2023-66) financiado por la Unión Europea, NextGenerationEU, UNIDIGITAL - PIEFI - Línea 3. Contenidos y programas de formación.

PARTICIPACIÓN EN LABORES DOCENTES

- Actividad docente en la asignatura "Química Orgánica" del Grado de Ingeniería Química de la Universidad de Las Palmas de Gran Canaria. 90 horas. Curso 2020/2021 y 2021/2022.

- Actividad docente en la asignatura “Fundamentos de Química” del Grado de Ciencias del Mar de la Universidad de Las Palmas de Gran Canaria. 24 horas. Curso 2021/2022.
- Actividad docente en la asignatura “Química Marina” del Grado de Ciencias del Mar de la Universidad de Las Palmas de Gran Canaria. 6 horas. Curso 2021/2022.
- Actividad docente en la asignatura “Química General” del Grado de Ciencias del Mar de la Universidad de Las Palmas de Gran Canaria. 24 horas. Curso 2022/2023.
- Actividad docente en la asignatura “Química Orgánica” del Grado de Ciencias del Mar de la Universidad de Las Palmas de Gran Canaria. 30 horas. Curso 2022/2023.
- Actividad docente en la asignatura “Química General” del Grado de Ingeniería Química de la Universidad de Las Palmas de Gran Canaria. 60 horas. Curso 2023/2024.
- Actividad docente en la asignatura “Química” del Grado de Física de la Universidad Nacional de Educación a Distancia. 24 horas. Curso 2021/2022 y 2023/2024.
- Actividad docente en la asignatura “Introducción a la Experimentación en Química Orgánica y Química Inorgánica” del Grado de Química de la Universidad Nacional de Educación a Distancia. 70 horas. Curso 2021/2022 y 2022/2023.
- Actividad docente en la asignatura “Fundamentos Químicos de la Ingeniería” del Grado de Ingeniería a de la Universidad Nacional de Educación a Distancia. 39 horas. Curso 2022/2023 y 2023/2024.
- Actividad docente en la asignatura “Principios Básicos de Química y Estructura” del Grado de Química de la Universidad Nacional de Educación a Distancia. 22 horas. Curso 2022/2023 y 2022/2023.

TUTORIZACIÓN DE TRABAJOS DE FIN DE GRADO

- Cotutora del Trabajo de Final de Grado titulado *Contenido de aminoácidos en microorganismos marinos en diferentes condiciones ambientales para su aplicación industrial*. Leído durante el curso 2020/2021 con calificación notable.
- Cotutora del Trabajo de Final de Grado titulado *Análisis de metabolitos en micro- y macroalgas*. Leído durante el curso 2022/2023 con calificación de sobresaliente.

TUTORIZACIÓN DE PRÁCTICAS DE EMPRESA

- Tutora de Empresa del alumnado perteneciente al 2º CFGM Química-Operaciones de Laboratorio (LOE) del Módulo de Formación en Centros de Trabajo en Formación Profesional. Con un total de 346 horas durante los cursos 2020/2021, 2021/2022 y 2022/2023.
- Tutora de Empresa de Prácticas Externas del Grado de Ingeniería Química de la Universidad de Las Palmas de Gran Canaria. Con un total de 240 horas durante el curso 2022/2023.

REFERENCIAS

Abinandan, S., Venkateswarlu, K., & Megharaj, M. (2021). Phenotypic changes in microalgae at acidic pH mediate their tolerance to higher concentrations of transition metals. *Current Research in Microbial Sciences*, *2*. https://doi.org/10.1016/j.crmicr.2021.100081

Afkar, E., Ababna, H., & Fathi, A. A. (2010). Toxicological response of the green alga *Chlorella vulgaris*, to some heavy metals. *American Journal of Environmental Sciences*, *6*(3), 230-237. https://doi.org/10.3844/ajessp.2010.230.237

Ajisaka, K., Agawa, S., Nagumo, S., Kurato, K., Yokoyama, T., Arai, K., & Miyazaki, T. (2009). Evaluation and comparison of the antioxidative potency of various carbohydrates using different methods. *Journal of Agricultural and Food Chemistry*, *57*(8), 3102-3107. https://doi.org/10.1021/jf804020u

Alia, & Saradhi, P. P. (1991). Proline accumulation under heavy metal stress. *Journal of Plant Physiology*, *138*(5), 554-558. https://doi.org/10.1016/S0176-1617(11)80240-3

Aliaga, C., & Lissi, E. A. (2000). Reactions of the radical cation derived from 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS·+) with amino acids. Kinetics and mechanism. *Canadian Journal of Chemistry*, *78*(8), 1052-1059. https://doi.org/10.1139/v00-099

Andjelković, M., Van Camp, J., De Meulenaer, B., Depaemelaere, G., Socaciu, C., Verloo, M., & Verhe, R. (2006). Iron-chelation properties of phenolic acids bearing catechol and galloyl groups. *Food Chemistry*, *98*(1), 23-31. https://doi.org/10.1016/j.foodchem.2005.05.044

Anusree, M. K., Manasa Leela, K., Sreehari, M., Raj, S., Sreenikethanam, A., & Bajhaiya, A. K. (2023). Chapter 14 - Marine microalgae: an emerging source of pharmaceuticals and bioactive compounds. En S. N. Meena, V. Nandre, K. Kodam, & R. S. Meena (Eds.), *New Horizons in Natural Compound Research* (pp. 251-265). Academic Press. https://doi.org/https://doi.org/10.1016/B978-0-443-15232-0.00025-4

Arnone, V., González-Santana, D., González-Dávila, M., González, A. G., & Santana-Casiano, J. M. (2022). Iron and copper complexation in Macaronesian coastal waters. *Marine Chemistry*, *240*. https://doi.org/10.1016/j.marchem.2022.104087

Bassi, R., & Sharma, S. S. (1993). Changes in proline content accompanying the uptake of zinc and copper by *Lemna minor*. *Annals of Botany*, *72*(2), 151-154. https://doi.org/https://doi.org/10.1006/anbo.1993.1093

Bastos, R., Coelho, E., & Coimbra, M. A. (2015). Modifications of *Saccharomyces pastorianus* cell wall polysaccharides with brewing process. *Carbohydrate Polymers*, *124*, 322-330. https://doi.org/10.1016/j.carbpol.2015.02.031

Bathula, S., & Kotra, K. K. (2019). Mixed ligand complexes of transition metal ions with selective bio-ligands in surfactant-water mixtures. *International Journal of Recent Technology and Engineering*, *8*(2 Special Issue 11), 3182-3190. https://doi.org/10.35940/ijrte.B1417.0982S1119

Benner, R. (2011). Loose ligands and available iron in the ocean. *Proceedings of the National Academy of Sciences of the United States of America*, *108*(3), 893-894. https://doi.org/10.1073/pnas.1018163108

Benzie, I. F. F., & Strain, J. J. (1996). The ferric reducing ability of plasma (FRAP) as a Measure of "Antioxidant power": The FRAP assay. *Analytical Biochemistry*, *239*(1), 70-76. https://doi.org/https://doi.org/10.1006/abio.1996.0292

Bhavya, P. S., Kim, B. K., Jo, N., Kim, K., Kang, J. J., Lee, J. H., Lee, D., Lee, J. H., Joo, H., Ahn, S. H., Kim, Y., Min, J.-O., Kang, M. G., Yun, M. S., Kang, C. K., & Lee, S. H. (2019). A review on the macromolecular compositions of phytoplankton and the implications for aquatic biogeochemistry. *Ocean Science Journal*, *54*(1), 1-14. https://doi.org/10.1007/s12601-018-0061-8

Blake, J. D., & Richards, G. N. (1968). Problems of lactonisation in the analysis of uronic acids. *Carbohydrate Research*, *8*(3), 275-281. https://doi.org/https://doi.org/10.1016/S0008-6215(00)82233-8

Blois, M. (1958). Antioxidant determinations by the use of a stable free radical. *Nature*, *181*, 1199-1200. https://doi.org/https://doi.org/10.1038/1811199a0

Blumenkrantz, N., & Asboe-Hansen, G. (1973). New method for quantitative determination of uronic acids. *Analytical Biochemistry*, *54*(2), 484-489. https://doi.org/https://doi.org/10.1016/0003-2697(73)90377-1

Borchard, C., & Engel, A. (2015). Size-fractionated dissolved primary production and carbohydrate composition of the coccolithophore *Emiliania huxleyi. Biogeosciences*, *12*(4), 1271-1284. https://doi.org/10.5194/bg-12-1271-2015

Bougherra, H., Berradj, O., Adkhis, A., & Amrouche, T. (2018). Synthesis, characterization, electrochemical and biological activities of mixed ligand copper(II) complexes with dimethylglyoxime and amino acids. *Journal of Molecular Structure*, *1173*, 280-290. https://doi.org/10.1016/j.molstruc.2018.06.088

Brown, M. R., Garland, C. D., Jeffrey, S. W., Jameson, I. D., & Leroi, J. M. (1993). The gross and amino acid compositions of batch and semi-continuous cultures ofIsochrysis sp. (clone T.ISO), *Pavlova lutheri* and *Nannochloropsis oculata*. *Journal of Applied Phycology*, *5*(3), 285-296. https://doi.org/10.1007/BF02186231

Callejón, R. M., Troncoso, A. M., & Morales, M. L. (2010). Determination of amino acids in grape-derived products: A review. *Talanta*, *81*(4-5), 1143-1152. https://doi.org/10.1016/j.talanta.2010.02.040

Cardoso, C., Pereira, H., Franca, J., Matos, J., Monteiro, I., Pousão-Ferreira, P., Gomes, A., Barreira, L., Varela, J., Neng, N., Nogueira, J. M., Afonso, C., & Bandarra, N. M. (2020). Lipid composition and some bioactivities of 3 newly isolated microalgae (*Tetraselmis* sp. IMP3, *Tetraselmis* sp. CTP4, and *Skeletonema* sp.). *Aquaculture International*, *28*(2), 711-727. https://doi.org/10.1007/s10499-019-00489-w

Çelekli, A., Kapi, M., & Bozkurt, H. (2013). Effect of cadmium on biomass, pigmentation, malondialdehyde, and proline of *Scenedesmus quadricauda* var. *longispina*. *Bulletin of Environmental Contamination and Toxicology*, *91*(5), 571-576. https://doi.org/10.1007/s00128-013-1100-x

Chang, E., & Kim, C. Y. (2018). Lipid peroxidation and antioxidant activities of the aqueous rhizome extract of *Rheum officinale Baillon*. *Journal of Food Quality*, *2018*, 1-7. https://doi.org/10.1155/2018/5258276

Chen, C. T., Chen, L.-M., Lin, C., & Kao, C. H. (2001). Regulation of proline accumulation in detached rice leaves exposed to excess copper. *Plant Science*, *160*(2), 283-290. https://doi.org/10.1016/S0168-9452(00)00393-9

Chia, M. A., Lombardi, A. T., da Graça Gama Melão, M., & Parrish, C. C. (2015). Combined nitrogen limitation and cadmium stress stimulate total carbohydrates, lipids, protein and amino acid accumulation in *Chlorella vulgaris (Trebouxiophyceae*). *Aquatic Toxicology*, *160*, 87-95. https://doi.org/https://doi.org/10.1016/j.aquatox.2015.01.002

Chokshi, K., Pancha, I., Ghosh, A., & Mishra, S. (2017). Salinity induced oxidative stress alters the physiological responses and improves the biofuel potential of green microalgae *Acutodesmus dimorphus*. *Bioresource Technology*, *244*, 1376-1383. https://doi.org/10.1016/j.biortech.2017.05.003

Cichoński, J., & Chrzanowski, G. (2022). Microalgae as a Source of Valuable Phenolic Compounds and Carotenoids. *Molecules*, *27*(24). https://doi.org/10.3390/molecules27248852

Coale, K. H., & Bruland, K. W. (1988). Copper complexation in the Northeast Pacific. *Limnology and Oceanography*, *33*(5), 1084-1101. https://doi.org/10.4319/lo.1988.33.5.1084

Cuevas, A., Kremer, E., Etcheverry, S. B., & Baran, E. J. (1998). Infrared spectra of the copper(II) complexes of amino acids with hydrophobic residues. *Acta Farnz. Borlaerense*, *17*(3), 213-221.

Curcuraci, E., Manuguerra, S., Messina, C. M., Arena, R., Renda, G., Ioannou, T., Amato, V., Hellio, C., Barba, F. J., & Santulli, A. (2022). Culture conditions affect antioxidant production, metabolism and related biomarkers of the microalgae *Phaeodactylum tricornutum*. *Antioxidants*, *11*(2). https://doi.org/10.3390/antiox11020411

Dai, Z., Wu, Z., Jia, S., & Wu, G. (2014). Analysis of amino acid composition in proteins of animal tissues and foods as pre-column o-phthaldialdehyde derivatives by HPLC with fluorescence detection. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*, *964*, 116-127. https://doi.org/10.1016/j.jchromb.2014.03.025

Danouche, M., El Ghatchouli, N., & Arroussi, H. (2022). Overview of the management of heavy metals toxicity by microalgae. *Journal of Applied Phycology*, *34*(1), 475-488. https://doi.org/10.1007/s10811-021-02668-w

Dao, L., Beardall, J., & Heraud, P. (2017). Characterisation of Pb-induced changes and prediction of Pb exposure in microalgae using infrared spectroscopy. *Aquatic Toxicology*, *188*, 33-42. https://doi.org/10.1016/j.aquatox.2017.04.006

De Ruiter, G. A., Schols, H. A., Voragen, A. G. J., & Rombouts, F. M. (1992). Carbohydrate analysis of water-soluble uronic acid-containing polysaccharides with high-performance anion-exchange chromatography using methanolysis combined with TFA hydrolysis is

superior to four other methods. *Analytical Biochemistry*, *207*(1), 176-185. https://doi.org/https://doi.org/10.1016/0003-2697(92)90520-H

Del Mondo, A., Smerilli, A., Ambrosino, L., Albini, A., Noonan, D. M., Sansone, C., & Brunet, C. (2021). Insights into phenolic compounds from microalgae: structural variety and complex beneficial activities from health to nutraceutics. *Critical Reviews in Biotechnology*, *41*(2), 155-171. https://doi.org/10.1080/07388551.2021.1874284

Duarte, C. M. (2014). Global change and the future ocean: a grand challenge for marine sciences. *Frontiers in Marine Science*, *1*. https://doi.org/10.3389/fmars.2014.00063

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., & Smith, F. (1956). Colorimetric method for determination of sugars and related substances. *Analytical Chemistry*, *28*(3), 350-356. https://doi.org/10.1021/ac60111a017

Dupont, C. L., & Ahner, B. A. (2005). Effects of copper, cadmium, and zinc on the production and exudation of thiols by *Emiliania huxleyi*. *Limnology and Oceanography*, *50*(2), 508-515. https://doi.org/10.4319/lo.2005.50.2.0508

Dupont, C. L., Nelson, R. K., Bashir, S., Moffett, J. W., & Ahner, B. A. (2004). Novel copper-binding and nitrogen-rich thiols produced and exuded by *Emiliania huxleyi*. *Limnology and Oceanography*, *49*(5), 1754-1762. https://doi.org/10.4319/lo.2004.49.5.1754

Engel, A., Thoms, S., Riebesell, U., Rochelle-Newall, E., & Zondervan, I. (2004). Polysaccharide aggregation as a potential sink of marine dissolved organic carbon. *Nature*, *428*(6986), 929-932. https://doi.org/10.1038/nature02453

Fal, S., Aasfar, A., Rabie, R., Smouni, A., & Arroussi, H. EL. (2022). Salt induced oxidative stress alters physiological, biochemical and metabolomic responses of green microalga *Chlamydomonas reinhardtii.* *Heliyon*, *8*(1). https://doi.org/10.1016/j.heliyon.2022.e08811

Fernandes, P. A. R., & Coimbra, M. A. (2023). The antioxidant activity of polysaccharides: A structure-function relationship overview. *Carbohydrate Polymers*, *314*, 120965. https://doi.org/https://doi.org/10.1016/j.carbpol.2023.120965

Field, C. B., Behrenfeld, M. J., Randerson, J. T., & Falkowski, P. (1998). Primary production of the biosphere: Integrating terrestrial and oceanic components. *Science*, *281*(5374), 237-240. https://doi.org/10.1126/science.281.5374.237

Filisetti-Cozzi, T. M. C. C., & Carpita, N. C. (1991). Measurement of uranic acids without interference from neutral sugars. *ANALYTICAL BIOCHEMISTRY*, *197*(1), 157-162. https://doi.org/10.1016/0003-2697(91)90372-Z

Fisher, N. S., & Fabris, J. G. (1982). Complexation of Cu, Zn and Cd by metabolites excreted from marine diatoms. *Marine Chemistry*, *11*(3), 245-255. https://doi.org/http://dx.doi.org/10.1016/0304-4203(82)90019-6

Franklin, N. M., Stauber, J. L., & Lim, R. P. (2001). Development of flow cytometry-based algal bioassays for assessing toxicity of copper in natural waters. *Environmental Toxicology and Chemistry*, *20*(1), 160-170. https://doi.org/10.1002/etc.5620200118

Gill, S. S., & Tuteja, N. (2010). Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. *Plant Physiology and Biochemistry*, *48*(12), 909-930. https://doi.org/10.1016/j.plaphy.2010.08.016

Goiris, K., Muylaert, K., Fraeye, I., Foubert, I., De Brabanter, J., & De Cooman, L. (2012). Antioxidant potential of microalgae in relation to their phenolic and carotenoid content. *Journal of Applied Phycology*, *24*(6), 1477-1486. https://doi.org/10.1007/s10811-012-9804-6

González, A. G., Cadena-Aizaga, M. I., Sarthou, G., González-Dávila, M., & Santana-Casiano, J. M. (2019). Iron complexation by phenolic ligands in seawater. *Chemical Geology*, *511*, 380-388. https://doi.org/10.1016/j.chemgeo.2018.10.017

González, A. G., Santana-Casiano, J. M., González-Dávila, M., & Pérez, N. (2012). Effect of organic exudates of *Phaeodactylum tricornutum* on the Fe (II) oxidation rate constant. *Ciencias marinas*, *38*(1B), 245-261.

Gouvêa, S. P., Vieira, A. A. H., & Lombardi, A. T. (2005). Copper and cadmium complexation by high molecular weight materials of dominant microalgae and of water from a eutrophic reservoir. *Chemosphere*, *60*(9), 1332-1339. https://doi.org/10.1016/j.chemosphere.2005.01.061

Gyurcsik, B., & Nagy, L. (2000). Carbohydrates as ligands: Coordination equilibria and structure of the metal complexes. *Coordination Chemistry Reviews*, *203*(1). https://doi.org/10.1016/S0010-8545(99)00183-6

Hajimahmoodi, M., Faramarzi, M. A., Mohammadi, N., Soltani, N., Oveisi, M. R., & Nafissi-Varcheh, N. (2010). Evaluation of antioxidant properties and total phenolic contents of some strains of microalgae. *Journal of Applied Phycology*, *22*(1), 43-50. https://doi.org/10.1007/s10811-009-9424-y

Halford, N. G., Curtis, T. Y., Muttucumaru, N., Postles, J., & Mottram, D. S. (2011). Sugars in crop plants. *Annals of Applied Biology*, *158*(1), 1-25. https://doi.org/10.1111/j.1744-7348.2010.00443.x

Halliwell, B., Murcia, M. A., Chirico, S., & Aruoma, O. I. (1995). Free radicals and antioxidants in food and in vivo: What they do and how they work. *Critical Reviews in Food Science and Nutrition*, *35*(1-2), 7-20. https://doi.org/10.1080/10408399509527682

Hamed, S. M., Selim, S., Klöck, G., & AbdElgawad, H. (2017). Sensitivity of two green microalgae to copper stress: Growth, oxidative and antioxidants analyses. *Ecotoxicology and Environmental Safety*, *144*, 19-25. https://doi.org/10.1016/j.ecoenv.2017.05.048

Hamed, S. M., Zinta, G., Klöck, G., Asard, H., Selim, S., & AbdElgawad, H. (2017). Zinc-induced differential oxidative stress and antioxidant responses in *Chlorella sorokiniana* and *Scenedesmus acuminatus*. *Ecotoxicology and Environmental Safety*, *140*, 256-263. https://doi.org/10.1016/j.ecoenv.2017.02.055

Hassler, C. S., Schoemann, V., Nichols, C. M., Butler, E. C. V, & Boyd, P. W. (2011). Saccharides enhance iron bioavailability to Southern Ocean phytoplankton. *Proceedings of the National Academy of Sciences*, *108*(3), 1076-1081. https://doi.org/10.1073/pnas.1010963108

Hays, G. C., Richardson, A. J., & Robinson, C. (2005). Climate change and marine plankton. *Trends in Ecology and Evolution*, *20*(6), 337-344. https://doi.org/10.1016/j.tree.2005.03.004

He, M., Yan, Y., Pei, F., Wu, M., Gebreluel, T., Zou, S., & Wang, C. (2017). Improvement on lipid production by *Scenedesmus obliquus* triggered by low dose exposure to nanoparticles. *Scientific Reports*, *7*(1), 15526. https://doi.org/10.1038/s41598-017-15667-0

Hodges, D. M., DeLong, J. M., Forney, C. F., & Prange, R. K. (1999). Improving the thiobarbituric acid-reactive-substances assay for estimating lipid peroxidation in plant

tissues containing anthocyanin and other interfering compounds. *Planta*, *207*(4), 604-611. https://doi.org/10.1007/s004250050524

Hogle, S. L., Barbeau, K. A., & Gledhill, M. (2014). Heme in the marine environment: from cells to the iron cycle. *Metallomics*, *6*(6), 1107-1120. https://doi.org/10.1039/C4MT00031E

Hossain, E. M., Mahmudul Hasan, M., Halim, M. E., Ehsan, M. Q., & Halim, M. A. (2015). Interaction between transition metals and phenylalanine: A combined experimental and computational study. *Spectrochimica Acta - Part A: Molecular and Biomolecular Spectroscopy*, *138*, 499-508. https://doi.org/10.1016/j.saa.2014.11.084

Hou, W., Ma, Z., Sun, L., Han, M., Lu, J., Li, Z., Mohamad, O. A., & Wei, G. (2013). Extracellular polymeric substances from copper-tolerance *Sinorhizobium meliloti* immobilize Cu^{2+}. *Journal of Hazardous Materials*, *261*, 614-620. https://doi.org/10.1016/j.jhazmat.2013.06.043

Huang, D., Boxin, O. U., & Prior, R. L. (2005). The chemistry behind antioxidant capacity assays. *Journal of Agricultural and Food Chemistry*, *53*(6), 1841-1856. https://doi.org/10.1021/jf030723c

Huang, W., Zhou, Y., Zhao, T., Tan, L., & Wang, J. (2022). The effects of copper ions and copper nanomaterials on the output of amino acids from marine microalgae. *Environmental Science and Pollution Research*, *29*, 9780-9791. https://doi.org/10.1007/s11356-021-16347-3

Hung, C. C., & Santschi, P. H. (2001). Spectrophotometric determination of total uronic acids in seawater using cation-exchange separation and pre-concentration by lyophilization. *Analytica Chimica Acta*, *427*(1), 111-117. https://doi.org/10.1016/S0003-2670(00)01196-X

Hunter, K. A., & Boyd, P. W. (2007). Iron-binding ligands and their role in the ocean biogeochemistry of iron. *Environmental Chemistry*, *4*(4), 221-232. https://doi.org/10.1071/EN07012

Jacquot, J. E., Kondo, Y., Knapp, A. N., & Moffett, J. W. (2013). The speciation of copper across active gradients in nitrogen-cycle processes in the eastern tropical South Pacific. *Limnology and Oceanography*, *58*(4), 1387-1394. https://doi.org/https://doi.org/10.4319/lo.2013.58.4.1387

Jacquot, J. E., & Moffett, J. W. (2015). Copper distribution and speciation across the International GEOTRACES Section GA03. *Deep-Sea Research Part II: Topical Studies in Oceanography*, *116*, 187-207. https://doi.org/10.1016/j.dsr2.2014.11.013

Janero, D. R. (1990). Malondialdehyde and thiobarbituric acid-reactivity as diagnostic indices of lipid peroxidation and peroxidative tissue injury. *Free Radical Biology & Medicine*, *9*(6), 515-540. https://doi.org/10.1016/0891-5849(90)90131-2

Jerez-Martel, I., García-Poza, S., Rodríguez-Martel, G., Rico, M., Afonso-Olivares, C., & Gómez-Pinchetti, J. L. (2017). Phenolic profile and antioxidant activity of crude extracts from microalgae and cyanobacteria strains. *Journal of Food Quality*, *2017*. https://doi.org/10.1155/2017/2924508

Jin, P., Wang, T., Liu, N., Dupont, S., Beardall, J., Boyd, P. W., Riebesell, U., & Gao, K. (2015). Ocean acidification increases the accumulation of toxic phenolic compounds across trophic levels. *Nature Communications*, *6*. https://doi.org/10.1038/ncomms9714

Kahler, P., Bjbrnsen, P. K., Lochte, K., & Antias, A. (1997). Dissolved organic matter and its utilization by bacteria during spring in the Southern Ocean. *Deep Sea Research Part II: Tropical Studies in Oceanography*, *44*(1-2), 341-353. https://doi.org/10.1016/S0967-0645(96)00071-9

Kahlon, S. K., Sharma, G., Julka, J. M., Kumar, A., Sharma, S., & Stadler, F. J. (2018). Impact of heavy metals and nanoparticles on aquatic biota. *Environmental Chemistry Letters*, *16*(3), 919-946. https://doi.org/10.1007/s10311-018-0737-4

Kapoor, S., Singh, M., Srivastava, A., Chavali, M., Chandrasekhar, K., & Verma, P. (2022). Extraction and characterization of microalgae-derived phenolics for pharmaceutical applications: A systematic review. *Journal of Basic Microbiology*, *62*(9), 1044-1063. https://doi.org/10.1002/jobm.202100458

Keyer, K., & Imlay, J. A. (1996). Superoxide accelerates DNA damage by elevating free-iron levels. *Proceedings of the National Academy of Sciences*, *93*(24), 13635-13640. https://doi.org/10.1073/pnas.93.24.13635

Koukal, B., Rossé, P., Reinhardt, A., Ferrari, B., Wilkinson, K. J., Loizeau, J. L., & Dominik, J. (2007). Effect of *Pseudokirchneriella subcapitata* (*Chlorophyceae*) exudates on metal toxicity and colloid aggregation. *Water Research*, *41*(1), 63-70. https://doi.org/10.1016/j.watres.2006.09.014

Kováčik, J., Klejdus, B., Hedbavny, J., & Bačkor, M. (2010). Effect of copper and salicylic acid on phenolic metabolites and free amino acids in *Scenedesmus quadricauda* (*Chlorophyceae*). *Plant Science*, *178*(3), 307-311. https://doi.org/10.1016/j.plantsci.2010.01.009

Kryukova, N. P., Frolov, V. Y., Kolokolov, F. A., Bolotin, S. N., & Panyushkin, V. T. (2005). Synthesis and study of copper(II) complexes with aspartic acid, serine, and valine. *Russian Journal of General Chemistry*, *75*(4), 503-506. https://doi.org/10.1007/s11176-005-0261-7

Kurzyna-Szklarek, M., Cybulska, J., & Zdunek, A. (2022). Analysis of the chemical composition of natural carbohydrates – An overview of methods. *Food Chemistry*, *394*, 133466. https://doi.org/10.1016/j.foodchem.2022.133466

Lattanzio, V., Kroon, P. A., Quideau, S., & Treutter, D. (2008). Plant Phenolics - Secondary Metabolites with Diverse Functions. En *Recent Advances in Polyphenol Research* (Vol. 1, pp. 1-35). wiley. https://doi.org/10.1002/9781444302400.ch1

Lee, D. E., Shin, G. R., Lee, S., Jang, E. S., Shin, H. W., Moon, B. S., & Lee, C. H. (2016). Metabolomics reveal that amino acids are the main contributors to antioxidant activity in wheat and rice gochujangs (Korean fermented red pepper paste). *Food Research International*, *87*, 10-17. https://doi.org/10.1016/j.foodres.2016.06.015

Li, H. Bin, Cheng, K. W., Wong, C. C., Fan, K. W., Chen, F., & Jiang, Y. (2007). Evaluation of antioxidant capacity and total phenolic content of different fractions of selected microalgae. *Food Chemistry*, *102*(3), 771-776. https://doi.org/10.1016/j.foodchem.2006.06.022

Li, C., Li, P., Fu, H., Chen, J., Ye, M., Zhai, S., Hu, F., Zhang, C., Ge, Y., & Fortin, C. (2023). A comparative study of the accumulation and detoxification of copper and zinc in *Chlamydomonas reinhardtii*: The role of extracellular polymeric substances. *Science of the Total Environment*, *871*, 161995. https://doi.org/10.1016/j.scitotenv.2023.161995

Lin, W., Su, F., Lin, M., Jin, M., Li, Y., Ding, K., Chen, Q., Qian, Q., & Sun, X. (2020). Effect of microplastics PAN polymer and/or Cu^{2+} pollution on the growth of *Chlorella pyrenoidosa.* *Environmental Pollution*, *265*, 114985. https://doi.org/10.1016/j.envpol.2020.114985

Liu, Q., Tang, X., Wang, Y., Yang, Y., Zhang, W., Zhao, Y., & Zhang, X. (2019). ROS changes are responsible for tributyl phosphate (TBP)-induced toxicity in the alga *Phaeodactylum tricornutum. Aquatic Toxicology*, *208*, 168-178. https://doi.org/10.1016/j.aquatox.2019.01.012

Lombardi, A. T., Hidalgo, T. M. R., & Vieira, A. A. H. (2005). Copper complexing properties of dissolved organic materials exuded by the freshwater microalgae *Scenedesmus acuminatus* (*Chlorophyceae*). *Chemosphere*, *60*(4), 453-459. https://doi.org/10.1016/j.chemosphere.2004.12.071

Lombardi, A. T., & Vieira, A. A. H. (1999). Lead- and copper-complexing extracellular ligands released by *Kirchneriella aperta* (*Chloroccocales, Chlorophyta*). *Phycologia*, *38*(4), 283-288. https://doi.org/10.2216/i0031-8884-38-4-283.1

López, A., Rico, M., Rivero, A., & Suárez de Tangil, M. (2011). The effects of solvents on the phenolic contents and antioxidant activity of *Stypocaulon scoparium* algae extracts. *Food Chemistry*, *125*(3), 1104-1109. https://doi.org/10.1016/j.foodchem.2010.09.101

López, A., Rico, M., Santana-Casiano, J. M., & González, A. G. (2015). Phenolic profile of *Dunaliella tertiolecta* growing under high levels of copper and iron. *Environmental Science and Pollution Research*, *22*, 14820-14828. https://doi.org/10.1007/s11356-015-4717-y

Luz, D. C., Zebral, Y. D., Klein, R. D., Marques, J. A., Marangoni, L. F. de B., Pereira, C. M., Duarte, G. A. S., Pires, D. de O., Castro, C. B. e., Calderon, E. N., & Bianchini, A. (2018). Oxidative stress in the hydrocoral *Millepora alcicornis* exposed to CO_2-driven seawater acidification. *Coral Reefs*, *37*(2), 571-579. https://doi.org/10.1007/s00338-018-1681-2

Maldonado, M. T., Allen, A. E., Chong, J. S., Lin, K., Leus, D., Karpenko, N., & Harris, S. L. (2006). Copper-dependent iron transport in coastal and oceanic diatoms. *Limnology and Oceanography*, *51*(4), 1729-1743. https://doi.org/10.4319/lo.2006.51.4.1729

Maldonado, M. T., Hughes, M. P., Rue, E. L., & Wells, M. L. (2002). The effect of Fe and Cu on growth and domoic acid production by *Pseudo-nitzschia multiseries* and *Pseudo-nitzschia australis. Limnology and Oceanography*, *47*(2), 515-526. https://doi.org/https://doi.org/10.4319/lo.2002.47.2.0515

Manach, C., Scalbert, A., Morand, C., Rémésy, C., & Jiménez, L. (2004). Polyphenols: food sources and bioavailability. *The American Journal of Clinical Nutrition*, *79*(5), 727-774. https://doi.org/10.1093/ajcn/79.5.727

Markou, G., Angelidaki, I., & Georgakakis, D. (2012). Microalgal carbohydrates: An overview of the factors influencing carbohydrates production, and of main bioconversion technologies for production of biofuels. *Applied Microbiology and Biotechnology*, *96*, 631-645. https://doi.org/10.1007/s00253-012-4398-0

Martin, O., Scholze, M., Ermler, S., McPhie, J., Bopp, S. K., Kienzler, A., Parissis, N., & Kortenkamp, A. (2021). Ten years of research on synergisms and antagonisms in chemical mixtures: A systematic review and quantitative reappraisal of mixture studies. *Environment International*, *146*, 106206. https://doi.org/10.1016/j.envint.2020.106206

Mason, E., Wieser, P. E., Liu, E. J., Edmonds, M., Ilyinskaya, E., Whitty, R. C. W., Mather, T. A., Elias, T., Nadeau, P. A., Wilkes, T. C., McGonigle, A. J. S., Pering, T. D., Mims, F. M., Kern, C., Schneider, D. J., & Oppenheimer, C. (2021). Volatile metal emissions from volcanic degassing and lava–seawater interactions at Kīlauea Volcano, Hawai'i. *Communications Earth and Environment*, *2*(1), 79. https://doi.org/10.1038/s43247-021-00145-3

Matysik, J., Bhalu, B., & Mohanty, P. (2002). Molecular mechanisms of quenching of reactive oxygen species by proline under stress in plants. *Current Science*, *82*(5), 525-532. https://www.jstor.org/stable/24105959

McIntyre, A. M., & Guéguen, C. (2013). Binding interactions of algal-derived dissolved organic matter with metal ions. *Chemosphere*, *90*(2), 620-626. https://doi.org/10.1016/j.chemosphere.2012.08.057

Mesu, J. G., Visser, T., Soulimani, F., Van Faassen, E. E., De Peinder, P., Beale, A. M., & Weckhuysen, B. M. (2006). New insights into the coordination chemistry and molecular structure of copper(II) histidine complexes in aqueous solutions. *Inorganic Chemistry*, *45*(5), 1960-1971. https://doi.org/10.1021/ic051305n

Millero, F. J., Woosley, R., Ditrolio, B., & Waters, J. (2009). Effect of ocean acidification on the speciation of metals in seawater. *Oceanography*, *22*(4), 72-85. https://doi.org/10.2307/24861025

Mira, L., Fernandez, M. T., Santos, M., Rocha, R., Florêncio, M. H., & Jennings, K. R. (2002). Interactions of flavonoids with iron and copper ions: A mechanism for their antioxidant activity. *Free Radical Research*, *36*(11), 1199-1208. https://doi.org/10.1080/1071576021000016463

Molderez, M., Nagels, L., & Parmentier, F. (1978). Time-course tracer studies on the metabolism of cinnamic acid in *Cestrum poeppigii. Phytochemistry*, *17*(10), 1747-1750. https://doi.org/10.1016/S0031-9422(00)88686-0

Morel, F. M. M., & Price, N. M. (2003). The biogeochemical cycles of trace metals in the oceans. *Science*, *300*(5621), 944-947. https://doi.org/10.1126/science.1083545

Morowvat, M. H., & Ghasemi, Y. (2016). Evaluation of antioxidant properties of some naturally isolated microalgae: Identification and characterization of the most efficient strain. *Biocatalysis and Agricultural Biotechnology*, *8*, 263-269. https://doi.org/10.1016/j.bcab.2016.09.010

Myklestad, S. M. (2001). Dissolved organic carbon from phytoplankton. En *Marine Chemistry* (pp. 111-148). https://doi.org/10.1007/10683826_5

Nielsen, E. S., & Wium-Andersen, S. (1970). Copper ions as poison in the sea and in freshwater. *Marine Biology*, *6*(2), 93-97. https://doi.org/10.1007/BF00347237

Nys, C., Janssen, C. R., Blust, R., Smolders, E., & De Schamphelaere, K. A. C. (2016). Reproductive toxicity of binary and ternary mixture combinations of nickel, zinc, and lead to *Ceriodaphnia dubia* is best predicted with the independent action model. *Environmental Toxicology and Chemistry*, *35*(7), 1796-1805. https://doi.org/10.1002/etc.3332

Obata, T., Schoenefeld, S., Krahnert, I., Bergmann, S., Scheffel, A., & Fernie, A. R. (2013). Gas-chromatography mass-spectrometry (gc-ms) based metabolite profiling reveals mannitol as a major storage carbohydrate in the coccolithophorid alga *Emiliania huxleyi*. *Metabolites*, *3*(1), 168-184. https://doi.org/10.3390/metabo3010168

Pérez-Almeida, N., González, A. G., Santana-Casiano, J. M., & González-Dávila, M. (2022). Ocean acidification effect on the iron-gallic acid redox interaction in seawater. *Frontiers in Marine Science*, *9*. https://doi.org/10.3389/fmars.2022.837363

Pinto, E., Sigaud-Kutner, T. C. S., Leitão, M. A. S., Okamoto, O. K., Morse, D., & Colepicolo, P. (2003). Heavy metal-induced oxidative stress in algae. *Journal of Phycology*, *39*(6), 1008-1018. https://doi.org/10.1111/j.0022-3646.2003.02-193.x

Pistocchi, R., Guerrini, F., Boni, L., & Balboni, V. (1997). Copper toxicity and carbohydrate production in the microalgae *Cylindrotheca fusiformis* and *Gymnodinium* sp. *European Journal of Phycology*, *32*(2), 125-132. https://doi.org/10.1080/09670269710001737049

Pistocchi, R., Mormile, A. M., Guerrini, F., Isani, G., & Boni, & L. (2000). Increased production of extra-and intracellular metal-ligands in phytoplankton exposed to copper and cadmium. *Journal of Applied Phycology*, *12*, 469-477. https://doi.org/10.1023/A:1008162812651

Pokrzywnicka, M., & Koncki, R. (2018). Disaccharides determination: A review of analytical methods. *Critical Reviews in Analytical Chemistry*, *48*(3), 186-213. https://doi.org/10.1080/10408347.2017.1391683

Priyadarshini, E., Priyadarshini, S. S., & Pradhan, N. (2019). Heavy metal resistance in algae and its application for metal nanoparticle synthesis. *Applied Microbiology and Biotechnology*, *103*(8), 3297-3316. https://doi.org/10.1007/s00253-019-09685-3

Quideau, S., Deffieux, D., Douat-Casassus, C., & Pouységu, L. (2011). Plant polyphenols: Chemical properties, biological activities, and synthesis. *Angewandte Chemie - International Edition*, *50*(3), 586-621. https://doi.org/10.1002/anie.201000044

Ragan, M. A., Smidsrod, O., & Larsen, B. (1979). Chelation of divalent metal ions by brown algal polyphenols. *Marine Chemistry*, *7*(3), 265-271. https://doi.org/10.1016/0304-4203(79)90043-4

Ran, L., Chi, Y., Huang, Y., He, Q., & Ren, Y. (2020). Synergistic antioxidant effect of glutathione and edible phenolic acids and improvement of the activity protection by coencapsulation into chitosan-coated liposomes. *LWT*, *127*, 109409. https://doi.org/10.1016/j.lwt.2020.109409

Raven, J. A., Evans, M. C. W., & Korb, R. E. (1999). The role of trace metals in photosynthetic electron transport in O_2-evolving organisms. *Photosynthesis Research*, *60*(2), 111-150. https://doi.org/10.1023/A:1006282714942

Rice-Evans, C., Miller, N., & Paganga, G. (1997). Antioxidant properties of phenolic compounds. *Trends in Plant Science*, *2*(4), 152-159. https://doi.org/https://doi.org/10.1016/S1360-1385(97)01018-2

Rico, M., López, A., Santana-Casiano, J. M., González, A. G., & González-Dávila, M. (2013). Variability of the phenolic profile in the diatom *Phaeodactylum tricornutum* growing under copper and iron stress. *Limnology and Oceanography*, *58*(1), 144-152. https://doi.org/10.4319/lo.2013.58.1.0144

Rocha, G. S., Parrish, C. C., Lombardi, A. T., & da G. G. Melão, M. (2016). Copper affects biochemical and physiological responses of *Selenastrum gracile (*Reinsch*). Ecotoxicology*, *25*(8), 1468-1477. https://doi.org/10.1007/s10646-016-1698-7

Romankevich, E. A. (1984). *Geochemistry of Organic Matter in the Ocean*. https://doi.org/doi.org/10.1007/978-3-642-49964-7

Ross, C., & Alstyne, K. L. V. (2007). Intraspecific variation in stress-induced hydrogen peroxide scavenging by the ulvoid macroalga *Ulva lactuca*. *Journal of Phycology*, *43*(3), 466-474. https://doi.org/10.1111/j.1529-8817.2007.00346.x

Roy, U. K., Nielsen, B. V., & Milledge, J. J. (2021). Tuning *Dunaliella tertiolecta* for enhanced antioxidant production by modification of culture conditions. *Marine Biotechnology*, *23*(3), 482-500. https://doi.org/10.1007/s10126-021-10041-8

Rue, E., & Bruland, K. (2001). Domoic acid binds iron and copper: a possible role for the toxin produced by the marine diatom *Pseudo-nitzschia*. *Marine Chemistry*, *76*(1), 127-134. https://doi.org/https://doi.org/10.1016/S0304-4203(01)00053-6

Sáez, C. A., Roncarati, F., Moenne, A., Moody, A. J., & Brown, M. T. (2015). Copper-induced intra-specific oxidative damage and antioxidant responses in strains of the brown alga *Ectocarpus siliculosus* with different pollution histories. *Aquatic Toxicology*, *159*, 81-89. https://doi.org/10.1016/j.aquatox.2014.11.019

Samperio-Ramos, G., Magdalena Santana-Casiano, J., González-Dávila, M., Ferreira, S., & A. Coimbra, M. (2017). Variability in the organic ligands released by *Emiliania huxleyi* under simulated ocean acidification conditions. *AIMS Environmental Science*, *4*(6), 788-808. https://doi.org/10.3934/environsci.2017.6.788

Santana-Casiano, J. M., González-Dávila, M., Fraile-Nuez, E., De Armas, D., González, A. G., Domínguez-Yanes, J. F., & Escánez, J. (2013). The natural ocean acidification and fertilization event caused by the submarine eruption of El Hierro. *Scientific Reports*, *3*(1). https://doi.org/10.1038/srep01140

Santana-Casiano, J. M., González-Dávila, M., González, A. G., Rico, M., López, A., & Martel, A. (2014). Characterization of phenolic exudates from *Phaeodactylum tricornutum* and their effects on the chemistry of Fe(II)-Fe(III). *Marine Chemistry*, *158*, 10-16. https://doi.org/10.1016/j.marchem.2013.11.001

Santana-Casiano, J. M., Gonzalez-Davila, M., Jesus Rodrıguez, M., & Millero, F. J. (2000). The effect of organic compounds in the oxidation kinetics of Fe (II). *Marine Chemistry*, *70*(1-3), 211-222. https://doi.org/10.1016/S0304-4203(00)00027-X

Santhanam, P., Begum, A., & Pachiappan, P. (2018). Basic and applied Phytoplankton biology. En *Basic and Applied Phytoplankton Biology*. Springer Singapore. https://doi.org/10.1007/978-981-10-7938-2

Shah, K., & Dubey, R. S. (1997). Effect of cadmium on proline accumulation and ribonuclease activity in rice seedlings: role of proline as a possible enzyme protectant. *Biologia Plantarum*, *40*(1), 121-130. https://doi.org/10.1023/A:1000956803911

Shaked, Y., & Lis, H. (2012). Disassembling iron availability to phytoplankton. *Frontiers in Microbiology*, *3*. https://doi.org/10.3389/fmicb.2012.00123

Sharma, S. S., & Dietz, K. J. (2006). The significance of amino acids and amino acid-derived molecules in plant responses and adaptation to heavy metal stress. *Journal of Experimental Botany*, *57*(4), 711-726. https://doi.org/10.1093/jxb/erj073

Sharma, S. S., Schat, H., & Vooijs, R. (1998). In vitro alleviation of heavy metal-induced enzyme inhibition by proline. *Phytochemistry*, *49*(6), 1531-1535. https://doi.org/10.1016/S0031-9422(98)00282-9

Shi, Z., Li, H., Li, Z., Hu, J., & Zhang, H. (2013). Pre-column derivatization RP-HPLC determination of amino acids in asparagi radix before and after heating process. *IERI Procedia*, *5*, 351-356. https://doi.org/10.1016/j.ieri.2013.11.115

Silva, J. C., Echeveste, P., & Lombardi, A. T. (2018). Higher biomolecules yield in phytoplankton under copper exposure. *Ecotoxicology and Environmental Safety*, *161*, 57-63. https://doi.org/10.1016/j.ecoenv.2018.05.059

Smerilli, A., Balzano, S., Maselli, M., Blasio, M., Orefice, I., Galasso, C., Sansone, C., & Brunet, C. (2019). Antioxidant and photoprotection networking in the coastal diatom *Skeletonema marinoi. Antioxidants*, *8*(6), 154. https://doi.org/10.3390/antiox8060154

Spurgeon, D. J., Jones, O. A. H., Dorne, J. L. C. M., Svendsen, C., Swain, S., & Stürzenbaum, S. R. (2010). Systems toxicology approaches for understanding the joint effects of environmental chemical mixtures. *Science of the Total Environment*, *408*(18), 3725-3734. https://doi.org/10.1016/j.scitotenv.2010.02.038

Stalikas, C. D. (2007). Extraction, separation, and detection methods for phenolic acids and flavonoids. *Journal of Separation Science*, *30*(18), 3268-3295. https://doi.org/10.1002/jssc.200700261

Strejckova, A., Dvorak, M., Klejdus, B., Krystofova, O., Hedbavny, J., Adam, V., & Huska, D. (2019). The strong reaction of simple phenolic acids during oxidative stress caused by nickel, cadmium and copper in the microalga *Scenedesmus quadricauda*. *New Biotechnology*, *48*, 66-75. https://doi.org/10.1016/j.nbt.2018.07.004

Suggett, D. J., Warner, M. E., Smith, D. J., Davey, P., Hennige, S., & Baker, N. R. (2008). Photosynthesis and production of hydrogen peroxide by *Symbiodinium* (*Pyrrhophyta*) phylotypes with different thermal tolerances. *Journal of Phycology*, *44*(4), 948-956. https://doi.org/10.1111/j.1529-8817.2008.00537.x

Takano, Y., Kashiyama, Y., Ogawa, N. O., Chikaraishi, Y., & Ohkouchi, N. (2010). Isolation and desalting with cation-exchange chromatography for compound-specific nitrogen isotope analysis of amino acids: Application to biogeochemical samples. *Rapid Communications in Mass Spectrometry*, *24*(16), 2317-2323. https://doi.org/10.1002/rcm.4651

Taraldsvik, M., & Myklestad, S. (2000). The effect of pH on growth rate, biochemical composition and extracellular carbohydrate production of the marine diatom *Skeletonema costatum. European Journal of Phycology*, *35*(2), 189-194. https://doi.org/10.1080/09670260010001735781

Tegeder, M. (2014). Transporters involved in source to sink partitioning of amino acids and ureides: Opportunities for crop improvement. *Journal of Experimental Botany*, *65*(7), 1865-1878. https://doi.org/10.1093/jxb/eru012

Thabet, J., Elleuch, J., Martínez, F., Abdelkafi, S., Hernández, L. E., & Fendri, I. (2023). Characterization of cellular toxicity induced by sub-lethal inorganic mercury in the marine microalgae *Chlorococcum dorsiventrale* isolated from a metal-polluted coastal site. *Chemosphere*, *338*, 139391. https://doi.org/https://doi.org/10.1016/j.chemosphere.2023.139391

Tonietto, A. E., Lombardi, A. T., Henriques Vieira, A. A., Parrish, C. C., & Choueri, R. B. (2014). *Cylindrospermopsis raciborskii* (*Cyanobacteria*) exudates: Chemical characterization and complexation capacity for Cu, Zn, Cd and Pb. *Water Research*, *49*, 381-390. https://doi.org/10.1016/j.watres.2013.10.025

Van de Waal, D. B., & Litchman, E. (2020). Multiple global change stressor effects on phytoplankton nutrient acquisition in a future ocean. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *375*(1798), 20190706. https://doi.org/10.1098/rstb.2019.0706

Vázquez, V., León, P., Gordillo, F. J. L., Jiménez, C., Concepción, I., Mackenzie, K., Bresnan, E., & Segovia, M. (2023). High-CO2 Levels rather than acidification restrict *Emiliania huxleyi* growth and performance. *Microbial Ecology*, *86*(1), 127-143. https://doi.org/10.1007/s00248-022-02035-3

Vickery, H. Bradford., & Schmidt, C. L. A. (1931). The history of the discovery of the amino acids. *Chemical Reviews*, *9*(2), 169-318. https://doi.org/10.1021/cr60033a001

Williford, T., Amon, R. M. W., Benner, R., Kaiser, K., Bauch, D., Stedmon, C., Yan, G., Walker, S. A., van der Loeff, M. R., & Klunder, M. B. (2021). Insights into the origins, molecular characteristics and distribution of iron-binding ligands in the Arctic Ocean. *Marine Chemistry*, *231*, 103936. https://doi.org/10.1016/j.marchem.2021.103936

Winder, M., & Sommer, U. (2012). Phytoplankton response to a changing climate. *Hydrobiologia*, *698*(1), 5-16. https://doi.org/10.1007/s10750-012-1149-2

Wu, J., Gu, X., Yang, D., Xu, S., Wang, S., Chen, X., & Wang, Z. (2021). Bioactive substances and potentiality of marine microalgae. *Food Science & Nutrition*, *9*(9), 5279-5292. https://doi.org/https://doi.org/10.1002/fsn3.2471

Yapo, B. M. (2012). On the colorimetric-sulfuric acid analysis of uronic acids in food materials: Potential sources of discrepancies in data and how to circumvent them. *Food Analytical Methods*, *5*(2), 195-215. https://doi.org/10.1007/s12161-011-9235-z

Yruela, I. (2013). Transition metals in plant photosynthesis. *Metallomics*, *5*(9), 1090-1109. https://doi.org/10.1039/c3mt00086a

Zhang, B., Xia, T., Duan, W., Zhang, Z., Li, Y., Fang, B., Xia, M., & Wang, M. (2019). Effects of organic acids, amino acids and phenolic compounds on antioxidant characteristic of Zhenjiang Aromatic Vinegar. *Molecules*, *24*(20), 3799. https://doi.org/10.3390/molecules24203799