detritus | Multidisciplinary Journal for Circular Economy and Sustainable Management of Residues

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ECOTOXICOLOGICAL ASSESSMENT OF WASTE SCRUBBER WATER IN UNICELLULAR ALGAE (*TETRASELMIS SUECICA*) AND BLUE MUSSEL (*MYTILUS EDULIS*) LARVAE

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Article Info:
Received:
26 June 2024
Accepted:
23 September 2024
Available online:
19 November 2024

Keywords:
Shipping wastes
Scrubber water
Metals
PAHs
Ecotoxicological tests
Tetraselmis suecica
Mytilus edulis

ABSTRACT

Wastes from shipping pose a substantial environmental challenge at local, regional and global scales, particularly in terms of adverse impacts on climate change and biodiversity. There is an increase in installations of exhaust gas scrubbers to reduce the emissions from ships following international regulations on sulphur content in marine fuel from 2020. Marine scrubbers can be classified into wet and dry scrubbers. Scrubber water (also known as washwater) from both wet scrubber systems has been found to release acidic water containing nutrients and contaminants back to the marine environment, including metals, aromatic hydrocarbons, and soot particles. This is especially true for the open-loop scrubbers that utilize the natural alkalinity of seawater and keep a high flow of process water in order to reduce SO2 in the exhaust and the washwater is discharged to sea, most often without substantial treatment. Little is known about potential impact of the discharged washwater on the marine environment. In ecotoxicological tests, a number of marine organisms have shown negative effects after acute and chronic exposures to varying concentrations of scrubber water, but the main pollutants involved in these responses are not clear yet. The aim of this study was to evaluate the effect of the main pollutants found in open-loop scrubber discharge water for survival, feeding and development of different species at the base of the food web after acute exposures to gas scrubber effluent. Toxicity, mortality, and physiology have been evaluated in marine microalgae, *Tetraselmis suecica*, and blue mussel (*Mytilus edulis*) larvae. Direct exposure to scrubber water appears to adversely affect biological and reproductive parameters in invertebrates, raising substantial concerns about ongoing open-loop exhaust gas scrubber system deployment.

1. INTRODUCTION

Waste materials from international shipping pose a significant threat to our oceans and their delicate ecosystems, particularly in terms of impacts on climate change and biodiversity. Ships produce oily waste from engines, machinery, and fuel systems. Oil spills harm marine life, contaminate water, and damage coastlines. Discharged washwater contains sulfur and other pollutants, impacting air and water quality. Ballast Water, used to stabilize ships during loading and unloading, introduces invasive species, disrupting local ecosystems. Sewage (so- called "black water"), from crew and passengers, contaminates seawater, spreads diseases, and harms aquatic organisms. Greywater (non-sewage wastewater from sinks, showers, and laundry) contains detergents and chemicals, affects localised water quality. Leftover cargo materials and cleaning water pollutes water, especially if cargo contains hazardous substances. Discarded rubbish from onboard activities, including plastics, paper, and food waste is ingested by marine animals, causing entanglement and habitat destruction. Efforts to manage ship-generated waste involve regulations, proper disposal, and sustainable practices to safeguard our oceans.

In addition, international shipping is responsible for the emissions of a variety of air pollutants, including volatile

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https://doi.org/10.31025/2611-4135/2024.19440

carbon-based gases, particulate matter (PM), sulphur oxides (SOx), and nitrogen oxides (NOx), with marine shipping currently predicted to account for 5-15% of global anthropogenic SOx emissions (Viana et al., 2014; Lindstad & Eskeland, 2016; Sofiev et al., 2018). Ship emissions are regulated by the International Maritime Organization (IMO) which has recently set a stricter limit on the sulphur content of shipping fuels. Exhaust gas cleaning systems, also known as scrubbers, remove SOx from exhaust gases before they are emitted to the atmosphere, with studies suggesting scrubbers are able to remove up to 95% of SOx from the exhaust gas (Andreasen & Mayer, 2007). Due to the increased costs of these higher quality fuels, many shipping companies opt for the use of scrubbers that offer a viable solution at a lower economic cost (Endres et al., 2018; Teuchies et al., 2020). The majority of scrubbers installed on marine vessels are wet scrubbers using either a 'closed-' or 'open- loop' system. Remnant washwater is discharged into the surrounding surface water at a flow rate between 200-500L/s for a 15MW vessel from an open-loop scrubber, and only ~0.5-3L/s for a 15MW vessel from a closed-loop scrubber due to the recirculation process (Teuchies et al., 2020). The washwater generated can contain a variety of pollutants, including polycyclic aromatic hydrocarbons (PAHs), trace metals and nitrates, thus displacing potential atmospheric pollutants to the marine environment (USEPA, 2011; Lunde Hermansson et al., 2021). Washwater is highly acidic, with the median pH of open-loop scrubber water reported as 5.63, due to the dissolved sulfuric and nitric acids (Comer, Georgeff & Osipova, 2020; Teuchies et al., 2020).

The chemical composition and concentrations of contaminants in discharged scrubber water are dependent on a number of factors including the type of scrubber, scrubber design, removal efficiency, composition of fuel and lubricant oil, and the operating conditions of the ship (Hassellöv, et al., 2020). Several PAHs - such as anthracene, phenanthrene, naphthalene, fluorene and fluoranthene - have been identified in multiple studies (Magnusson, et al., 2018; Schmolke, et al., 2020), and may originate from the fuel itself or the fuel combustion process. Metals are an important component of scrubber water with the highest recorded concentrations reported as vanadium, zinc, copper and nickel (Teuchies, et al., 2020). Vanadium and nickel originate from, and according to previous studies, correlate strongly to the sulfur content of the used fuel (Hansen & Aalborg, 2012; Teuchies, et al., 2020). However, the high concentrations of copper and zinc may be related to the composition of other equipment such as a ship's anti-fouling system, the sampling tube, and corrosion protection anodes (Turner, et al., 2017; Ushakov, et al., 2020). A report by Carnival corporation & PLC (2019) found similar metals in their washwater effluents, with additional metals such as lead (Pb) and chromium (Cr) present at lower concentrations.

Despite the rapid increase in the use of scrubbers, knowledge about the impacts of scrubber effluent on the marine environment remains insufficient (Lange & Markus, 2015). Very few studies have investigated the effects of scrubber water on marine organisms. Some argue that the environmental impacts of the discharge of scrubber water will be negligible, with the resulting concentrations of hazardous substances in the surface water predicted to be orders of magnitude below levels of concern (Kjølholt et al., 2012). However, this modelling was completed on the assumption that background concentrations of contaminants remain constant. This scenario is unlikely to be representative, especially close to ports, marinas, and areas where sources of diffuse pollutants may accumulate, and as substances such as PAHs and metals can persist for long periods in the environment, thus increasing in concentration over time. Initial studies have also demonstrated lethal and sub-lethal effects on various marine organisms when exposed to different dilutions of scrubber washwater (Koski, Stedmon & Trapp, 2017; Magnusson, Thor & Granberg, 2018; Ytreberg et al., 2019). Furthermore, there is particular concern over the effects of scrubber washwater within ports, estuaries, and coastal waters, that experience low water exchange rates and are some of the most heavily trafficked areas (Ytreberg et al., 2019; Teuchies et al., 2020; Thor et al., 2021). Studies analysing the contents of scrubber discharge suggest that the use of scrubbers directly conflicts with the Water Framework Directive's goals of achieving good environmental status within European waters (Lunde Hermansson et al., 2021). Therefore, understanding the potential effects of scrubber water discharge, as well as some of the main pollutants such as PAHs and heavy metals, on phytoplankton and zooplankton is important for assessing the environmental risks associated with increased scrubber usage in the future.

Many studies that have investigated the effects of PAHs and metals have used concentrations of individual compounds that are orders of magnitude higher than those found in scrubber water. Existing studies tend to focus on assessing the impact of single, isolated chemicals on aquatic organisms. This approach, while informative, does not reflect the real conditions in the environment faced by marine life when exposed to scrubber washwater. Scrubber discharge contains complex mixtures of various contaminants, including metals, sulfur compounds, polycyclic aromatic hydrocarbons (PAHs), and other potentially harmful substances. As a result, marine organisms are often subjected to combined chemical interactions and synergistic effects that can amplify toxicity beyond what would be expected from exposure to individual compounds alone. This complexity highlights the need for our study; original research that evaluates the cumulative and interactive effects of multiple pollutants in scrubber washwater in order to provide a more comprehensive understanding of its true impact on aquatic ecosystems. Our study also examines the effects of individual pollutants present in scrubber wash water, such as phenanthrene, anthracene, and fluoranthene, compared to their combined impact.

A multispecies approach toward ecotoxicological testing is fundamental for the purposes of risk assessment and getting accurate environmental management and ecological risk assessment procedures. For this reason, the ecotoxicological experiments in this report include two different species reflecting different trophic levels in the marine food web. Part I of this paper provides evidence of the effects of scrubber water and some of the main pollutants

on the growth and mortality of marine microalgae, *Tetraselmis suecica*. Part II shows results obtained with the blue mussel larvae exposed to different dilutions of scrubber water. These results provide information about the potential effect of scrubber water discharges in the marine environment and which trophic levels could be more sensitive to the components of this water.

2. PART I: EVALUATION OF THE TOXICITY OF SCRUBBER WATER AND SOME OF THE MAIN POLYCYCLIC AROMATIC HYDROCARBONS (PAHS) AND HEAVY METALS FREQUENTLY FOUND IN SCRUBBER EFFLUENTS ON THE GROWTH AND MORTALITY OF THE MARINE MICROALGAE, *TETRASELMIS SUECICA*

2.1 Introduction

Marine microalgae are important components of the aquatic environment due to their role as primary producers in the aquatic food web and as key players in biogeochemical cycling (Ebenezer & Ki, 2013). They can be one of the first groups affected by contamination and can thus be sensitive indicators of environmental change (Debelius et al., 2009; Kumar & Shin, 2017). For this reason, they have frequently been used in ecotoxicological assays, results of which have been widely used as a basis of risk assessment and the development of environmental regulations (Kumar & Shin, 2017).

Tetraselmis suecica is a species of unicellular green algae with a high growth rate and a global distribution (Ebenezer & Ki, 2013; Kumar & Shin, 2017). *T. suecica* is a commercially important algae species due to its widespread use as a basic food organism in aquaculture (Kumar & Shin, 2017). Due to its unicellular nature, it is likely to be sensitive to potentially toxic compounds (Debelius et al., 2009). Microalgae are often the main primary producers within aquatic environments and thus they represent the main energy input into the ecosystem (Campanella, et al., 2001). Therefore, the impacts of the components of scrubber washwater on a species such as *T. suecica* are of great interest, due to the potential wider environmental impacts on the marine environment, as detrimental effects to algal species are likely to affect organisms at higher trophic levels, and thus potentially impact ecosystem health (Chen et al., 2018). This species shows short generation times (Cid, et al., 2012), is easily cultivated within a laboratory (McCormick & Cairns, 1994), and is closely connected with other marine organisms (Champan, 2013), making it an ideal test species.

The objectives of the ecotoxicological assays using *T. suecica* were:

- To determine the effect of scrubber water at different concentrations on the survival and growth of *T. suecica*.
- To evaluate the effect of phenanthrene, anthracene and fluoranthene as single compounds and combined on the survival and growth of *T. suecica*.
- To assess the effect of heavy metals on the survival and growth of *T. suecica*.

2.2 Methods

2.2.1 Algae culture conditions

The green unicellular marine algae *T. suecica* was cultured in the laboratory under standardised conditions (24 hours of continuous light at 20°C), following the internal standard protocol at the National Oceanographic Centre, Southampton. The subculturing process was carried out using 20 L bottles containing filtered seawater, which was enriched with nutrient solution. Cultures of *T. suecica* were supplied with 0.2 µm-filtered air and were routinely monitored by microscope observation to confirm their growth and check for contamination.

2.2.2 Experimental solutions

All glassware was acid-washed (HNO_3 10% vol.) and rinsed with distilled water and acetone prior to the experiments.

Scrubber water discharge

The scrubber water was collected from the open scrubber system of "Catherine C" by DANAOS. Sample was taken on 19th of August 2021; location: at sea; position: 51 04.6N; 001 37.9W. The scrubber discharge water was collected from the overboard unit of the ship (scrubber outlet) directly into acid-washed glass containers; at the same time, inlet water was collected from the seawater pump system onboard (scrubber inlet). All containers were shipped to Southampton, UK, upon returning to the harbour and were subsequently stored at 4°C and dark until use in experiments. The scrubber water concentrations for the experiments were 0.001, 0.01, 0.1, 1.0, 2.0, 5.0, 10.0, 20.0, 40.0 and 100% of the original outlet scrubber discharge water. The dilutions were made using autoclaved and filtered sea water. Each treatment was replicated three times and the whole experiment was repeated twice.

PAHs

PAHs were tested individually at concentrations equivalent to those identified in scrubber water. Phenanthrene and fluoranthene are some of the most prevalent PAHs identified in washwater (USEPA, 2011; Teuchies et al., 2020). Anthracene has been identified as a priority substance by the European Water Framework Directive (Teuchies et al., 2020), and studies have suggested it is one of the most toxic hydrocarbons (Aksmann & Tukaj, 2004). Multiple studies have reported median effect concentrations at the magnitude of micrograms per litre (Gala & Giesy, 1992; Aksmann & Tukaj, 2004, 2008), with one study reporting a median lethal concentration (LC50) of 1.93 µg/L (Weinstein & Polk, 2001). This low lethal concentration is a similar concentration to those recorded in scrubber water, suggesting that anthracene could be one of the more potent pollutants present within scrubber discharge.

Analytical grade phenanthrene, fluoranthene and anthracene (Sigma-Aldrich) were dissolved in 100% acetone to produce stock solutions at concentrations of 1 mg/mL. These were subsequently diluted using autoclaved seawater, to obtain a final concentration of 100 µg/L of phenanthrene, and 20 µg/L for both fluoranthene and anthra-

cene. Appropriate volumes of final stock solutions were then added to experimental vials, and diluted with artificial seawater, to obtain desired concentrations of PAHs. The concentrations used in this study were selected to reflect those found in scrubber water at various levels of dilution, equating to a 1%, 10%, 40% and 100% concentration of washwater. The nominal test concentrations were therefore 0.05, 0.5, 2 and 5 µg/L for phenanthrene, and 0.01, 0.1, 0.4 and 1 µg/L for both anthracene and fluoranthene. Each treatment was replicated three times and the whole experiment was repeated twice.

When assessing the toxicity of PAH combinations, the ratio of the individual mixture components was kept constant while the total concentration of the mixture was varied.

Metals

Four metals commonly found in scrubber effluents were our focus for the tests. These are V, Ni, Zn and Cu. All metal sources were of analytical grade (≥99% purity) and purchased either from Merk (Germany) or Sigma-Aldrich (Germany). Substitutes were used as sources for each metal to simplify the homogeneity of the solution. Vanadyl sulphate hydrate (H_2O_6 SV) was used as a vanadium source; copper sulphate pentahydrate ($CuSO_4.5H_2O$) was used as a copper source; zinc sulphate heptahydrate ($ZnSO_4.7H_2O$) was used as a zinc source; nickel sulphate hexahydrate ($NiO_4.6H_2O$) was used as a nickel source.

A stock solution of each metal was produced with an initial concentration of 1000 ppm. This initial stock solution was serial diluted in autoclaved seawater to obtain stock solutions of: 500 µg/l for V; 500 µg/l for Zn; 100 µg/l for Ni; 50 µg/l for Cu. The appropriate volume of each solution was added to a final solution of 20 ml. The final concentrations of metals were: 350 µg/l for V; 250 µg/l for Zn; 50 µg/l for Ni; 40 µg/l for Cu. These concentrations were determined from the average of metals found in open loop systems noted by Teuchies et al. (2020) as well as an preliminary results obtained by the EMERGE consortium. The [100%] concentration represents the concentration of metals taken directly from a tap near the scrubber outlet. Three additional diminishing concentrations were tested to consider the dilution of the effluent over time as well as analyse the trend in cell concentration and viability. These concentrations are: [40%]; [10%] and [1%]. Each treatment was replicated three times and the whole experiment was repeated twice.

2.2.3 Experimental design

Toxicity assays were performed following the proposed standard protocol for conducting ecotoxicological studies on algae from ISO standard 8692 (ISO, 1989). Toxicity testing was carried out over a 96-hour period for scrubber water discharge and 72-hour period for PAHs and metals exposure. Static, non-renewal tests were carried out at 20±0.7°C, under continuous illumination. Tests were conducted in 50 mL beakers; test solution volume was 20 mL. Stock cultures in logarithmic growth phase (cell density of 1×10^5 cells mL^{-1}) were used as inoculum. During exposure, beakers were shaken daily. Two independent tests were carried out with triplicate repeats for each treatment. A negative control (i.e. a group that does not receive any type of treatment and, therefore, should not show any change during an experiment) was included with autoclaved seawater only. A solvent control was included with a diluted acetone for PAHs exposure and a solution of seawater with 2% of HNO_3 for metals exposure. 40 mg/L copper(II) sulfate pentahydrate ($CuSO_4.5H_2O$) and 200µg/L tributyltin chloride (TBT-Cl) were used as the reference toxicants for PAHs and metals experiments, respectively (Tam, Wong and Chong, 2001).

2.2.4 Cell concentration

At the end of the 72-hour exposure period, growth was determined by cell counts using a Neubauer Improved haemocytometer at 10 x magnification under an optical microscope.

2.2.5 Trypan blue cell viability test

Growth was determined by cell counts using a Neubauer Improved haemocytometer and a light microscope. After the incubation period of 72 hours, 10 µl of Trypan blue solution was added to 10 µl of each treatment to stain the sample for microscopic evaluation. The number of viable and non-viable individual algal cells were identified and enumerated using a haemocytometer under a light microscope (Olympus CH30) with 20X magnification. Non-viable algae cell were distinguishable from viable cells as they were stained blue unlike the green viable cells without the penetration of the stain. In an attempt to avoid unconscious bias when counting and identifying viable cells, this was performed blind, and treatments and concentrations were only made known post-counting.

2.2.6 Statistical analysis

The data were tested for normality and homogeneity of variance. One-way analysis of variance (ANOVA) was subsequently utilised to test for significant differences in cell concentration and mortality between treatment groups. Where a significant difference was observed, Tukey's HSD post hoc test was used to assess pairwise differences. When data did not meet the assumptions of normality and homogeneity of variance, the non-parametric Kruskal-Wallis one-way ANOVA was employed, followed by Dunn's test, to compare individual treatment groups. Percentage mortality data was arcsine transformed before any statistical analysis was carried out (Bellas et al., 2008).

2.3 Results

2.3.1 Effect of exposure to scrubber water on cell concentration and mortality of T. suecica

After a 96-h exposure, a significant difference in algal cell concentration between treatment groups ($p < 0.001$; Figure 1a) was observed. Cell concentration and mortality showed a concentration-dependent effect with the maximum reduction observed for the highest concentrations of scrubber water. The 96-h LC_{50} was calculated as 60.17% at the end of the experiment.

2.3.2 Effect of exposure to PAHs on cell concentration and mortality of T. suecica

After the 72-hour exposure, there were no significant differences in the algal cell concentration or mortality between the negative seawater controls and the acetone controls, in the second experiment ($p > 0.05$). A significant difference in algal cell concentration between treatment groups ($F = 15.61$, $p < 0.001$; Figure 2a) was observed. Exposure of *T. suecica* to all concentrations of phenanthrene, fluoranthene and anthracene resulted in a significant reduction in the cell concentration, compared to the control treatment ($p < 0.01$). However, cell concentration did not decrease with increasing concentration of toxicant, with no significant differences in cell growth between concentrations of all the PAHs tested ($p > 0.05$).

The mortality significantly differed between treatment groups ($F = 11.8$, $p < 0.001$; Figure 2b). None of the PAH treatment groups resulted in a significantly different mortality compared to the negative controls ($p > 0.05$). However, the resulting mortality upon exposure to 100%, 10% and 1% phenanthrene, as well as 100% fluoranthene, significantly differed to all other PAH treatments ($p < 0.05$; Figure b). One of the individual PAHs tested (phenanthrene) showed significant toxicity (significantly greater algal mortality than the solvent control, p=<0.05) at the 100% concentration, however the other two PAHs (anthracene and fluoranthene) did not show significant toxicity at the any of the tested concentrations.

All 4 possible combinations of the 3 PAHs at all concentrations were significantly different (p=<0.05) to the control. Kruskal-Wallis tests were performed at a 95% confidence interval to determine if there was a significant difference in mean percentage mortality between the individual PAHs and when in combination at 4 relative concentrations. Each individual PAH was compared to its respective combinations (e.g., Anthracene (A) 1% was compared to A+F 1%, A+P 1%, and A+F+P 1%) (Table 2).

Post-hoc Dunn tests were performed at a 95% confidence level. In the case of all 3 PAHs, the effects of PAHs in combination were significantly different from the individual effect (Figure 3). For each combination tested there appeared to be a positive correlation between increasing concentration and the level of toxicity on Tetraselmis. The resulting p-values were all <0.05 demonstrating that the concentration of each combination tested had a significant impact on the toxicity of that combination.

The post-hoc tests demonstrated significant differences between the 1% and 100% concentrations for all four

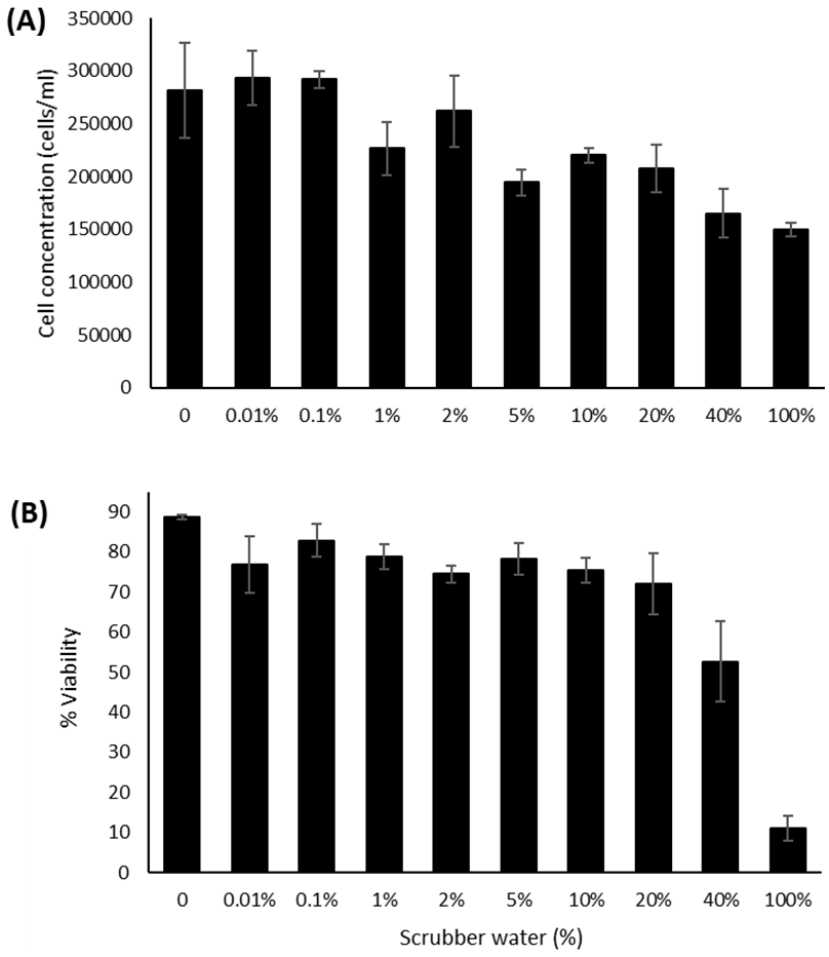

FIGURE 1: The effect of scrubber water on (A) the cell concentration and (B) mortality of the marine algae, *Tetraselmis suecica*, over a 96-hour period. Values plotted indicate the mean ± SD (n=3).

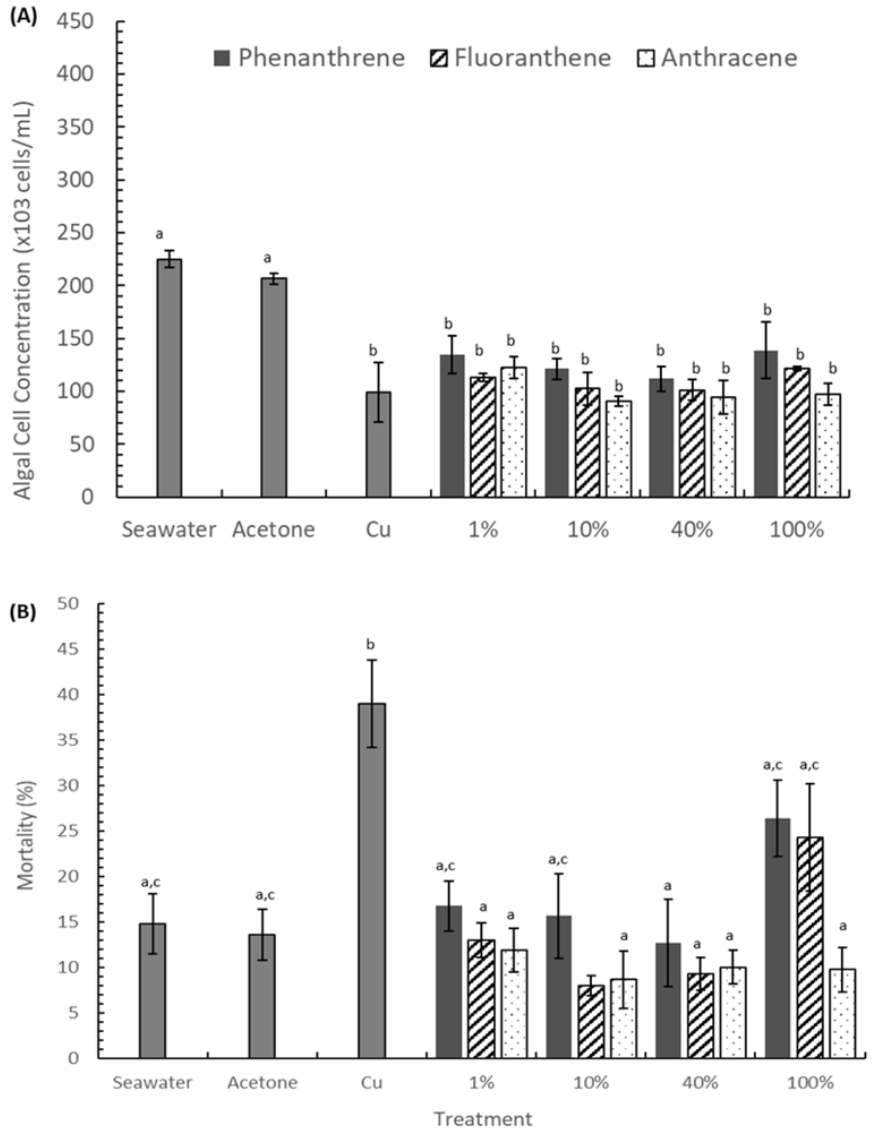

FIGURE 2: The effect of the polycyclic aromatic hydrocarbons (PAHs), phenanthrene, anthracene and fluoranthene on (A) the cell concentration and (B) mortality of the marine algae, *Tetraselmis suecica*, over a 72-hour period. Values plotted indicate the mean ± SD (n=3). Letters represent significant differences ($p < 0.05$) between treatments, whereby if bars share the same letter, they are not significantly different.

combinations illustrating this large variation in concentration has a significant impact on their toxicity. However, the toxicity of the various intermediate concentrations differed between the PAH combinations. The toxicity of both the A+F and F+P mixtures was significantly different between the 1-10%, 1-40%, and 10-100% treatments. This suggests there is a significant relationship between increased concentration and toxicity, however the impact of increasing concentration appears to decrease at the highest concentrations. The toxicity of A+P is only significantly different between the 1-10% treatments. This suggests that for this combination of PAHs, the greatest increase in toxicity occurs between 1-10% concentration, and the toxicity of the three highest concentrations does not differ significantly, illustrating a weaker relationship between concentration increase and toxicity, with a plateau in toxicity apparent across these higher concentrations. Finally, the A+F+P mixture shows no significant difference between the 1-10% treatments unlike the other 3 mixtures, however, does show significant difference between all other combinations of concentrations. Hence concentration significantly impacts the toxic effects of this PAH combination above 10% with each increase in concentration resulting in an even greater toxic effect.

To assess the interactions between the PAHs examined, the extent to which the effect of the PAHs in combination exceeds the effect of each PAH considered individually can be explored, a method outlined by Rothman (1986). This extent can be measured utilising the equation below:

$$p_{03+n} - p_{01} - p_{02} - p_n + p_{00} \quad (1)$$

where:

p = probability of the outcome from 0-1 (in this case, the probability of algal mortality)

03+n = effect of the toxicants in combination

01 = effect of first individual toxicant

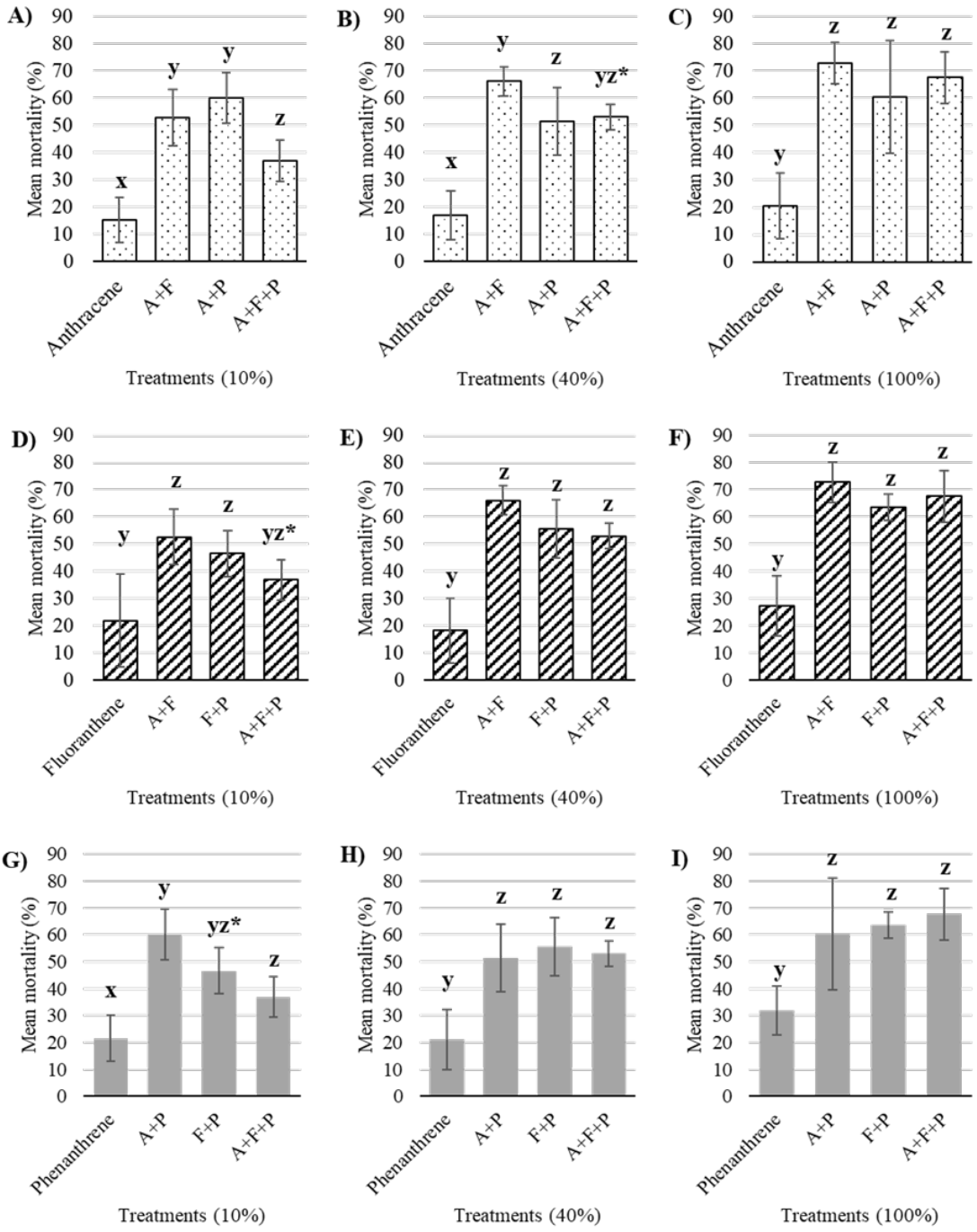

FIGURE 3: Mean percentage algal mortality of different treatments under a range of concentrations and combinations of PAHs. Anthracene and respective mixtures at A) 10%, B) 40%, C) 100%. Fluoranthene and respective mixtures at D) 10%, E) 40%, F) 100%. Phenanthrene and respective mixtures at G) 10%, H) 40%, I) 100%. Error bars = +/- 1 standard deviation. Different letters signify significant difference. *These treatments are unusually not significantly different from 2 treatments which ARE significantly different from one another, thus are labelled accordingly.

02 = effect of second individual toxicant
n = effect of nth individual toxicant
00 = effect resulting from no toxicant (e.g., solvent control)
If $p_{03+n} - p_{01} - p_{02} - p_n + p_{00} > 0$, the interaction is said to be positive or "super-additive".
If $p_{03+n} - p_{01} - p_{02} - p_n + p_{00} < 0$, the interaction is said to be negative or "sub-additive".
A value of exactly 0 would represent a standard additive effect.

The interaction effect was calculated at all 4 concentrations in order to get a broader understanding of the interactions between PAHs (Table 2).

Table 2 gives insight into the nature of the interaction of PAHs within mixtures at different concentrations. A+F shows super-additive (aka synergistic) effects at all four concentrations alongside A+P and F+P at 10, 40 and 100% concentration, and A+F+P at 40% concentration. This suggests that at many concentrations, the relative toxic effects

TABLE 1: Effects of individual and mixtures of PAHs on algal mortality at the 4 relative concentrations. Kruskal-Wallis p-values at 95% are displayed with levels of significance illustrated (* = p ≤ 0.05, ** = p ≤ 0.01, *** = p ≤ 0.001).

PAH (& respective combinations)	Relative concentration	Kruskal-Wallis p-value
Anthracene	1%	0.07
	10%	0.0005 ***
	40%	0.0005 ***
	100%	0.004 **
Fluoranthene	1%	0.06
	10%	0.008 **
	40%	0.0007 ***
	100%	0.002 **
Phenanthrene	1%	0.5
	10%	0.0003 ***
	40%	0.007 **
	100%	0.009 **

TABLE 2: Interaction index for the 4 PAH mixtures using equation above. >0 represents a super-additive effect, <0 represents a sub-additive effect (highlighted in grey). Percentage mean mortality was converted to probability from 0-1 for calculation purposes.

PAH mixture	Relative concentration	Interaction calculation result
Anthracene + Fluoranthene	1%	0.004
	10%	0.249
	40%	0.402
	100%	0.342
Anthracene + Phenanthrene	1%	-0.014
	10%	0.327
	40%	0.226
	100%	0.172
Fluoranthene + Phenanthrene	1%	-0.002
	10%	0.124
	40%	0.257
	100%	0.137
Anthracene + Fluoranthene + Phenanthrene	1%	-0.186
	10%	-0.432
	40%	0.061
	100%	-0.028

of the PAHs are greater in combination than when tested individually. However, this finding was not universal with both A+P and F+P at 1%, and A+F+P at 1, 10, and 100% concentrations showing sub-additive (aka antagonistic) effects.

2.3.3 Effect of exposure to heavy metals on cell concentration and mortality of T. suecica

For cell viability, the negative and solvent controls were non significantly different (Dunn Test: p= 0.76) whilst they were both significantly different to the positive control (Dunn Test negative-positive: p= 0.0054; Dunn Test solvent-positive: p=0.0086).

Significant differences in cell density among the treatments for 100, 40, 10 and 1% of the scrubber effluent metal concentrations were observed. The cell density for metals which had concentrations equal to 100% of scrubber effluent were significantly different from the negative control (Kruskal-Wallis: n= 6, p =0.001624). All the treatments had lower cell concentrations than the negative control (from ~100,000 to ~40,000 cells/ml) but weren't significantly different from one another (Figure 4).

The cell density of treatments when concentrations were at 40% of the reported scrubber effluent concentration were significantly different from one another (Kruskal-Wallis: n= 6, p =0.000403). All the 40% treatments had significantly different and lower averages compared to the negative control, except Cu (Dunn test Cu-negative: p = 0.287). Whilst observing the treatments, V had the lowest cell density (33,000 cells/ml) but was not significantly different to Zn (48,500 cells/ml; Dunn test V-Zn: p= 0.029) or Ni (52,100 cells/ml; Dunn test V-Ni: p= 0.029). V was, however, significantly different to Cu (83,125 cells/ml; Dunn test V-Cu: p= 0.0082). The other treatments (Ni, Cu, Zn) were not significantly different from one another.

The cell density of treatments where concentrations were at 10% reported significant differences between themselves (Kruskal-Wallis: n= 6, p =0.002471). Ni, Cu, Zn and the negative control were not significantly different from one another. V was significantly different to both Cu and the negative control (Dunn test V-Cu: p= 0.0015; Dunn test V-negative control: p= 0.0004).

Finally, when metal concentrations were at 1% of the reported scrubber effluent concentrations, cell density of the treatments were significantly different (Kruskal-Wallis: n= 6, p =0.0420). The only significant difference was between Cu and V (Dunn test V-Cu: p=0.003). Every other treatment was not significantly different to the negative control.

Significant differences in cell viability among the treatments for 100, 40, 10 and 1% of the scrubber effluent metal concentrations were observed (Figure 5). Viability of cells when the metal concentrations were equal to 100% of scrubber effluent had significant differences in their average (Kruskal-Wallis: n=6, p =0.00034). At 100% concentrations, all of the metals had cell viability ranging from 0.35 to 0.5 compared to the negative control, which had a viability of 0.89. Whilst all of the metals were significantly different to the negative control, no significant differences were found between themselves (Dunn test: p > 0.05).

The cell viability of treatments when concentrations were at 40% of the reported scrubber effluent concentration were significantly different (Kruskal-Wallis: n= 6, p =0.0075). Just like for the 100% concentration, all the treatments had significantly different and lower averages compared to the negative control. The metals had similar viabilities (~ 0.65) and were not significantly different from one another.

The cell viability of treatments when concentrations were at 10% reported significant differences between themselves (Kruskal-Wallis: n= 6, p =0.01155). Just like the 40% and 100% concentrations, Metals had similar viabilities (~ 0.75) and were not significantly different from one another. However, they all had lower and significantly dif-

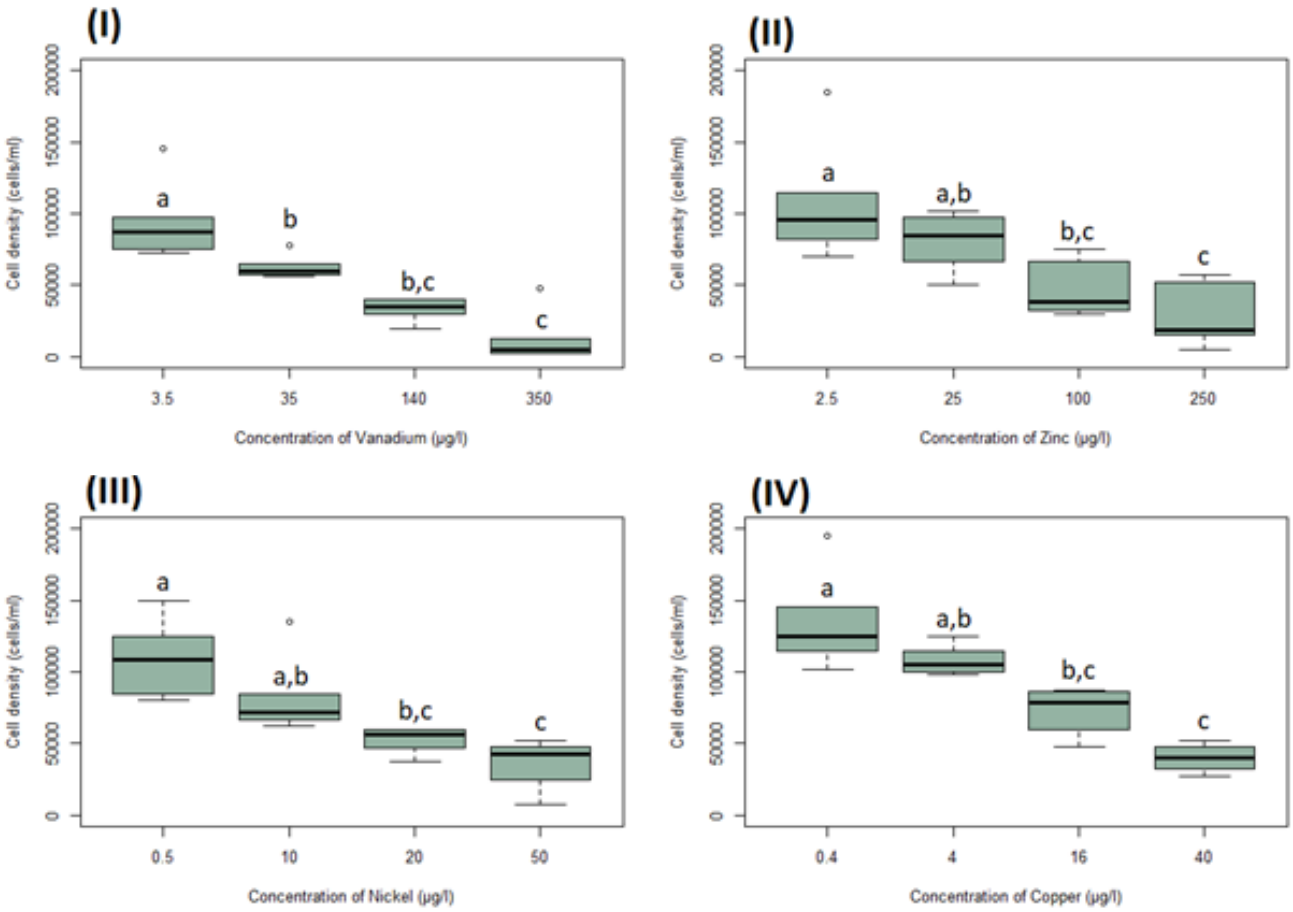

FIGURE 4: *T. suecica* average cell density after 72h of exposure to different concentrations of vanadium, zinc, copper and nickel. Average obtained from the three culture replicates per bioassay done twice. Different letters designate values that are statistically significantly different from one another (ANOVA and Tukey's test with a $p \leq 0.05$). (I). Vanadium; (II). Zinc; (III). Nickel; (IV). Copper.

ferent averages in viability when compared to the negative control.

Finally, when metal concentrations were at 1% of the reported scrubber effluent concentrations, cell viability of the treatments were still overall significantly different (Kruskal-Wallis: n= 6, p =0.01429). Zn, Cu and the negative were not significantly different from one another. However, the negative control and Zn were significantly different to V (Dunn test V-negative control: p =0.0123; Dunn test V-Zn: p=0.021) and Ni (Dunn test Ni-negative control: p =0.007; Dunn test Ni-Zn: p=0.014). Cu, V and Ni had averages of cell viability that weren't negatively different from on another (Dunn test $p > 0.05$).

3. PART II: EVALUATION OF THE TOXICITY OF SCRUBBER WATER ON THE EARLY DEVELOPMENT OF THE BLUE MUSSEL (*MYTILUS EDULIS*)

3.1 Introduction

It has been reported that contaminants present in scrubber discharge water could induce biological effects at low concentrations (Koski et al., 2017; Ytreberg et al., 2019). Negative effects of scrubber water discharges on plankton have been reported with more toxic effects compared to single compounds (Koski et al., 2017). This indicates synergistic effects of the scrubber discharge water mixture, particularly with direct - not dietary - exposure to discharge water (Koski et al., 2017). If this is a common situation for organisms at the basal levels of the food web, it could have substantial consequences for the food web structure and ecosystem functioning.

For many aquatic species, early life stages are the most vulnerable to environmental stressors, and therefore embryos and larvae are commonly used for toxicity tests to determine the sensitivity of organisms to pollutants (Connor, 1972; Martin et al., 1981). Mussels are an ideal test species due to their high sensitivity to pollutants, ecological importance, vital ecological niches, susceptibility to pollutants uptake and close connection with marine predators and human health. However, for the purposes of risk assessment, ecotoxicological tests and organisms used should be standardized.

Embryos and larvae are less tolerant to toxic compounds than adults of the same species and therefore represent the critical life stages for toxicity tests (Connor, 1972; Martin et al., 1981). For this reason, embryo-larval bioassays have proved to be very sensitive indicators of seawater contamination (Bay et al., 1983). Mytilus larvae has been frequently used in different studies due to its

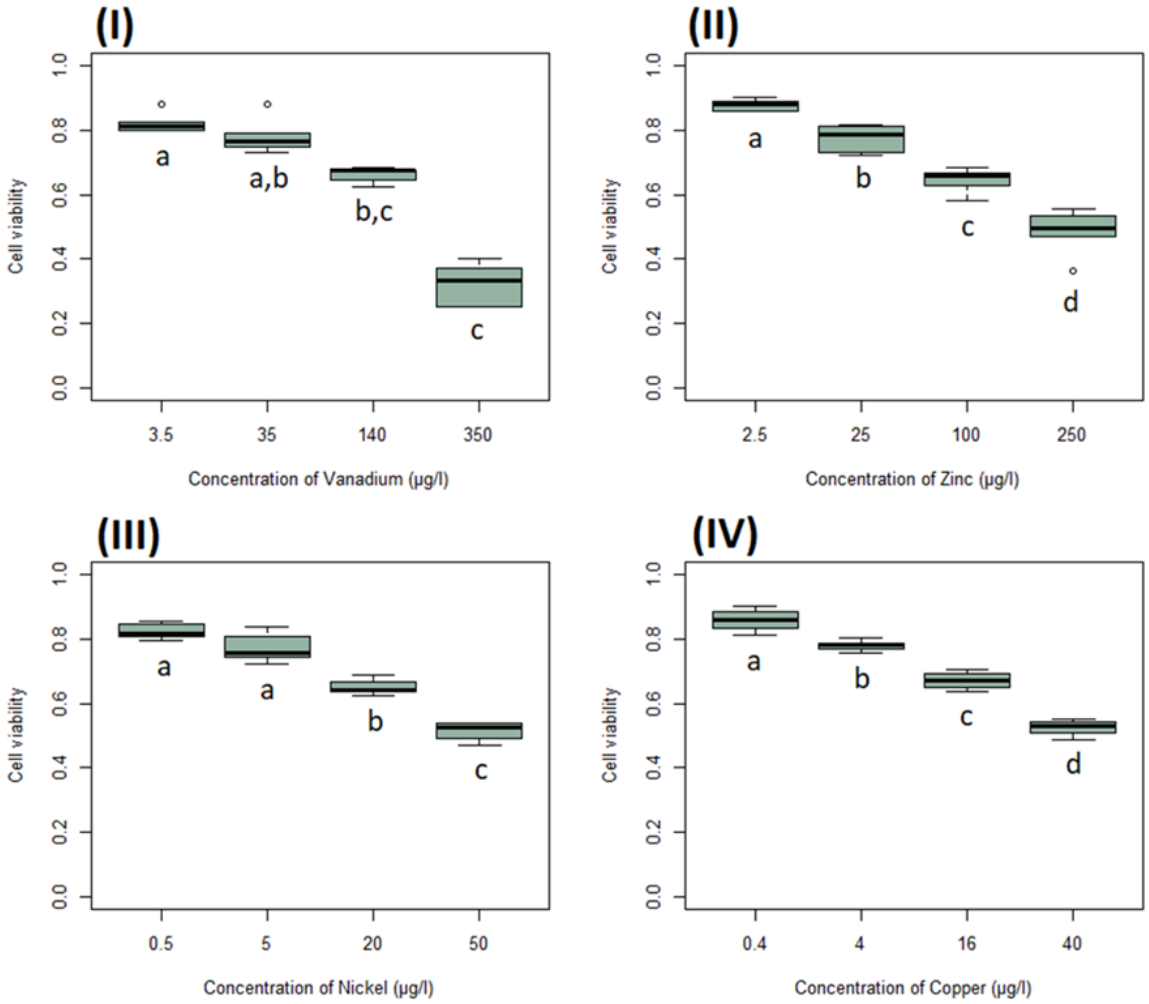

FIGURE 5: *T. suecica* average cell viability after 72h of exposure to different concentrations of vanadium, zinc, copper, and nickel. Average obtained from the three culture replicates per bioassay done twice. Different letters designate values that are statistically significantly different from one another (ANOVA and Tukey's test with a $p \leq 0.05$). (I). Vanadium; (II). Zinc; (III). Nickel; (IV). Copper.

good response to environmental contaminants (Annamaria et al., 2005; Beaumont et al., 1987).

The aim of our study using *M. edulis* larvae was to investigate the effects of open-loop scrubber discharge water on physiological parameters of *M. edulis* developing larvae in order to find the potential effect on their survival and development after exposure to this discharge water.

3.2 Methods

3.2.1 Animals

Adult blue mussels (shell length 4.15 ± 0.25 cm) were collected from the pontoon at the National Oceanography Centre Southampton (NOCS) and were transported to the hatchery. The mussels were cleaned of any epifauna, epifloral, and other attached materials with a plastic brush before acclimating them indoors in a tank with filtered Sea Water under a static condition and with continuous aeration. Eighty percent of the water was exchanged twice per week and before the addition of microalgae. Animals were kept at 14°C and salinity 32 ppt corresponding to the natural conditions of seawater at the collection site, and were fed with a combined diet of *Tetraselmis suecica* and *Isochrysis galbana*. Microalgae were cultured in 20-L flasks in the algae room under standardised conditions (24 hours of continuous light at 20°C), following the standard protocol at the National Oceanographic Centre, Southampton. The subculturing process was carried out using filtered seawater enriched with nutrient solution. Cultures were supplied with 0.2 µm-filtered air, and were routinely monitored by microscope observation to confirm their growth and check for contamination.

3.2.2 Spawning induction

The mussels were induced to spawn by the temperature shock method. Six blue mussels were placed in a spawning tank containing filtered seawater at 30 ppt, with continuous aeration. Every 30 min, the mussels alternate between warm seawater tanks (25°C) and cold sweater tanks (14°C). Once the spawning of the mussels had completed, the adults were returned to the acclimation tank. Fertilised eggs were collected using a 30 µm sieve, placed and maintained in a glass beaker (5 L) filled with SW, filtered through 1-µm filter, with continuous aeration. Before starting the exposure to the different dilutions, fertilised

eggs were counted using a counting chamber under a microscope, diluted to a density of 200 eggs·cm^{-2} in seawater into a flat-bottomed glass dish (See Annex 1).

3.2.3 Scrubber water discharge

The scrubber water was collected from the open scrubber system of "Catherine C" by DANAOS. Sample was taken on 19th of August 2021; location: at sea; position: 51 04.6N; 001 37.9W. The scrubber discharge water was collected from the overboard unit of the ship (scrubber outlet) directly into acid-washed glass containers; at the same time, inlet water was collected from the seawater pump system onboard (scrubber inlet). All containers were shipped to Southampton, UK, upon returning to the harbour and were subsequently stored at 4°C and in the dark until use in experiments. The scrubber water concentrations for the experiments were 0.001, 0.01, 0.1, 1.0, 2.0, 5.0, 10.0, 20.0, 40.0 and 100% of the original outlet scrubber discharge water. The dilutions were made using autoclaved and filtered sea water. Each treatment was replicated five times and the whole experiment was repeated twice.

3.2.4 Experimental design

The fertilized eggs of *M. edulis* under the different treatments were kept undisturbed in the dark without aeration and food addition for a period of 48 h at 16°C. Experiments were performed using a final volume of test solution of 20 mL. Five replicates for each treatment were tested. Due to the end of the spawning season, we had a lack of spawning animals and just one experiment could be performed. Copper sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$) was used as reference substance with test concentrations included in the range 0 µg/L to 100 µg/L of $CuSO_4 \cdot 5H_2O$. The acceptability of test results was based on negative control for a percentage of normal D-shaped larvae ≥80% (His et al., 1999).

3.2.5 Biological measurements

Larvae were inspected under a stereoscope and an optical microscope after approximately 48 h and assessed for the percentage which exhibited normal morphology (normality, D-veliger shape) from random samples of approximately 100 larvae (direct sampling, using a light microscope (Olympus CH30) with 20X magnification). Morphological deformity was determined. Deformities were considered when shell abnormalities, hypertrophy of the mantle, and/or hinge abnormalities were found.

3.2.6 Statistical analysis

Data on the percentage of developing veliger larvae were arcsine-square root transformed before analysis. ANOVA or the Student t-test were used to determine if there were significant differences in the mean percentage of normal D-larvae and larval growth rate among treatments. Data analysis was carried out using the software SPSS version 27.

3.3 Results

Morphologically normal veliger larvae developed at all treatments (Figure 6), although the relative proportion

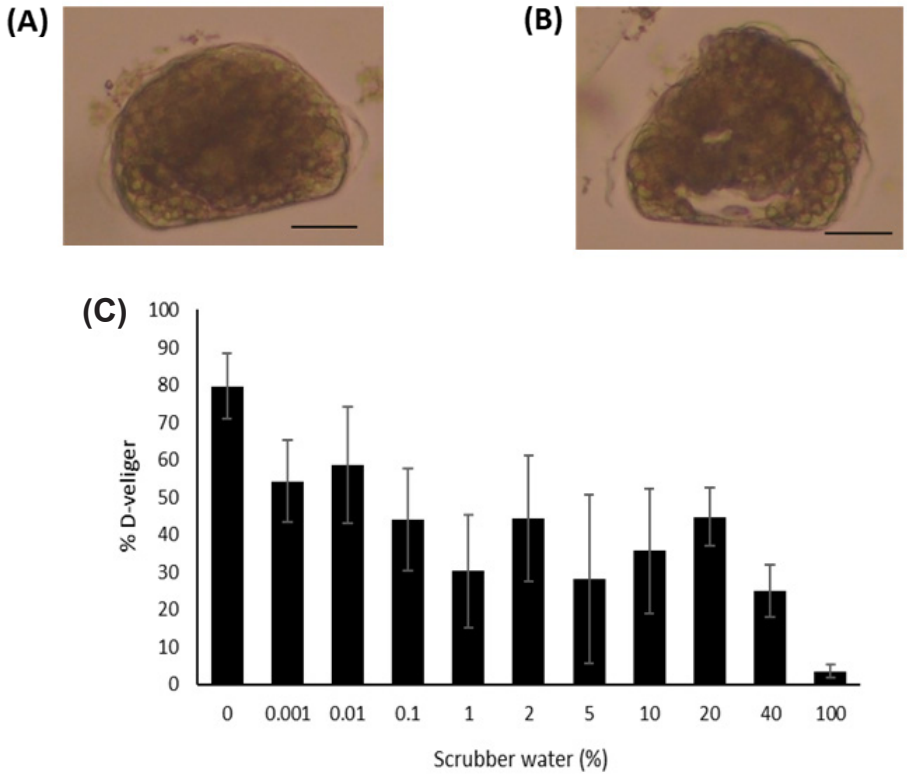

FIGURE 6: *M. edulis* (A) Normal D-shaped larvae and (B) Abnormal D-shaped larvae with hypertrophy of the mantle. (C) Percentage of D-veliger larvae after 48 h exposure of *M. edulis* fertilized eggs to different dilutions of scrubber water. Error bars represent standard deviations. Scale bar: 20µm.

statistically ($p<0.05$) varied among treatments. Figure 6 shows the percentage of normal morphological development of veliger *M. edulis* 48 h after fertilisation at different scrubber water dilutions. All the dilutions inhibited the embryonic development of *M. edulis* in a concentration-dependant manner. The EC_{50} after 48 h exposure to scrubber water was 0.94%.

4. DISCUSSION

4.1 Effects of scrubber water on *Tetraselmis suecica* and *Mytilus edulis* larvae

The results demonstrate that the normal *Mytilus edulis* larvae development can be affected by exposure to scrubber water at different dilutions. A high sensitivity of embryo-larval stage of the blue mussel larvae was found. This high sensitivity has been reported for Mytilus by other authors confirming the use of this species as a bioindicator useful for ecotoxicological testing focused on ecological risk assessment (Boukadida et al., 2016). On the other hand, the high LC50 value obtained for *T. suecica* exposed to scrubber water indicates that this species is more tolerant to this kind of pollution. Different sensitivities can be expected between species (Croxton, Wikfors & Schulterbrandt-Gragg, 2015) making a multispecies approach fundamental for the purposes of risk assessment and environmental management. Marine invertebrates are particularly sensitive to environmental pollutants. Exposure to scrubber water can lead to physiological stress, impaired reproductive success, and even increased mortality rates (Koski et al., 2017; Thor et al., 2021; Picone et al, 2023). The acidic nature of the wash water can disrupt shell formation in mollusks by altering calcium availability (Gazeau et al., 2013), while the presence of toxic metals and PAHs can interfere with enzyme function and cause oxidative stress in a variety of invertebrate species (Letendre et al., 2011; Vlahogianni & Valavanidis, 2007; Vitorino, 2023). Given their role as key components of marine ecosystems and their position in the food web, negative impacts on invertebrates from scrubber discharge can have cascading effects on biodiversity and ecosystem health, making it crucial to carefully manage and regulate scrubber water emissions in marine environments.

4.2 Effects of PAHs on algae growth and mortality

The results demonstrate that algal growth was impacted by exposure to PAHs, where exposure to all concentrations of each PAH resulted in a reduction in cell concentration. This is consistent with the responses shown in many other studies, whereby PAH exposure results in reduced cell densities and growth inhibition (Gala & Giesy, 1994; Jiang et al., 2002; Šepič, Bricelj & Leskovšek, 2003; Wang, Zheng & Meng, 2008). However, this did not occur in a dose-dependent manner, as cell growth did not significantly differ with increasing toxicant concentration. This contrasts with the majority of the literature, where growth is inhibited in a dose-dependent manner to PAH exposure (Aksmann & Tukaj, 2004, 2008; Wang, Zheng & Meng, 2008).

The apparent lack of toxicity of PAHs presented in this study could be due to the fact that toxicity assessments using marine algae can be more challenging compared to assessments using freshwater species (Ebenezer & Ki, 2013). The tolerance of *T. suecica* to chemical exposure likely affected the toxicity of these chemicals. *T. suecica* specifically has been shown to be more tolerant to chemical stress, due to its ability to bioaccumulate higher concentrations, compared to other species of marine algae (Ebenezer & Ki, 2016). For example, *T. suecica* has the ability to withstand total PAH concentrations approaching 400 µg/L (Garr, Laramore & Krebs, 2014), which is multiple orders of magnitude greater than the concentrations used in this study. It has been reported that marine algae could have a higher tolerance to toxicant contamination compared to freshwater species, and other species such as invertebrates, especially at lower concentrations (Gala & Giesy, 1992; Ebenezer & Ki, 2013).

Cell size has also been shown to be an important factor in determining PAH toxicity in phytoplankton, with smaller cells being the most sensitive to chemical exposure (Echeveste, Agustí & Dachs, 2011). This is a result of smaller cells having an increased capability to incorporate contaminants into their cells, due to their higher surface to volume ratio (Echeveste, Agustí & Dachs, 2010). This has been demonstrated by the LC_{50} of phenanthrene ranging from 30.2 to 189 µg/L, depending on the cell size of the phytoplankton species (Echeveste, Agustí & Dachs, 2010).

Another factor that is likely to have significantly affected the toxicity of the PAHs in this study is the light conditions. Treatments were stored in an incubator where they were exposed to standard light conditions. However, many studies have shown that the toxicity of PAHs increases upon the exposure to UV light, with the sensitivity of organisms increasing two to three-fold under UV light exposure (Grote, Schüürmann & Altenburger, 2005; Okay & Karacik, 2007). The growth rate upon exposure to anthracene of the green algae Selenastrum capricornutum was inversely correlated to the intensity of UV-A radiation, with EC_{50} ranging from 3.9 to 37.4 µg/L (Gala & Giesy, 1992). This is due to photomodification, which causes the formation of multiple oxygenated products which are typically more toxic than the parent PAH, and photosensitisation; this causes the generation of oxygen species, resulting in oxidative damage (Aksmann & Tukaj, 2004). Anthracene is predicted to be one of the strongest and most efficient photosensitisers, and its photomodification results in the formation of more than 20 products, the majority of which are quinones (Aksmann & Tukaj, 2004, 2008). Due to their similarity with naturally occurring components of living cells, they can often disrupt cellular processes, such as the electron transport chain, resulting in impaired photosynthesis (Tukaj & Pokora, 2006). This in turn leads to the production of reactive oxygen species production (Aksmann & Tukaj, 2004; Tukaj & Pokora, 2006). Therefore, this study likely underestimates the toxicity of the PAHs tested at the chosen concentrations, especially anthracene, as in the marine environment, these chemicals will be exposed to UV light, thus increasing their toxicity. Analysis has estimated a threshold of photo-induced toxicity as low as 1.5 µg/L (Gala & Giesy, 1992), therefore the concentrations which occur in this study are likely to result in more significant

effects on morality and growth, when combined with UV-light exposure.

The PAHs tested in this study are all lower weight PAHs, and are therefore more volatile in nature (Croxton, Wikfors & Schulterbrandt-Gragg, 2015). This implies that evaporation needs to be considered, which could have resulted in lower toxicity of PAHs, as they may have evaporated at a higher rate within the crucial period for their toxicity.

Scrubber water is known to comprise of a multitude of pollutants, including various PAHs, metals, nitrates, and is also highly acidic (USEPA, 2011). Exposure to a multiple stressors has the potential to cause toxic effects, despite individual contaminants being below levels of concern (Beyer et al., 2014). For example, when testing the toxicity of scrubber effluent to copepods, none of the contaminants occurred at concentrations that could individually explain the toxic effects observed (Thor et al., 2021). This demonstrates that scrubber water toxicity cannot be predicted by comparing the concentrations of individual pollutants to toxicity thresholds (Magnusson, Thor & Granberg, 2018).

Exposure to multiple PAHs has been shown to have varying effects, with a combination of phenanthrene and anthracene, as well as paired exposure to anthracene and fluoranthene, resulting in synergistic effects on algal cell growth (Wang, Zheng & Meng, 2008).

The results of this study show a significant difference between the toxic effects of the 4 PAH mixtures at different concentrations, illustrating the dose-response we might have expected to see with the individual PAHs, with the greater PAH concentrations resulting in greater algal mortality. Specific studies into the effects of PAHs on plant cells have shown anthracene is especially disruptive to the photosynthetic elements of plant cells, causing distinct changes in the pigment composition (Jajoo, et al., 2014). However, the phenanthrene-containing mixtures did not appear to be significantly more toxic which may be explained by the fact phenanthrene is not as toxic as anthracene and fluoranthene, thus a greater concentration does not necessarily correlate with greater toxic effects as illustrated by greater effect and lethal concentration endpoints in several other studies (Abernethy, et al., 1986; Spehar, et al., 1999; Verbruggen, 2012). This suggests when in combination, the individual toxicity of PAHs is less important, and the combined effects/interactions dominate the toxicity of the mixture.

Additionally, we may have expected the treatments containing 3 PAHs to produce greater toxic effects at all concentrations due to the predicted interactive effects of the PAHs. However, at the highest and lowest concentrations, adding a third PAH to the mixture did not result in significantly different levels of toxicity, while unexpectedly at the 10% and 40% concentrations, the mixture containing 3 PAHs demonstrated weaker toxic effects than mixtures containing 2 PAHs.

The lack of difference between mixture toxicity at 1% may be due to insufficient concentration and chemical exposure to result in mortality, and thus equal mortality was observed across all mixtures from natural causes or potentially from exposure to the acetone solvent which was shown to result in a small increase in algal mortality (Verbruggen, 2012). Furthermore, the lack of differences between mixture toxicity at the 100% concentration may be a result of excessive exposure time resulting in all 4 mixtures reporting equal algal mortality. Toxicity begins to decrease and plateau after a certain length of time, allowing less-toxic mixtures to 'catch up' with the other more rapid-acting toxic treatments (Connell, et al., 2016). In future, shorter exposure experiments may be beneficial to get a better understanding of the magnitude of toxicity of these different mixtures at the 100% relative concentration.

The effects of scrubber water on marine organisms are difficult to predict based on individual chemical action. While it is important to compare the effects of different mixtures with each other, the results above have illustrated that it is equally important to understand how the effects of these mixtures differ from the individual effects, and furthermore to understand the interactive effects/mechanisms of PAHs in combination with one another.

PAHs are considered to be general narcotics and are presumed to be similarly acting substances, meaning their toxicity in combination is expected to be additive. This additive interactive effect has been demonstrated in numerous previous studies (Swartz, et al., 1995; Landrum, et al., 2003). Deviation from additive toxicity has also been reported, with both synergistic and less than additive effects have been found in both marine and freshwater organisms (Boese, et al., 1997; Olmstead & LeBlanc, 2005), and further illustrated in the present study, indicating that the concentration addition model may not accurately represent the toxicity of PAHs in combination. Numerous studies suggest the variation in interactive effects may be due to the differing toxic mechanisms of chemicals within mixtures (Chen & Lu, 2002), however in the present study this should not be the case as the toxic mechanisms of the three PAHs are presumed to be identical. Environmental pollutants can disrupt the antioxidant enzymes system through closely related mechanisms, indirect interactions, and cascade effects that influence processes from pre-transcriptional stages to catalytic activity (Regoli et al., 2014). Chemical pathways can significantly increase the intracellular production of reactive oxygen species (ROS) and, at the same time, excessive oxyradical levels can suppress xenobiotic metabolism, leading to significant environmental consequences for organisms exposed to complex chemical mixtures. Although some studies have shown up-regulations in genes such as CYP450s, GST, SOD, GPx, CAT, and HSPs, establishing their characterization as PAH-sensitive genes (Onyena et al., 2024), the mechanisms are still unclear because PAHs can affect organisms depending on exposure time, kind, matrix, and pollutant dose. In microalgae cells, it seems like exposure to PAHs (as single compounds or in mixture) can result in a reduction in growth rates and biomass, alter photosynthetic activity, cause damage in the membrane integrity, chloroplast and thylakoids, increase lipid cellular content and antioxidant-response (Othman et al., 2023). The joint action modes of PAH mixtures are complicated and thus the reasons behind the complex pattern of the joint action mode of PAH mixtures requires further research.

4.3 Effects of metals on algae growth and mortality

Metals such as As, Cr, Co, Cu, Ni, Se, Va and Zn are essential as nutritional requirements at trace amount for many organisms but are toxic when present in greater amounts (Inthorn, 2001). We observed an elevated reduction in cell density and cell viability of T.suicica when exposed to [100%] of the metal concentrations found in open loop scrubber effluents. Further dilutions of the metal concentrations resulted in no significant reduction in cell density. However, V and Ni still had significant differences in average cell viability when compared to the control even at concentrations of [1%].

Dilution occurs at a higher rate in open sea systems than in inland systems. In open sea, washwater will be diluted up to 2000 times when 50 m behind the vessel, and 1750 times whilst in a port at low speed (Buhaug et al., 20006). At this dilution rate, the acute toxicity of the metals is not expected. However, ports contain high volumes of vessels at a single time which could result in an increase in overall discharge and thus increase the concentration found in the port. Whilst open water systems might not be directly affected by the scrubber effluents, closed inland systems such as harbours or rivers might be more affected by the washwater. Teuchies et al. (2020) reported that metal concentrations that originated from scrubber washwater is higher in inland waterbodies (i.e.: estuaries, harbours) than in open marine systems. For example, it is expected that the concentration of V could increase by 46% when in docks. The overall concentration of these metals depends on the speed and manoeuvre/activity of the vessel. Lower use of fuel results in a lower volume of scrubber water discharge, and vice versa.

Whilst studies can be found regarding the EC50s of T. suicica with some of these metals such as copper (Davarpanah and Guilhermino, 2015), not all of the metals have their EC50s reported. Examining the combined effect of these metals on T. suicica could help paint a better picture of the overall effects of scrubber water.

The co-exposure to metals and PAHs has frequently reported synergistic action, with nearly 45% of research on their combined toxic effects demonstrating synergistic mortality (Gauthier et al., 2014). Multiple studies have shown additive or synergistic effects of anthracene and cadmium, both of which are found in scrubber water, on the growth of algal species (Tukaj & Pokora, 2006; Pokora & Tukaj, 2010; Baścik-Remisiewicz et al., 2011). A synergistic effect between copper and a mixture of PAHs has been reported, which adversely affected the development of kelp species (Espinoza-González et al., 2021). It is believed that the effect of PAHs on membrane integrity influences permeability to metals, and that metals inhibit the detoxification of PAHs (and vice-versa), therefore resulting in increased toxicity upon combined exposure in aquatic systems (Gauthier et al., 2014). However, the combined action of PAHs and metals can depend on the concentrations, as the effects of cadmium and phenanthrene switches from synergism to antagonism with increasing concentrations (Zhu, Lu & Chen, 2008). Therefore, further research is required to understand how the contaminants present in scrubber interact to affect their toxicity on the marine environment.

Overall, our results have given reason for concern regarding the discharge of scrubber water due to the potential detrimental impacts to the Tetraselmis species tested. As previously mentioned, this algae species was used as it is widely distributed and it not only plays its own important ecological role, but also represents the role of a multitude of other algal species. In the same manner, invertebrate larvae together with algae species form the basis of a food chain/web, occupying the position of producer. This role makes them immensely important as they act as a food source to numerous aquatic organisms, which in turn are eaten by larger organisms further up the food chain. We have identified the toxic potential of a handful of components of scrubber water discharge resulting in increased mortality of the test algae species and abnormal development of blue mussel larvae. On a larger scale, such as the increasing use and associated washwater discharge of scrubbers worldwide, these observations have the potential to massively impact marine food webs and subsequently marine ecosystems. Even at low concentrations, the discharge of scrubber wash water has the potential to effect marine organisms via the processes of bioaccumulation and biomagnification. The results report a lower mortality rate at lower PAH concentrations, however the hydrophobic character and low biodegradability of PAHs cause accumulation in organisms beyond their concentration levels within the environment (Van der Oost, et al., 1988). Those algae which don't die at low PAH concentrations still have the potential to absorb or take up the toxic PAHs, meaning, if consumed in large volumes, PAHs can accumulate within higher consumers (Alexander, 1999) and transfer these pollutants through the food chain.

The concentrations *T. suecica* were exposed to in this study represent the PAH levels found in scrubber effluent from a single ship. However, marine organisms will be exposed to scrubber discharge from multiple ships, as their installations increase. One study found that 30 scrubber-equipped ships emitted approximately 35 million tonnes of scrubber water in one year, which was set to increase to 47 million tonnes as a result of the introduction of the global sulphur cap (Comer, Georgeff & Osipova, 2020). Furthermore, coastal regions often already have higher background concentrations of pollutants, as they are susceptible to larger inputs of PAHs from other sources, including atmospheric deposition, direct spillage of petroleum from vessels and industrial emissions (Wolska et al., 2012; Cerezo & Agustí, 2015). The drastic rise in the application of scrubber technology on marine vessels may result in the increased accumulation of contaminants to already polluted areas, which in the long term may see PAHs, and other pollutants, reaching toxic concentrations (Kjølholt et al., 2012; Ytreberg et al., 2019). Additionally, coastal marine environments are home to some of the greatest biodiversity and ecologically protected areas, which can be highly sensitive to chemical stress (Thor et al., 2021). Therefore the increase in substances such as metals and PAHs, from scrubber effluent, will likely have an adverse effect on these sensitive species and habitats (Lange &

Markus, 2015). Therefore, as a result of a combination of these factors, scrubber water may present a greater threat than this study initially suggests, and its effects on the marine environment should not be dismissed.

Although this study provides valuable insight into the potential effects of scrubber effluent, further experimentation is required to gain a better understanding of scrubber water toxicity. Additional studies should investigate the interaction between multiple pollutants identified in scrubber water, as mentioned above, due to their combined action having the potential to be more toxic than the exposure to single pollutants. Furthermore, the effects of the components of scrubber water, and their mixtures, need to be tested on more sensitive species.

More sensitive techniques are proposed using marine microalgae species such as flow cytometry to determine cell density, changes in cell size, formation of ROS, Mitochondrial membrane potential, and changes in DNA content. Additional experiments exposing blue mussel and sea urchin larvae to scrubber water will be carry out. This includes more experiments to confirm EC_{50}, fertilization success, and reduce the range of scrubber water concentrations to clarify the effects obtained below 0.94%.

5. CONCLUSIONS AND RECOMMENDATIONS

Wastes from shipping may be very damaging at local, regional, and global scales, particularly in terms of impacts on climate change and biodiversity. The use of both closed- and open-loop scrubbers on marine vessels is increasing rapidly, resulting in the discharge of large volumes of potentially toxic scrubber water into the marine environment. This study has successfully evaluated the cumulative and interactive effects of multiple pollutants in scrubber washwater to provide a more comprehensive understanding of its true impact on aquatic ecosystems. We assessed the toxicity of a small proportion of PAHs found within scrubber water, which as a group, constitute an even smaller proportion of the total number of pollutants found within scrubber water. Selected metals were tested in conjunction with PAHs to understand the potential environmental impacts on key organisms. The characteristics of discharged water, such as its pH and temperature, further complicate the combined effects of chemicals, and thus the full scrubber water toxicity. Despite only testing a very small proportion of the chemicals found within scrubber water, this study has demonstrated increased algal mortality rates when exposed to concentrated scrubber water, PAHs in combination (and more so than when exposed to individual PAHs) and selected shipping-related metals. Blue mussel larvae were highly sensitive to scrubber water showing effects on development. This raises concern over the impacts scrubber water may have on the marine environment both now and in the future as the installation of scrubbers to vessels continues to increase. If scrubbers continue to be considered an effective and preferred method to meet emission restrictions, then the need for significant investment in technological advances, improved monitoring protocols, and evidence-based regulations on scrubber water discharge limits and restrictions is extremely urgent to prevent further potentially irreversible damage to the marine environment.

ACKNOWLEDGEMENTS

This paper has been produced as part of the European Union project “EMERGE: Evaluation, control and Mitigation of the EnviRonmental impacts of shippinG Emissions” (referred to as “EMERGE”). The EMERGE project has received funding from the European Union’s Horizon 2020 Programme Research and Innovation action under grant agreement No 874990. We thanks the staff of the “Catherine C” and DANAOS.

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