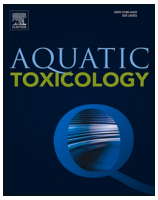
Aquatic Toxicology 252 (2022) 106299

Contents lists available at ScienceDirect

Aquatic Toxicology

journal homepage: www.elsevier.com/locate/aqtox



Toxicity of tyre wear particle leachates to marine phytoplankton

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ARTICLE INFO

Keywords:
Phytoplankton
Tyre wear particles
Leachates

ABSTRACT

Tyre wear particles (TWP) are some of the dominant sources of microplastics in the aquatic environment. Once TWP enter aquatic systems, they can leach certain plastic additives that can be potentially toxic to biota. However, little is known about the impact of TWP lixiviates on marine phytoplankton, the base of marine food webs. This study aims to determine the acute toxic effect of leachates derived from TWP on three phytoplankton species: the cryptophyte *Rhodomonas salina*, the diatom *Thalassiosira weissflogii* and the dinoflagellate *Heterocapsa steinii*, using the median effect concentration (EC_{50}) for specific growth rate as endpoint. Leachates were obtained by incubating 1 g L^{-1} of < 250 μm TWP in artificial seawater for 3 days. Each phytoplankton species was exposed to leachates at five different concentrations, and cell concentrations were measured every 24 h over 3 days. Leachates from TWP were toxic to marine phytoplankton. The dinoflagellate *H. steinii* was the most sensitive species, with 72-h EC_{50} of 23% leachate concentration, whereas *R. salina* and *T. weissflogii* exhibited EC_{50} values of 64% and 73%, respectively. Our results suggest that TWP leachates have a negative effect on phytoplankton growth, although more field data on the concentration of TWPs and their leachates is needed to fully evaluate the environmental impact of TWP.

1. Introduction

Particles from car tyre wear on roads are likely the greatest contributors to aquatic microplastic pollution, with an estimated 500,000 tonnes generated annually in the EU alone (Hann et al., 2018). Of these tyre wear particles (TWP), an estimated 50–140 thousand tonnes are emitted into EU surface waters annually. TWP compose a substantial portion of the marine microplastics (MPs), estimated to contribute between 28 and 60% of total microplastic pollution in the marine environment (Lassen et al., 2015; Boucher and Friot, 2017).

Once TWP enter the oceans, they can leach several substances including toxic metals such as zinc, lead and cadmium, along with organic compounds such as hydrocarbons and benzothiazole derivatives (Wik and Dave, 2009). Previous studies have shown significant effects of TWP and these leachates on various physiological and behavioural characteristics of zooplankton and mussels, including feeding, embryonic development, growth rates and survival (Capolupo et al., 2020; Halle et al., 2021; Koski et al., 2020), although only at TWP concentrations exceeding environmentally relevant conditions (>1000 TWP L^{-1}; Koski et al., 2020). Specifically, high zinc concentrations in TWP have been shown to directly correlate with increased toxicity towards aquatic primary producers such as the freshwater microalgae *Pseudokirchneriella subcapitata* (Gualtieri et al., 2005) and marine macroalgae *Ulva lactuca* (Turner and Rice, 2010). When treated with a leachate solution originating from 80 g TWP L^{-1}, the marine microalgae, diatom *Skeletonema costatum* showed a 72-h median effect concentration (EC_{50}) of 0.5% (Capolupo et al., 2020). Based on these results, there is reason to believe that TWP could pose a threat to certain aquatic organisms, including phytoplankton.

Marine phytoplankton form the base of the marine food web and are largely responsible for the net primary production in the ocean, to which they contribute 45–50 Pg C per year (Field et al., 1998). Their abundance, species composition and biomass vary greatly with seasonality and geographical distribution. Diatoms such as *Thalassiosira* spp. generally dominate spring blooms in Arctic and temperate areas, while flagellates and dinoflagellates are more dominant during the summer and autumn, respectively (McQuatters-Gollop et al., 2007). Phytoplankton vary significantly in their size, morphology and growth rate, which might influence the resistance of the cells to harmful substances. For instance, the structure and composition of their cell wall differs.

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https://doi.org/10.1016/j.aquatox.2022.106299
Received 13 July 2022; Received in revised form 14 September 2022; Accepted 16 September 2022
Available online 17 September 2022

Diatoms have porous silica frustules (De Tommasi et al., 2017) whereas cryptomonads such as *Rhodomonas* have a proteinaceous periplast surrounding the cell (Brett et al., 1994) and dinoflagellates have a cellulosic amphiesma cell covering, which in certain species such as *Heterocapsa,* support cellulose theca plates (Gagat et al., 2014). Due to their strong cell wall, diatoms could be better protected against TWP than dinoflagellates and cryptophytes. Also, due to the uptake of chemicals through diffusion, cells with larger surface area could be more susceptible, as could cells that have a slow growth rate and therefore perhaps a longer retention time of harmful substances.

The purpose of this study was to investigate the acute effects of TWP leachates on the growth rates of phytoplankton species representing the three major groups of marine eukaryotic phytoplankton: flagellates, diatoms, and dinoflagellates. The diatom *Thalassiosira weissflogii*, the flagellate *Rhodomonas salina,* and dinoflagellate *Heterocapsa steinii* (formerly known as *Heterocapsa triquetra*) were used as model species. These species represent different phases of the seasonal plankton cycle in mid-latitudes and have different sizes, morphologies, and growth rates. We wanted to assess (1) whether the TWP leachates are toxic for phytoplankton and (2) whether phytoplankton species with different traits (cell size, morphology, and surface area) differ in their response to TWP leachates. We used median effect concentration (EC_{50}) for population growth rate as an endpoint to TWP leachate toxicity.

2. Methods

2.1. Leachate extraction

Leachates were extracted from particles cut from an unused winter car tyre (Imperial 145/70-13 71T-Snowdragon HP) following a lixiviation protocol for aquatic toxicity testing (Almeda et al., 2022). The protocol involves five steps: micronization, size fractioning, lixiviation, filtration, and toxicity testing. For the micronization process, the outer layer of the tyre was cut into small pieces with a stainless-steel knife. The fragments were ground into fine particles using a pneumatic milling cutter and collected in a stainless-steel tray. In a slight modification to the protocol, liquid nitrogen was then added to separate the particles, allowing them to be dry-sieved through steel mesh with a pore size of 250 μm. A subsample of the fractioned particles was suspended in a Tween-80 0.01% solution, a surfactant which reduces surface tension between particles and water, thereby separating the particles from one another and allowing their size and concentration to be measured using a Beckman Multisizer3 Coulter Counter. These measurements showed that 98% of the TWP were $<$ 250 μm in equivalent spherical diameter (ESD) (Supp. Fig. S1).

For lixiviation, these particles were incubated in glass bottles containing artificial seawater (ASW) with 25.2‰ salinity, at a concentration of 1 g TWP L^{-1}. The bottles were covered with aluminium foil and placed on a plankton wheel rotating at a speed of 1 rpm for 72 h at 20 °C. Finally, the TWP were filtered out using 0.8 μm Whatman GF/F filters on a vacuum filtration unit. pH of the resulting stock solution was measured using a VWR pH 100 pH-meter, to ensure that it was similar to that of the FSW used to grow the phytoplankton. As this was the case (pH = 7.51 ± 0.1), it was not necessary to buffer the leachate stock. Leachates were stored refrigerated in 1 L glass bottles for a maximum of 24 h before use in toxicity testing. Remaining leachates were frozen at -18 °C to be used in chemical analyses.

2.2. Chemical analysis of TWP and their leachates

Triplicate samples of the leachate solution were analysed along with solid TWP. Inductively coupled plasma mass spectrometry (ICP-MS) was used for the analysis of metals. The analyses of samples for organic compounds were performed with a gas chromatograph coupled to a mass spectrometer GC System 7890B and Triple Quad 7010 The analysis of the selected persistent organic pollutants (POPs) and organophosphorus flame retardants (OPFRs) in the tire samples was performed following the analysis methodology presented in Camacho et al. (2019). For solid-liquid extraction, 5 mL of a chlorhexidine:ethyl acetate mixture (1:1, v/v) was added to an amber glass vial containing 1 ± 0.05 g of TWP. After that, the vials were shaken vigorously and subjected to ultrasound for 30 min. The samples were then shaken for 24 h on a rotary shaker. Next, the 5 mL of solvent was transferred to a new vial and the process was repeated two more times until a final volume of 15 mL of the extraction mixture was reached after 72 h. Finally, 1 mL of this mixture was filtered through a 0.20 μm Chromafil® PET filters (Macherey-Nagel, Düren, Germany) to an amber glass chromatography vial and analysed by GC-MS/MS. The leachate samples were placed on an orbital shaker for 2 h. Then, 20 mL were filtered through a 0.20 μm Chromafil® PET filters. Next, an aliquot of 1 mL was evaporated in a vacuum concentrator RVC 2-25 CD plus to dryness. Finally, the samples were resuspended in 1 mL CHX and analysed by GC-MS/MS.

2.3. Experimental setup

Phytoplankton cultures were grown in B1 media (Hansen, 1989) and kept at 18 °C with an illuminance of 60 lux and 14:10 day: night cycle. Cell diameter, volume, and surface area of the three species in an exponential growth phase were determined using a Multisizer Coulter Counter. The experimental species and their characteristics are shown in Table 1.

Phytoplankton growth inhibition tests were performed following the OECD guidelines for the testing of chemicals (OECD, 2011). The only exception to the guidelines was that the phytoplankton were grown at a lower temperature of 18 °C, as the species in question are from temperate waters and their stock cultures were also grown at 18 °C. Initial concentrations at the start of the experiments were ca. 2×10^4 cells mL^{-1} for *Rhodomonas salina* and *Thalassiosira weissflogii* and 3×10^3 cells mL^{-1} for *Heterocapsa steinii*.

A preliminary test was performed to determine a range for exposure concentrations. This test was performed on *R. salina* using leachates obtained from TWP concentrations of 1 and 5 g L^{-1}, at a concentration of 90% leachates. Based on the results from the preliminary experiment, five different leachate concentrations of 1 g L^{-1} TWP were selected for 72 h growth inhibition tests: 50, 25, 10, 1 and 0%.

Phytoplankton cultures were inoculated with leachates in airtight 200 mL glass bottles filled to 150 mL. B1 media was added to each sample at a concentration of 1.1 mL L^{-1} and in the case of *T. weissflogii*, silica was also added at 1 mL L^{-1}. Three replicates were used for each

Table 1
Concentration of organic additives and metals in the TWP (μg g^{-1}) and in leachates (ng L^{-1}; mean ± SD) (n.d.= not detected).

Organic compounds	Type	Concentration (μg g^{-1}; TWP)	Concentration (ng mL^{-1}; leachates)
Hexachlorobenzene	OCP	0.01	n.d.
Tributylphosphate	OPFR	0.01	n.d.
Tris (2-chloroisopropyl) phosphate	OPFR	0.21	0.11 ± 0.16
Tri(2-ethylhexyl) phosphate	OPFR	0.02	n.d.
Acenaphthene	PAH	0.35	n.d.
Benzo [ghi] perylene	PAH	0.94	n.d.
Fluoranthene	PAH	3.88	n.d.
Naphthalene	PAH	0.45	4.32 ± 0.23
Phenanthrene	PAH	1.22	n.d.
Pyrene	PAH	10.97	n.d.
PCB 52	PCB	$<$ 0.005	n.d.
PCB 101	PCB	$<$ 0.005	n.d.
Metals			
66 Zn		20,057.15	1053.26 ± 74.04
88 Sr		8.38	4351.20 ± 36.14

test concentration and control. Bottles were placed on a plankton wheel at a rotatory speed of 1 rpm. Illuminance was measured at four points around the wheel, with an average value of 94 lux, and 14:10 h light: dark cycle. The cell density and mean cell diameter were measured every 24 h for a total of 72 h, using the Coulter Counter. pH was also measured to ensure that it did not deviate more than 1.5 units during the experiment.

2.4. Calculations and statistics

The specific growth rate was calculated assuming an exponential increase in cell concentration and averaged for each treatment concentration. Growth rates were then plotted both as a function of time and leachate concentration. The percentage inhibition of growth rate in the treatments compared to the control was calculated to facilitate a comparison. Statistical significance of differences between control and treatment growth rates was evaluated using Dunnett's multiple comparison test at 95% confidence level (Dunnett, 1955). EC_{50} values were determined by performing a non-linear regression analysis of the concentration-response relationship, based on individual responses of each replicate. Specific growth rates at each point of measurement (24, 48, 72 h) were normalised to percentages of the growth rates in the control. The means of the normalized three replicates for each leachate concentration were then fitted to an inhibitor vs normalised response curve using Prism 9, following the equation $Y = 100/(1 + (EC_{50}/X)^b)$, where Y is the percentage inhibition, X is the leachate concentration and b is the slope. This model was used to calculate EC_{50} values for each day during the exposure.

3. Results

3.1. Chemical characterisation of TWP and leachates

A number of organic compounds were found in TWP (Table 1), including organochlorine pesticides (OCPs), OPFRs, polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs). High concentrations of PAHs were present in the TWP, of which pyrene was the highest, at 10.97 μg g^{-1}. Ranking from highest to lowest concentrations, the other PAHs found were fluoranthene, phenanthrene, benzo[ghi] perylene, naphthalene and acenaphthene. OCPs, OPFRs and PCBs were detected in lower concentrations compared to the PAHs (Table 1). Most of the compounds found in the TWP were not detected in the leachates, with only naphthalene and tris (2-chloroisopropyl) phosphate being found at concentrations of 4.32 ± 0.23 and 0.11 ± 0.16 ng mL^{-1}, respectively.

Metals were detected in TWP in a range of concentrations (Supp. Table S1). Aluminum (Al), vanadium (V), manganese (Mn), iron (Fe), zinc (Zn), titanium (Ti), chromium (Cr), nickel (Ni), copper (Cu), barium (Ba), bismuth (Bi) and lead (Pb) were found at concentrations > 5 μg g^{-1}. Some of these metals were also detected in the TWP leachates with Zn and strontium (Sr) present at the highest concentrations (Table 1). Other metals such as Mn, Ni, molybdenum (Mo), Ba, Pb, and uranium (U) were found in lower concentrations in the leachates. All metals with quantification limits are listed in Table S1 in the Supplementary material.

3.2. Growth inhibition tests

During the preliminary test, leachate concentrations equal to or greater than 90% of 1 g L^{-1} TWP caused 100% mortality in every sample within 72 h (Supp. Fig. S2).

Exponential growth was observed in the control treatments of all species (Fig. 1). *T. weissflogii* had the highest growth rate at 0.70 ± 0.018 d^{-1}, followed by *R. salina* at 0.60 ± 0.004 d^{-1} and *H. steinii* at 0.36 ± 0.002 d^{-1}. TWP leachates at 50 and 25% concentration had a substantial negative effect on the growth rate of all three species, with the 50% treatment causing 100% mortality of *H. steinii* after 72 h. During the first 24 h, *R. salina* had a consistently lower growth rate compared to *T. weissflogii* at all leachate concentrations. *H. steinii* maintained a consistently lower growth rate compared to the other species, and even showed a negative growth rate (i.e., cell mortality) during certain time intervals at higher leachate concentrations. The control populations had an average generation time of 45.9 ± 2.2 h, compared to just 27.7 ± 3.0 and 24.5 ± 4.8 h for *R. salina* and *T. weissflogii*, respectively.

In most cases, growth inhibition did not appear to increase with increased exposure time. The growth rate of *T. weissflogii* decreased over time, but as it also happened in the controls, the decrease was not related to the exposure to leachates (Fig. 2).

Inhibition of growth rate increased with higher leachate concentrations for all three species (Table 3) (Fig. 3). *H. steinii* had similar inhibition percentages at 1 and 10% leachate concentrations (3.2 and -0.6%) but showed a large increase in inhibition at 25 and 50% concentrations (73.4 and 100%), indicating a threshold effect. The model was unable to calculate confidence intervals for *H. steinii*, meaning the EC_{50} values should be interpreted with caution. Although there was a clear inhibitory effect of leachate on *R. salina* and *T. weissflogii* at most concentrations, only the 50 and 25% treatments resulted in a statistically significant difference from control growth rates (Dunnett's test; $p < 0.05$). For *H. steinii*, the growth rates were significantly lower at 50, 25 and 10% treatments than in the control (Dunnett's test; $p < 0.05$). *R. salina* and *T. weissflogii* showed similar 72 h EC_{50} of 64% and 73%, respectively, while *H. steinii* had a substantially lower EC_{50} of 23% (Table 4).

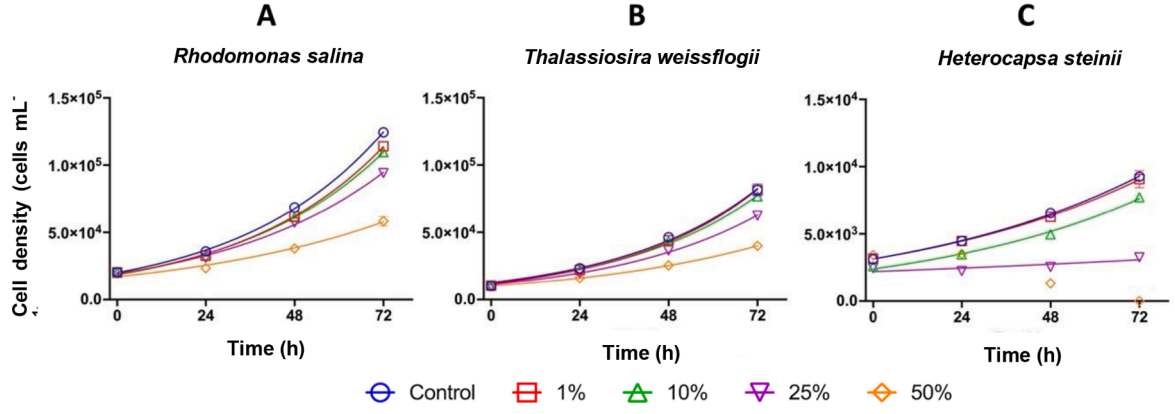

Fig. 1. Concentration (mean ± SD, cells mL^{-1}) of *Rhodomonas salina* (A), *Thalassiosira weissflogii* (B) and *Heterocapsa steinii* (C) at different leachate concentrations (1 g L^{-1} TWP) over time. Note the different scale on the vertical axis for *H. steinii*.

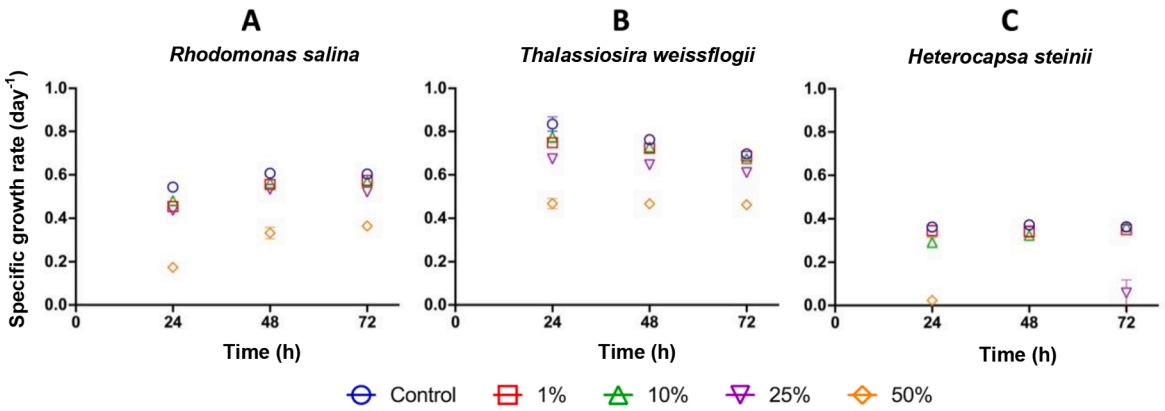

Fig. 2. Specific growth rates (d^{-1}) of *Rhodomonas salina* (A), *Thalassiosira weissflogii* (B) and *Heterocapsa steinii* (C) (mean ± SD) after being exposed to different concentrations of leachates and exposure time. Negative growth rates are not shown.

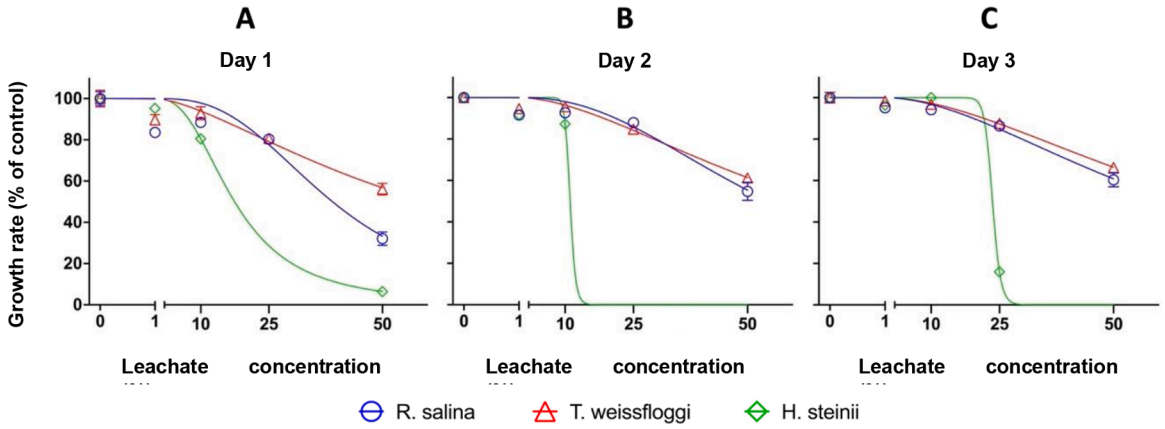

Fig. 3. Non-linear fit of normalised growth rates (mean ± SD) of *Rhodomonas salina* (A), *Thalassiosira weissflogii* (B) and *Heterocapsa steinii* (C) (mean ± SD) as a function of leachate concentration for each day. Negative growth rates are shown as 0%.

4. Discussion

Capolupo et al. (2020) quantified the organic compounds contained in marine TWP leachates and showed that leachates contained highly significant levels of Acetophenone, Benzothiazole, *n*-cyclohexylformamide and Phthalide as well as metals including Mn, Co, Zn and Sr, with Zn being by far the most pervasive at a concentration of 5240 μg L^{-1}, accompanied with a decreased pH. Leachates in Capolupo et al. (2020) were produced from 80 g TWP L^{-1} and were incubated for 14 days, in comparison to our study where 1 g TWP L^{-1} was incubated for 3 days. This explains why the concentrations of chemical compounds were substantially higher than in our experiments. It is also possible that the lower pH due to very high initial TWP concentrations contributed to increased inhibition in Capolupo et al. (2020). The size of TWP used to produce leachates was also larger (1–2 mm compared to < 250 μm in the present study). This could have an effect on toxicity as Halsband et al. (2020) showed increased concentrations of metals such as Zn, Co and Mn with decreasing particle size of crumb rubber granulate. Particle size influences the rate at which chemicals leach from plastic (Town and van Leeuwen, 2020). Smaller particles have higher SA:Vol ratios, causing faster desorption rate of chemicals. This emphasises the importance of using standard protocols for leachate preparation, as proposed by Almeda et al. (2022).

In our study, the PAH naphthalene was the most abundant organic compound detected in the TWP leachates. As an important component of crude oil, the inhibition of naphthalene towards *H. steinii* has previously been tested, resulting in EC_{50} of 1653 μg L^{-1} (Ozhan and Bargu, 2014) whereas Østgaard et al. (1984) observed an EC_{50} of 400 μg L^{-1}. Both concentrations are much higher than that found in our leachates, possibly suggesting that another compound was causing the high toxicity, or that the low EC_{50} in our experiment was a result of a cocktail effect. Ozhan and Bargu (2014) also found the diatom *Ditylum brightwellii* to be more vulnerable to naphthalene than the dinoflagellate *H. steinii*, which contrasts with the results found in our study.

Zn and Sr were the predominant metals detected in the leachates (Table 1). Sr has only been shown to inhibit phytoplankton growth at much higher concentrations than those found in TWP leachates (> 0.51 g L^{-1} for *Platymonas subcordiformis*) (Mei et al., 2006), while zinc and zinc oxides have an effect at lower concentrations (ZnO no-effect concentration of 223 μg L^{-1} for *Thalassiosira pseudonana*) (Miller et al., 2010; Miao et al., 2005), which is lower than the concentration measured in our study (1053.26 ± 74.04 μg L^{-1}). This could suggest that, among the compounds detected in the leachates, Zn is primarily responsible for the observed growth inhibition. Also, other studies point to Zn and Benzothiazole derivatives as likely contributors to toxicity towards marine phytoplankton and copepods (Capolupo et al., 2020; Halsband et al., 2020).

Table 2
Measurements of mean cell diameter (μm) and surface-area-to-volume ratio (SA:Vol) (μm^{-1}) of each phytoplankton species.

Species	Cell diameter (μm)	Volume (μm^3)	SA:Vol (μm^{-1})
R. salina	7.35 ± 1.11	219	0.79
T. weissflogii	12.70 ± 1.11	1131	0.46
H. steinii	16.64 ± 1.10	2513	0.35

H. steinii appeared to be most susceptible to inhibition by TWP leachates. This was despite it having the lowest SA:Vol ratio (Table 2), which could have increased its resilience towards the toxic compounds in TWP leachates. This could suggest that the characteristics of the cell wall are more important than the size or SA:Vol ratio when it comes to resilience towards toxic substances. The susceptibility of *H. steinii* could also have been caused by its slower growth rate, which resulted in a potential exposure time of each generation to leachates for almost twice the duration of the other species. Including relevant species with distinctly different traits would be useful both considering the relevance of TWP toxicity and its mechanisms. For example, the cyanobacteria *Synechococcus* sp. is dominant in many ocean regions, has a higher growth rate compared to the other species tested (Liu et al., 1998) and has a large SA:Vol ratio. Other knowledge-gaps include the effects of leachates over a longer exposure time, to clarify whether growth inhibition persists or if the phytoplankton adapt or acclimatise, and the effect of additional stressors, including changes in temperature, salinity, pH or light levels.

The solid to liquid ratio (S:L) influences the toxicity of plastic leachates. The effect of plastic load on lixiviate toxicity was investigated by Beiras et al. (2019). Leachates obtained at higher S:L ratios were more toxic to the zooplankton. However, the increase in toxicity was not linear with the increase in plastic load. A 10-fold increase in the load of particles used to make up the leachate from 1 to 10 g L^{-1} produced a 3.4-fold increase in toxic units only. The explanation is the potential saturation of hydrophobic components leached into the aqueous medium, causing lower toxicity (in mass per volume units) of leachates obtained using higher plastic loads (Beiras et al., 2019).

Gualtieri et al. (2005) tested the effects of TWP leachates on the freshwater chlorophyte *Raphidocelis subcapitata*, finding an EC_{50} of 0.93% for 50 g L^{-1} leachates. These leachates were prepared at pH 3 and were incubated for 24 h at 50 rpm, as opposed to pH 7.5 and 72 h at 1 rpm in our experiments. This makes it difficult to compare EC_{50} values, as chemical concentrations could vary significantly between the two studies.

Toxicity of TWP leachates could also vary between tyre brands, type, and their condition. For instance, unused tyres are more toxic to marine copepods compared to used tyres (Koski et al., 2020); and slightly worn tyres are more toxic to the freshwater phytoplankton *P. subcapitata* than heavily worn tyres (Wik and Dave, 2009).

Overall, there are still many uncertainties regarding how compounds leach from TWP (Halsband et al., 2020), especially when considering variables such as initial concentration, particle size, incubation time, temperature, and salinity. Standard protocols for leachate extraction from TWP would allow for more reliable comparisons of effect concentrations between studies (Almeda et al., 2022).

When discussing whether toxic effects are significant at environmentally relevant concentrations of TWP, one must therefore factor in likely variation between observed concentrations and actual toxicity levels in a given environment. While it is difficult to draw a fair comparison between concentrations measured in particles per volume and mass per volume, it can be assumed that the concentration of leachates used in the present study are above what could be found in open surface waters. However, this does not account for particles that have leached out their additives prior to entering sediment and would therefore not be found in the water column. An example of where the abundance of TWP could pose a threat in the environment is the concentration of 0.013 g L^{-1} calculated for road runoff (Wik and Dave, 2006), which falls within the range used in this study and did cause a slight inhibition towards each species. Tian et al. (2020) has also shown that leached plastic additives from TWP can have ecological impacts at environmental concentrations. The quinone transformation product 6PPD caused acute toxicity towards Coho salmon at concentrations found in road runoff (Tian et al., 2020). When it comes to measured TWP concentrations in open waters, there is a considerable lack of data (Mennekes and Nowack, 2022).

Overall, there is still a lack of data concerning both the concentration and composition of TWP leachates, and the identification of compounds responsible for toxicity towards marine planktonic species. Therefore, more environmental chemical data is needed to evaluate the potential ecological impacts of TWP on plankton food webs.

Table 4
EC_{50} (% concentration of leachates) for each species at each time interval. Values in square brackets represent 95% confidence intervals. Confidence intervals for *H. steinii* could not be calculated.

Species	*R. salina*	*T. weissflogii*	*H. steinii*
Day 1	39% [34, 46]	60% [50, 85]	11%
Day 2	55% [49, 67]	65% [59, 76]	11%
Day 3	64% [57, 75]	73% [67, 80]	23%

5. Conclusions

In conclusion, there was a significant negative effect of TWP leachates on the growth rate of the three marine phytoplankton species at TWP concentrations found in coastal environments after road runoff.

Among the studied phytoplankton species, the dinoflagellate *H. steinii* was more sensitive to TWP than the other two species, perhaps due to its lack of protective cell wall or its slow growth rate. For future studies, the effects of TWP leachates on a broader range of phytoplankton should be considered to resolve the species-specific differences in response to leachates.

Since primary production plays such a key role in the marine food web, even a slight reduction in growth rate could result in a major ecosystem imbalance. While microplastic concentrations in open waters may not currently be high enough to cause ecotoxicity to phytoplankton, their levels should be continually monitored, especially in places where plastics like TWP may congregate, such as estuaries, water bodies close to roads and urban wastewater discharges.

CRediT authorship contribution statement

Thomas Suurlan Page: Methodology, Formal analysis, Investigation, Writing – original draft, Visualization. **Rodrigo Almeda:** Conceptualization, Methodology, Validation, Writing – review & editing, Supervision, Funding acquisition. **Marja Koski:** Conceptualization, Methodology, Validation, Writing – review & editing, Supervision. **Evanthia Bournaka:** Investigation, Writing – review & editing. **Torkel Gissel Nielsen:** Conceptualization, Methodology, Validation, Writing –

Table 3
Growth rate and growth rate inhibition (% difference from control growth rate) at each leachate concentration. For cases where average growth rate was negative, inhibition is shown as 100%.

	Growth rate (d^{-1})			$\%I_r$		
Leachate conc.	*R. salina*	*T. weissflogii*	*H. steinii*	*R. salina*	*T. weissflogii*	*H. steinii*
0%	0.61	0.70	0.36			
1%	0.58	0.69	0.35	5.2%	0.4%	3.2%
10%	0.57	0.68	0.36	6.0%	2.7%	-0.6%
25%	0.52	0.61	0.10	14.0%	11.5%	73.4%
50%	0.37	0.46	-2.71	40.5%	32.1%	100%

review & editing, Supervision.

Declaration of Competing Interest

The authors report no declarations of competing interest.

Data Availability

Data will be made available on request.

Acknowledgments

We thank Delove Asiedu and Rocio Torres for their assistance in measuring cell counts, Jack Melby for his help with the phytoplankton cultures, Hans Hansen for helping us with grinding car tyres, and Octavio Pérez Luzardo and Andrea Acosta for their support with the chemical analyses. This study was funded by the JPI Oceans RESPONSE project through the Innovation Fund-Denmark, the ULPGC- Science and Technology Park Foundation (subcontract DTU-ULPGC, C2020/65 to RA), the Spanish Ministry of Science and Innovation through a Ramón y Cajal Program grant (RYC2018-025770-I) and the MICROPLEACH project (PID2020-120479GA-I00) to RA.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.aquatox.2022.106299.

References

Almeda, R., Kuddithamby, G., Alonso-López, O., Vilas, A., Christelle, C., Loisel, T., Nielsen, T., Cachot, J., Beiras, R., 2022. A Protocol for Lixiviation of Microplastics for Aquatic Toxicity Testing. SSRN. https://ssrn.com/abstract=4056818.

Beiras, R., Tato, T., López-Ibáñez, S., 2019. A 2-tier standard method to test the toxicity of microplastics in marine water using *Paracentrotus lividus* and *Acartia clausi* larvae. Environ. Toxicol. Chem. 38, 630–637.

Boucher, J., Friot, D., 2017. Primary Microplastics in the Oceans: A Global Evaluation of Sources. IUCN.

Brett, S., Perasso, L., Wetherbee, R., 1994. Structure and development of the cryptomonad periplast: a review. Protoplasma 181, 106–122.

Camacho, M., et al., 2019. Organic pollutants in marine plastic debris from Canary Islands beaches. Sci. Total Environ. 662, 22–31.

Capolupo, M., Sørensen, L., Jayasena, K.D.R., Booth, A.M., Fabbri, E., 2020. Chemical composition and ecotoxicity of plastic and car tire rubber leachates to aquatic organisms. Water Res. 169, 115270.

De Tommasi, E., Gielis, J., Rogato, A., 2017. Diatom frustule morphogenesis and function: a multidisciplinary survey. Mar. Genom. 35, 1–18.

Dunnett, C.W., 1955. A multiple comparison procedure for comparing several treatments with a control. J. Am. Stat. Assoc. 50 (272), 1096–1121.

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.

Gagat, P., Bodył, A., Mackiewicz, P., Stiller, J.W. (2014). Tertiary Plastid Endosymbioses in Dinoflagellates. In: Löffelhardt, W. (eds) Endosymbiosis. Springer, Vienna. https://doi.org/10.1007/978-3-7091-1303-5_13.

Gualtieri, M., Andrioletti, M., Vismara, C., Milani, M., Camatini, M., 2005. Toxicity of tire debris leachates. Environ. Int. 31 (5), 723–730.

Halle, L.L., Palmqvist, A., Kampmann, K., Jensen, A., Hansen, T., Khan, F., 2021. Tire wear particle and leachate exposures from a pristine and road-worn tire to Hyalella azteca: comparison of chemical content and biological effects. Aquat. Toxicol. 232, 105769.

Halsband, C., Sørensen, L., Booth, A.M., Herzke, D., 2020. Car tire crumb rubber: does leaching produce a toxic chemical cocktail in coastal marine systems? Front. Environ. Sci. 8, 125.

Hann, S., Sherrington, C., Jamieson, O., Hickman, M., Kershaw, P., Bapasola, A., Cole, G., (2018): Investigating options for reducing releases in the aquatic environment of microplastics emitted by (but not intentionally added in) products. Final Report. London/Bristol.

Hansen, P., 1989. The red tide dinoflagellate Alexandrium tamarense: effects on behaviour and growth of a tintinnid ciliate. Mar. Ecol. Prog. Ser. 53, 105–116.

Koski, M., Søndergaard, J., Christensen, A.M., Nielsen, T.G., 2020. Effect of environmentally relevant concentrations of potentially toxic microplastic on coastal copepods. Aquat. Toxicol. 230, 105713.

Lassen, C., Hansen, S.F., Magnusson, K., Hartmann, N.B., Rehne Jensen, P., Nielsen, T.G., Brinch, A., 2015. Microplastics: Occurrence, Effects and Sources of Releases to the Environment in Denmark. Danish Environmental Protection Agency.

Liu, H., Campbell, L., Landry, M., Nolla, H., Brown, S., Constantinou, J., 1998. Prochlorococcus and synechococcus growth rates and contributions to production in the Arabian sea during the 1995 southwest and northeast monsoons. Deep Sea Res. Part II Top. Stud. Oceanogr. 45 (10), 2327–2352.

McQuatters-Gollop, A., Raitsos, D., Edwards, M., Attrill, M., 2007. Spatial patterns of diatom and dinoflagellate seasonal cycles in the NE Atlantic ocean. Mar. Ecol. Prog. Ser. 339, 301–306. MAR ECOL-PROGR SER.

Mei, L., Xitao, X., Renhao, X., et al., 2006. Effects of strontium-induced stress on marine microalgae Platymonas subcordiformis (Chlorophyta: Volvocales). Chin. J. Oceanol. Limnol. 24, 154–160.

Mennekes, D., Nowack, B., 2022. Tire wear particle emissions: measurement data where are you? Sci. Total Environ. 830, 154655. ISSN 0048-9697.

Miao, A.J., Wang, W.X., Juneau, P., 2005. Comparison of Cd, Cu, and Zn toxic effects on four marine phytoplankton by pulse-amplitude-modulated fluorometry. Environ. Toxicol. Chem. 24 (10), 2603–2611.

Miller, R.J., Lenihan, H.S., Muller, E.B., Tseng, N., Shannon, H.K., Arturo, K.A., 2010. Impacts of metal oxide nanoparticles on marine phytoplankton. Environ. Sci. Technol. 44 (19), 7329–7334.

OECD, 2011. Test No. 201: Freshwater Alga and Cyanobacteria, Growth Inhibition Test. OECD.

Ozhan, K., Bargu, S., 2014. Can crude oil toxicity on phytoplankton be predicted based on toxicity data on benzo(a)pyrene and naphthalene? Bull. Environ. Contam. Toxicol. 92 (2), 225–230.

Tian, Z., Zhao, H., Peter, K.T., Gonzalez, M., Wetzel, J., Wu, C., Hu, X., Prat, J., Mudrock, E., Hettinger, R., Cortina, A.E., Biswas, R.G., Kock, F.V.C., Soong, R., Jenne, A., Du, B., Hou, F., He, H., Lundeen, R., Gilbreath, A., Sutton, R., Scholz, N.L., Davis, J.W., Dodd, M.C., Simpson, A., McIntyre, J.K., Kolodziej, E.P., 2022. A ubiquitous tire rubber-derived chemical induces acute mortality in Coho salmon. Science 371 (6525), 185–189. https://doi.org/10.1126/science.abd6951, 2021 Jan 8Epub 2020 Dec 3. Erratum in: ScienceFeb 18;375(6582):eabo5785. PMID: 33273063.

Town, R.M., van Leeuwen, H.P., 2020. Uptake and release kinetics of organic contaminants associated with micro- and nanoplastic particles. Environ. Sci. Technol. 54 (16), 10057–10067. https://doi.org/10.1021/acs.est.0c02297. Aug 18Epub 2020 Aug 7. PMID: 32700905.

Turner, A., Rice, L., 2010. Toxicity of tire wear particle leachate to the marine Macroalga, Ulva lactuca. Environ. Pollut. 158 (12), 3650–3654.

Wik, A., Dave, G., 2006. Acute toxicity of leachates of tire wear material to daphnia magna—variability and toxic components. Chemosphere 64 (10), 1777–1784.

Wik, A., Dave, G., 2009. Occurrence and effects of tire wear particles in the environment – a critical review and an initial risk assessment. Environ. Pollut. 157 (1), 1–11.

Østgaard, K., Eide, I., Jensen, A., 1984. Exposure of phytoplankton to Ekofisk crude oil. Mar. Environ. Res. 11 (3), 183–200. IssueISSN 0141-1136.