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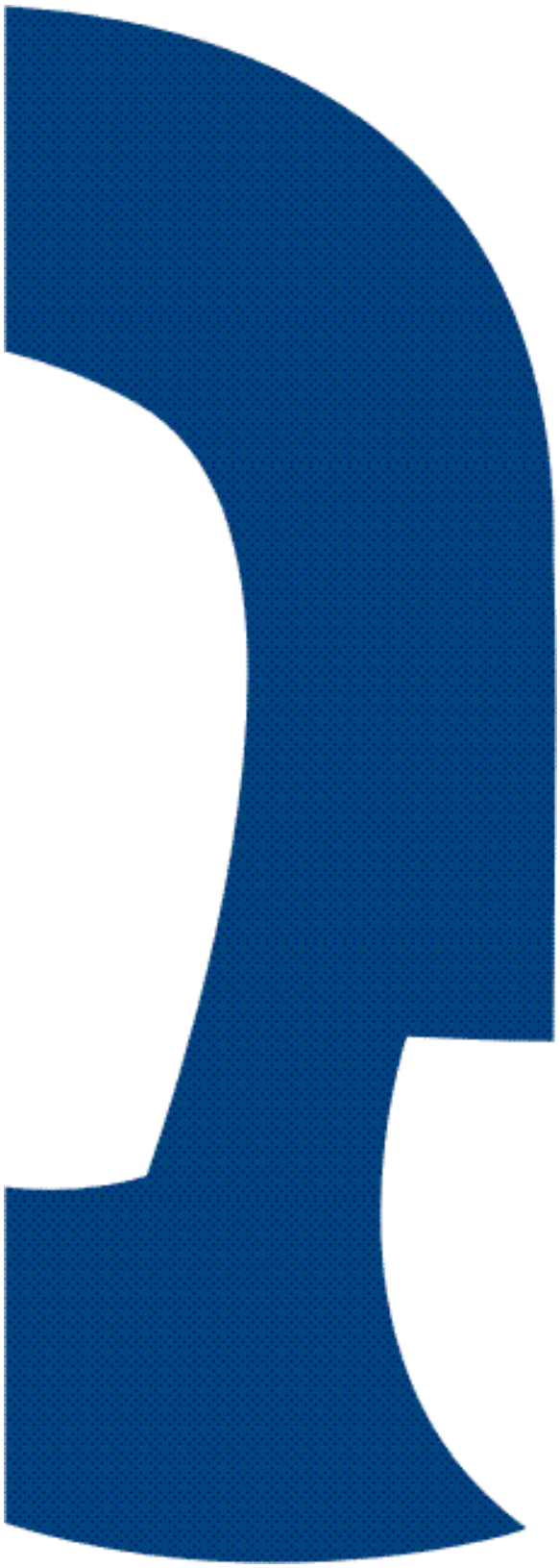

Analysis of the production of compounds of biomedical interest from symbiont microalgae cultures

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Abstract

The genus *Zoanthus* has been studied over the years due to new incorporations of bioactive compounds with great pharmacological potential, such as the zoanthamine-alkaloid family, secondary metabolites with a novel chemical structure. The complexity of the biosynthesis of these compounds may be due to the peculiar relationship that the coral establishes especially with the genus *Symbiodinium sp*. To evaluate the capacity of the symbiont zooxanthellae ad bacterial to biosynthesise alkaloids, the chemical profiles of the extracts from the coral *Z. pulchellus* and the dinoflagellate *Symbiodinium* sp. isolated from it, as well as extracts from the laboratory culture enriched with different carbon sources (glucose, glycerol, and sodium acetate) of *Symbiodinium sp*., were analysed by HPLC-UV/DAD. The result was the isolated and identification of two major compounds, zoanthamine and norzoanthamine only in *Symbiodinium* sp. cells isolated from field-collected coral and from the zoanthid itself. We observed a change in the behaviour of the symbiont reflected in the chemical composition of the profiles when it is cultured in the laboratory, including in cultures enriched with carbon sources, without successfully achieving the presence of the desired alkaloids and cell growth. To rule out any involvement of other symbiont organisms, 3 bacterial colonies of zooxanthellae, identified as *Marinobacter* sp., *Pseudoalteromonas* sp., *Labrenzia* sp. were isolated, cultured, and co-cultured. These were negative for the presence of alkaloid, but in comparison, changes in chemical profiles suggested by the interaction of zooxanthellae with bacteria in co-culture were also observed. The analyses were supported by H-RMN and Dragendorff revealed TLC tests that qualitatively reflected the existence of alkaloids in the enriched *Symbiodinium* sp. cultures, although no target metabolite was isolated. Therefore, it is possible that these symbiotic microalgae may have a precursor role in the biosynthesis of future metabolites. Ultimately, this study provides information on the scarce literature covering the unresolved debate: the real involvement of symbiotic organisms in marine natural products and to shed light on the origin of zoanthamine alkaloids.

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1. Introduction

Historically, both terrestrial flora and fauna have been major elements in the exploitation and study of natural products for pharmaceutical, medicinal and/or industrial purposes (Fenical, 1982; Joffe & Thomas, 1989;) but the decline of new compounds (Jensen & Fenical, 2000) has been key in initiating interest in marine resources. Initially, in the 1930s, the focus was on shallow and easily accessible areas (Carté, 1993) but the development of scuba diving (Fenical, 1982) and progressive improvements in culture, genomics or bioinformatics techniques have enabled major advances in marine research (the isolation of novel organic compounds, the discovery of new gene clusters or new pathways for biosynthesis among other examples) (Lewis et al.; 2010; Onaka, 2017). This is important because marine ecosystems are considered a prolific resource of biological and chemical with bioactive potentials (Jimeno et al., 2004; Ameen et al.; 2021) comprising around 70-80% of global biodiversity (Simmons et al.; 2005; Mitra & Zaman, 2016) and currently harbouring 500,000 marine species, of which 9 out of 28 phylum have been studied for isolation of natural products endowed with novel structures compared to those produced by terrestrial organisms (Lu et., al 2021).

The diversity of environmental conditions of the marine environment itself contemplates a wide thermal range, different hydrostatic pressures, salinity levels, light intensities and even eutrophic or oligotrophic spaces, thus generating specific habitats for organisms with unique characteristics (Jha & Zi-Rong, 2004; Xie et al., 2018). This set of actions has caused organisms to develop different evolutionary strategies such as the synthesis of secondary metabolites between them alkaloids, steroids, lactones, phenols, lipids, polyketides, terpenes, peptides, polysaccharides, macrolides and anthraquinones depending on their biosynthesis pathways (Jiménez, 2018; Chen et al.; 2020).

Secondary metabolites, also called natural products, are organic compounds with low molecular weight and high structural diversity produced by living organisms, e.g., plants, fungi, bacteria, or animals. They are not as directly involved in the growth and development of the organism as primary metabolites (Croteau et al., 2000). But they generally are responsible for conferring adaptive roles and increasing ability to survive, for example, by functioning as defence compounds or signalling molecules in ecological interactions, symbiosis, competition, and so on (Paul, 1992; Engel et al.; 2002). Finally, research has resulted in the isolation of more than 32,000 products derived from marine sources (Ghareeb et al. in 2020) and many of these have biotechnological applications such as anticancer, anti-inflammatory, antidiabetic, antibiotic, cytotoxic and others (Faulkner 2000, Proksch et al., 2002). However, there

is a low percentage approved as drugs (Ghareeb et al., 2020) although many of them are involved in important clinical trials (Minh et al., 2005).

1.1. Marine invertebrates and associated microorganisms

Marine invertebrates comprise 60% of marine animals and are generally sessile organisms or organisms with reduced mobility. Many lack morphological defence structures, therefore, over the course of evolution, many organisms have been able to generate their own chemical defences such as secondary metabolites, ensuring a higher probability of survival against predators and/or competitors (McClintock & Baker, 2001). One of the main resources that marine benthic organisms compete for is space. Space is a limited resource posing an intense competition, especially in the coral reef habitat due to the high species diversity and population density (Proksch, 1994) suggesting the production of compounds with antifouling, anti-algal, antifungal activity (Höller et al.; 2000), in general, inhibitors of growth or cell division of other species. For example, in sponges have demonstrated their ability to synthesise such products to disable bacterial colonisation has been demonstrated (Kelly et al.; 2005).

One of the most common and studied natural products from marine invertebrates are toxins due to their pharmacological and marketing value (Ebada et al., 2008). For example, the alkaloid ecteinascidin-743 or ET-743 from the colonial tunicate *Ecteinascidia turbinata* contains anti-tumour effects in advanced stage cancers (Zewail-Foote & Hurley, 1999; Rinehart, 2000). However, with the ongoing exploration of the oceans, research has focused on microorganisms due to the wide selection in the environment (bacteria, cyanobacteria, fungi, dinoflagellates), which have a great capacity to produce such compounds (Bhatnagar & Kim, 2010). A clear example is the interest associated with microalgae and toxic red tides, with the negative economic and human health effects they cause (Anderson et al., 2012). Similarly, the importance of invertebrate-microbe symbiotic associations has been studied.

The associated microorganisms are able to utilise CO_2 and nitrogenous waste from the host, while the latter is favoured due to large inputs of additional energy (up to 90%) through photosynthesis (Stanley, 2006). They are also responsible for the abundance of many corals and anemones in poor water and high light regions (Muller-Parker & Davy, 2001; Birkeland, 2015) and play a role in the loss of resilience of coral reefs (Baker, 2003). Despite this, today the vast majority of symbiotic species have not been described or cultured (Santo et al. 2001; Baker, 2003) or certain functions are unknown, such as their participation in the biosynthetic pathways of some marine products, leaving unresolved gaps in the literature as in the case of the endosymbiont dinoflagellate genus *Symbiodinium* sp (one of the most studied) and the corals of the genus *Zoanthus* sp.

1.2. *Zoanthus* and marine natural products

The genus *Zoanthus* (Anthozoa: Hexacorallia: Zoantharia: Zoanthidae) is a class of soft coral invertebrates widely distributed in subtropical and tropical water regions of the Indian, Pacific and Atlantic Oceans (Risi et al., 2016). Zoanthids are highly competitive due to their ability to repopulate due to high reproductive rates (Rabelo et al., 2013; Albinsky et al.; 2018), where their clonal proliferation provides a significant benefit in their ability to compete for resources (Jackson, 1977; Geller & Walton, 2001). They also use toxic substances for direct inhibition of certain species, which is called allelopathy (Sammarco et al., 1985).

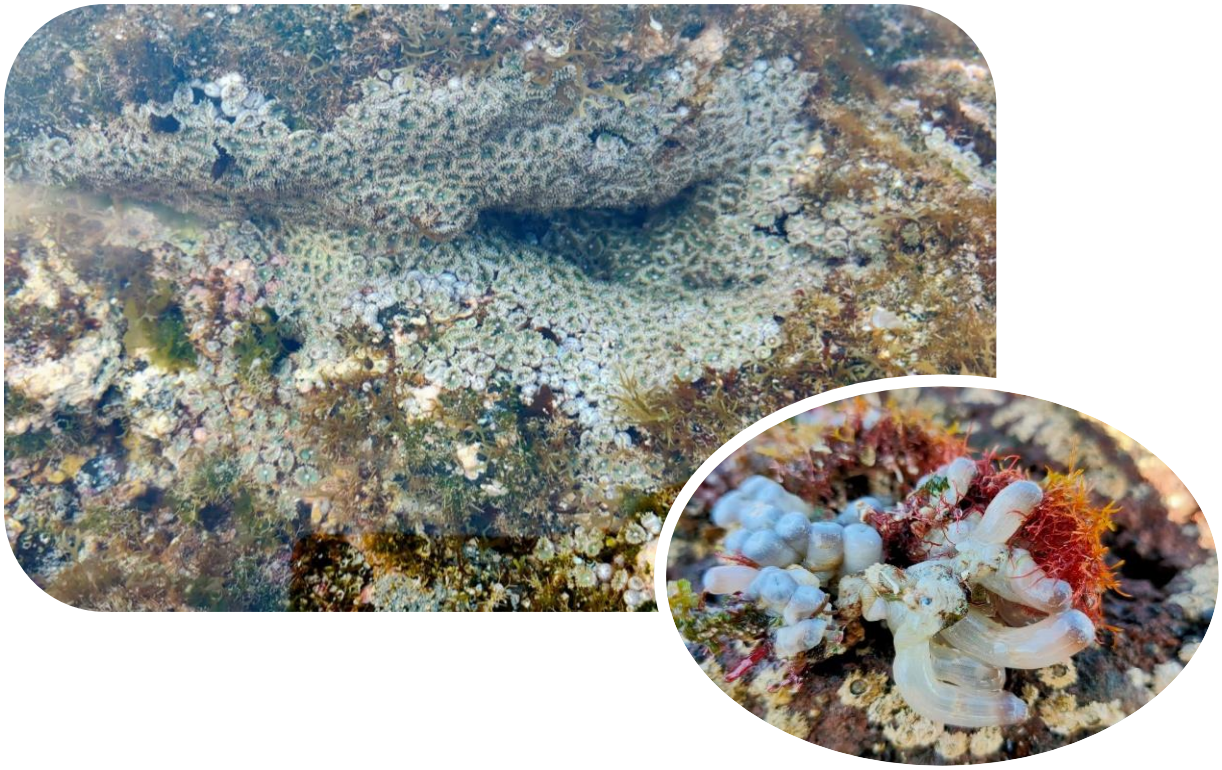


Figure 1. Photograph detailing the soft coral *Z. pulchellus* its polyps in Tenerife, Canary Islands.

This genus corresponds to one of the corals more investigated for the isolation of different marine products due to its rich chemical diversity. Among its compounds, the production of alkaloids stands out, a class of organic compounds that contain nitrogen in their molecular structure and have an important physiological activity (Li et al.; 2016), specifically the zoanthamine family $(C_{30}H_{42}NO_5)$ (fig. 2A).

Zoanthamine (ZN) (fig. 2A) was first isolated from the colonial zoanthid *Zoanthus* sp. in 1984 off the coast of India (Rao et al.; 1984) and has a novel structure that was unrelated to previously studied alkaloids, thus opening a new line of research of biotechnological interest.

Over the years, zoanthamine-type alkaloids have demonstrated biological activity with anti-inflammatory (Hsu et al.; 2016), antibacterial (Venkateswarlu et al.; 1998) and cytotoxic effects such as growth inhibition of P388 leukaemia (Fukuzawa et al.; 1995). For example, Norzoanthamine (NZ) ($C_{29}H_{39}NO_5$) (fig. 2A) (Fukuzawa et al.,1995) contemplates an anti-osporosis ability which is able to reduce the production of Interleukin-6 (IL-6) protein responsible in the formation of this skeletal disease (Inoue et al.; 2014).

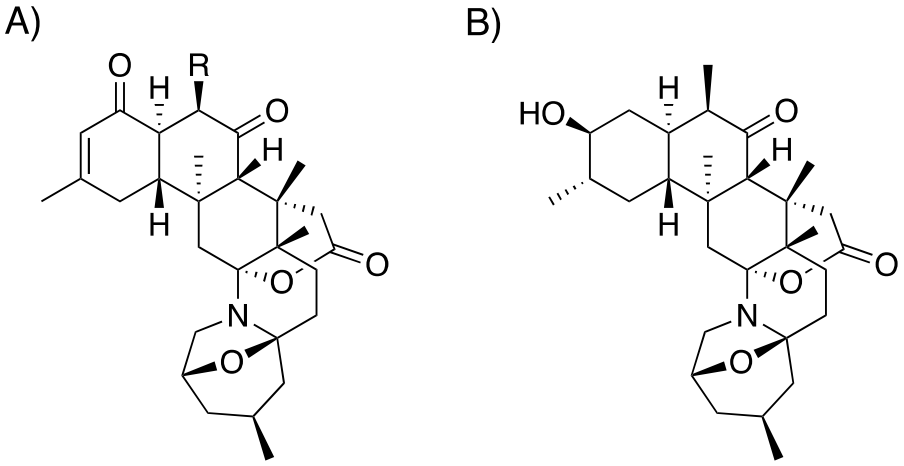


Figure 2. A) Molecular structure of Zoanthamine (R=Me) and Norzoanthamine(R=H) B) Zooxanthellamine.

Although almost 40 years have gone by since the discovery of this family of alkaloids, the literature on their complex biosynthesis is scarce due to the close relationship with the previously named dinoflagellate genus *Symbiodinium sp.* These symbiotic organisms may very often be involved in the metabolic complementation/discharge of small molecules that can become building blocks for the resulting metabolites, leading to a greater diversity of natural products (Van Oppen and Medina, 2020). Different studies have investigated whether they alone can produce metabolites. Nakamura et al. in 1998 found new alkaloids such as zooxanthellamine (fig. 2B) (in high concentrations) secreted by symbiont dinoflagellates in laboratory culture. This fact could explain the algal origin of the alkaloids in symbiotic mechanisms and thus call into question whether it is really the *Zoanthus* that produces the metabolites or the symbionts, due to the great similarity between zooxanthellamine and zoanthamine.

Therefore, one of the main objectives of this study is to contribute to the investigation of the unresolved debate: the involvement of symbiotic organisms, in particular isolates of the colonial zoanthid *Z. pulchellus*, in the production of natural products, and thus shed light on the behaviour of microorganisms such as symbiotic dinoflagellates in culture and the origin of zoanthamine alkaloids.

2. Metodology

2.1. Collection of biological material

Fieldwork was carried out in the intertidal zone of Punta de Hidalgo, Bajamar, Tenerife (28°34′35 06″; 16°19′43 64″W) (fig.3). Around 70-100 polyps from 2 different colonies of the zoanthid *Z. pulchellus* (fig.1) were collected and immediately strained into a 90x120 mm crystallizer (VWR®), polyps were placed in 1L wide-mouth bottles (Nalgene®) and 15-20 polyps were separated from the basal disc using dissecting forceps. The samples were placed in seawater and transported in a cooler at 4°C to the Instituto Universitario de Bio-Orgánica Antonio González (IUBO-AG).

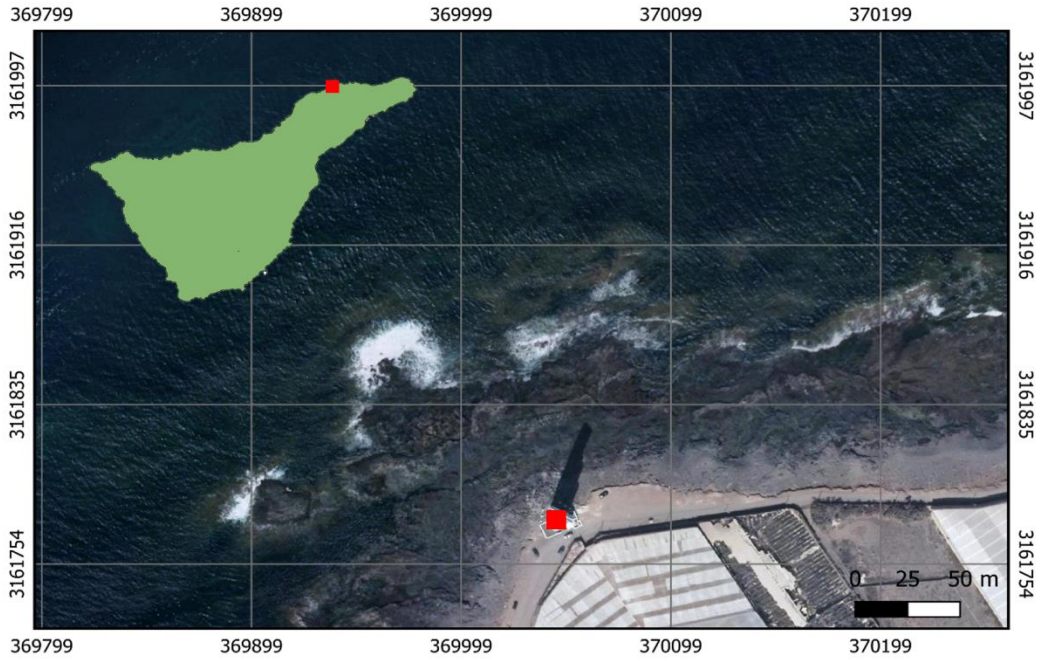


Figure 3. Location of the sampling area, La Punta de Hidalgo, Bajamar, Tenerife.

2.2. Isolation of *Zoanthus pulchellus* symbiont

2.2.1. Zooxanthellae isolation

In a stereoscopic microscope, the polyps of *Z. pulchellus* were dissected with a scalpel from the apical to the basal area, this sagittal plane was opened and placed on a glass slide to show the internal structure of the mesoglea and gastrodermis, under inverted microscope (Motic® K-500L), where they were observed embedded its zooxanthellae of *Z. pulchellus*. Two polyps were preserved in a 15 mL conical tube (Falcon) at -80 ºC for the molecular identification of zooxanthellae. The rest of the polyps were dissected and a scraping of the apical area tentacles, mouth, and pharynx (where the highest number of symbionts was observed), the released cells were immersed in a mucus matrix where only

symbiont-containing host cells were found. An aliquot of 5 mL was separated for the isolation in culture of zooxanthellae, the remaining sample was concentrated for the extraction of metabolites. The cells were resuspended in 50 mL of sterile seawater at 8 ± 2 ºC, manually sieved in 100 and 20 µm mesh to remove solids and mucus. Then this suspension was centrifuged (Eppendorf® 5418R) at 1,000 rpm for 10 min at 4 ºC in mini cartridges (PARASEP® SF-EU) of 15 mL to remove tissue debris and impurities. The supernatant was recovered with the zooxanthellae. Under the inverted microscope (Motic® AE31E) with a camera (Moticam ProS5 Lite) single-cell the isolation with capillary micropipette in 96-well plates and 50 mL Erlenmeyer flasks with the culture media GK (Guillard, 1973) at 34 ‰ de salinity, 20 ºC de temperature, 1040 lux de irradiance and light-darkness cycle 18:6 h (fig 4).

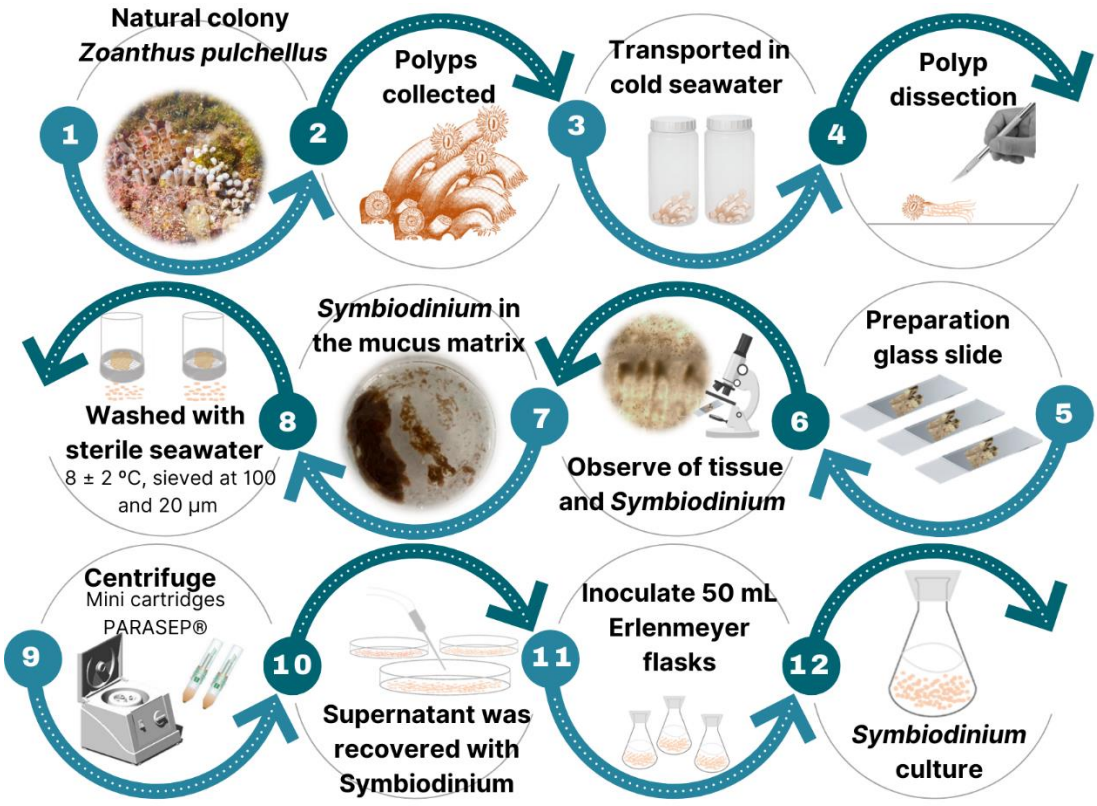


Figure 4. Schematic representation of the zooxanthellae (*Symbiodinium*) collection and isolation process.

2.2.2. Isolation of prokaryotes associates to the zooxanthellae

One mL of zooxanthellae culture was poured in duplicate into Petri dishes containing solid Marine Agar Media 2216 (Panreac). Inoculated Petri dishes were incubated at 20 °C and microbial growth was monitored periodically over 6 months. In total, the colonies that have grown colonies were isolated in pure culture, and they were

grown in liquid media and cryopreserved at -80 °C in 20% glycerol and belong to the Marine Microbial Collection of IUBO-ULL.

2.3. Characterization of symbionts

DNA of *Z. pulchellus* tissue, supernatant, zooxanthellae in culture and the isolated bacterial colonies were extracted from liquid cultures after centrifugation (5 min at 18,000 × g) and the cell pellet was recovered by re-suspending in 200 mL of lysis buffer. Samples were incubated for 5 min at room, and 5 M NaCl and chloroform sequentially added. After centrifugation (18 000 × g, 2 min), the aqueous phase was recovered, DNA was precipitated with cold absolute ethanol and washed twice with 70% ethanol. DNA pellets were dried at room temperature and then suspended in 50 mL of H2O. For samples *of Z. pulchellus* tissue, supernatant, zooxanthellae the 23S rRNA gene was amplified using primers 23SHYPERUP (5′-TCAGTACAAATAATATGCTG-3′) and 23SHYPERDNM13 (5′-GGATAACAATTTCACACAGGTTATCGCCCCAATTAAACAGT-3′) (Sampayo et al. 2009). Already for the bacteria samples the16S rRNA gene was amplified using universal primers E8F (50-TAGAGTTTGATCMTGGCTCAG-30) and 1492R (50-TACGGYTACCTTGTTACGACTT-30) (Weisburg et al., 1991). Commercial PCR kit AmpONE Taq DNA polymerase (GeneAll Biotech) was performed using a PTC200 MJ Research Thermal Cycler and PCR products were purified using the EXO-SAP-IT kit (Affymetrix-USB) following manufacturer's instructions. Sanger Sequencing was carried out using the BigDyeTM Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific) and 3500 Series Genetic Analyzer automatic sequencer (Thermo Fisher Scientific). The SeqScape (Thermo Fisher Scientific) software was used for the analysis of sequencing data and filtering out low quality reads.

Sequences were analyzed using Chromas (Chromas software 2.6.6, Technelysium, Pty. Ltd.1) and Seaview5 (Gouy et al., 2010) programs and they were compared to the GenBank database by the Blast(n) algorithm to establish their closest relatives.

All DNA extraction, rRNA gene amplification and sequencing was carried out at the Genomic Service of the General Research Support Services of Universidad de La Laguna.

2.4. Laboratory Cultures

2.4.1. Carbon-enriched culture of symbiotic zooxanthellae

The symbiont dinoflagellate culture (zooxanthellae) isolated from *Z. pulchellus* has been cultivated in laboratories of the Group of Marine Products from ULL, preserved with a constant conditions (temperature (20º), photoperiod (Light:Dark (18:6)), irradiation (355 Lux)). For this experiment, culture of zooxanthellae was cultivated in Guillard-K (GK) medium (Keller et al.; 1987) were enriched with different carbon sources (glycerol, glucose, and sodium acetate) and a control (only GK culture medium). All experiments were performed in triplicate. As shown in figure 5, firstly, 1.2 L of GK medium with was prepared by adding stock solution (table 1) in filtered water for each experiment.

Table 1. GK medium composition (Keller et al.; 1987, Cruz, 2007; Oliveira, 2021)

Additives		Stock solutions (g L^{-1} of distilled water – dH_2O)	Medium (m L^{-1} of seawater)
$NaNO_3$		75	1
NH_4Cl		2.67	1
NaH_2PO_4. H_2O		1.38	1
TRIS		121.10	1
Trace metal	$Na_2EDTA.2H_2O$	33.6	1
	$FeEDTA\ .3H_2O$	4.30	1
	$MnCl_2$. $4H_2O$	0.178	1
	$ZnSO_4$. $7H_2O$	0.02	
	$CoCl_2$. $6H_2O$	0.014	
	$Na_2 2MoO_4$. $2H_2O$	0.007	
	H_2SeO_4.	0.00129	
	CuSO4. 5H2O	0..250	
Vitamins	Biotin	0.01	1
	Vitamin B12	1	
	Thiamine. HCl	0.2	

For each experiment, was added to the required amount of carbon source (0.071 mL of glycerol, 0.1730 g of glucose and 0.0790 g of sodium acetate, respectively), each having a final concentration of $0{,}064 \cdot 10^{-5}$ mol/L. Once this mixture was obtained, 400 mL were poured into 3 different Erlenmeyer flasks containing 100 mL of the

dinoflagellate culture. The final volume was 500 mL per flask. The material used was previously autoclaved and disinfected by UV rays and in a sterile area to avoid contamination of the cultures, which were left in a culture chamber for 20 days (fig.6) with fixed with fixed conditions (described in the previous paragraph). Finally, cells from each experiment were counted to evaluate cell growth with respect to the different carbon sources (table 5).

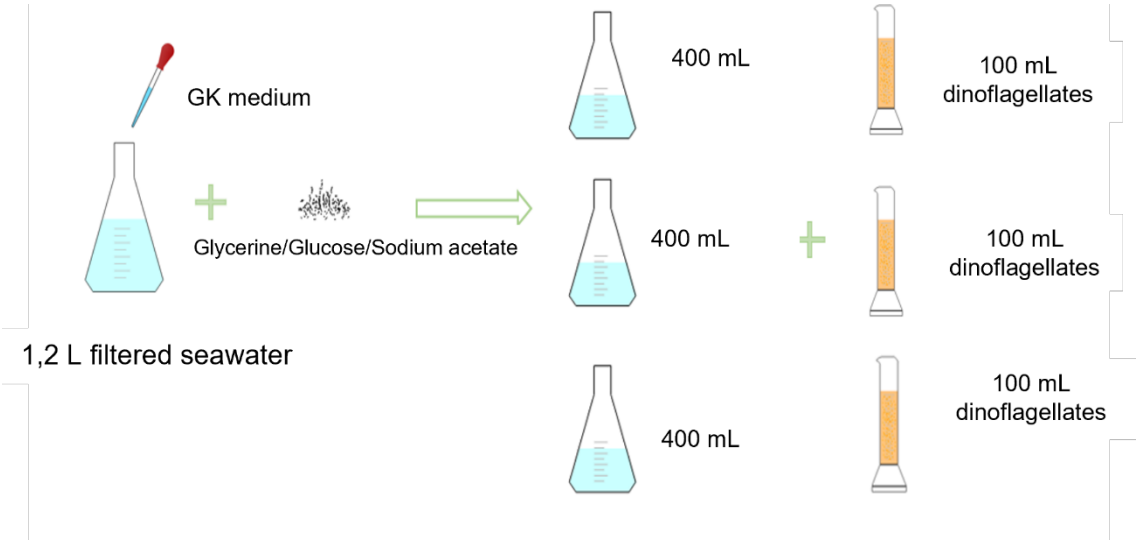


Figure 5. Summary diagram of the cultivation process of the experiment. It is carried out in triplicate for each carbon source

Figure 6. Comparative photograph, A: the first day of culture (t=0 days). B: last day of culture (t=20 days). A darkening of the culture due to cell growth of microalgae can be seen.

2.4.2. Bacteria Culture

The bacteria associated with symbiont zooxanthellae (*Marinobacter* sp.., *Pseudoalteromonas* sp., *Labrenzia* sp.) were cultured in 1.5 L of distilled H_20 added to 60 g of Marine broth (PanReac AppliChem®) dehydrated culture medium Subsequently, 500mL were poured into 3 different flasks and 3 mL previous inoculation of bacteria was added to each flask. Finally, the bacteria were cultured for 10 days (fig. 7) with the help of a shaker and a dark place at constant temperature (20º C). The procedure was the same for all 3 bacteria.

Figure 7. Bacterial culture (t=10 days). All three share a gelatinous appearance and orange colours.

2.4.3. Co-Culture between zooxanthellae and bacteria

A co-culture of the 3 bacteria mentioned in the previous section was carried out with zooxanthellae maintained in laboratory cultures. In this case, 3mL of previous bacteria inoculation were poured separately for 1.5 L of zooxanthellae culture grown previous for 7 days in medium GK as the methodology detailed in the section 2.4.1. Also, a co-culture with the 3 bacteria together was carried out, in which 1mL of each of them was added for 1.5 L of culture of the zooxanthellae. Finally, the cultures were for a total of 10 days in the same conditions as stated in section 2.4.1).

2.5. Preparation of Extracts

2.5.1. *Zoanthus pulchellus* colonies

The seawater was withdrawn from the colonies by decantation and macroalgae, crustaceans and mollusks that are attached to the rock were separated manually, after cleaning the colonies were deposited in a borosilicate glass desiccator 250 x 350 mm (BRAND™), and the extraction was carried out by adding 1 L of dichloromethane (DCM) for 24 h, this step was repeated 4 times. The extract was dryness with a rotary evaporator (Buchi® B-490 Labortechnik AG, Switzerland) and stored at 5ºC.

2.5.2. Symbiont zooxanthellae

The mucus matrix was concentrated in a 250 mL bottle (Duran®) and it was resuspended and washed 4 times with 100 mL of sterile seawater at 8 ± 2 ºC, in centrifugation at 3000 rpm, for 5 min at 4ºC, the supernatant was removed and the cell pellet was preserved, extraction was carried out to the cell pellet by adding 50 mL of DCM (three times). Before discarding the supernatant, it was observed under the microscope and contained a significant number of zooxanthellae cells ≥ 1,000 cells mL^{1}; therefore, the recovered volume 400 mL^{-1} was extracted a liquid: liquid extraction with DCM in a 1:1 ratio. The zooxanthellae extracts (mucus cells and supernatant) were concentrated in a rotary evaporator and kept at 5 ºC.

2.5.3. Laboratory culture experiments

The approach used was liquid-liquid or solvent extraction, which consisted of a process of separation of the components between two liquid phases (Müller et al.; 2000) and was performed the same for all 3 types of culture (carbon-enriched culture of symbiotic zooxanthellae, bacteria, and co-cultures).

Cultures were stirred to homogenise and suspend cells deposited at the bottom of the flask. The extracting agent was dichloromethane (DCM) in 1:1 ratio (DCM: culture medium). Therefore, DCM was added to the cultures in a separating funnel, which was

energetically agitated, releasing the gas produced, thus starting the mixing between the two liquids. Finally, the separation between the two liquid phases was observed (fig. 8) and the DCM phase was collected separately. The procedure was repeated twice to obtain a better purification and efficiency of the method. Finally, the solvent was evaporated with the help of a rotary evaporator (Buchi® B-490 Labortechnik AG, Switzerland) at a temperature of 40ºC and a vacuum pump (Buchi® R-200 Labortechnik AG, Switzerland). Samples were preserved at -5ºC.

Figure 8. Photograph of the process of culture extractions (A) Enriched *Symbiodinium sp*, (B) Bacterias.

2.6. Analysis of chemical profile by HPLC-UV/DAD, isolation, and structural characterization of alkaloids

High performance liquid chromatography (HPCL) was used for the analysis and isolation of the samples, which allowed us to examine the samples with low concentration, by separating the components between the mobile phase (the solvent) and the stationary phase (the column packing) (Bélanger et al.;1997). The model used was the Infinitely Better Agilent 1260 Series Quaternary Pump in conjunction with an ultraviolet diode array detector (UV-DAD) (fig.9). The separation conditions used are described in table 2.

Table 2. Conditions employed in the analytical and semi-preparative method

	Conditions				
	Time (min)	Solvents		Flow (mL min^{-1})	Total Time (min)
Analytical		A: H_2O	B: AcN	1	50 o 65
	0	100	0		
	30	40	60		
Semipreparative	40	0	100	2.5	100
	50-65-100	0	100		

Figure 9. HPLC used for chemical profile and alkaloids isolation.

2.6.1. HPLC-UV/DAD analytical for the chemical profile

The chemical profile of all extracts was performed with HPLC equipped with a Luna® C18 column (250x4.6mm, 5 µm, 100 Å). Mobile phase eluents were water HPLC grade (A) and Acetonitrile HPLC grade (AcN) (B) which gradually went from 100:0 to 0:100 from A: B as described in table 2.

Firstly, the extracts were diluted in 10 mg/mL AcN and filtered on 0.45 µm PTFE syringe filters (Chromafil®). Subsequently, these were poured into 1.5mL glass vials and deposited in the HPLC injection zone to start. The flow rate was 1 mL/min, the sample injection volume was 20 µL and the analysis time was 50 minutes for fresh *Zoanthus* and zooxanthella extracts and 65 minutes for laboratory cultures (table 2). The DAD data were recorded in a range from 190 to 400 nm, and the samples were detected at 235 nm.

2.6.2. HPLC-UV/DAD semipreparative for the isolation of metabolites

Once the chemical profile and the majority peaks of the extracts were observed, a fractionation was performed, specifically of extracts obtained of *Z. pulchellus* colonies and the glucose-enriched culture. The same procedure as the previous point was followed, the 15 mg of the samples were diluted in 200 µL of AcN and with a 0.45 µm PTFE syringe filter (Chromafil®) the solution was filtered. All solvents were at HLPLC purity grade. The same HPLC model was used, but with a Luna® C18 column (250x100mm, 5 µm, 100 Å). The fractions were collected in the automatic collector. The collection time was 0.5 min/test tube due to a flow rate of 2.5 mL/min. Total analysis time was 100 minutes (table 1). The analysis was carried out at a wavelength of 235 nm.

The structure of the compounds was determined using nuclear magnetic resonance (NMR) and MS spectroscopy. NMR experiments were performed in Bruker AVANCE 600 MHz instrument is equipped with a 5 mm TCI inverse detection cryoprobe (Bruker Biospin, Falländen, Switzerland). Standard Bruker NMR pulse sequences were utilized. Dichloromethane-d2 (CD2Cl2, purity 99.98%, Sigma-Aldrich®) was used as solvent to measure 1H, 13C and 2D NMR experiments. The resulting NMR spectra were analyzed by MestReNova software (Mestrelab Research). The chemical shifts are reported relative to solvent signals for 1H (∂H 2.49) and 13C (∂C 53.50), in which the chemical shift is expressed in parts per million (ppm). HR-ESI-MS data were obtained on a Waters LCT Premier XE Micromass (Manchester, UK).

2.6.3. Thin-Layer chromatography (TLC) with specific reagent to detect alkaloids

Thin layer chromatography was used to observe the presence of alkaloids in the extracts and to analyse and unite by similarity the fractions obtained in the separation from the previous semi-preparative method. This method is based on a separation of components by means of a silica gel adsorbent layer (TLC Silica gel 60 F25) and a combination of a stationary phase and mobile phase.

In this study, the TLC plate was 5x5 cm in size and the samples were dissolved with DCM. Subsequently, small amounts of sample were deposited on the plate, which was

immersed in a shallow chamber with CH_2Cl_2:CH_3OH (9.5:0.5) for the mobile phase. For alkaloid detection, first, fluorescence was observed in ultraviolet (UV) light at a wavelength of 235 nm and then Dragendorff aerosol reagent was applied. Dragendorff's reagent is a colour reagent to detect alkaloids.

2.7. Statistical analysis

The data were subjected to homoscedasticity tests to verify normality to evaluate the effect of different carbon sources on the cell growth of the *Symbiodium sp.* culture through a one-way analysis of variance (ANOVA) using the statistical package Rstudio (vers. 2022.02.3).

3. Results

3.1. Taxonomic identification of symbionts

Cells isolated from *Z. pulchellus* coral were analysed and based on their morphological (fig. 10-12) and genomic characteristics, the zooxanthellae cultured in the laboratory were identified as *Symbiodinium sp.*

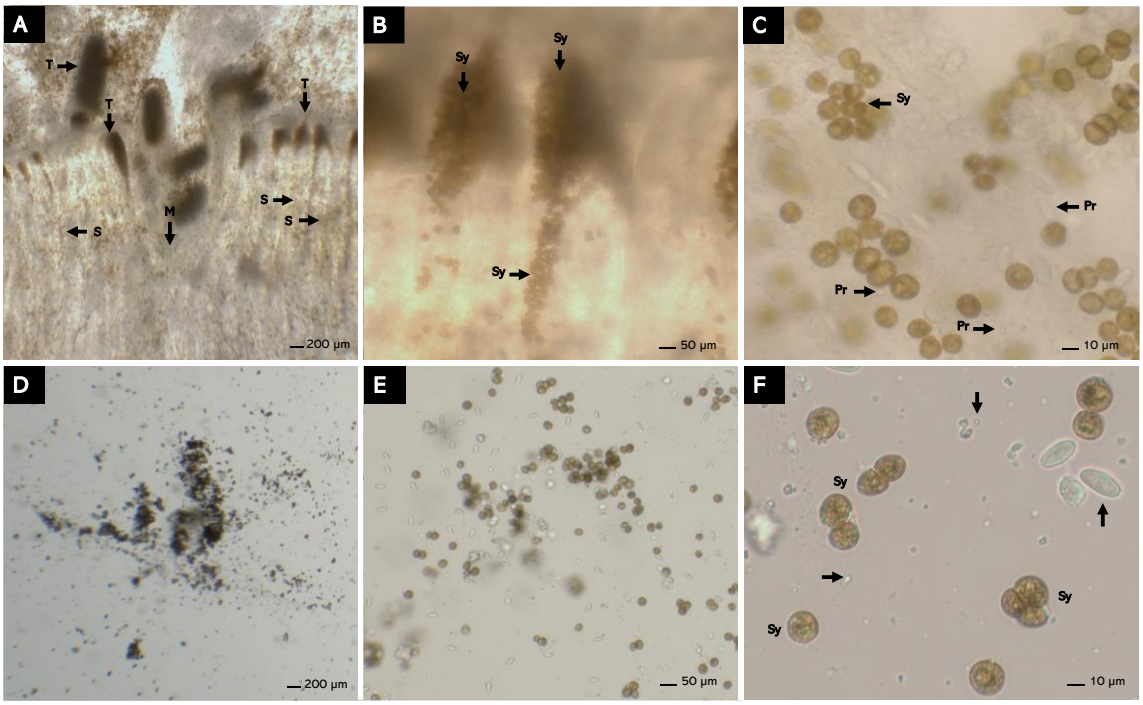


Figure 10. Micrographs of the sagittal plane *Z. pulchellus*, A) arrows (T) tentacles of the polyp, dark area shows symbiont zooxanthellae, (S) septum inside the polyp with zooxanthellae, (M) mouth; B) arrows (Sy) show the insertion of zooxanthella inside the tentacles in the endodermis and mesoglea tissue; C) endosymbionts of the *Z. pulchellus*, arrows (Sy) zooxanthellae and (Pr) prokaryotes; D) mucus matrix with the core microorganisms of *Z. pulchellus;* E) endosymbiotic microorganisms released from *Z. pulchellus,* F) individual and dividing cells of zooxanthellae (Sr), arrows show 3 different types of endosymbiotic prokaryotes associated with the core of *Z. pulchellus*

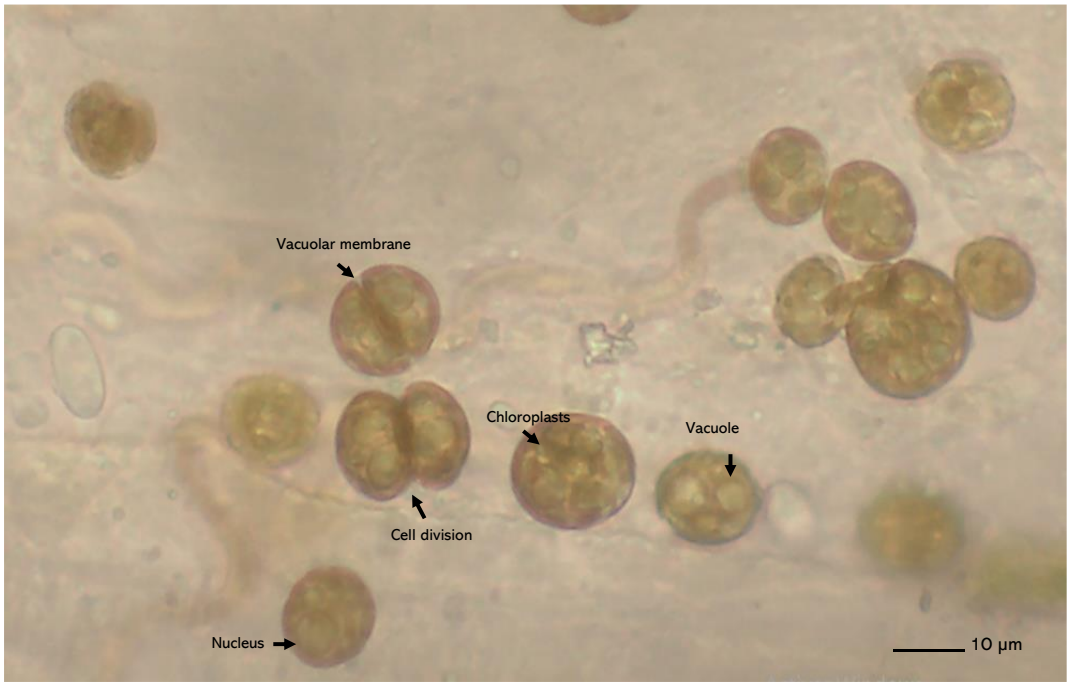


Figure 11. Morphological diversity and cells organelles of Symbiodinium within the endodermis and mesoglea of *Z. punchellus*.

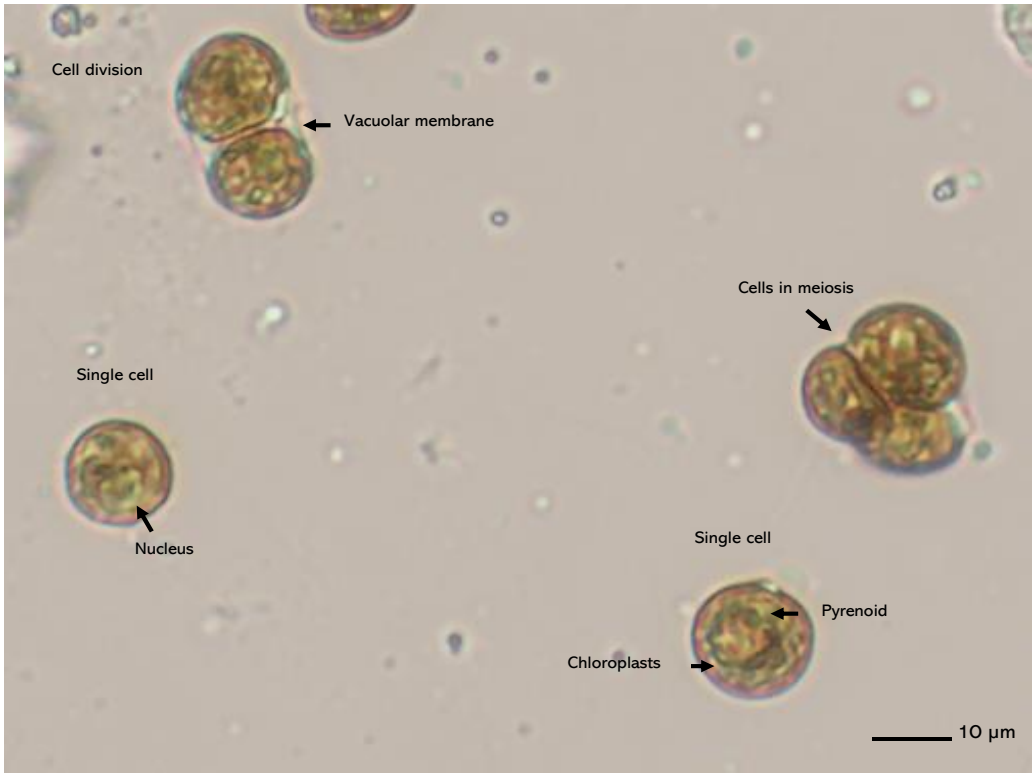


Figure 12. Morphological diversity and cells organelles of Symbiodinium isolated from *Z. punchellus*

On the other hand, genomic techniques were used to identify 3 bacterial colonies (*Marinobacter* sp., *Pseudoalteromonas* sp, *Labrenzia* sp.) isolated (fig. 13) from the symbiont.

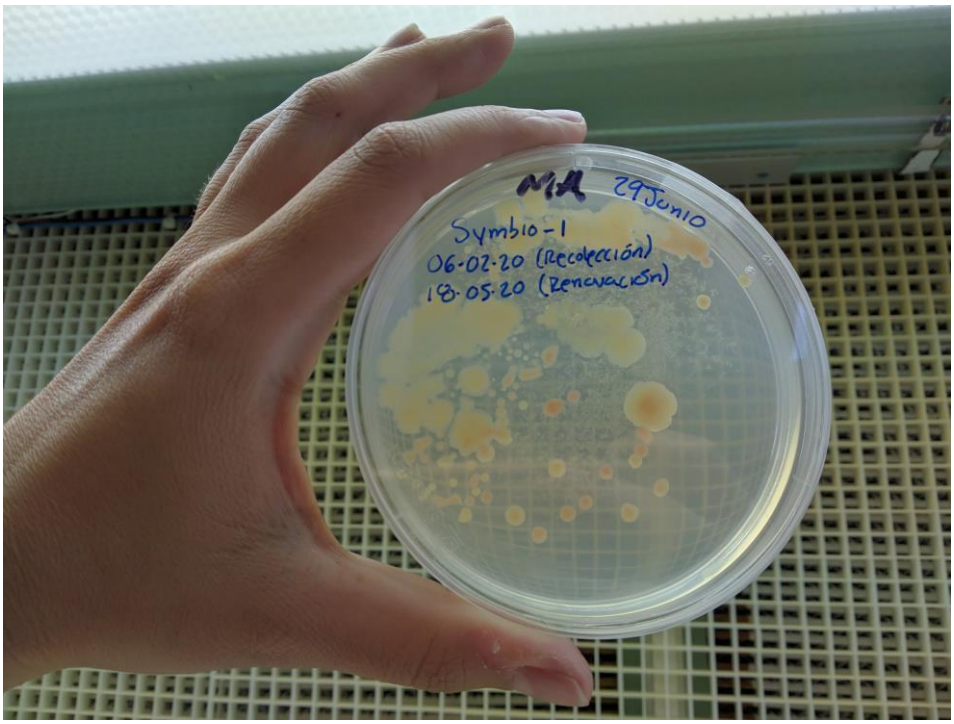


Figure 13. Culture plate for the isolation of the bactéria.

3.2. Chemical analysis

3.2.1. Extracts from *Z. pulchellus* and *Symbiodinium* sp.

This study aims to provide information on the poor knowledge currently available on the biosynthesis of marine natural products. Firstly, the chemical profile of the extracts obtained from soft coral, cell mucus and supernatant (fig. 14) were analysed using high-performance liquid chromatography coupled with an ultraviolet diode array detector (HPLC-UV/DAD). This type of instrument evaluates UV absorbance over time of the samples due to the separation of the component by the assigned column described in the methodology. All 3 samples obtained a similar profile at one hour with two major components. The retention time (RT) of the first one (**1**) was RT= 24.82 min and of the second component (**2**) was RT= 25.24 min. The supernatant extract showed a lower signal intensity ($1{,}45 \cdot 10^{13}$ mUa) compared to the other two extracts (~$5 \cdot 10^{13}$ mUa).

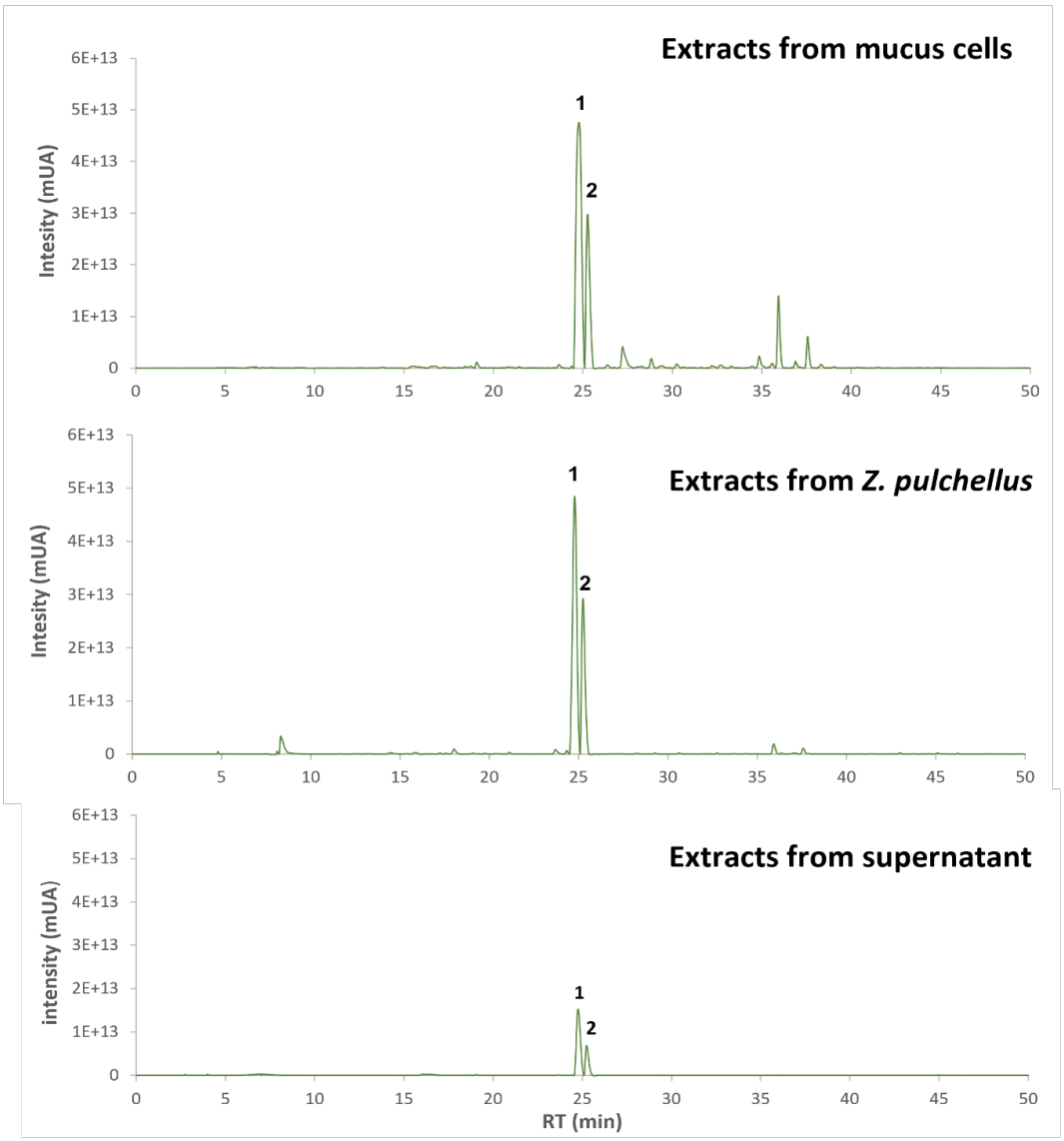


Figure 14. Chromatograms (HLPC) from *Z. pulchellus* colonies and zooxanthellae (cell pellets and supernatant) at 235 nm. Component 1 corresponds to NZ and component 2 is ZN.

The peak 1 (RT= 24.82 min) and 2 (RT= 25.24 min) could be isolated from extracts of *Z. pulchellus* colonies thanks to the purification of these from the HPLC system with a semi-preparative column as described in the methodology. Furthermore, they were subjected to 1D and 2D NMR and MS spectroscopy and the data were compared with the available literature (Rao, 1984; Fukuzawa et al.;1995; Kuramoto et al.;1997). The structure of the compounds corresponded to known and important alkaloids previously isolated from *Zoanthus* (Rao,1984). The identified compounds are norzoanthamine (**1**) and zoanthamine (**2)** (fig. 15)

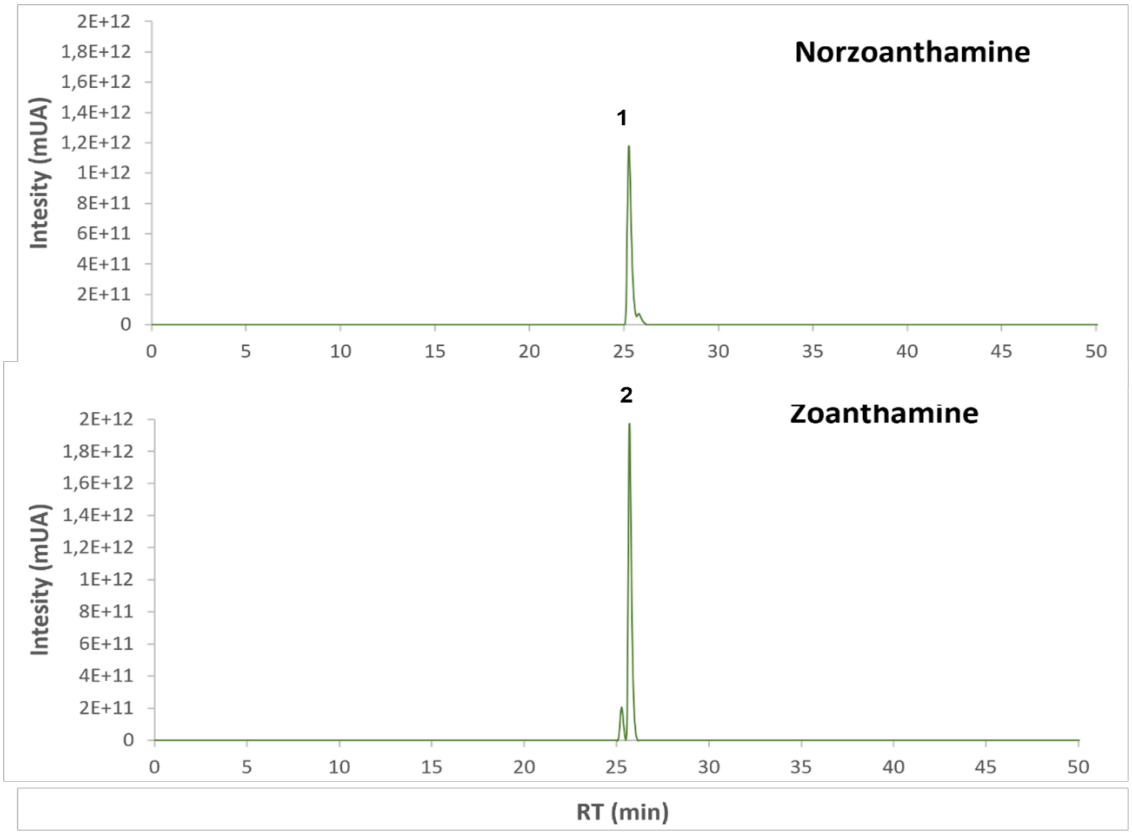


Figure 15. Chromatography of the isolated pure components. Secondary metabolites shown in the graph correspond to (1) NZ and (2) ZN.

3.2.2. Extracts from carbon-enriched culture of *Symbiodinium* sp.

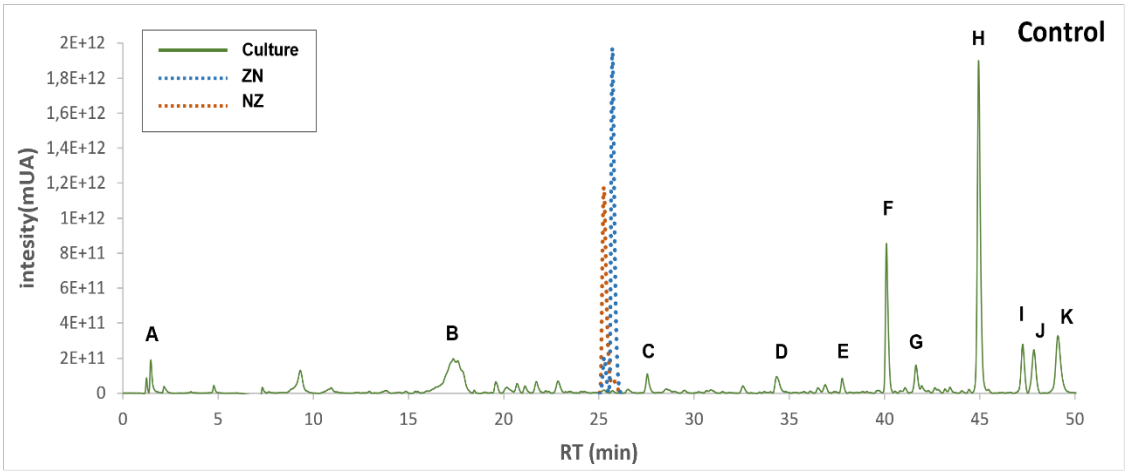
To analyse the behaviour of the enriched culture, first of all, a cell count was carried out (table 3), despite observing a higher density for the culture with glucose (12259 ± 21033), on the contrary, the culture with sodium acetate was the sample with the lowest growth (78889 ± 8389) even lower than the control culture (95185 ± 18736), which only contained GK medium, the different carbon sources did not lead to any significant increase in cell growth (F= 3.69, p=0.06).

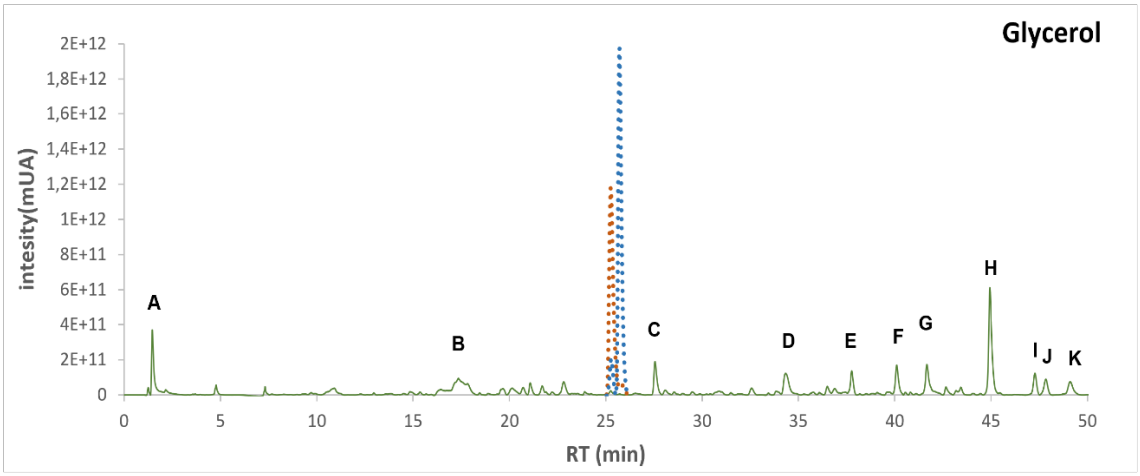
Table 3. Cell count of dinoflagellate cultures and amount of extract obtained from these cultures.

Cultive	Cells* (cel mL^{-1})	Extracts (gr)
Control	95185 ± 18736	0.020
Glycerol	98889 ± 13922	0.023
Glucose	122593 ± 21033	0.015
Sodium acetate	78889 ± 8389	0.0153

*Statistical analysis: one way ANOVA (F=3,69, p=0,06).

Regarding the chemical profile, the results indicate that all the samples enriched with a carbon source and even the control presented qualitatively similar profiles, where the presence of 11 main peaks (fig. 16) stands out (RT: A=1.49 min, B=17.60 min, C=27.60 min, D=34.36 min, E=37.70 min), F= 40,13 min, G=41.71 min, H=45.02 min, I =47.81 min, J=47.91 min, K=49.17 min). However, a quantitative variation is observed in all chemical profiles. It is worth noting the H peak as the major peak, whose maximum value occurs in the culture with glucose ($1{,}93{\cdot}10^{12}$ mUA). On the other hand, if we examine the chemical profile of figure 14 and figure 16, it can appreciate a change indicating that the symbiont zooxanthella behaves differently and generates different compounds than when it is analysed freshly extracted from *Zoanthus*.





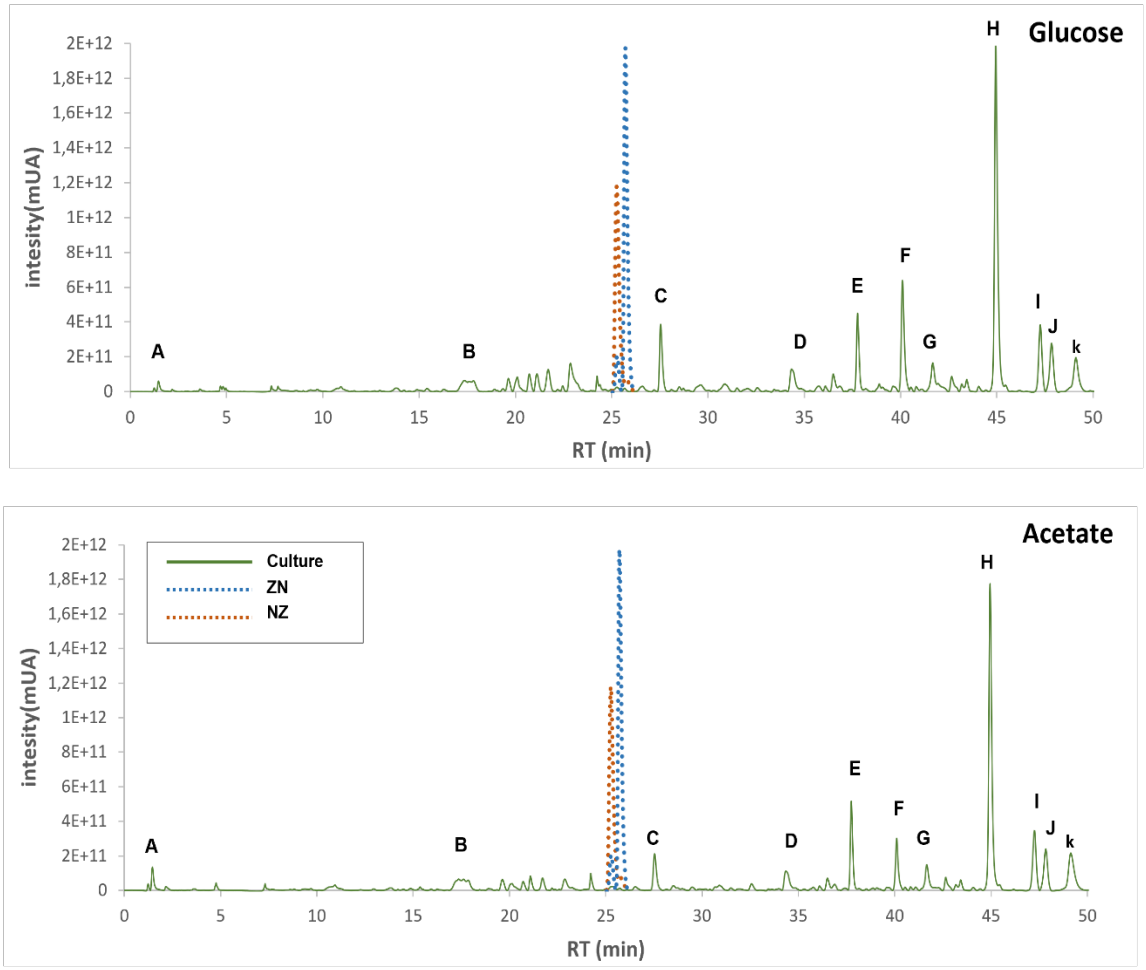


Figure 16. Chemical profile by HPLC-UV/DAD (235nm) of *Symbiodinium* sp. in the different enriched cultures.

The samples were subjected to TLC to qualitatively demonstrate the occurrence of alkaloids as natural products. The appearance of fluorescence (fig 19) in the samples which were subsequently revealed with Dragendorff spray reagent resulted in an orange-yellowish colour indicating the presence of alkaloids (Roper et al.; 1965). However, the ^{1}H-NMR spectroscopy of all experiments did not shed light on the signal manifestation of the targeted alkaloids: ZN y NZ (fig.17).

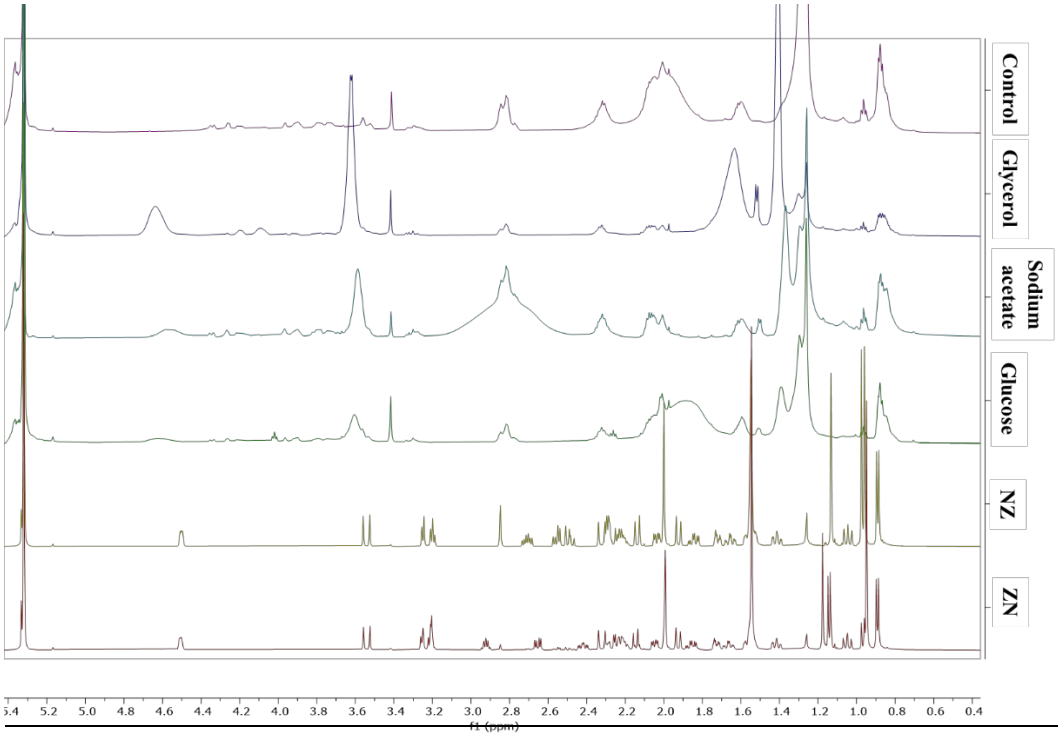


Figura 17. RMN de ^{1}H in CD_2Cl_2 of culture extracts of *Symbiodinium* sp.

Glucose-enriched culture was subjected to the semipreparative HPLC-UV/DAD technique described in section 2.2 with the intention of isolating least the major metabolites, but, at least in that initial attempt at separation, it was not possible to achieve it.

3.2.3. Bacterial culture and co-culture

The 3 bacteria isolated from the symbiont were studied to observe their possible role in the biosynthesis of the alkaloid family. The chemical profile by HPLC-UV/DAD neither reflect NZ (1) (RT= 24.82 min) or ZN (2) (RT= 25.24 min) production. The figure 18 reveals that the peak L (RT= 40.19 min) is the major component of these profiles, very similar in RT to peak F (RT= 40.13 min) of the symbiont *Symbiodinium* sp. culture, where the highest production of this metabolite (peak L, RT= 40.19 min) is found in SymG. If we compare these chromatograms with the co-cultures in figure 20, we confirm that there is a reduction in the intensity with low intensities for the zooxanthellae culture. This becomes apparent if we compare the SymG bacterial colony with the SymG-coculture. Furthermore, the majority peak (L) is not generated in any co-culture in the SymG-coculture. When all three bacterial colonies are put together in a single culture, a high intensity of any one component is also not observed.

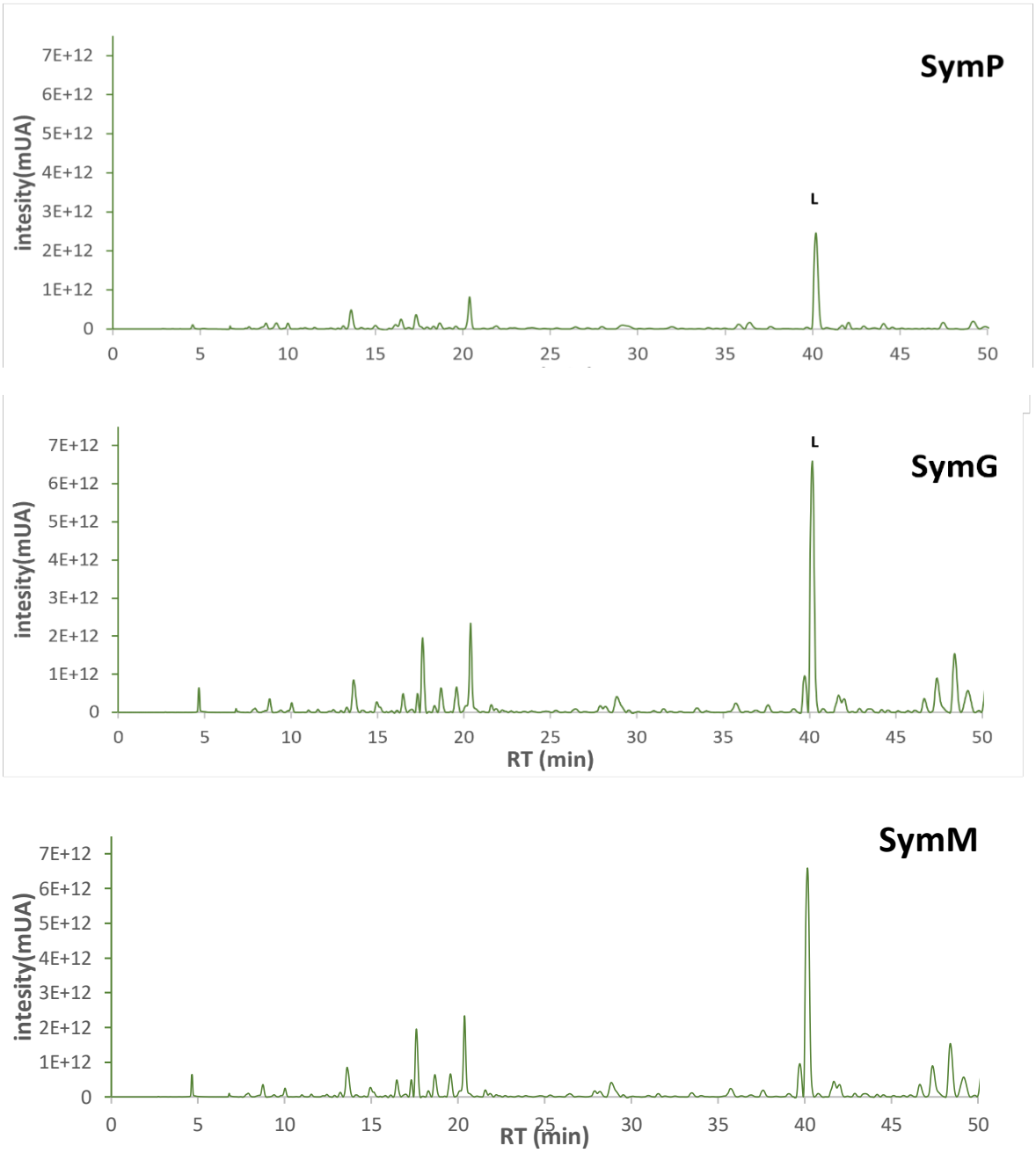


Figure 18. Chemical profile from SymP (*Labrenzia* sp.), SymG *(Pseudoalteromonas* sp.) and SymM (*Marinobacter* sp.) by HPLC-UV/DAD (235nm) of the 3 isolated bacteria.

All extracts from the co-cultures were subjected to TLC, the results of which (fig. 19) reinforce the intuition. No alkaloids were found in the samples.

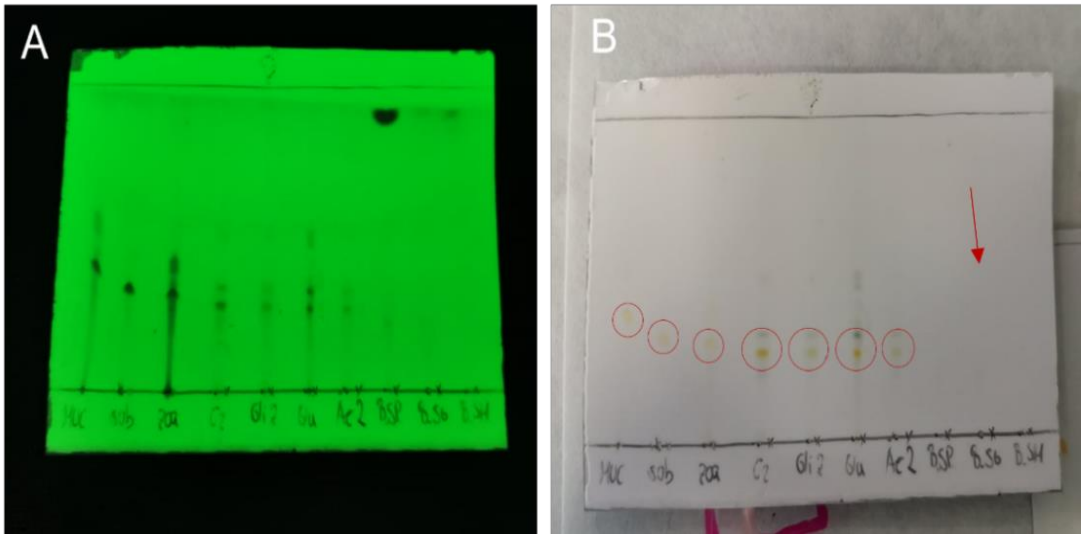
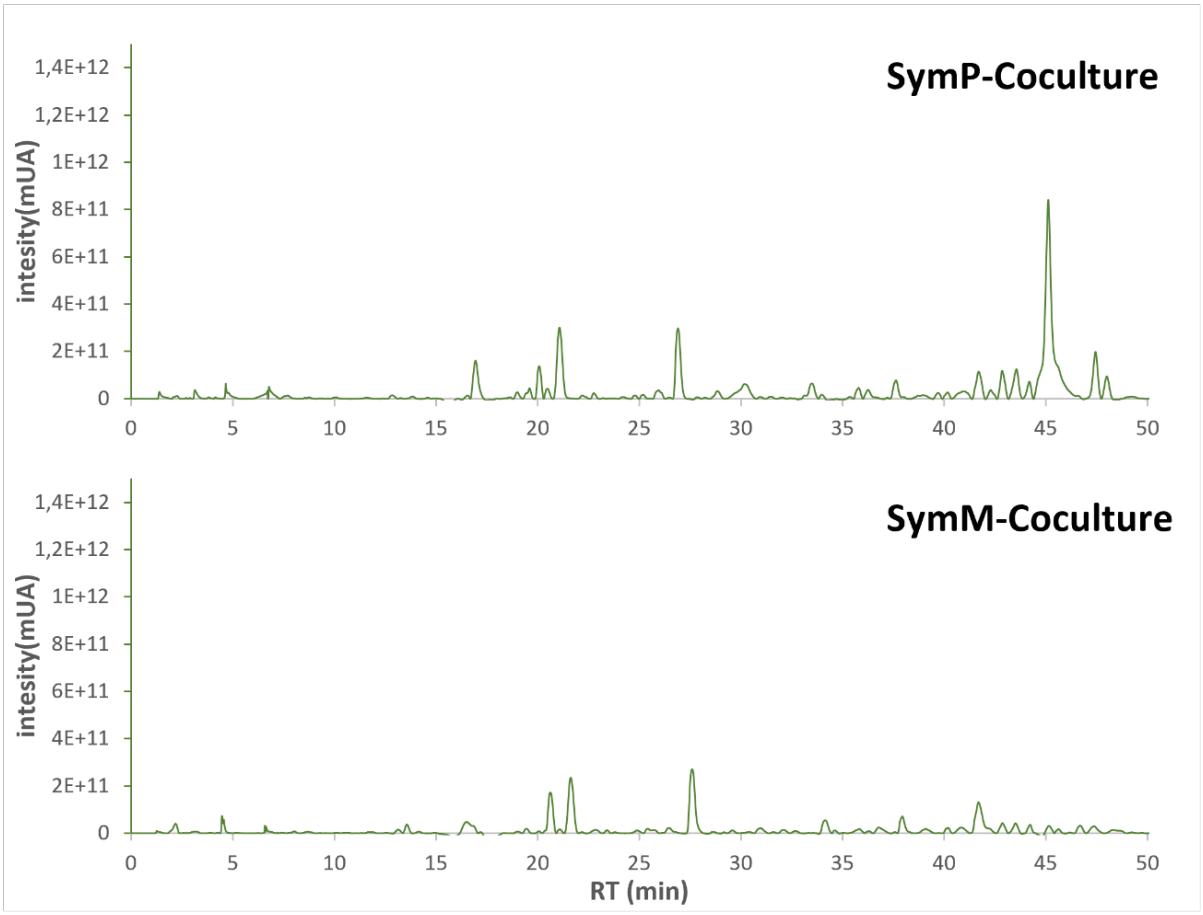


Figure 19. TLC of the isolated *Zoanthus* and dinoflagellate samples. Those marked in the photograph are identified as MUC (cell mucus), supernatant (supernatant), zoa (*zoanthus*), the dinoflagellate culture: C2 (Control = culture without enrichment), Gli2 (culture with glycerol), Glu (culture with glucose), Ace2 (culture with acetate) and the bacterial culture: BSP (*Labrenzia* sp.), BSG (*Pseudoalteromonas* sp) and BSM (*Marinobacter* sp) A) TLC by UV reflecting fluorescence B) TLC revealed with Dragendorff spray reagent, the development of which is in yellow-orange colour.



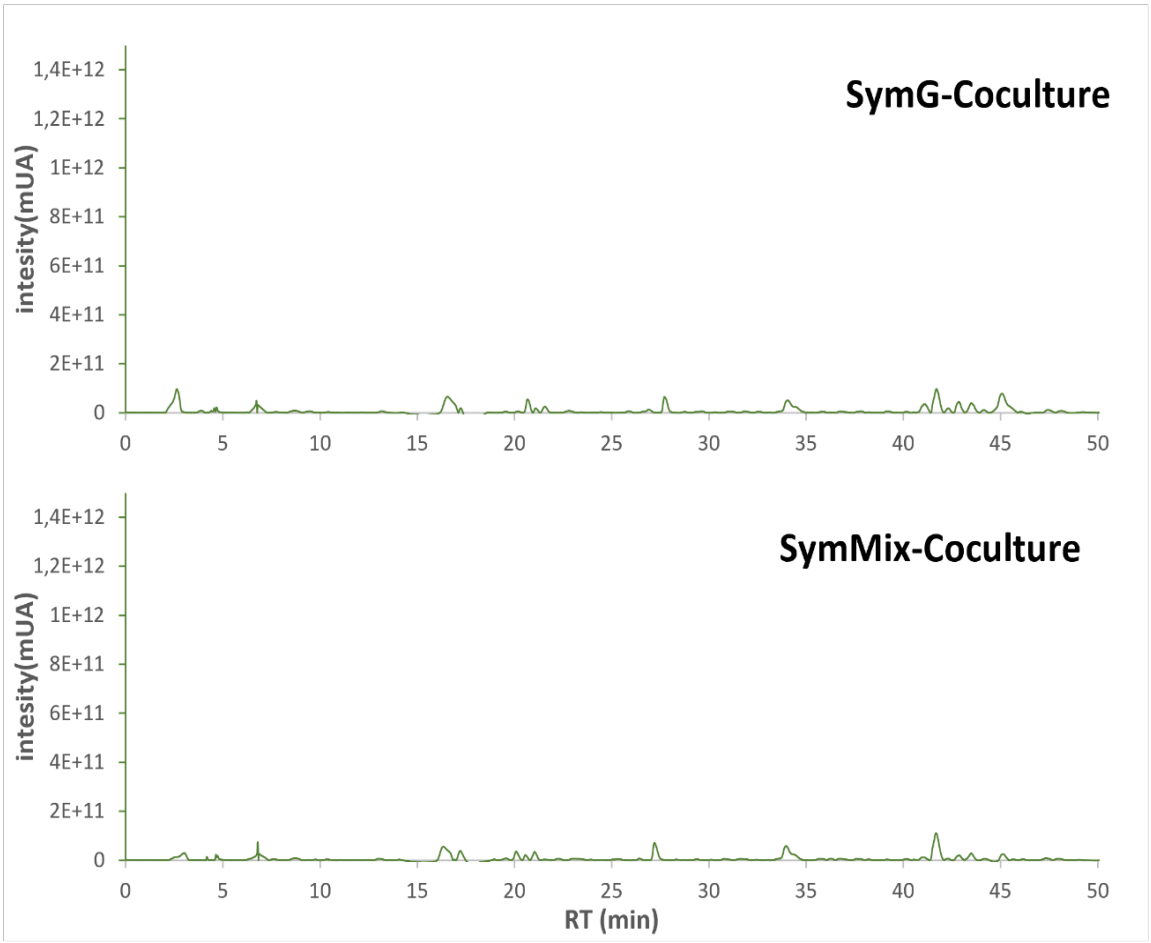


Figure 20. Chemical profiles by HPLC-UV/DAD (235nm) of the samples SymM-Coculture (co-culture with SymM bacteria (*Marinobacter sp.*)), SymP-Coculture (co-culture with SymP bacteria (*Labrenzia sp.*)), SymG (co-culture with SymG bacteria (*Pseudoalteromonas* sp.)), SymMix-Coculture (co-culture with the three above mentioned bacteria). Cocultures with the Symbiodinium strain maintained in laboratory culture.

4. Discussion and conclusion.

In this study, the involvement of zooxanthella and bacterial symbiont, specifically the isolates from the colonial zoanthid *Z. pulchellus*, in the production of zoanthamine-type alkaloids. The results have been favourable with the isolation of two major compounds, which could be identified as NZ and ZN in *Symbiodium* sp. cells isolated from field-collected coral and from the zoanthid itself, sharing a similar chemical profile. In contrast, when the symbiont genus *Symbiodinium* sp. was cultured in the laboratory the chemical profile was different, including in cultures enriched with carbon sources, without successfully achieving the presence of the desired alkaloids. In addition, 3 bacterial colonies found on zooxanthellae were isolated and identified, all of which tested negative for the presence of alkaloids both when cultured and when co-cultured with the symbiont.

The isolation and identification of ZN and NZ from Z. *pulchellus* extracts again encourages that these family alkaloids are characteristic features of the genus *Zoanthus*, which, thanks to them, be able to identify the coral more easily and accurately in future research. From this coral, other studies have previously demonstrated the presence of derivatives of the zoanthamine family (norzoanthamine, norzoanthaminone, oxyzoanthamine, 3-hydroxynorzoanthamine, zoanthaminone, 11-hydroxyzoanthamine, 11-hydroxynorzoanthamine, 30-hydroxynorzoanthamine, epioxyzoanthamine and zoanthenol) (Daranas et al.; 1999; Villar et al., 2003; García-García et al.2022). Despite this, there is a scarce literature on the interaction that the symbiont has on the biosynthesis of the components.

In our case, zooxanthellae extracted from o Z. *pulchellus* share a similar chemical profile with their host and ZN and NZ can be isolated from them. This changes when the symbiont is cultured in laboratory, including with culture enriched with different carbon sources, as a completely different chemical profile composition is observed compared to fresh samples with no apparent observations of ZN and NZ. In other words, zooxanthellae do not act in the same way as in the freshly extracted sample as in the in-situ trials. On the one hand, this change could be affected by carbon supply, but the results obtained from the control culture (only culture medium contained) were again similar to the enriched cultures. Furthermore, statistical analysis using a one-factor ANOVA revealed that the cell growth of the samples was not statistically significant with respect to the carbon sources, with similar growth in all cultures including the control, indicating the irrelevance of these carbon sources in zooxanthellae. Thus, we suspect that the change in the chemical composition of the profiles is due to the impossibility of the symbiont to interact with its host.

Qualitative analysis in TLC with Dragendorff developer on spay performed on the enriched cultures, did show a presence of alkaloid, but it was not possible to isolate these alkaloids with the semi-preparative HLPC technique. Therefore, a hypothesis made is the possibility that these reflected alkaloids are precursors for the biosynthesis of future metabolites like NZ and ZN. Previous studies such as Kita et al. (2007) have isolated different secondary metabolites from cultures of *Symbiodinium* sp. in different invertebrates, as in the case of the marine flatworm, *Amphiscolops* sp., in which the metabolite symbiodinolide was isolated. Also, zooxanthellatoxin-A and zooxanthellatoxin-B, whose purpose is vasoconstrictor, have been isolated (Nakamura et al., 1995). In 1998, Nakamura et al. reported zooxanthellamine (fig. 2B) from cultured *Symbiodinium* sp. whose structure is similar to zoanthamines isolated from *Zoanthus* sp. coral, casting doubt on the true origin of the alkaloids.

The 3 types of bacterial colonies found in the microalgae were isolated, identified and the culture was analysed to contemplate any possibility, as it is known that bacteria are faithful generators of bioactive compounds (Andryukov et al., 2018). Extractions of the samples did not show any presence of alkaloid, but it was still cultured again, although this time, in conjunction with zooxanthellae to study the behaviour of this one. The chemical profiles changed compared to when the bacteria were alone, therefore, there is indeed a bacteria-zooxanthella interaction, but not at the level of generating the desired alkaloids.

Marine natural products are really a new representation of scientific advancement as biotechnology, specifically in the field of pharmacology (Daniotti and Re, 2021). The increase in bioactive components has led to several new drugs and clinical trials (Newman and Cragg, 2016; Jiménez, 2018) for the improvement and combat of diseases. The zoanthamine alkaloid family, being a type of nitrogenous component with novel and unique structure (Li et al.; 2016). The supply of these compounds, over the years, has generated numerous concerns among researchers, because as it is known, corals today are at high risk due to marine pollution (caused by agriculture, industrialization, urbanization) (Osborne et al., 2017), rising temperatures and ocean acidification due to increased CO2 (Albright, 2018) among other factors are proving the decline of coral reefs and even the decline of symbiotic organisms (Weis, 2008).Therefore, to avoid as little damage as possible, the study of symbiont zooxanthellae could be key to ensure the supply of the compounds of interest. The cell growth and maintenance of micro-organism cultures is much easier due to the way they reproduce and in view of industrial thermals, as they would produce a larger amount of raw material at lower costs.

Future investigations will have to extract zooxanthellae cells from *Z.pulchellus* and observe the chemical behaviour of the coral in both in situ and fresh assays, in order to determine whether laboratory conditions really generate changes in the chemical composition of the profiles and in the synthesis of metabolites. On the other hand, the carbon sources supplied in this study (glucose glycerol and sodium acetate) to the culture of *Symbiodinium* sp. did not obtain the expected results, therefore, carrying out the same study, but with different carbon sources such as sodium bicarbonate and/or CO2 injection, could give us an idea of the behaviour at the time of cell growth and thus establish a concrete and precise methodology for the optimisation of the culture. In addition, to verify again the presence of alkaloids in their extracts and thus to know or shed light on the true origin of the alkaloids.

Finally, the conclusions drawn from this study are as follows:

- The zooxanthellae cell culture extracted from *Z. pulchellus* were identified as *Symbiodinium* sp.
- Cell growth and qualitative chemical characteristics of the *Symbiodinium* sp. culture was not affected by the carbon sources (glucose, glycerol, and sodium acetate) supplied.
- Isolation of two major compounds (ZN and NZ) in fresh sample extracts of *Z.pulchellus* and symbiont zooxanthellae.
- Neither NZ nor NZ could be isolated from enriched culture of *Symbiodinium* sp but showed qualitative but not quantitative changes in the chemical composition of the profile compared to fresh samples.
- Three bacterial colonies (*Marinobacte*r sp., *Pseudoalteromonas* sp, *Labrenzia* sp) were isolated and identified from the zooxanthellae.
- Both the culture of the 3 bacterial colonies and the co-culture of these with the zooxanthellae did not reflect alkaloids, but a field in the chemical profile was observed.

5. Acknowledgements

This study has been developed in the laboratory of the Marine Products research group of the IUBO-AG, therefore, I would like to thank all the people involved, especially Nathalia and Victor for helping me and teaching me absolutely everything I needed. Also, I would like to thank my family and friends for accompanying me on this long road.

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• Descripción detallada de las actividades desarrolladas durante la realización del TFT

Las actividades desarrolladas durante el TFT han sido las siguiente:

- Recolección del *Z. pulchelles.*
- Aislamiento e identificación de las zooxantelas *Symbiodinium* sp. a partir del *Z.pulchelles* .
- Cultivo enriquecido del *Symbiodinium* sp. con 3 fuentes de carbono diferentes (glucosa, glicerol y acetato de sodio)
- Aislamiento, identificación y cultivo de las 3 colonias de bacterias: *Marinobacter* sp*., Pseudoalteromonas sp., Labrenzia sp.*
- Co-cultivo de las 3 colonias de bacterias (*Marinobacter* sp*.*, *Pseudoalteromonas* sp., *Labrenzia* sp.) con *Symbiodinium* sp simbionte
- Extracción líquido-líquido de los diferentes cultivos.
- Realización de diferentes técnicas cromatográficas: HPLC – UV/ DAD y TLC con revelador Dragendorff de los extractos obtenidos.
- Realización de espectrometría de RMN de los extractos obtenidos.
- Interpretación de las diferentes técnicas nombradas anteriormente.
- Aislamiento del NZ y ZN de los extractos procedentes del *Z. pulchelles* y *Symbiodinium* sp*.*
- Conteo celular de los cultivos enriquecido de *Symbiodinium* sp*.*
- Realización de análisis estadístico (ANOVA) mediante el programa Rstudio.
- Realización del mapa sobre la zona de recolección mediante el programa Qgis.

• Formación recibida (cursos, programas informáticos, etc.)

- La formación recibida para este TFT ha sido amplia. En primer lugar, antes de la realización de mis experimentos, me ofrecieron observar y ayudar en diferentes procedimientos que implicarían en mi investigación. Posteriormente, se me enseñó a realizar diferentes técnicas de cultivo, extracción y de cromatografías como HPLC – UV/ DAD y TLC para la identificación y el aislamiento de los diferentes metabolitos secundarios que se buscaban.

- **Nivel de integración e implicación dentro del departamento y relaciones con el personal.**

    - El nivel de integración e implicación del grupo de investigación desde el primer momento ha sido excelente. Siempre me han hecho participe de todas las decisiones y han contado con mi opinión.

- **Aspectos positivos y negativos más significativos relacionados con el desarrollo del TFT**

    - Los aspectos positivos del TFT se resume en todo el aprendizaje que he tenido en todo este tiempo, observar cómo funciona un grupo de investigación y un laboratorio en un ámbito más profesional.
    - Los aspectos negativos del TFT vienen de la mano a la obligación de exponer en una lengua extranjera, ya que los créditos de inglés necesarios que deberían de estar repartidos por las diferentes asignaturas a lo largo del grado se establecen todos en el TFT, sumándole una dificultad y estrés adicional totalmente innecesario.

- **Valoración personal del aprendizaje conseguido a lo largo del TFT.**

    - En aspectos generales, el TFT me ha resultado bastante positivo y me ayudado a esclarecer sobre mi futuro profesional.