

RESEARCH ARTICLE

Combined effects of microplastics and nitrogen on bivalve-mediated biogeochemical cycling

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Abstract

Estuary health is declining globally due to an increase in anthropogenic stressors including microplastics and nutrient loading. These stressors are often studied individually; however, it is vital to understand their combined effects on coastal ecosystems to inform ecosystem-based management. This is the first study to evaluate the multi-stressor responses of microplastics (polyethylene microbeads) and nitrogen loading in intertidal soft sediment communities. In this laboratory experiment, the individual and combined effects of microplastics and nitrogen were assessed on biogeochemical processes mediated by the bivalve, *Macomona liliana*. This deep-dwelling bivalve feeds on microphytobenthos at the sediment surface and augments porewater nutrients through its behavior. Ammonium porewater concentrations increased ~ 260% with the addition of nitrogen; however, this porewater increase did not carry over to the multiple stressor treatment. Further, sediment-water nitrogen effluxes tended to be higher with the addition of single stressors under light conditions, with a significant increase in the multiple stressor treatments. These findings suggest that nitrogen processing responses emerge under multiple stressor conditions. Furthermore, sediment profile image analysis revealed a significant negative effect of multiple stressors on sediment redox conditions. Lastly, bivalves exposed to multiple stressors had approximately 1% more docosahexaenoic acid (DHA, 22:6n-3) as a proportion of their total fatty acids in their tissue compared to those under control conditions, reflecting the low levels of DHA found in the microphytobenthos under multiple stressor conditions. The study highlights the complex interactions between subtle shifts in food quality and infaunal behavior that affect nutrient cycling at the sediment–water interface in coastal ecosystems.

Estuaries are highly dynamic and productive habitats that provide valuable ecosystem services, notably nutrient cycling and food production (Costanza et al. 1997; Thrush et al. 2013). However, increased coastal development has caused a marked decline in the overall health and ecosystem functioning of estuarine systems worldwide—including loss of biodiversity, excess sedimentation, and pollution (e.g., heavy metals and microplastics) and an increase in nutrient loading beyond levels that can be processed (Halpern et al. 2019; Lotze

et al. 2006). While several studies have evaluated the effects of individual stressors, there is a paucity of research into the cumulative effects of microplastics with other stressors on coastal and estuarine ecosystems (Crain et al. 2008; Stockbridge et al. 2020). Microplastic pollution and nitrogen loading individually impact ecosystem health (Ladewig et al. 2023; Plew et al. 2018). However, there is limited research on how these two anthropogenic stressors interact (although see Serra et al. 2020) despite their frequent co-occurrence and potential to interact in nonlinear ways (Crain et al. 2008; Crotty et al. 2017). Interacting effects of excess nitrogen and mud content (Douglas et al. 2018; Ellis et al. 2017) and organic matter and microplastics (Cluzard et al. 2015) have demonstrated substantial effects on benthic infauna, which in turn affects overall ecosystem functioning. To date, the combined effects of excess nitrogen and microplastics have not been studied in soft sediment habitats,

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despite their increasing burden in many estuarine ecosystems worldwide.

Nitrogen loading from the intensification of land-use in catchments (Heggie and Savage 2009) often leads to excess nutrients (Plew et al. 2018) and increased risk of eutrophic symptoms. Coastal eutrophication can lead to increased nuisance macroalgae and phytoplankton blooms, depleted bottom-water oxygen, and alterations in benthic microalgal communities (Cloern 2001; Howarth et al. 2011). Microphytobenthos (MPB), a thin layer of photosynthetic organisms on the sediment surface, is often the main primary producer in shallow soft sediments and an important energy source for estuarine species (Thrush et al. 2013). Microphytobenthos play a key role in nitrogen cycling between the sediment and overlying water column (Hope et al. 2020c; Miller et al. 1996). However, due to increasing nutrient concentrations, the community composition, abundance, and interactions of MPB with other components of the ecosystem may shift (Hope et al. 2020b), resulting in changes to ecosystem functioning (Thrush et al. 2014). Excess nitrogen loads can change the relationship between MPB and heterotrophic bacteria, so bacteria preferentially assimilate excess inorganic nutrients rather than recycling MPB-derived nutrients. This increases labile organic matter in the sediment and causes a concurrent decrease in nitrogen efflux from the sediment into the overlying water (Oakes et al. 2020; Riekenberg et al. 2020). Further, increased nutrient inputs may result in reduced oxygen concentrations in bottom-waters and surficial sediment due to reduced MPB photosynthesis and increased bacterial respiration, thereby negatively impacting macrobenthos (Diaz and Rosenberg 1995). Estuaries have a substantial capacity to process nutrient loads (Costanza et al. 1997) in part due to the complex matrix of burrows and bio-irrigation driven by infaunal behavior (Birchenough et al. 2012; Crawshaw et al. 2019; Gilbertson et al. 2012). A corollary, therefore, is that shifts in infaunal behavior can have indirect effects on sediment oxygenation, microphytobenthic production, nutrient cycling, and ecosystem functioning.

The presence of microplastics, defined as plastic particles < 5 mm in diameter (Arthur et al. 2009), has been documented in every environment and trophic level (Ajith et al. 2020; Thompson et al. 2004) and pose a significant risk to the health and behavior of marine biota (Galloway et al. 2017; Lusher et al. 2017). The individual effects from microplastic pollution are dependent on polymer type, shape, size, and chemical additives (Foley et al. 2018), with effects varying across taxa and environment (Setälä et al. 2016). Microplastics can significantly alter biogeochemical cycling in shellfish beds (Cluzard et al. 2015; Green et al. 2017; Ladewig et al. 2023) due to decreases in fitness and activity of large bivalves, as documented for the tellinid clam, *Macomona liliana*, in New Zealand estuaries (Hope et al. 2020a; You et al. 2020). These infaunal bivalves reside at approximately 10 cm depth and are facultative deposit

feeders that use a large inhalant siphon to feed on detritus and MPB on the sediment surface (Cook et al. 2010). The movement and feeding of this species enhance nutrient regeneration and transport to the sediment surface (Woodin et al. 2016), which in turn stimulates MPB growth at a rate exceeding the removal rate by their feeding (Hope et al. 2020a). Accordingly, any changes in *M. liliana* behavior from exposure to microplastics may result in shifts in biogeochemical cycling. Additionally, increasing nitrogen loads (Oakes et al. 2020; Riekenberg et al. 2020) or microplastic burdens can shift the community structure of MPB (Hope et al. 2020a; You et al. 2020), the bivalve's food source, towards greater cyanobacteria dominance and lower nutritional value. Due to the surface properties of microplastics, chemical compounds, bacteria, and nutrients may be absorbed into these polymers (Galloway et al. 2017). Indeed, microplastics have been found to harbor microbial communities that are distinct from the surrounding environment (Zettler et al. 2013) both in species composition and their ability to process nutrients (Dai et al. 2024; Liu et al. 2025). As these microbes can alter nutrient cycling, this suggests that microplastics may interact with nitrogen loading in unexpected ways.

This mesocosm study investigates the combined effects of multiple stressors on biogeochemical cycling in soft sediment habitats. Specifically, the individual and co-occurring effects of microplastics and nitrogen loading on biogeochemical cycling mediated by the functionally important bivalve, *M. liliana*, were investigated using a controlled laboratory experiment. We hypothesized that (1) microplastic presence in the sediment will decrease the burrowing activity and feeding rate of *M. liliana* and consequently alter sediment oxygenation and reduce the porewater ammonium concentrations from bivalve excretion; (2) nitrogen loading will increase MPB biomass and result in lowered nitrogen efflux; and (3) nitrogen loading and microplastic burden together will have a larger combined effect compared to their individual effects; however, due to complex feedbacks, the direction and magnitude are difficult to predict.

Methods

Study design and experiment

A laboratory mesocosm experiment was conducted at the Portobello Marine Laboratory, Otago University, Dunedin, New Zealand. A total of 30 4-L square clear polypropylene tanks (H: 190 mm, W: 180 mm, D: 180 mm) were filled with 10 cm of sieved sediment. Each tank had an inflow pipe 1 cm above the sediment surface and an outflow pipe 7 cm above the sediment surface. Four experimental mesocosms ($n = 6$ replicates each) were established as follows: (1) control, (2) nitrogen addition, (3) microplastic addition, and (4) multiple stressor addition (nitrogen + microplastics), with each mesocosm containing $n = 3$ bivalves (equivalent

to 125 ind m⁻²), which represents naturally occurring densities in New Zealand estuaries (Supporting Information Fig. S1). To assess the effect of the bivalves on sedimentary biogeochemistry, an additional six tanks were added with sieved sediment and no bivalves to establish baseline conditions. The mesocosms are adapted from the methods of Hope et al. (2020a) with each tank receiving 1 cm of treated or control sediment on top of the sieved sediment (Supporting Information Fig. S2 for more details).

The sediment was collected at low tide from an intertidal sandflat in Pounaweia Estuary, Catlins, Otago, in April 2022 at a site with an abundant *M. liliana* community. Pounaweia is classified as a relatively healthy estuary based on eutrophication modeling (Plew et al. 2020) and a pilot study conducted in 2022 (Supporting Information Table S1). Sediment was sieved through a 500 μm mesh to exclude macrofauna and large shell hash and homogenized. Care was taken to ensure limited loss of fine particles during sieving. The sediment was divided between tanks to a consistent volume (11 cm depth) and allowed to settle (18 d) to establish natural biogeochemical gradients.

Red polyethylene microplastic beads (Cospheric 212–250 μm , 1.070 g cm⁻³) were used to dose the sediment treatment layer. Microplastics were incubated in ambient seawater ($\sim 13^\circ\text{C}$) for 21 d prior to use to develop a biofilm (Harrison et al. 2014) and to simulate time spent in the water column. A single batch of microplastic dosed sediment (3.5 g microplastic kg⁻¹ sediment) was prepared and divided between the appropriate tanks for a final dosage of 0.32 g microplastic kg⁻¹ sediment. This dosage was chosen to be comparable to similar mesocosm studies on bivalves, which used dosages of 0.01–0.5 g kg⁻¹ (Hope et al. 2020a), 0.2–2.0 g kg⁻¹ (Green et al. 2016), and 0.1–0.5 g kg⁻¹ (Urban-Malinga et al. 2021). While it was not possible to verify the distribution of microplastics in each of the mesocosms, the sediment was well mixed for 10 min until a visibly homogeneous distribution of particles was observed.

For the nutrient enrichment treatments, a commercial quick-release nitrogen-only fertilizer (Egmont Rapid Growth Fertilizer: Urea) was used. Sediment was enriched by evenly distributing fertilizer pellets 1 cm below the surface at a dose of 150 g N m⁻². This dose represents a nitrogen load of a moderately degraded estuary and has been used in similar estuarine research (Douglas et al. 2018; Thrush et al. 2014).

Tanks were randomly distributed under lighting (Phillips Master de Luxe 30 W/940) suspended 38 cm above the sediment surface, providing approximately 100 μmol photons m⁻² s⁻¹ as measured with an Apogee underwater quantum meter (MQ-510). The tanks were incubated for 18 d under a 12 h light : 12 h dark regime to promote MPB growth. After the initial incubation, three bivalves (37.62 mm \pm 0.33 mm; mean \pm SE), hand collected in April 2022 from the top 10 cm of sediment in Blueskin Bay, Dunedin, Otago (Supporting Information Table S1), were randomly allocated to each

treatment tank and left overnight to bury. After 24 h, unburied bivalves were removed and replaced with fresh individuals. The experiment was left to run for 25 d after the bivalve additions. Bivalves continued to feed throughout the experiment and were retrieved from 9 to 11 cm sediment depth at the end of the experiment (personal observation; Supporting Information Fig. S3, Video S1).

Post-exposure sampling

At the end of the 25-d experiment, biogeochemical cycling was evaluated using a benthic flux method adapted from previous studies (Douglas et al. 2018; Thrush et al. 2014). In brief, the flux method compares the rate of change in dissolved solute concentrations in the overlying water in each tank during periods of photosynthesis (light conditions) and no photosynthesis (dark conditions). An efflux refers to a net movement of solutes from the sediment to the overlying water column while an influx refers to a net drawdown into the sediment. Tanks were left to acclimate to the light for approximately 2.5 h before stopping water flow from the flow-through system and isolating each tank. Initial samples were taken prior to isolation by drawing 50 mL of water from the inflow pipe and using a calibrated Ecosense ODO200 meter to measure oxygen concentrations. Next, 10 mL of the sample was filtered through a 45 μm polypropylene filter and stored at -20°C until nutrient analysis. Tanks were left to incubate for approximately 2 h before taking final samples. Water was not stirred during this time. One full syringe was taken and discarded to flush out the sampling tube before repeating the steps used for the initial sampling. This process was repeated under dark conditions (2.5 h after turning off the lights) later the same day. Because all initial water was from the inflow pipe before tanks were isolated for the incubations, an average of all tanks was used for initial conditions, but individual tanks were sampled for the concentrations at the end of the incubation. From this data, gross primary production was calculated by subtracting the oxygen flux under dark conditions from the oxygen flux under light conditions.

Sediment sampling was conducted after all incubations were complete. Photographs of the sediment surface and one side-profile were taken of each tank for later analysis of total MPB cover and apparent redox potential discontinuity depth respectively. Next, a surface scrape of the top 1 mm of the sediment was taken from three random locations within the tank and pooled to assess the fatty acid profiles of the MPB. Finally, three syringe cores (2.6 cm diameter) were taken from the top 0–2 cm sediment in each tank and pooled and homogenized for analysis of chlorophyll *a* (Chl *a*) (stored at -20°C until analysis) and porosity. Similarly, three syringe cores (1.5 cm diameter) were sampled at 0–2 cm depth and pooled for total organic carbon (TOC) and porewater ammonium concentration.

Bivalves were retrieved and digestive tissue was dissected for fatty acid analysis. Although microplastic particles remained within the tanks for the duration of the trial and

were regularly consumed by the bivalves (personal observation; Video S1), ingestion of beads by the bivalves was not quantified as it would provide only a snapshot of behavior.

Laboratory analysis

Porewater nutrient samples were processed within 24 h of sampling by adding 4 mL of MilliQ water and centrifuging the sediment aliquots at 3300 rpm for 10 min. The resulting supernatant was filtered through disposable 45 μm polypropylene filters and stored at -20°C until analysis. Nutrient flux samples and porewater samples were analyzed for dissolved ammonia on an autoanalyzer using standard methods for seawater nutrient analysis (Lachat QuikChem[®] 8500 Series 2 flow injection analysis auto-analyzer).

The porosity of the sediment was calculated using the formula:

$$\text{Porosity} = (\text{wet weight} - \text{dry weight}) / (1 \text{ g cm}^{-3} \times \text{wet sediment volume}).$$

Sediment samples were analyzed for TOC (%) by drying at 60°C until constant weight and combusting at 550°C for 3 h (Parker 1983) and using loss-on-ignition as a proxy for TOC.

Sediment for Chl *a* analysis was freeze-dried and homogenized. An aliquot of approximately 1.5 g was ground with a mortar and pestle for 30 s before adding 6 mL of 90% acetone and sonicating the sample for 30 s in an ice bath. Samples were left to soak for approximately 20 h in dark, refrigerated conditions. After 12 h, and prior to measurement, the samples were agitated before being centrifuged for 5 min at 1500 rpm. Absorbance was measured at 665 nm and 750 nm wavelengths on a spectrophotometer (Cary UV mini, 1240). An acidification step was performed to separate the phaeophytin from Chl *a* pigments by adding 0.3 mL 0.1 N HCl and waiting 90 s before measuring fluorescence again (Arar and Collins 1997). Chlorophyll *a* concentrations were calculated using the methods outlined by Lorenzen (1967).

Fatty acids from the digestive tissue of the bivalves and from surficial sediment scrapes were extracted, transesterified, and measured as fatty acid methyl esters on a gas chromatograph (full methods are described in the Supporting Information Fatty Acid Methods). All fatty acids were standardized to the sample dry weight. Individual fatty acids identified were expressed as the proportion of the total fatty acids (% total fatty acids [TFAs]). In particular, the omega-3 fatty acids (eicosapentaenoic acid [EPA], 20:5n-3) and (docosahexaenoic acid [DHA], 20:6n-3) were identified and reported as they are essential fatty acids for bivalve health and reproduction (Fearman et al. 2009; Tan et al. 2023). Since bivalves cannot synthesize these fatty acids themselves, they must obtain them through their diet. We did not measure total lipid content. Bivalve wet weights were standardized (i.e., measured after blot drying for a fixed period, with size accounted for by

shell length) and used as an approximate condition index post-experiment. A *proper* condition index could not be calculated, as an aliquot of the bivalve tissue was saved for another experiment.

Photographic analysis of sediment profiles

The percent surface cover of visible MPB and the extent of black sediment for each tank were assessed with ImageJ software (Version 1.53a) using the lasso tool. To quantify the percentage cover of MPB on the sediment surface, the percentage of visible algae cover in each photo was calculated (Fig. 1). All images were cropped to exclude any sides or corners of the tanks, as well as any disturbance to the sediment caused by the inflow pipe, to ensure accurate measurements. A subset of photos was analyzed a second time to ensure consistent measurements from beginning to end of analysis. The percentage of black sediment was determined by measuring the total area of dark sediment in each side-view photograph. Black sediment is a latent response to sulfate reduction and may therefore indicate hypoxic conditions, making visual assessments of sediment profiles effective as a qualitative proxy of redox state transitions. (Abdelrhman and Cicchetti 2012; Madell et al. 2024; O'Meara et al. 2018; Simone and Grant 2017).

Statistical analysis

The effect of bivalves on the system was assessed using *t*-tests between the baseline tanks and the bivalve addition tanks for the following parameters: ammonium porewater, ammonium flux, MPB surface cover, and sediment TFA. Normality was assessed with Shapiro–Wilk tests per treatment group, and homogeneity of variances was tested using Levene's test. When normality and equal variances were met, a *t*-test was used; if variances were unequal, Welch's *t*-test was applied; and if normality was violated, a Wilcoxon rank-sum test was performed.

Ammonium flux and oxygen flux were analyzed using linear mixed-effect models with light-condition and stressor-treatment as fixed effects and tank as a random effect. Ammonium porewater concentrations, percentage of visible black sediment, and relative fatty-acid proportions (EPA and DHA) in the sediment were assessed using linear models. The relative fatty-acid proportions in the bivalve tissue were assessed using a linear mixed model with tank as a random factor. The percentage of MPB surface cover was assessed using a linear mixed model, incorporating stressor-treatment and date as fixed effects, with tank as a random effect to account for repeated measures. The ratio of Chl *a* to pheophytin in surface sediment was used as an indicator of fresh benthic production and assessed using a linear model. Assumptions of the models were assessed as follows: normality of residuals using QQ plots and the Shapiro–Wilk test, homoscedasticity using the Breusch-Pagan test, autocorrelation using the Durbin-Watson test, and multicollinearity using variance inflation factors. Data were log-transformed if assumptions were not met. All

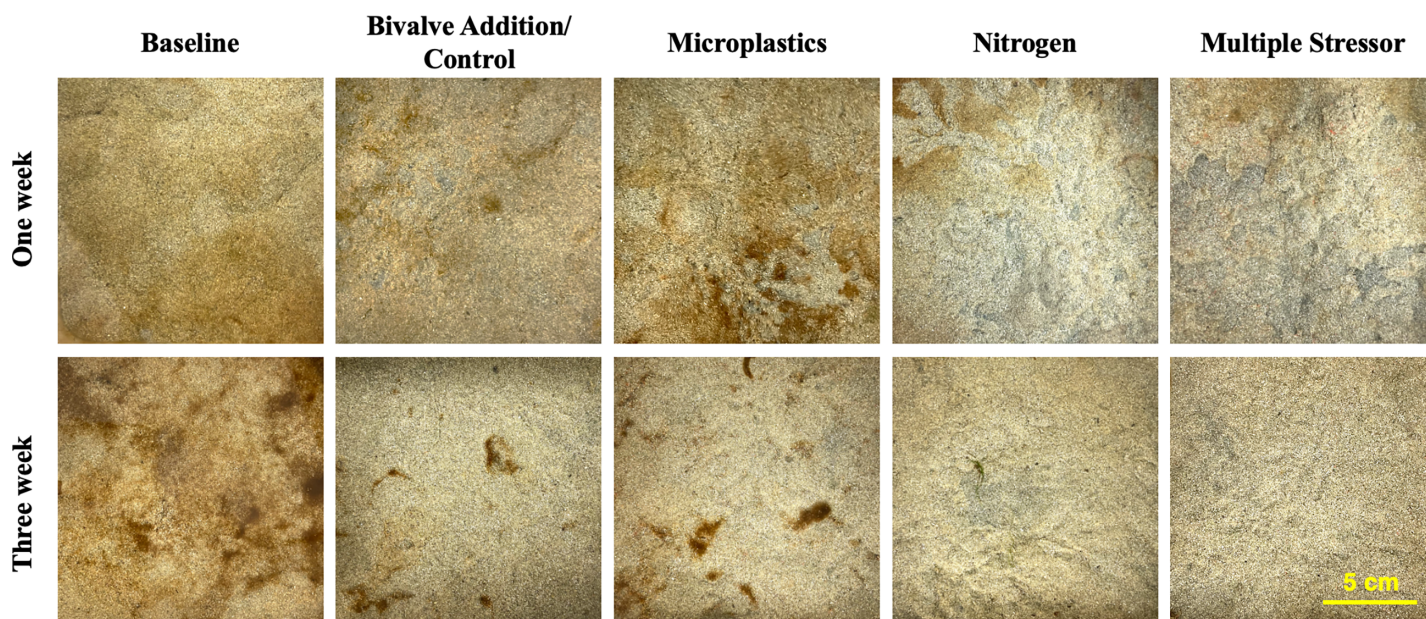


Fig. 1. Representative surface photograph of each mesocosm group 1 week and 3 weeks after experiment start. All groups contained three bivalves except for the Baseline group. Photographs cropped to exclude tank edges and shadows.

statistical analyses were conducted using R (version 4.3.3) with model outcomes summarized in Table 1.

Due to 83% bivalve mortality and distinct bacterial colony formation on the sediment surface in two out of the six nitrogen addition tanks (28% total mortality for the nitrogen treatment), these replicates were removed from the data analysis. No further bivalve mortality was observed.

Results

Bivalve additions

The addition of bivalves significantly reduced porewater ammonium concentrations by over 50% compared to baseline sediment ($t = 291$, $df = 9$, $p = 0.017$; Fig. 2a); however, the addition of bivalves had no discernable effect on ammonium flux between the sediment–water interface in either light or dark conditions (Fig. 2c). Furthermore, TFA content of MPB in the sediment was marginally lower with the presence of bivalves ($t = -2.34$, $df = 5.6$, $p = 0.06$; Fig. 2b), consistent with the significantly lower MPB surface cover observed in the photographic analysis of the sediment containing bivalves compared to the baseline sediment at both the 1-week ($t = 2.90$, $df = 10$, $p = 0.02$) and 3-week time points ($t = 8.25$, $df = 10$, $p < 0.01$; Fig. 2d).

Porewater nutrients

The addition of nitrogen into the surficial sediment resulted in elevated ammonium porewater concentrations (Fig. 3a) 3 weeks after nutrient addition ($1449.36 \mu\text{g/L} \pm 297.15$; mean \pm SE), with a concentration approximately 260% higher (adjusted $R^2 = 0.4103$, $F_{16} = 5.41$, $p = 0.009$)

than the control. This concentration was within the expected range for the enrichment dosage and within the range reported globally in estuaries with anthropogenically modified catchments (Douglas et al. 2016). Conversely, the addition of microplastics showed little effect on ammonium porewater compared to control treatments. The ammonium porewater concentration in the multiple stressor treatment also showed no significant difference compared to the control.

Benthic-pelagic solute flux

The mean sediment–water efflux of ammonium was higher under dark conditions compared to light conditions (log estimate = 1.32, $F_{18} = 3.11$, $p = 0.006$; Fig. 3b). Under light conditions, there were no significant differences among the control and the single stressor treatments; however, ammonium efflux in the multiple stressor treatment was nearly two-fold greater than the efflux measured in the control (estimate = 2.02, $t_{25} = 2.77$, $p = 0.01$). Variability among replicates was higher under dark conditions, especially in the two treatments with added nitrogen. There was a net efflux of oxygen from the sediment under light conditions and a net influx of oxygen under dark conditions; however, no statistical significance was detected among treatments (Supporting Information Fig. S5).

Sediment profiles

The presence of black sediment was evident in several tanks throughout the sediment column. The percentage area in the downcore profiles that had black sediment is presented as a qualitative indicator of sediment oxygen conditions. In controls, sediment was well mixed and black sediment was not

Table 1. Summary statistics from linear mixed-effects models (LMMs) and linear models (LMs) for all response variables measured in the experiment. LMMs include tank as a random intercept, while LMs were fitted without random effects. Fixed-effect estimates \pm standard errors (SE) are reported, with associated p values; $p < 0.05$ are shown in bold. Estimates for log-transformed variables are presented on the transformed scale.

Response	Effect	Estimate (SE)	p
Log ammonium flux	Intercept (control—light)	1.34 (0.52)	0.015
	Dark	1.32 (0.43)	0.006
	Microplastics	1.16 (0.73)	0.125
	Nitrogen	1.57 (0.81)	0.065
	Multiple stressor	2.02 (0.73)	0.010
	Dark + microplastics	-0.47 (0.60)	0.442
	Dark + nitrogen	-0.88 (0.67)	0.209
	Dark + multiple stressors	-1.06 (0.60)	0.096
Oxygen flux	Intercept (control—light)	2.26 (0.11)	< 0.001
	Dark	-121 (0.16)	< 0.001
	Microplastics	-0.13 (0.16)	0.422
	Nitrogen	-0.12 (0.18)	0.507
	Multiple stressor	-0.22 (0.16)	0.188
	Dark + microplastics	0.19 (0.23)	0.415
	Dark + nitrogen	-0.21 (0.25)	0.407
	Dark + multiple stressors	0.19 (0.23)	0.417
MPB cover	Intercept (control—week 1)	22.46 (5.77)	< 0.001
	Week 3	-16.92 (6.14)	0.012
	Microplastics	-1.86 (8.12)	0.821
	Nitrogen	2.57 (8.16)	0.755
	Multiple stressor	-1.16 (8.16)	0.888
	Week 3 + microplastics	2.07 (8.68)	0.814
	Week 3 + nitrogen	2.60 (8.68)	0.768
	Week 3 + multiple stressor	7.77 (8.68)	0.381
Ammonium porewater	Intercept (control)	564.30 (147.10)	0.001
	Microplastics	119.70 (208.00)	0.573
	Nitrogen	885.00 (232.60)	0.002
	Multiple stressor	189.90 (232.60)	0.433
Log black sediment	Intercept (control)	-11.55 (2.10)	< 0.001
	Microplastics	7.79 (2.98)	0.017
	Nitrogen	13.01 (3.33)	0.001
	Multiple stressor	13.48 (2.98)	< 0.001
GPP	Intercept (control)	6.84 (0.96)	< 0.001
	Microplastics	-1.29 (1.36)	0.352
	Nitrogen	-0.48 (1.51)	0.753
	Multiple stressor	-1.84 (1.36)	0.192
Log Pheo : Chl a	Intercept (control)	-0.14 (0.18)	0.431
	Microplastics	-0.06 (0.25)	0.820
	Nitrogen	0.46 (0.27)	0.105
	Multiple stressor	0.46 (0.25)	0.086
Chl a	Intercept (control)	1.25 (0.29)	< 0.001
	Microplastics	0.30 (0.42)	0.483
	Nitrogen	0.44 (0.44)	0.155
	Multiple stressor	-0.17 (0.42)	0.687
Bivalve TFA	Intercept (control)	32.66 (4.79)	< 0.001
	Microplastics	1.33 (6.77)	0.847

(Continues)

Table 1. Continued

Response	Effect	Estimate (SE)	<i>p</i>
Bivalve DHA %	Nitrogen	−3.85 (7.84)	0.629
	Multiple stressor	7.31 (6.83)	0.300
	Intercept (control)	3.39 (0.21)	< 0.001
	Microplastics	−0.02 (0.29)	0.958
	Nitrogen	0.55 (0.34)	0.117
Bivalve EPA %	Multiple stressor	1.04 (0.29)	0.002
	Intercept (control)	11.53 (0.63)	< 0.001
	Microplastics	0.24 (0.89)	0.792
	Nitrogen	0.51 (1.04)	0.633
	Multiple stressor	0.61 (0.90)	0.509
Sediment TFA	Intercept (control)	0.09 (0.01)	< 0.001
	Microplastics	0.02 (0.02)	0.330
	Nitrogen	0.00 (0.02)	0.897
	Multiple stressor	−0.01 (0.02)	0.564
	Intercept (control)	0.08 (0.06)	0.200
Sediment EPA %	Microplastics	−0.01 (0.08)	0.948
	Nitrogen	−0.06 (0.09)	0.534
	Multiple stressor	0.10 (0.01)	0.282

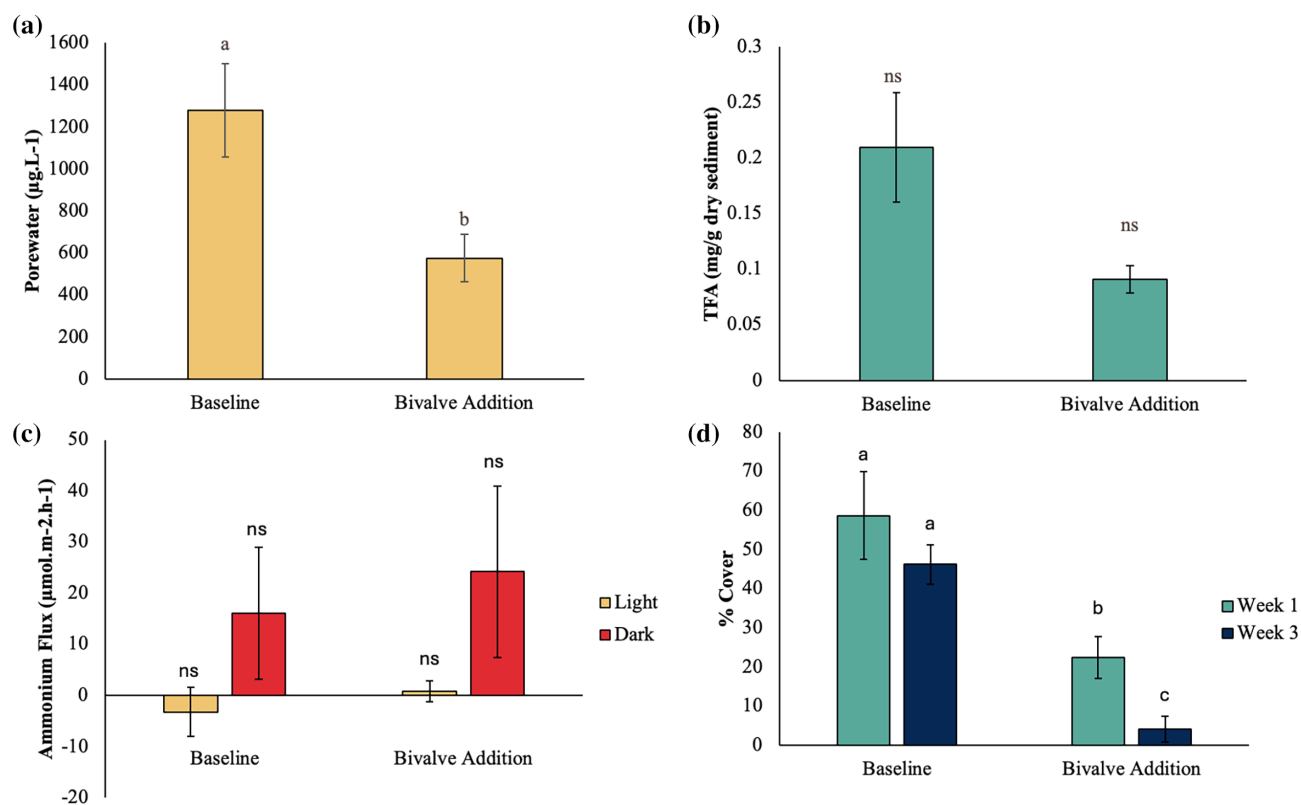


Fig. 2. Mean (\pm SE) response of baseline mesocosm conditions to the addition of bivalves for: **(a)** ammonium concentrations in sediment in porewater, **(b)** total fatty acids in surficial sediment, **(c)** ammonium flux under light and dark conditions, and **(d)** percent cover of apparent MPB. Letters above bars denote significant differences based on *t*-tests with significant differences at $p < 0.05$. ns = not significant.

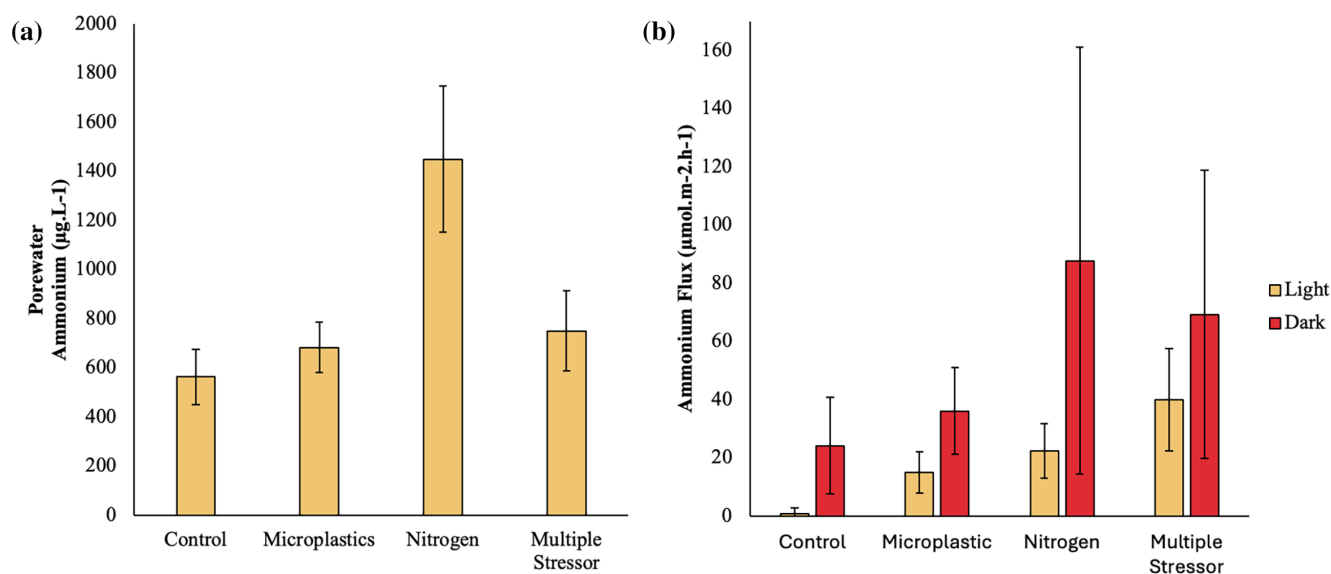


Fig. 3. Mean (\pm SE) ammonium response 3 weeks after stressor additions: (a) Porewater ammonium concentrations in mesocosms, and (b) ammonium flux between the sediment and overlying water column in light (yellow) and dark (red) conditions. All tanks contained bivalves ($n = 3$).

evident, while in stressor treatments the percentage of black sediment was high in some tanks (Fig. 4a). The multiple-stressor treatment on average exhibited twice as much black sediment as the sum of the individual stressors and, along with the individual stressor treatments, was found to be

significantly higher than the control (adjusted $R^2 = 0.5116$, $F_{18} = 8.33$, $p < 0.01$).

Sediment porosity and TOC (based on loss-on-ignition) were not significantly different across treatments. However, the stressors had individual and different effects on the

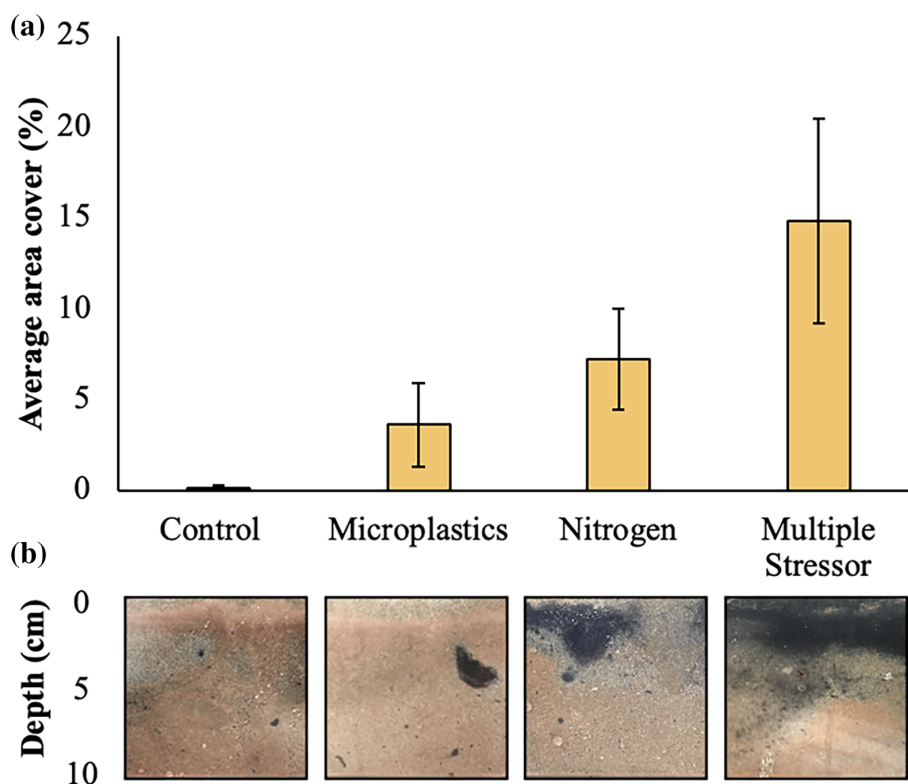


Fig. 4. Mean (\pm SE) percent of black sediment in down-core profiles for control and treatment mesocosms ($n = 6$) containing bivalves ($n = 3$) after a 3-week experiment (a), and representative photographs of down-core sediment conditions (b; digitally enhanced for clarity).

Table 2. Mean (\pm SE) response to mesocosm parameters 3 weeks after stressor additions. Values denote the mean with the coefficient of variance in brackets ($n = 6$).

		Control	Microplastic	Nitrogen	Multiple stressor
Sediment properties	Total organic carbon (%)	1.72 (4.70)	1.75 (7.16)	1.66 (11.13)	1.77 (12.74)
	Porosity	1.77 (8.26)	1.75 (15.60)	1.94 (5.56)	1.80 (14.24)
	Black sediment (%)	0.14 (244.95)	3.62 (156.70)	7.99 (90.26)	14.82 (93.44)
Ammonium	Porewater ($\mu\text{g L}^{-1}$)	564.34 (49.14)	684.01 (36.69)	1449.37 (41.00)	751.28 (43.37)
	Light flux ($\mu\text{mol m}^{-2} \text{h}^{-1}$)	0.81 (630.97)	15.06 (115.80)	22.30 (84.49)	40.00 (107.61)
	Dark flux ($\mu\text{mol m}^{-2} \text{h}^{-1}$)	24.21 (169.01)	36.07 (100.86)	87.72 (167.32)	69.29 (175.03)
MPB	GPP ($\text{O}_2 \text{ mM m}^{-2} \text{h}^{-1}$)	3.72 (35.10)	3.02 (40.75)	2.74 (38.63)	2.72 (53.73)
	Week 1 MPB (% cover)	22.46 (59.13)	20.59 (47.04)	21.48 (46.76)	21.30 (43.41)
	Week 3 MPB (% cover)	5.54 (146.86)	5.75 (64.84)	1.76 (128.29)	12.15 (237.45)
	Chl <i>a</i> ($\mu\text{g/g}$ dry sediment)	1.04 (66.85)	2.08 (68.93)	1.46 (85.04)	2.38 (136.76)

variability of sediment properties (see coefficients of variance in Table 2). Specifically, adding nitrogen nearly doubled the variability in TOC levels in both the nitrogen-only and multiple stressor treatments. In contrast, microplastics nearly doubled the variation in porosity in both treatment groups with microplastics.

Microphytobenthic cover and nutritional value

The ratio between MPB Chl *a* and pheophytin (the degraded form of Chl *a*) was used as a proxy for fresh benthic primary production. This ratio was nonsignificant among treatments (adjusted $R^2 = 0.1914$, $F_{3,15} = 2.42$, $p = 0.11$); however, the two treatments containing added nitrogen had notably higher average ratios than all other treatments (Fig. 5c). Furthermore, the surface cover of MPB decreased in all treatments over time (estimate = -13.81 , SE = 2.92 , $p < 0.01$; Fig. 5b).

No significant differences in TFA concentrations in sediment MPB and bivalve tissue were measured across any of the stressor treatments (Fig. 6a). Additionally, post-experiment standardized bivalve wet weights showed no significant differences among treatments. The nutritional quality of MPB was assessed by measuring the proportion of essential fatty acids including EPA and DHA in the surface sediment MPB. DHA was only detected in sediment MPB that had nitrogen additions (Fig. 6b); however, the proportion of EPA remained consistent across all treatments and the control. The proportion of DHA in the bivalve tissue reflected the available DHA in the sediment and was significantly higher in the multiple stressor treatment group (estimate = 1.04 , SE = 0.29 , $p = 0.002$; Fig. 6e). The ratio between essential fatty acids DHA and EPA was also significantly increased in the multiple stressor treatment compared to the microplastic treatment (Supporting Information Fig. S4C).

Discussion

This study demonstrates that anthropogenic stressors that commonly co-occur within estuaries (i.e., excess nitrogen and

microplastic pollution) influence ecosystem functions performed by a key bivalve, *M. liliiana*, under both single stressor and multiple stressor exposures. *Macomona liliiana* is an ecosystem engineer and is integral to healthy ecosystem functioning in soft-sediment habitats, influencing nutrient cycling and primary production through its behaviors (Woodin et al. 2016). Specifically, a significantly lower porewater ammonium concentration was measured when bivalves were present compared to baseline sediment, indicating a bivalve-driven removal of ammonium from the sediment (Fig. 2a). Porewater pressurization from *M. liliiana* during its burrowing and feeding facilitates solute transport from the sediment into the overlying water column (Crawshaw et al. 2019; Volkenborn et al. 2012; Woodin et al. 2016), which in turn influences bacterial activity and organic matter remineralization (Bertics and Ziebis 2009; Gilbertson et al. 2012; Ming-Yi et al. 1993) and stimulates benthic primary production. Accordingly, these bivalves both directly and indirectly control ecosystem functions, with sub-lethal effects of stressors likely having impacts on overall ecosystem functioning of estuarine soft sediments.

The amount of black sediment present in the tanks was affected by both individual and multiple stressor exposures. Increased amounts of visibly black sediment, an emergent symptom of iron and sulfate-reducing microbes, are a qualitative indicator of a breakdown of oxygenation pathways in the sediment and a shift to more anaerobic processes (Fenchel and Riedl 1970; Freitag et al. 2003). Microplastics impact sediment oxygen penetration and microbial habitats by altering sediment permeability (Carson et al. 2011; Xu et al. 2020) and porosity (Table 2), whereas nitrogen loading often decreases bottom-water oxygen through increased microbial decomposition of excess organic matter (Cloern 2001; Howarth et al. 2011). In this study, both microplastics and nitrogen independently increased between-tank variation in sediment properties—microplastics affecting the variation in sediment porosity and nitrogen influencing the variation in TOC (Table 1). Furthermore, these stressors can decrease the

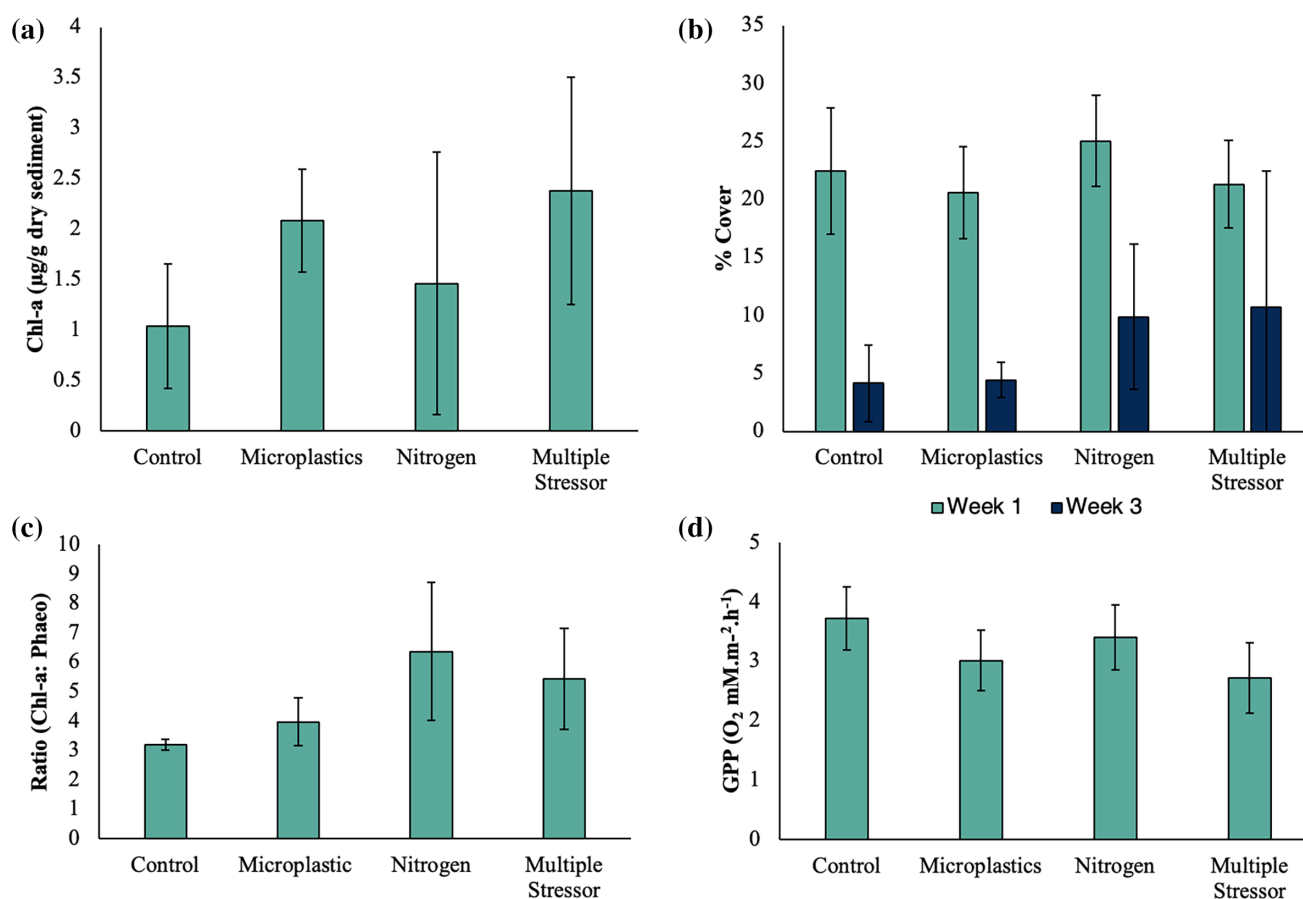


Fig. 5. Mean (\pm SE) response of microphytobenthos to stressor additions in mesocosm tanks ($n = 6$), shown for: **(a)** Chlorophyll *a* content of the surficial 2 cm of sediment, **(b)** percentage apparent microphytobenthic surface cover, **(c)** ratio of chlorophyll *a* to pheophytin in the surficial 2 cm of sediment, and **(d)** gross primary production.

burrowing activity of macrofauna, which further controls sediment's oxygen concentrations (Diaz and Rosenberg 1995; Hope et al. 2020a). The matrix of macrofaunal burrows in estuaries is important for increasing the oxic-anoxic interface of sediment, allowing for nitrifying and denitrifying bacteria to coexist and promoting the removal of large amounts of nitrogen through denitrification (Seitzinger 1988). Importantly, the elevated amount of black sediment observed under the multiple stressor treatment suggests the combination of these two stressors may exacerbate individual stressor effects on sediment condition. Because sediment conditions are vital for infauna and ecosystem processes, understanding the combined effects of multiple stressors is key to establishing environmental thresholds for stressor loads. This study shows how single-stressor models may be inadequate for predicting critical thresholds when stressors co-occur.

The multiple stressor exposure likely caused a shift in nitrogen cycling and diffusion processes as there was a higher ammonium efflux in the combined stressor treatment (Fig. 3b) while porewater ammonium concentrations were not significantly different from the nitrogen only treatment

(Fig. 3a). Several concurrent mechanisms may contribute to these patterns. Microplastics may alter sediment permeability and diffusion pathways (Aminzadeh et al. 2025)—with the observed increase in the variability of sediment porosity being consistent with such modification—and may also affect *M. liliانا* feeding and excretion (Hope et al. 2020a), together reducing ammonium porewater concentrations and facilitating an increase of ammonium efflux. Additionally, increased variability in MPB surface cover under multiple stressor scenarios suggests variable uptake of ammoniacal nitrogen, while the increased amount of black sediment is indicative of more reduced redox conditions, potentially suppressing nitrification while enhancing anaerobic nitrogen processing pathways (Fenchel and Riedl 1970; Freitag et al. 2003). Together, these interconnected processes may contribute to the observed response in ammonium porewater and ammonium efflux in the multiple stressor treatment. Previous research has also shown that microplastic presence (Cluzard et al. 2015; Green et al. 2016) and excess nitrogen (Oakes et al. 2020; Riekenberg et al. 2020; Zhang et al. 2023) individually affect nitrogen cycling within soft-sediment habitats. Microplastic presence

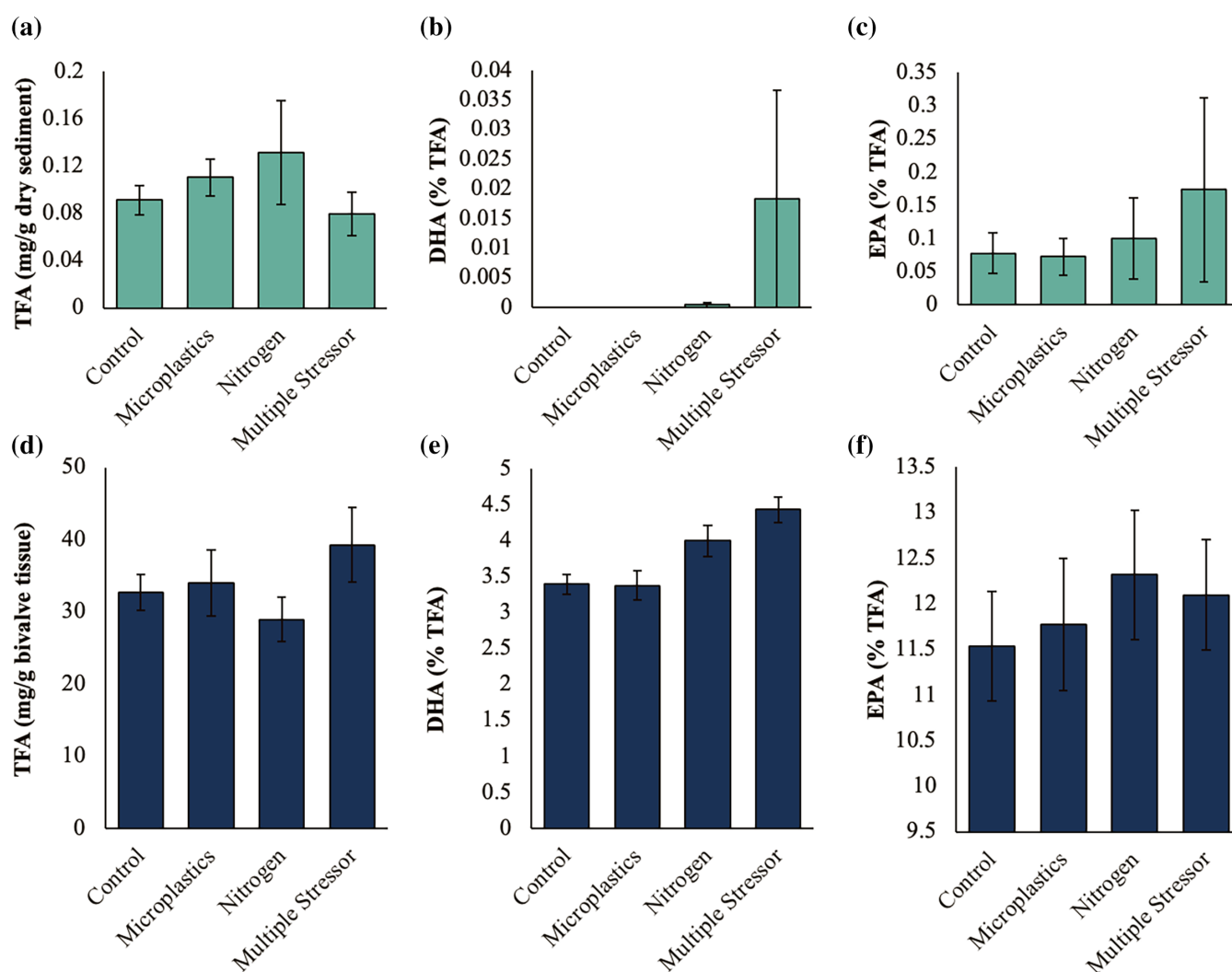


Fig. 6. Mean (\pm SE) post-exposure fatty acid measurements shown for: (a) total fatty acids (TFA) in the MPB sediment profile, (b) proportion of essential fatty acid DHA in sediment MPB, (c) proportion of essential fatty acid EPA in sediment MPB, (d) total fatty acids in the bivalve tissue, (e) proportion of the essential fatty acid DHA in bivalve tissue, and (f) proportion of the essential fatty acid EPA in bivalve tissue.

can influence feeding and therefore excretion rates in several benthic species including marine worms (Green et al. 2016; Wright et al. 2013) and bivalves (Green et al. 2017; Woods et al. 2018), leading to further changes in sedimentary nutrient pools and solute transport (Gilbertson et al. 2012). Microplastics may also interrupt nitrogen cycling due to the physical and chemical properties of plastics that affect bacterial growth and community structure (Li et al. 2020a; Zettler et al. 2013) or alter microenvironments where microbial processes occur (Li et al. 2020b). Excess nutrients may also affect microbial processes with feedback loops for MPB and infauna. Heterotrophic bacteria, for example, preferentially use inorganic nutrients when available, rather than recycle organic nutrients from decomposing MPB, allowing for organic nitrogen to accumulate in the sediment (Riekenberg et al. 2020). Indirectly, excess nitrogen can reduce invertebrate feeding

and movement by damaging their tissues, resulting in decreased functioning (Zhang et al. 2023). Ultimately, both nitrogen loading and microplastic presence can individually modify infaunal behavior and bacterial processes resulting in changes to ammoniacal processing. This study highlights how the combination of both stressors adds increased variability to the response of ammonium flux at the sediment–water interface and MPB Chl *a* content and patchiness, supporting research that shows increased variability as a symptom of stress in marine ecosystems (Warwick and Clarke 1993).

The presence of deep-dwelling surface-deposit feeding bivalves had a significant effect on MPB concentrations indicating feeding was an important driver of benthic primary production standing stock. By contrast, there was no significant difference in the surface cover of MPB among treatments, either due to consistent bivalve feeding across all treatments,

reduced feeding in some treatments paired with reduced algal growth, or increased feeding in some treatments paired with increased algal growth. Irrespective of the mechanism, TFA content and standardized wet weights of *M. liliiana* did not differ significantly among treatments or the control, indicating the bivalves maintained their condition and were meeting their nutritional requirements despite the presence of stressors. However, in multiple stressor groups, the proportion of the essential fatty acid DHA within bivalve tissue increased slightly while the proportion of EPA remained the same. This reflects improved nutritional quality of their food source, as indicated by the presence of DHA in the nitrogen groups and multiple stressor groups (Fig. 6). Because DHA and EPA tend to partition across algal taxa (e.g., DHA in dinoflagellates, EPA in diatoms) (Peltomaa et al. 2019) and nitrogen enrichment can favor dinoflagellates (Kang and Kang 2022), this pattern may indicate a nitrogen-driven MPB community shift. Previous work has found *M. liliiana* to persist in estuaries with low levels of anthropogenic contamination, suggesting this species is relatively tolerant to low levels of eutrophication impacts (Ellis et al. 2017) and maintains key functioning, such as bio-turbation and sediment mixing (You et al. 2023) at low single stressor levels. However, as stressor levels increase and multiple stressors occur, their ability to maintain ecosystem functioning is likely to be affected. Mesocosm experiments with *M. liliiana* have observed decreases in health and functioning including energy reserve and reburial rates in bivalves exposed to microplastics (Hope et al. 2020a; You et al. 2020). This could be attributed to bivalves experiencing a false sense of satiation and consuming less when microplastics are present, or microplastics triggering a reduction in the growth or nutritional quality of the MPB (Green et al. 2017; Hope et al. 2020a). In contrast, low doses of nitrogen can be beneficial in facilitating the growth and nutritional quality of MPB (Hope et al. 2020b); whereas high doses of nitrogen can be highly toxic to invertebrates, resulting in reduced feeding and death (Zhang et al. 2023), and can trigger eutrophic symptoms (Howarth et al. 2011) that diminish oxygen conditions. In the present study, the addition of nitrogen resulted in some bivalve mortality, an increase in nutritional quality of MPB (which is reflected in the bivalves' tissues), and an overall increase in MPB variability. Further research may provide a more nuanced understanding of the relationship between *M. liliiana* feeding and MPB standing stock when exposed to these two stressors. Understanding the relationship between these stressors and MPB is important since any shift in food quality or quantity will affect higher trophic levels and may disrupt the bivalve's capacity to stimulate solute fluxes through reduced excretion and solute pumping.

While this experiment provides valuable insights into the potential effects of co-occurring anthropogenic stressors on ecosystem functioning, several limitations should be acknowledged. Firstly, the study was conducted in a controlled laboratory setting, which may not fully replicate the complex

interactions of natural environments. Consequently, the observed impacts on *M. liliiana* and associated ecosystem processes may differ under field conditions. However, care was taken to use natural densities of *M. liliiana* and light conditions that mimic natural settings for MPB growth. Throughout the experiment, *M. liliiana* were observed feeding, indicating quasi-normal behavior despite the controlled laboratory setting. Additionally, the specific concentrations of microplastics and nitrogen used in this study were based on prior research and provide insights into potential interactions at this dose; however, further research using multiple dosages would provide additional insight into threshold responses. Lastly, although this study did not explicitly test for interactive effects between microplastics and nitrogen, the results suggest that emergent responses may occur when these two stressors are both present. Despite these limitations, this is the first study to investigate these two stressors simultaneously in a marine environment, and the findings emphasize the necessity of considering multiple stressors when assessing ecological health.

This study demonstrates that combined microplastics and nitrogen exposure can alter key ecosystem functions, nutrient cycling, and primary production in soft sediments, mediated through alterations in infaunal bivalve behavior. This provides the first description of the joint effects of microplastics and nutrient loading on an estuarine system and indicates potential for further interruption to ecosystem functioning that should be investigated in natural settings. Furthermore, the observed effects of the multiple stressor treatment emphasize the importance of considering multiple, co-occurring stressors when managing at-risk ecosystems.

Author Contributions

Saskia E. Foreman: Conceptualization, methodology, investigation, data curation, formal analysis, writing – original draft, visualization, validation, software, project administration, funding acquisition, writing – review and editing. Bridie J. M. Allan: Methodology, resources, writing – review and editing. Amandine Sabadel: Methodology, resources, writing – review and editing. Candida Savage: Supervision, conceptualization, methodology, resources, project administration, funding acquisition, writing – review and editing.

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Conflicts of Interest

None declared.

Data Availability Statement

The data that support the findings of this study are openly available in the data repository “figshare” at <http://doi.org/10.6084/m9.figshare.31021639>.

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Supporting Information

Additional Supporting Information may be found in the online version of this article.

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