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ORIGINAL PAPER

Effects from maritime scrubber effluent on coastal metazooplankton

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Abstract
Exhaust Gas Cleaning Systems, i.e., "scrubbers", have been installed on ships as an alternative to low sulfur maritime fuels. The effluent that open-loop systems discharge is a cocktail of low pH seawater with high concentrations of PAHs, metals and particles, and poses concern to the marine environment. Metazooplankton are the first metazoan responders to exogenic pressures, thus understanding their responses to scrubber effluents will provide insights of shipping impacts on higher trophic levels of marine ecosystems. In the present community ecotoxicology study, experimental jars were utilized to examine scrubber effluent effects on the small-size fraction of coastal metazooplankton populations (Thessaloniki Bay, Eastern Mediterranean Sea). Treatments were set up by applying two scenarios: (a) low scrubber effluent content (1% v/v; LS) and (b) high scrubber effluent content in seawater (10% v/v; HS) compared with seawater; Control (C). Overall, a non-significant change in copepod's developmental stages (nauplii) was documented in LS treatments compared to controls (C: 1,179; LS: 988 ind per experimental jar), contrary to the significant effects detected in HS treatments (674 ind per experimental jar). The responses were species-specific, with *Oithona* responding positively and exhibiting positive growth rates across treatments (C: 0.3; LS: 0.2; HS: 0.2 d^{-1}). Community network analysis revealed greater connectivity of metazooplankton species and groups in the Control and LS treatments compared to HS. Overall, no adverse effects in the 1% scrubber effluent treatments were reported in the plankton copepods community in our community ecotoxicological experiment, complementing the respective results on natural bacterioplankton and phytoplankton communities.

Keywords Scrubber water · Community ecotoxicology · Zooplankton · Copepods · Pollutants · PAHs

Introduction

Maritime transport activity has increased globally in recent years, as a result of the rising demand for goods, bigger ship cargo capacity and the market interconnection across continents (UNCTAD 2019; Fratila et al. 2021). However, this increase has raised concerns about its impact on the escalating air pollution problem (Gössling et al. 2021; Beecken et al. 2023; Rapkos et al. 2023). Maritime transport contributes significantly to greenhouse gases, nitrogen oxide (NOx) and sulphur oxide (SOx) emissions, and other pollutants including particulate matter (PM) and black carbon (BC) (Grigoriadis et al. 2021; Aakko-Saksa et al. 2023; Rapkos et al. 2023). These emissions not only worsen the global warming problem but are also known to threaten human health (Valavanidis et al. 2008; Mueller et al. 2023), especially in coastal and port regions where air pollution levels are often elevated (Fameli et al. 2020; Teuchies et al. 2020; Mueller et al. 2023). Projections are indicating that these emissions will continue to rise if effective measures are not implemented (Zhao et al. 2020; Aakko-Saksa et al. 2023).

In recent years, several regulations have been introduced to address the environmental impact of ship emissions.

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According to the International Convention for the Prevention of Pollution from Ships (MARPOL) Annex VI rule, the maximum Sulphur (S) content of fuels used on board should not exceed 0.1% in the established Sulphur Oxide Emission Control Areas (SECAs), while in the non-SECA areas the max S-content in fuels is limited to 0.5%. Shipping companies had two options in order to comply with these regulations; either use low-sulfur fuels or include alternative emission control that can reduce emissions to an equivalent level (Contini and Merico 2021; Aakko-Saksa et al. 2023). The latter includes the installation of Exhaust Gas Cleaning System (EGCS), also known as “scrubbers”. Since lighter low-sulfur fuels are more expensive than heavy fuels, scrubbers have received attention, and their installation has increased significantly over the last few years (Teuchies et al. 2020; Karatuğ et al. 2022). Through the scrubbing process, up to 95% of SOx and up to 60–90% of PM can be removed from ship exhaust before being released into the atmosphere (Andreasen and Mayer 2007; Yang et al. 2021). The vast majority of installed scrubbers refers to open-loop ones, i.e. the ones that directly discharge the effluent to the sea after this has washed off the exhaust. Various factors, including engine type, scrubber type, fuel and lube oil, seawater properties, alkaline chemical properties and quantity and specific ship operation by the crew, may affect scrubber emission performance (Grigoriadis et al. 2024).

Although scrubbers can contribute to the reduction of atmospheric pollution from shipping, this solution may lead to secondary impacts. Especially the use of open-loop systems, results to a shift of the environmental impact of sulphur emissions from the atmosphere directly into marine ecosystems. As ship engines burn heavy fuels, they release pollutants such as sulphur oxides (SOx), high concentrations of several polycyclic aromatic hydrocarbons (PAHs), particles and certain metals, which are included in the scrubber effluent and discharged in the sea surface water (Teuchies et al. 2020), affecting, firstly, all the size fractions and components that make up the plankton communities. The composition of scrubber effluent being discharged, particularly the ranges of concentrations for key pollutants like PAHs and heavy metals, varies between ships and this variation can be attributed to many different factors including fuel origin, fuel sulphur content, type of scrubber (i.e. open, closed, hybrid), engine load and/or additional treatment of the effluent before discharged (Teuchies et al. 2020). Study of García-Gómez et al. (2023) based on gas chromatography coupled with high-resolution mass spectrometry focused on the identification of PAHs and their alkylated derivatives, which are among the most frequent and potentially toxic organic contaminants detected in scrubber effluents, showed that all the detected compounds are present in crude oil and petroleum contaminated sites. Among the 16 priority PAHs, naphthalene and phenanthrene were the compounds present at the highest concentrations, ranging from 3 to 13 µg L^{-1}. Also, scrubbers may also remove significant quantities of other pollutants such as NOx but the maximum removal efficiency should not exceed 12% to avoid high discharge of nitrates to the marine environment (IMO 2021).

Several recent studies that have been published, regarding the effect of scrubber effluent on marine plankton organisms, report diverse effects (Table S1). Koski et al. (2017) investigated the impact of scrubber effluent on survival, feeding and reproduction of the copepod *Acartia tonsa*. The results indicated increased adult mortality and reduced feeding, which have been probably due to the synergistic effects of heavy metals and other pollutants in the scrubber effluent since the mixture itself was considered more harmful than the toxicity of single compounds. Furthermore, Koski et al. (2017) reported an increase in growth of the phytoplankter *Rhodomonas* sp. compared to the control treatment. This increase could be attributed to the NOx content in scrubber effluents, which can serve as a stimulant for phytoplankton growth, linked with potential eutrophication consequences (Genitsaris et al. 2023). The last was also noted by Ytreberg et al. (2019) who investigated the effects of scrubber discharge on natural microplankton community from the Baltic Sea and recorded increased Chl-a and particulate organic matter concentrations in the 10% scrubber treatment. Thor et al. (2021) observed high mortality rates on the juvenile life stages of the calanoid *Calanus helgolandicus* exposed to scrubber water originated from open-loop and closed-loop systems. Impacts of scrubber effluent on several single-species planktonic biological indicators were also examined by Picone et al. (2023), whose toxicity tests evidenced acute effects on algal growth and copepod survival at 10% and 20% scrubber water, while significant effects on the reproductive success of the copepod *Acartia tonsa* were observed at < 1% scrubber effluent concentration.

Overall, limited research has been published investigating the scrubber effluent impacts on natural plankton communities. However, the most critical gap lies in community ecotoxicology studies. Although these studies are complex and often difficult to interpret, they reveal the multiple interactions within ecosystems which scale from individual to population and community level (Clements and Rohr 2009). Ecosystem based approaches underline the interconnections within the ecosystem, recognizing the importance of interactions among many target species and other non-target species (Borja et al. 2009). Recently, towards this direction, Genitsaris et al. (2023) investigated the impact of scrubber effluent on the phytoplankton and bacterioplankton functioning and their multitrophic interactions, sampled from natural plankton communities. Despite the acute (within 24 h) adverse effects of scrubber effluent on large-sized phytoplankton, which were only reported at high scrubber concentrations (10% v/v), a rapid taxonomic shift of bacterial communities towards known PAHs-degrading genera was also observed, indicating that the bacterioplankton buffering effects can enhance the resilience of phytoplankton communities against stressors. This underscores the significance of ecotoxicological approaches that integrate multiple species' responses and can better assess the real environmental consequences resulting from scrubber effluent discharges in the marine environment.

Complementing the research by Genitsaris et al. (2023), the present study is the first investigating the effects of scrubber effluent inputs on a natural metazooplankton (i.e. metazoan plankton or multicellular zooplankton) populations. Metazooplankton have a pivotal role in aquatic ecosystems serving as an intermediate link to the transfer of primary production biomass to higher trophic levels, contributing to biogeochemical cycling and vital ecosystem services (Calbet and Saiz 2016). In recent years, mainly after the recognition of zooplankton as a descriptor by the European Marine Strategy Framework Directive (MSFD) for assessing and monitoring the ecological health of aquatic systems, its use in biomonitoring and ecotoxicology studies have increased significantly (Gorokhova et al. 2016; Chiba et al. 2018; Ndah et al. 2022 and the references therein; Stamou et al. 2022). Marine metazooplankton, especially copepods, are the most abundant metazoan organisms and outnumber every other group of multicellular animals on earth (Turner 2004; Suthers et al. 2019), and after bacterioplankton and phytoplankton, are some of the first organisms to potentially be affected by the scrubber water discharge. Metazooplankton can influence the environmental fate of PAHs through metabolic processes (e.g., transfer of PAHs to fecal pellets and eggs), contributing this way to the vertical fluxes of PAHs from surface waters (Tsapakis et al. 2006; Berrojalbiz et al. 2009). Research investigating the impact of high concentrations of PAHs on metazooplankton is mostly on naphthalene (e.g., Calbet et al. 2007; Hansen et al. 2008; Saiz et al. 2009) and the several ecotoxicology studies testing the single-substance and mixture PAHs toxicity have reported narcotic effects (e.g., Barata et al. 2005), reduced feeding (e.g., Saiz et al. 2009), reduced reproductive rates (e.g., Calbet et al. 2007) and negative effects on larval development of single-species (e.g., Picone et al. 2023). However as marine metazooplankton constitutes a diverse community, whose representatives exhibit significantly different ecological characteristics and functions, these responses often differ depending on the species, stage of development, feeding type and environmental conditions although in most cases, they are commonly considered as a whole, like a "black box" (Zhang et al. 2023; and the references therein).

The present community ecotoxicology study investigates the responses of taxa, groups and also the changes in the structure of a natural metazooplankton community after scrubber effluent inputs, through a community lab-experiment, where open-loop scrubber effluent of different % content (10% v/v and 1% v/v) was added to natural seawater. The experiment was simultaneously performed with the two sets of experiments described by Genitsaris et al. (2023), by sampling natural plankton communities originating from the same site and using the same scrubber effluent. The natural community was collected from a site located in the urban coastal area of Thessaloniki Bay, which experiences anthropogenic eutrophication events and is exposed to multiple sources of pollution. The sampling area is next to the Port of Thessaloniki, one of the main Mediterranean ports, that receives a significant amount of commercial maritime transport (www.marinetraffic.com). The Mediterranean Sea is one of the busiest seas in the world and will become a SECA by 2025 (IMO 2022), offering an ideal model system to study scrubber effluent effects on marine environment. We focused on the smaller size fractions, namely the metazoan microzooplankton community (hereafter metazooplankton), largely represented by the developmental stages of copepods that are as considered the most sensitive indicators of pollutants and ecotoxicological test organisms (Hussain et al. 2020) and small-sized copepod species, since they represent the major part of the metazooplankton abundance and biomass of the sampling area. Our working hypothesis are:

1. Scrubber effluent effects on metazooplankton will be species-, developmental stage- and scrubber effluent content- specific.
2. Scrubber effluents affect negatively the community composition and abundance of the metazooplankton community leading to shifts in the overall community structure.

To investigate these hypothesis two experimental approaches were studied representing the impact of (a) high content (10% v/v) and (b) low content (1% v/v) of scrubber effluent in seawater, specifically aiming to investigate the impacts on the low content scenario which is more relatable to the actual dilution of the scrubber effluent in the surrounding sea water.

Methods

Scrubber effluent origin

The scrubber effluent used for the ecotoxicological experiment was obtained from the discharge outlet of an open-loop system on board the DANAOS Leo C container ship during an on-board campaign of EMERGE project at the Mediterranean Sea. The effluent was collected in acid/acetone-washed 16 L glass bottles and stored in a cold (4–8 ºC) and dark environment until the start of the experiment.

Experimental design

Surface seawater (0–2 m, in situ; salinity 38.4 PSU, temperature 12 ± 1 °C) was collected from a coastal site (40°37'57.5"N, 22°56'11.3"E) located in Thessaloniki Bay, in the inner part of Thermaikos Gulf, on 21 January 2022 (Fig. S1). The collected seawater was filtered through a 50 μm mesh plankton net to exclude the metazooplankton grazers/predators and was transferred within an hour to the lab and left to settle for a two-hour relaxation period in acid and acetone-washed glass bottles. Additionally, zooplankton samples were collected by multiple horizontal hauls, using a 50 μm net, to obtain sufficient metazooplankton density, and these samples were then used to enrich with metazooplankton the experimental jars. Our natural metazoan plankton community was experimentally manipulated in densities of natural populations to balance between observing significant effects and mimicking natural population densities of the area. After collection, the several zooplankton samples (containing animals and seawater) were left for relaxation in separate open glass beakers for 2 h. Then the samples were combined in a single homogeneously mixed metazooplankton sample, in order to be distributed to the experimental jars.

The experimental set up consisted of 9 experimental jars of 4 L (final volume). Experimental jars were set up to represent three different conditions: (a) seawater characterized as control (C), (b) low scrubber effluent content in seawater (1% v/v) characterized as LS and (c) high scrubber effluent content (10% v/v) characterized as HS (Table S2). The levels of scrubber effluent content applied in the present experiment align with the PAHs concentrations measured in coastal areas of the Eastern Mediterranean affected by high maritime traffic (Valavanidis et al. 2008). The filtered through 50 μm seawater (salinity 38.4 PSU), consisting of the natural community of bacteria, phytoplankton and protozoa, was distributed to each of the 9 experimental jars. Then, the homogenized metazooplankton community from the net catches was divided into 10 subsamples (100 ml each). Nine of them were used to enrich the experimental jars, while the last one was preserved with 4% formalin solution and used for the estimation of the initial community composition and abundance (Day 0). Thus, each experimental jar contained in average 548 individuals on Day 0. At last, the scrubber effluent was added to the scrubber treatments.

No supplementary food was added during the experiment. In total, the ecotoxicological experiment was conducted for 7 days (Day 0 was considered the seawater prior to scrubber effluent addition), and then till Day 6, signifying the end of the experiments.

The seawater used for the experimental jars' filling, originated from the same sampling site as the phytoplankton-bacterioplankton experiment by Genitsaris et al. (2023) (in which the metazooplankton taxa were excluded) and the same scrubber effluent was used. The two experiments were conducted simultaneously, at the same controllable conditions, having also the same duration. The concentrations of PAHs, alkyl-PAHs, and metals were determined in seawater and scrubber effluent according to the methodology described in detail in the Supplementary Material by Genitsaris et al. (2023).

Environmental parameters during the experiment

The experimental jars were set up in temperature-controlled glass bottles (12 ± 1.0 ºC), indoors, and were exposed to natural light conditions [day-night cycle being 9 h 58 min: 14 h 02 min at the start of the experiment (21st January)]. Water temperature, salinity and pH were monitored throughout the duration of the experiment. More specifically, water temperature (°C) and conductivity (μS/cm) were measured daily using the YSI Pro 1030 instrument (YSI Inc., Ohio, USA). Conductivity was transformed to salinity (PSU) based on the equation in Weyl (1964). Also, pH was measured using the 913 pH Meter (Metrohm AG, CH-9100 Herisau, Switzerland), which was properly calibrated for use in seawater (HELCOM 2017; Anes et al. 2019). The experimental jars' water mass was gently stirred closed once a day to maintain homogeneous mixing of the water column. Also, sub-samples were collected daily, using a manual bilge pump, from each experimental jar to reassure its viability throughout the experiment duration.

Microscopic analysis

At the end of the experiment on Day 6, the experimental jars' water mass was sieved through a 50 μm nylon mesh. Exposure to PAHs can induce narcotic effects in copepods, leading to alterations in their swimming behavior (decreased swimming activity) and physiological functions (e.g., disorientation, immobilization) (e.g., Barata et al. 2005). Therefore, the specimens from each experimental jar that were retained by the mesh were transferred to a Petri dish to be checked for swimming activity under a stereoscope. Animals that were not actively swimming were considered dead and therefore not included in the results. Species identification and abundance estimation were carried out under a microscope (Leica, Leitz Laborlux S). Taxonomic identification of individuals was based on appropriate taxonomic keys (Tregouboff and Rose 1957; Conway 2012a, b), as well as the classification system proposed by Razouls et al. (2005–2023). When possible, adult copepods were identified at the species level, whereas copepodites were grouped together and not further identified. Adult female individuals of the copepod genus *Oithona* were identified at the species level based on morphological features of the rostrum, mandible, maxillule, and urosome, as well as the number of exopod spines on the swimming legs, while *Oithona* males were grouped together.

Data analysis

The dataset was tested for normality using the Shapiro-Wilk test (Shapiro and Wilk 1965) and for homogeneity of variance using Levene's test (Levene 1960); appropriate transformations of the data (log-transformation) were employed in cases where this assumption was not met. The residuals were normally distributed ($P > 0.05$), and the variances were homogeneous. One-way analysis of variance (ANOVA) was then used to determine if there were statistically significant differences in the response variables; metazooplankton taxa abundance among the treatments and the pairwise differences were statistically tested through the post hoc Tukey's test (ANOVA). All above analyses were carried out using the PAST software (version 4.03).

The populations' net growth rate (r) was calculated for the main taxa/groups of the metazooplankton community to determine changes in abundance between the last day of the experiment (Day 6) and Day 0 divided by the number of days between those two, according to the equation below:

$r = \frac{\text{In}\, B_6 - \text{In}\, B_0}{\text{D}_6 - \text{D}_0}[d^{-1}]$, where B6 is the abundance on Day 6 and B0 is the abundance on Day 0,while D represents the Day of the measurement.

To quantify the differential sex responses (between males and females) of *Oithona* spp. and *Oncaea media*-group, the increase rate was calculated to determine the percentage change by the end of the experiment in relation to the initial abundance, according to the equation below:

$\text{Increase rate} = \frac{\text{B}_6 - \text{B}_0}{\text{B}_0} \times 100\%$, where B6 is the species abundance at Day 6 and B0 the species abundance at Day 0.

These two species were chosen since they were the dominant adult copepods.

In addition, to compare the initial metazooplankton community (Day 0) and the metazooplankton communities of each mesocosm (C, LS, and HS) on Day 6, the Bray-Curtis similarity coefficients were calculated based on untransformed abundance values of metazooplankton taxa/groups to identify interrelationships between samples and construct the Non-Metric Multidimensional Scaling (NMDS) plot. Also, the Similarity Percentage Analysis (SIMPER) was carried out to identify sources of variance between the metazooplankton assemblages of the different groups of the NMDS plot. The above analyses were performed using the Plymouth Routine in Multivariate Ecological Research (PRIMER) v.6 software package (Clarke and Gorley 2006).

Lastly, network analysis was performed for the metazooplankton community of each treatment to explore and visualize potential alteration of their structure after the exposure to scrubber effluent. The relationships were characterized through Maximal Information-based Non-parametric Exploration (MINE) statistics by computing the Maximal Information Coefficient (MIC) between each pair of taxa (Reshef et al. 2011), based on abundance data matrix. MIC is a non-parametric method that captures associations (linear and non-linear) between data pairs. It provides a score that represents the strength of the relationship. Unlike traditional correlation measures (e.g., Spearman, Pearson), MIC can identify a wide range of dependencies, making it a suitable correlation tool for cross-sectional data and versatile for complex datasets (Weiss et al. 2016). The matrix of MIC scores corresponding to *P-values* < 0.01, based on pre-computed p-values of various MIC scores at different sample sizes, was used to visualize the networks via Cytoscape 3.9.1. Also, by using the Network Analyzer option of Cytoscape, the topological parameters that are key measures to assess and quantify the structural properties of each network (Newman 2003; Doncheva et al. 2012), were calculated.

Results

Physical parameters during the experiment

Temperature and salinity levels of the experimental jars were monitored throughout the duration of the experiment. Their variation, across all treatments, was within the range of short-term fluctuations of in situ conditions in the study area at the time of sampling; 12 ± 1.0 °C and 38.39 ± 0.2 PSU (Table S3).

Scrubber effluent and seawater chemical characterization

The pH of the seawater collected from Thessaloniki Bay (Day 0) was 8.06 whereas the pH of scrubber was 2.95. The addition of scrubber effluent resulted in slight decrease of pH in LS treatments (pH = 7.90) whereas a higher decrease (pH = 7.18) was observed for HS (Table S3). In Control treatments, the pH after Day 4 the pH showed a slight decrease till the end of the experiment (Day 6; 7.79) (Table S3), that could be attributed to the relevant biological activities in seawater (e.g., metabolic byproducts due to microbial activity leading to a decrease in pH, and/or carbon dioxide dissolution originating from respiration results in carbonic acid form that lowers the pH).

Measurements in the seawater at the start of the experiment (Day 0) indicated that the values of total PAHs and their alkylated derivatives were < 21.38 ng L^{-1} and 4.29 ng L^{-1}, respectively, which were much lower than the high concentrations of PAHs and alkyl-PAHs determined in the scrubber effluent; 12,736.87 ng L^{-1} and 22,211.83 ng L^{-1}, respectively (Table S4). Regarding metals, their total concentrations in the seawater of Day 0 were low (35.09 μg L^{-1}), while the scrubber effluent contained considerably high levels of them (405.69 μg L^{-1}) (Table S5). More detailed information regarding the PAHs, alkyl-PAHs and metals values is provided in Genitsaris et al. (2023).

Lastly, nitrate concentrations in the scrubber effluent were 56.08 μM and in the seawater was 5.71 μM.

Responses based on abundance and species richness

Differential responses among species and groups of the metazooplankton community were detected at the different treatments after scrubber effluent inputs (one-way ANOVA followed by Tukey's post hoc test; $P < 0.05$) (Fig. 1). Among the developmental early-stages of copepods, for nauplii, significantly negative effects were not observed at the LS (1% v/v) (C: 1,179.33; LS: 988 ind per experimental jar) while adverse effects were observed at the HS treatment (674.33 ind per experimental jar) compared to the control. The same pattern was observed for copepodites (C: 354.33; LS: 294.66; HS: 150 ind per experimental jar). Negative effects were not reported in total adults' copepod abundance (C: 256.6; LS: 229.3; HS: 181 ind per experimental jar), as well as in the most abundant copepod species, *Oithona nana* and *Oithona davisae*, which seemed to be less affected by scrubber effluents (*O. nana* C: 76; LS: 54.3; HS: 56.3 ind per experimental jar; *O. davisae* C: 82.3; LS: 84.3; HS: 69.3 ind per experimental jar). The meroplankton larvae were noted to be more sensitive to scrubber inputs since significant effects were detected on both scrubber effluent contents. However, at LS, the observed impact was low, taking into consideration the "positive" growth rates at LS at the end of the experiment (C: 0.20 d^{-1}; LS: 0.15 d^{-1}; HS: -0.014 d^{-1}). Regarding growth rates, they were positive for all metazooplankton taxa/groups, life-stages and also in all treatments, except for the case of meroplankton larvae in the high scrubber content (10%) which exhibited a "negative" growth rate (-0.01 d^{-1}). Interestingly, the growth rates of *O. nana* and *O. davisae* were very similar among treatments regardless of the scrubber effluent inputs (*O. nana*; C: 0.34 d^{-1}; LS: 0.28 d^{-1}; HS: 0.29 d^{-1}, *O. davisae*; C: 0.06 d^{-1}; LS: 0.06 d^{-1}; HS: 0.03 d^{-1}) (Fig. 1).

The total metazooplankton community abundance followed the trend of the most abundant group -the developmental stages of copepods- and significant effects were noted only at HS (Fig. 2; Left panel). These results are consistent with the community ecotoxicological experiment on phytoplankton by Genitsaris et al. (2023). However, comparing the growth rates for both experiments, they were positive across all treatments for metazooplankton (C: 0.19 d^{-1}; LS: 0.16 d^{-1}; HS: 0.09 d^{-1}), while phytoplankton was more sensitive to high scrubber effluent inputs, noting negative growth rate in HS (-0.20 d^{-1}) (Fig. 2).

The species composition of the initial metazooplankton community (Day 0) and the metazooplankton communities of each mesocosm (C, LS, and HS) on Day 6 are presented in Table S6. The average number of the recorded taxa increased till the end of the experiment in the Control and LS experimental jars (Day 6; C: 16.3 taxa; LS: 16.3 taxa) compared to the initial species richness (Day 0; 14.5 taxa), while in HS the species richness remained rather unaffected (12.3 taxa) (Fig. S2). According to the one-way ANOVA analysis investigating differences in species richness among experimental jars for Day 6, followed by Tukey's post hoc test, significant differences were noted between C-HS treatments (Fig. S2).

This difference was also depicted via the multivariate analysis by the NMDS plot, where the initial metazooplankton community (Day 0) was separated from the communities developed in the treatments (C, LS and HS) on Day 6. Furthermore, among the different treatments, C and LS were grouped together in 80% similarity clusters and both separated from HS (Fig. S3; Table S7). Moreover, the sex-responses of male and females copepods to scrubber inputs were investigated. For this, *Oncaea media*-group and *Oithona* species were chosen, as the most abundant copepods among the metazooplankton community. Females of *O. nana* and *O. davisae* were grouped together since their males cannot be further distinguished during the enumeration process. In the case of *O. media*-group, both females and males showed similar responses among treatments.

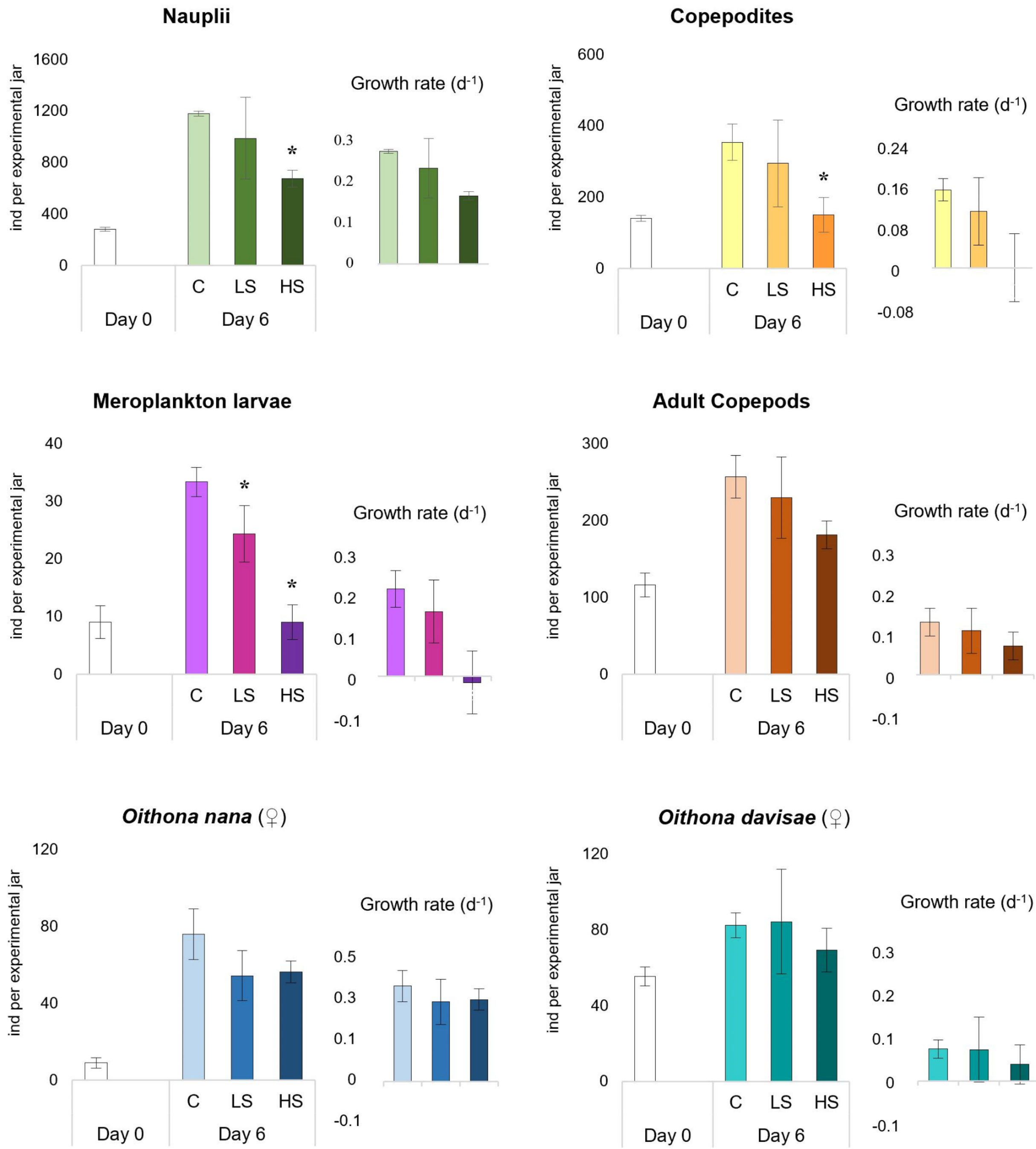

Fig. 1 Abundance (individuals per experimental jar) and growth rates (d^{-1}) of the metazooplankton group taxa in the three different treatments; Controls (C), low scrubber effluent content (1% v/v) (LS) and high scrubber effluent content (10% v/v) (HS) after seven days of exposure. Error bars represent standard deviations and the asterisk (*) represents significant difference between the scrubber treatments and control (one-way ANOVA followed by Tukey's post hoc test; $P<0.05$)

However, the females' increase rate was higher in the Control and LS treatments (C: 49.19%; LS: 49.19%) contrary to males (C: 25.18%; LS: 9.28%), while both males and females exhibited negative increase rates in the HS treatments (Males: -17.36%; Females: -3.19%) (Table S8). Regarding *Oithona* spp. (including *O. davisae* and *O. nana*), both females and males abundance responses were positive regardless the scrubber inputs after the 7 days of exposure

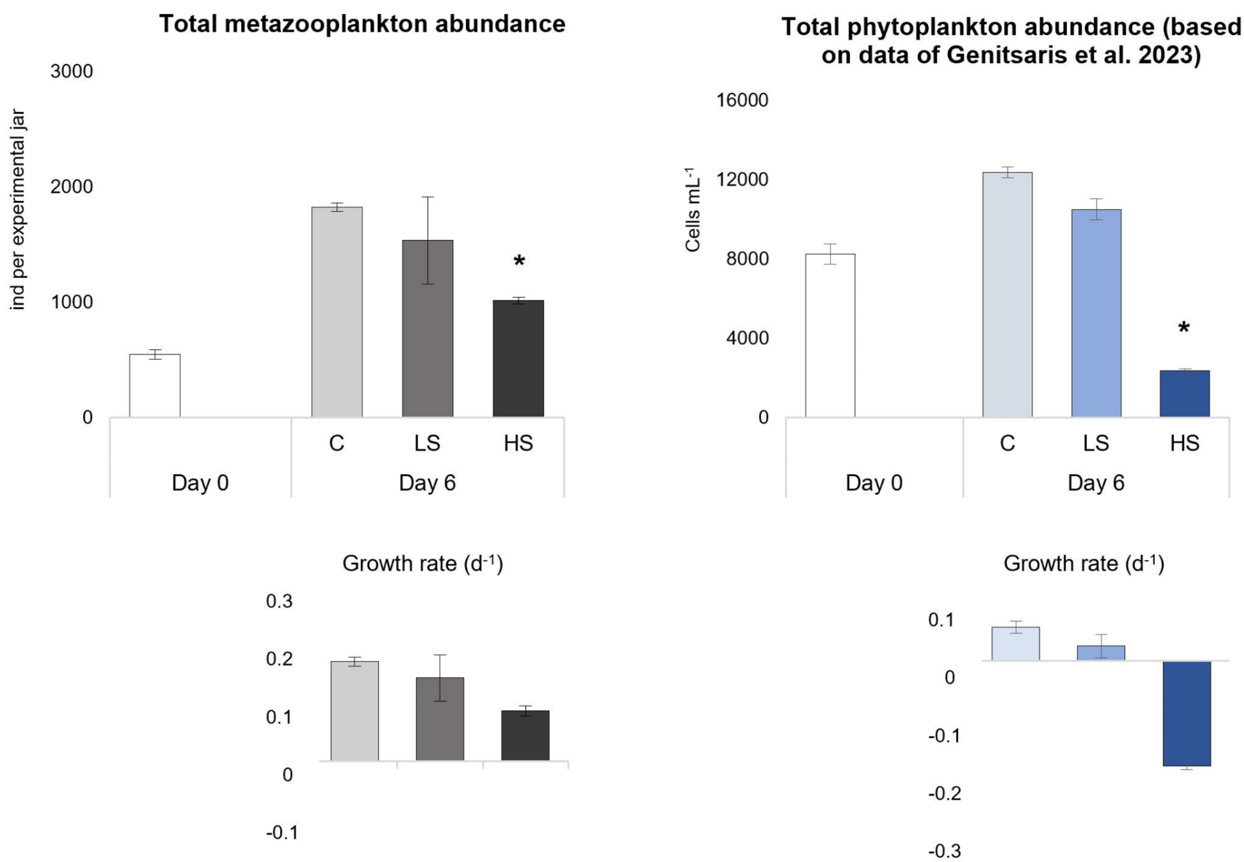

Fig. 2 Total metazooplankton abundance (individuals per experimental jar) (; present study) and total phytoplankton abundance (Cells mL^{-1}) (based on the data of Genitsaris et al. 2023) and the growth rates (d^{-1}) for each experiment in the three different treatments; Controls (C), low scrubber effluent content (1% v/v) (LS) and high scrubber effluent content (10% v/v) (HS) after seven days of exposure. Error bars represent standard deviations and the asterisk (*) represents significant difference between the scrubber treatments and control (one-way ANOVA followed by Tukey's post hoc test; $P < 0.05$)

[(Females C: 145.42%; LS: 114.97%; HS: 94.82%), (Males C: 233.33%; LS: 206.66%; HS: 108.84%)] (Fig. 3; Table S8). Significant difference among treatments of Day 6 was only noted for males of *Oithona* spp. between Control and HS treatments according to the one-way ANOVA followed by Tukey's post hoc test; $P < 0.05$ (Table S8).

Based on the network analysis of interconnections within the metazooplankton communities of the different treatments (Fig. 4) and the topological parameters calculated for each network (Table 1), more complex associations between the metazooplankton taxa were observed in the C and LS treatments compared to HS, in terms of number of nodes and edges, indicating differences in community structure and response to the variable scrubber effluent inputs. Similarly, higher average number of neighbors, reflecting the number of direct connections each node has with other nodes in the network, appeared in C and LS treatments implying greater connectivity of metazooplankton species and groups within the communities. The rest topological parameters of clustering coefficient (measure of local cohesiveness), characteristic path length (shortest path length between two nodes averaged over all pairs of nodes), network density (the proportion of actual edges compared to the maximum possible edges) and network heterogeneity (the tendency of a network to contain hub nodes) were similar among treatments.

Discussion

The present community ecotoxicological study evaluated the impacts of scrubber effluent inputs on a metazooplankton community, highlighting differential responses among taxa and groups and detectable disruptions in community structure at high scrubber effluent content (HS; 10% v/v). The results of our lab-experiment on a natural metazooplankton community align with the findings of Genitsaris

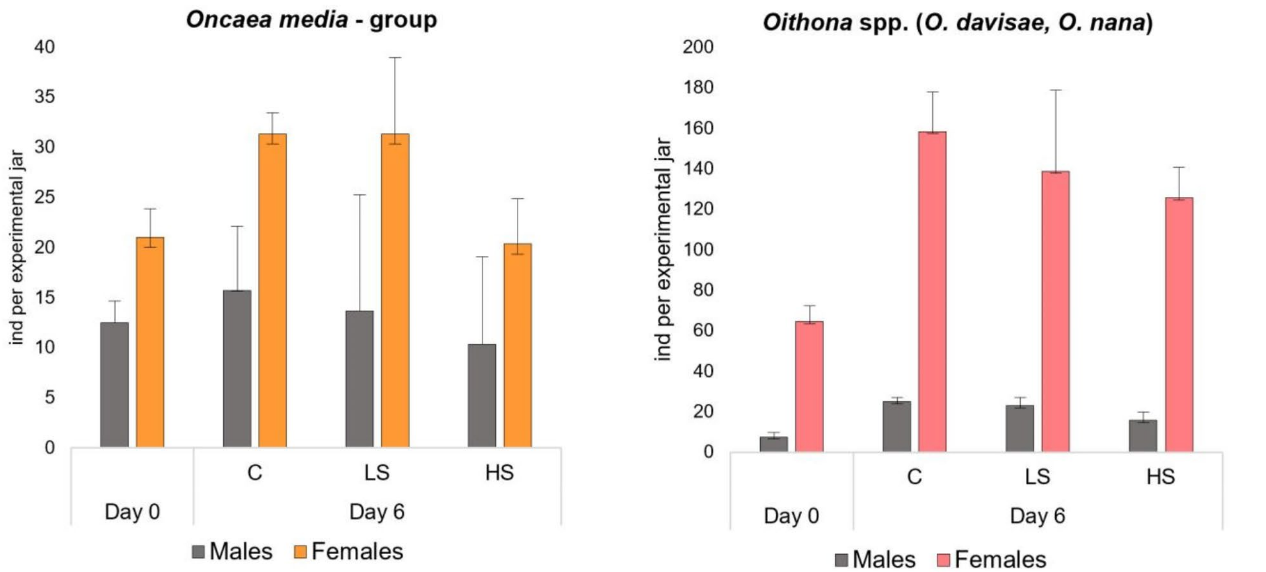

Fig. 3 Male and female abundance (individuals per experimental jar) of *Oncaea media*-group and *Oithona* spp. in the three different treatments; Controls (C), low scrubber effluent content (1% v/v) (LS) and high scrubber effluent content (10% v/v) (HS) after 7 days of exposure. Error bars represent standard deviations

et al. (2023) on natural bacterioplankton and phytoplankton communities, confirming these results at a higher trophic level. Together, these studies emphasize the importance of examining the effects of scrubber effluents on whole plankton communities, which are the first responders in the marine environment and play a key role in determining the environmental fate of pollutants in aquatic environments. This underscores the significance of ecotoxicological approaches that integrate multiple species' responses and can better assess the real environmental consequences resulting from scrubber effluent discharges in the marine environment.+

Hypothesis 1, which predicts that the effects on metazooplankton will be species- and developmental stage-specific, was partly confirmed only at the HS treatments (10% v/v). The copepod developmental early stages, nauplii and copepodites, did not exhibited significantly negative effects at the LS treatment compared to the control. In adult copepod abundance both at the HS treatment and LS treatments (1% v/v) no adverse effects were observed. This difference at HS could be attributed to the increased sensitivity of the early life-stages of copepods to chemical compounds at high concentrations, which has been documented in several studies (e.g., Green et al. 1996; Medina et al. 2002; Hack et al. 2008; Saiz et al. 2009; Kadiene et al. 2019). This age-related variation in sensitivity can be further explained by the allometric differences in weight and size, the increased capacity of the adults for detoxification, and the molting and metamorphosis processes in the early life-stages of copepods, which are critical events that take place in a short period of time (Andersen et al. 2001; Medina et al. 2002). This sensitivity during the early stages is commonly observed in many other invertebrates (Rivera-Duarte et al. 2005; Bellas 2006). The meroplankton communities of the experimental jars, mainly represented by Polychaeta larvae, were also sensitive to scrubber effluent inputs and significant effects were detected in both the LS and HS treatments, however, with low effect at LS while keeping positive growth rate. Taxa in meroplankton are widely used as bioindicators (both as adults and larvae) of marine pollution and as ecotoxicological test species to evaluate the effects of various contaminants. Several studies have reported the impacts of pollutants e.g., heavy metals (Reish et al. 1974; Gopalakrishnan et al. 2007), PAHs mixtures (Chandler et al. 1997) and Benzo(a)pyrene (Jha et al. 1996) on polychaetes, concluding that exposure to contaminants, both at early life- and adult- stages, has been linked to several disruptions in (larval) survival, developmental abnormalities, and instability (as pointed also by Lewis et al. 2008; García-Alonso et al. 2014). However, the mentioned studies focused on single-species experiments, using stock cultures, thus overlooking the resilience fostered by the broader marine communities that surround these species, particularly the pollutants biodegrading capacity of natural bacterioplankton (Genitsaris et al. 2023). In a recent study by Jönander et al. (2023), the importance of natural plankton communities was underscored through an investigation into the effects of scrubber effluents from closed-loop systems on a natural mesozooplankton community. The authors report that copepods' reproduction and survival were affected at the lowest tested concentration of 1.5% of scrubber effluent. However,

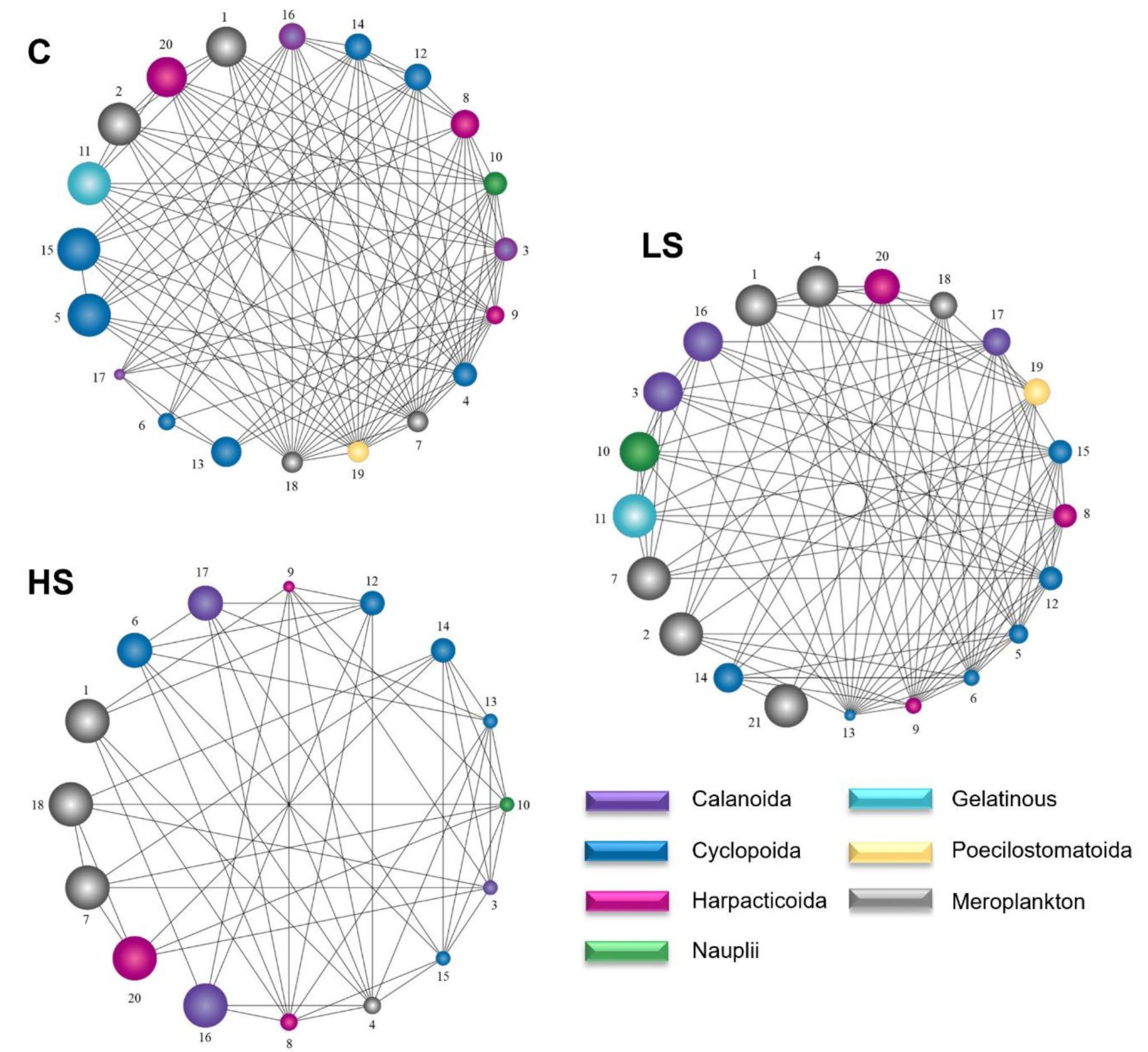

Fig. 4 Network diagrams of the metazooplankton taxa (nodes) showing correlations (edges) with highly significant connections (*P-values* < 0.01) based on the maximal information coefficient (MIC) scores for the different treatments. The edges are undirected and the size of each node is proportional to its clustering coefficient. Different colors represent the different metazooplankton taxonomic groups. The coding for the networks' nodes is presented in Table S9

Table 1 Topological parameters characterizing the networks of the different treatments

Topological parameters	C	LS	HS
Number of nodes	20	21	16
Number of edges	111	107	54
Average number of neighbors	11.100	10.190	6.750
Characteristic path length	1.426	1.624	1.750
Clustering coefficient	0.775	0.831	0.762
Network density	0.584	0.510	0.450
Network heterogeneity	0.287	0.275	0.231

due to variations in the composition of scrubber effluents between closed-loop and open-loop systems, particularly in terms of higher concentrations of metals and organic pollutants in closed-loop systems, direct comparison with the findings of the present study is difficult.

Among adult copepods, *O. nana* and *O. davisae* seemed to be less affected by scrubber effluents, implying differences in tolerance and resilience among copepod populations. This is not surprising, as oithonoids, particularly *O. nana*, are frequently linked to high anthropogenic pollution and are considered bioindicators of marine ecosystem degradation (Richard and Jamet 2001; Serranito et al. 2016; Drira et al. 2018). Their physiological features, such as short life-cycle, low respiratory rate, high metabolism, flexible diet, and high reproduction rate compared to other copepods, can explain their higher tolerance to anthropogenic pressures (Lampitt and Gamble 1982; Castellani et al. 2005). These ecological characteristics facilitate *O. davisae* in becoming a successful invasive species, keystone to the indigenous metazooplankton communities of the colonized areas, including the Thessaloniki Bay. Saiz et al. (2009) carried out singled-species experiments investigating the short-term effects of two PAHs, naphthalene and dimethylnaphthalene, on naupliar and adult stages of stock cultured specimens of *O. davisae* and concluded higher adult tolerance to naphthalene compared to nauplii in terms of lethal effects. Regarding naphthalene, *O. davisae* adults did not exhibit significant mortality at 10 mg L^{-1} (Saiz et al. 2009), a concentration that was notably greater than that detected in the HS effluent used in our community lab-experiments (about 0.00005 mg L^{-1}). Furthermore, the LC_{50} concentration of naphthalene for *O. davisae* reported by Saiz et al. (2009) and Barata et al. (2005) was higher than the one found for the calanoid copepods *Paracartia grani* ($LC_{50}=2.54$ mg L^{-1}, Calbet et al. 2007), *Eurytemora affinis* ($LC_{50}=3.80$ mg L^{-1}, Michalec et al. 2013) and *Calanus finmarchicus* ($LC_{50}=7.02$ mg L^{-1}, Neverdal et al. 2006) confirming again the differences of tolerance across copepods at very high concentrations of PAHs and the adaptability of oithonoids to contaminants. Overall, it has already been reported that cyclopoids are more tolerant to hydrocarbons than calanoids (Lee and Nicol 1977).

The environmental fate of PAHs is closely related to the organic carbon cycle (Duran and Cravo-Laureau 2016), based on bacterioplankton and phytoplankton functions (Genitsaris et al. 2023) and followed by metazooplankton which also plays an important role. The eggs of metazooplankton are important vectors for the transport of PAHs in the marine environment (Berrojalbiz et al. 2009). Indeed, Hansen et al. (2017) reported evidence of maternal PAH transfer in eggs, which was also reported to occur in vertebrates (Vignet et al. 2015). As only females produce eggs, it is possible that females can have a competitive advantage against males in terms of PAHs bioaccumulation and the effects that this brings on. Taking this into account, the sex-specific response to scrubber effluent was also investigated. Results indicated that the responses are rather species-specific. More specifically, the responses of *Oncaea media*-group were similar between males and females across all treatments, however in the case of *Oithona* spp. males exhibited higher increase rates compared to females across all treatments, contrary to the assumption above. Consequently, no definitive conclusions can be drawn from these results and further investigation is needed to fully resolve this issue and particularly in experiments not excluding natural seawater microbial communities by their removal (filtering) or use of synthetic seawater medium.

Hypothesis 2, suggesting that the toxic cocktail of scrubber effluent contaminants can alter the composition and abundance of the metazooplankton community leading to shifts in the overall community structure, is also partly confirmed, only for HS treatments. According to network analysis, the Control and LS networks appear to be more diverse and interconnected because of the higher number of nodes, edges, and average number of neighbors, which from an ecological perspective may be interpreted as higher stability and resilience to disturbances (Borrett et al. 2018). In contrast, HS treatments had a less linked network, indicating probable vulnerability to ecological stress. The fact that the rest of the topological parameters was very similar among networks/treatments, indicates that despite the differences in the overall network size and composition, the nodes in all three networks tend to interact with their neighbors in a consistent manner and exhibit similar patterns of how compact a network is on average. Reduced connectivity and shifts in the overall community structure have also been documented by several mesocosm studies that investigated via network analysis the impact of contaminants like PAHs (Álvarez-Barragán et al. 2022; Yan et al. 2023) and heavy metals (Lawes et al. 2017) on natural communities.

The high concentration of scrubber effluent inputs (HS; 10% v/v) had a more pronounced negative effect on the large phytoplankton (Genitsaris et al. 2023) compared to the effects on the metazooplankton community presented here. More specifically, the nano-and micro-phytoplankton community and their dominant species exhibited an immediate (24 h) and sharp decline after the scrubber inputs and noted ¨negative¨ growth rates in the HS treatments in relation to the critical time (the time of maximum growth of the control treatment). However, in our case all the metazooplankton taxa/groups exhibited ¨positive¨ growth rates, (expect the meroplankton larvae at HS) even after 7 days of exposure. This is no surprise, since generally contaminants bioaccumulation factors (BAF) for metazooplankton are lower compared to phytoplankton due to (a) to the egestion and metabolism processes and/or the higher depuration rates (Harris et al. 1977; Berrojalbiz et al. 2009) and (b) bacterioplankton's fast biodegradation of the most abundant PAHs of the scrubber effluent (Genitsaris et al. 2023). Furthermore, high doubling rates/short generation times of phytoplankton compared to zooplankton can make them more susceptible to initial high contaminants concentrations before these contaminants can fully break down to harmless compounds. This means that pollutants are quickly degraded, and therefore there are smaller concentrations to affect the growth of the zooplankton and their developmental stages, since these are relatively slower than the growth of phytoplankton that were affected (Genitsaris et al. 2023). Considering the results to date, the responses of phytoplankton and metazooplankton to scrubber effluent at 1% v/v treatments, are not expected to result in shifts in trophic interactions and energy flow, suggesting no significant ecosystem-level implications. Nevertheless, the acute population decline of large phytoplankton in the first 24 h of exposure at 10% v/v treatments, short-term disruption of the aquatic food web is expected which appears to be damped at long-term since: (a) the decline was followed by a stationery phase (Genitsaris et al. 2023), and (b) natural phytoplankton communities are diverse allowing shifts in dominance (Genitsaris et al. 2023), thereby not significantly affecting primary productivity in the long term. Additionally, the diversity of natural metazooplankton communities, including varying tolerance among copepod interacting species, as presented in our findings, indicates higher stability and resilience to disturbances, suggesting not significant cascading effect through the food web. It is underlined that bacterioplankton communities, comprising the highest abundance of pelagic biota and the first responders to the effluent, thus affecting the exposure levels to the rest of the marine organisms from phytoplankton to multicellular biota, and reducing the potential adverse effects on them (Genitsaris et al. 2024).

In practical terms, the scrubber discharge when emitted 50 m in the wake of the vessels, undergoes a dilution of 2000-fold when vessels operate in the open sea, and a 1750-fold dilution during slower-speed operations at ports (Teuchies et al. 2020). However, for in-port scrubber operation where the vessel is stationary, much lower dilutions may be present in the vicinity of the vessel, depending on local conditions. This dilution is up to 20 times higher than what was applied in the LS treatment of the present community lab-experiment study.

Conclusions

Overall, no adverse effects of scrubber effluent inputs in the responses of the metazooplankton community of the Thessaloniki Bay (Eastern Mediterranean Sea), in terms of species composition, abundance, impact on early life-stages, and community interconnection were detected at low scrubber effluent content (LS; 1% v/v). Slightly significant impacts on positively growing meroplankton larvae might be considered, indicating further investigation needed for this group. The adverse effects of scrubber effluent inputs in the responses of the metazooplankton community were only detected at high scrubber effluent content (HS; 10% v/v).

It is important that, the real level of dilution of the scrubber effluents in the surrounding surface sea water where they are discharged, must be also taken into strong consideration when addressing the actual impact of scrubber discharge in marine environments. This dilution is up to 20 times higher than what was applied in the LS treatment of the present community lab-experiment study, and at this dilution level, no adverse effect on the natural plankton copepod populations was observed. In conclusion, this is the first metazooplankton community ecotoxicology study that contributes valuable information to the field of ecotoxicology and highlights the complexity of ecological responses to stressors under potential buffering effects of natural microbiota. There is growing concern for the implications of open-loop scrubber discharge into the sea, particularly for areas with high marine traffic (Hassellöv 2023). Knowing the natural remediation capacity of microbial communities which constitute the basis of food-webs, and the ecosystem engineers of biochemical cycles may enable assessment of contaminant availability at higher trophic levels of the ecosystem food-webs (Artigas et al. 2012; Genitsaris et al. 2023). The key role of plankton bacterial biodegradation should be considered in scrubber effluent ecotoxicological experiments; otherwise, the results cannot be extrapolated to assess impacts of scrubber effluent's discharge to the real, bacterial dominated, marine environments. The absence of natural marine bacteria in the growth media or matrix of single-species ecotoxicological experiments, which typically are sterile or synthetic seawater, could be a key point of ecological relevance in the experimental set-up for studying species sensitivity and risk assessment (Genitsaris et al. 2024). Considering the above, further investigation is needed before any policy making and management of scrubbers use.

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Data availability The authors confirm that the data supporting the findings of this study are available within the article and its supplementary material.

Declarations

Conflict of interest The authors declare that they have no known competing financial interests or personal relationships and no conflict of interest that could have appeared to influence the work reported in this paper.

Ethics approval None required.

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