# Stoichiometry of regenerated nutrients differs between native and invasive freshwater mussels with implications for algal growth

**RUNNING HEAD**: Invasive mussels shift nutrient benthic fluxes

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**KEYWORDS**

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**Summary**

1. Filter-feeding mussels couple benthic and pelagic environments and create biogeochemical hot spots. Mussels may exert either top-down control (via filtration) or bottom-up stimulation (via biodeposition and excretion) of primary producers. Mussel metabolism may be species-specific and the disappearance of native species or their replacement by invasive species may affect ecosystem functioning, notably gas exchange, nutrient fluxes and stoichiometry at the sediment-water interface.
2. In this study, we tested experimentally how native (unionids) and invasive (dreissenids) mussels, singly and in association, affect benthic fluxes of dissolved gas and nutrients in the light and in the dark, and indirectly phytoplankton growth from a freshwater estuary, the Curonian Lagoon, where the two species of mussel coexist.
3. We show that native and invasive mussels stimulated $O_2$ consumption and the production of total dissolved inorganic carbon (DIC) and $N_2$ increasing sediment heterotrophy and nutrient regeneration, with species-specific effects on nutrient stoichiometry and algal growth. Ammonium and $SiO_2$ exchanges were similar for both species, while $PO_4^{3-}$ fluxes and excretion rates were significantly higher in sediments with dreissenids. Phytoplankton growth was also significantly higher in the presence of dreissenids as compared to unionids. Dreissenid mussels decreased the dissolved inorganic N to P ratio of regenerated nutrients and may favour the growth of cyanobacteria.
4. Hence the replacement of native with invasive mussels may produce large changes in benthic nutrient cycling and in phytoplankton growth and community composition.

# 1 INTRODUCTION

Native mussel communities are considered at present as the most endangered group of organisms globally (Strayer & Dudgeon, 2010). The decline of their diversity and abundance depends upon multiple factors, including water quality and habitat deterioration and the invasion of fast spreading alien species (Schloesser, Nalepa, & Mackie, 1996; Bowers & De Szalay, 2004; Strayer, 2010). The replacement of native with alien mussels may trigger changes in species diversity and ecosystem functioning of whole aquatic environments. Such consequences are poorly understood as they may vary from early to late invasion stages due to compensation responses, changes in community composition and food web structures (Schloesser, Nalepa, & Mackie, 1996; Caraco et al., 1997; Bowers & De Szalay, 2004; Ozersky, Evans, & Barton, 2012).

In pristine environments, freshwater mussels may comprise a major portion of the biomass of benthic macrofauna and are an important functional component of the macrofauna community (Vaughn & Hakenkamp, 2001; Atkinson, Vaughn, Forshay, & Cooper, 2013; Burlakova, Karatayev, Pennuto, & Mayer, 2014; Cyr, Collier, Clearwater, Hicks, & Stewart, 2017; Ruginis, Zilius, Vybernaite-Lubiene, Petkuviene, & Bartoli, 2017). Mussels displace pelagic primary production at the benthic level, fertilize sediments via labile biodeposits and fasten nutrient turnover, by conversion of particulate nutrients into reactive inorganic forms (Strayer, 1999; Gergs, Rinke, & Rothhaupt, 2009; Benelli et al., 2017). In nutrient-poor aquatic ecosystems native mussel beds may alleviate via excretion nitrogen (N) limitation and produce N, phosphorus (P) or silica (Si) co-limitation, depressing cyanobacteria and favouring diatom growth (Atkinson, Vaughn, Forshay, & Cooper, 2013; Strayer, 2014; Cyr, Collier, Clearwater, Hicks, & Stewart, 2017).

Invasion by dreissenid mussels have stimulated many studies focusing on the functional role of invading organisms (Caraco et al., 1997; Higgins & Vander Zanden, 2010; Strayer, 2010; Ruginis et al., 2014). Dreissenids were shown to effectively decrease chlorophyll concentration in lakes and rivers due to their high densities and filtration rate (Strayer, 2009; Cha, Stow, & Bernhardt, 2012). Some authors postulated the possibility to manage such invasion (e.g., with periodic removal of mussels biomass and associated N and P) in order to counteract eutrophication, but the amount of nutrient stocked in biomass is generally much lower than that circulated as particulate or dissolved forms (Stańczykowska & Lewandowski, 1993; Heat, Fahnenstiel, Gardner, Cavaletto, & Hwang, 1995; Arnott & Vanni, 1996; Higgins & Vander Zanden, 2010). Different studies suggested that top-down control of phytoplankton by zebra mussels (*Dreissena polymorpha*) is site-specific and depends upon a number of other factors such as water depth, stratification, turbidity and background nutrient concentration (Conroy et al., 2005; Caraco, Cole, & Strayer, 2006; De Stasio, Schrimpf, Beranek, & Daniels, 2008). In Lake Erie, Zhang et al. (2008, 2011) predicted small top-down

control of phytoplankton by zebra mussels but large, mussels-mediated effects on the mobilization of inorganic N and P. Phytoplankton may peak even under elevated grazing pressure by dreissenids, suggesting complex mechanisms regulating blooms (Caraco et al., 1997). Dominance of cyanobacteria in dreissenid invaded lakes were interpreted on the basis of selective filtration or alteration of nutrient cycles and ecological stoichiometry, e.g., enhanced N removal via denitrification and/or enhanced reactive P mobilization via excretion or release from sediment (Bykova, Laursen, Bostan, Bautista, & McCarthy, 2006; De Stasio, Schrimpf, Beranek, & Daniels, 2008; Makhutova et al., 2013).

Zebra mussel biodeposits increase benthic heterotrophic activity via increased bacterial numbers and benthic secondary production (Gergs, Rinke, & Rothhaupt, 2009; Ozersky, Evans, & Barton, 2012; Benelli et al., 2017). Increased heterotrophy, together with mussel metabolic requirements, resulted in decreased oxygen concentrations in river reaches (Caraco et al., 2000; Higgins & Vander Zanden, 2010). Simultaneously, dreissenid activity may stimulate mechanisms that compensate for large benthic oxygen demand. Elevated filtration rates and nutrient mobilization in fact increase light penetration and stimulate the activity of benthic primary producers. There is no univocal agreement on the net effect of dreissenids on ecosystem autotrophy or heterotrophy (Caraco, Cole, & Strayer, 2006; Stadmark & Conley, 2011). It likely depends upon different regulating factors such as the density of filter feeders (patchy colonies versus reefs), their metabolism and nutrient excretion rates, the morphometry of the invaded ecosystem (shallow *versus* deep), the background nutrient concentration, water turbidity and hydrodynamic factors (stratified or well-mixed) – Caraco et al., (1997); Yu & Culver, (1999); Conroy et al., (2005); Caraco, Cole, & Strayer, (2006).

Native and invasive mussels may co-occur in aquatic ecosystems during the early stages of invasions or due to combinations of available trophic resources and niches, by minimizing competition and allowing coexistence (Russert Kraemer, 1979). The co-occurrence allows investigating how benthic biogeochemistry varies depending on the dominance of native or invasive species. In this study, we investigated benthic biogeochemistry in a shallow, generally turbid and well-mixed area of the Nemunas River Delta (Curonian Lagoon, Baltic Sea) where native (the unionids *Unio tumidus* and *Anodonta anatina*) and invasive (*D. polymorpha*) mussels coexist. Unionids and dreissenids can be found as single individuals or in small clumps, respectively, or in association, with dreissenid colonies fouling unionid shells (Zaiko, Daunys, & Olenin, 2009; Dzierzynska-Białonczyk, Jermacz, Mackiewicz, Gajewska, & Kobak, 2018). Dreissenids may take advantage of growing on unionids, and in particular in the proximity of their siphons, due to large water flows and higher oxygen and nutrient availability (Hörmann & Maier, 2006); however, this can be different in sites with limited food availability and where competition can be high (Baker & Hornbach, 2008). Previous studies reported that dreissenids may outcompete unionids a few years

after the invasion, resulting in drastic decrease of their abundance and diversity (Bowers & De Szalay, 2004). This is not the case in the Curonian Lagoon, were dreissenids invasion was documented decades ago and where dreissenids coexist with unionids (Zettler & Daunys, 2007). We analysed the exchange of dissolved $O_2$, total inorganic carbon (DIC), $N_2$, $CH_4$ and nutrients ($NH_4^+$, $SiO_2$, $PO_4^{3-}$) in sediments with unionids and dreissenids when present alone and in association. All fluxes were measured in the light and in the dark in order to evaluate the effects of native and invasive mussels on benthic autotrophic and heterotrophic processes. We also determined the filtration and excretion rates of the mussels and tested their effect on phytoplankton growth.

We predicted higher heterotrophic activity in sediments colonised by the small, invasive *D. polymorpha* than in sediments with unionids due to higher filtration rates, biodeposition, microbial-mediated oxygen consumption and nutrient regeneration. This should result in higher potential stimulation of pelagic algal growth by *D. polymorpha*. It was also expected that benthic primary producers play an important role in attenuating nutrient recycling by mussels, due to benthic uptake and retention (Vaughn & Hakenkamp, 2001; Atkinson, Vaughn, Forshay, & Cooper, 2013).

## 2 METHODS

In this study, we performed 3 sets of experiments each one targeting a specific objective. In experiment 1, light and dark incubations of intact sediments with native and invasive mussels addressed the effects of mussels on gas and nutrient exchange. In experiment 2, incubations of native and invasive mussels without sediments addressed their filtration, respiration and nutrient excretion rates. Finally, experiment 3 addressed the effects of native and invasive mussels on phytoplankton growth.

### Sampling procedure and incubation setup

Intact sediment cores were collected by means of Plexiglass liners (height=30 cm, inner diameter=8 cm) from a shallow (1 m depth) sandy area within the Nemunas River Delta (55°20'25.9"N, 21°11'24.4"E) in September 2016. Low discharge and transparent water allowed collecting sediments with and without two families of mussels: Dreissenidae (*D. polymorpha*, an invasive species) and Unionidae (*U. tumidus* and *A. anatina*, two native species). Unionids were clearly visible, with half body burrowed within sediments; some were devoid of *D. polymorpha* while others were fouled by dreissenids. The sediment surface hosted also patchy colonies of *D. polymorpha* attached on any debris. Encrusting algae were commonly found growing close to the upper portion of unionids and over dreissenid clumps.

We collected 16 intact sediment cores, 4 replicates for 4 treatments: control sediment without mussels (C), sediment with a clump of *D. polymorpha* (D), sediment with an individual of unionids (U) and sediment with a unionid fouled by dreissenids (D+U). After sampling, all liners were provided with a bottom lid and a magnetic bar fixed 10 cm above the sediment interface to stir the water and avoiding sediment resuspension. They were transferred in a cool box filled with *in situ* water and transported to the laboratory within 2 hours. Then, all the cores were submerged with the top open in a temperature-controlled (17 ± 0.1 °C) tank (100 L), containing aerated and well-stirred lagoon water. The tank was provided with a central magnet rotating at 40 rpm and driving all magnets inside the cores in order to ensure water exchange with the tank and to supply phytoplankton to mussels. The tank cover was provided with halogen lamps (Osram Decostar, 35 W), positioned above each core and producing an irradiance of 96 ± 12 µE $m^{-2}$ $s^{-1}$ measured at the sediment-water interface with an underwater quantum sensor (Li-COR LI-192) and over a 12 hours light and 12 hours dark period. The cores underwent an overnight preincubation and were then processed to measure benthic fluxes (Experiment 1).
Besides intact sediment cores, nearly 200 L of *in situ* water and additional sets of mussels, including native and invasive species, were collected from the site to measure filtration, respiration and excretion rates (Experiment 2) and to measure phytoplankton growth with dialysis bags (Experiment 3).

**Experiment 1: benthic respiration and nutrient fluxes**

The day after the sampling, light and dark fluxes of dissolved gas and nutrients were measured via short-term batch incubations, as detailed in Benelli et al. (2018). Incubations lasted 2 hours, in order to keep $O_2$ concentration within ± 20 % of the initial value. Longer incubation times may result in large drops of $O_2$, creating limiting conditions for aerobic processes and resulting in wrong estimation of rates (Dalsgaard et al., 2000). Incubations started when transparent, gas-tight lids were positioned on the top of each core. At the beginning and at the end of the incubations, dissolved $O_2$ concentration was measured with a microelectrode and initial and final water samples (100 ml) were collected with plastic syringes from each core from an one-way valve located in the lids (Figure 1a). The collected volume was replaced with an equivalent amount of tank water entering the cores from another valve. Each water sample underwent the same processing: an aliquot of 40 ml was transferred to two 12 ml exetainers (Labco Scientific, UK), the first for DIC, which was immediately titrated and the second for dissolved $O_2$, $N_2$ and $CH_4$ analyses. The latter were added with 100 µl of 7 M $ZnCl_2$ to stop microbial activity (analytical methods are reported below). An aliquot of 20 ml was filtered (GF/F glass-fiber filters, 0.7 µm) and transferred into 20 ml plastic vials in order to analyse ammonium ($NH_4^+$) and dissolved inorganic silica ($SiO_2$). Another aliquot

of water was filtered and transferred into 5 ml glass vials to measure soluble reactive phosphorus ($PO_4^{3-}$). Details on the analytical techniques are reported below. Dissolved gas and nutrient fluxes were calculated according to the equation:

$$Flux_x = \frac{([x]_f - [x]_i) \times V}{A \times t}$$

where $[x]_f$ and $[x]_i$ are the concentrations (µM or mM) of the solute *x* at the end and at the start of the incubation, respectively, *V* (L) is the volume of the core water phase, *A* ($m^2$) is the area of the sediment and *t* (hr) is the incubation time.

Daily fluxes (mmol $m^{-2}$ $day^{-1}$) were calculated according to the following equation:

$$Daily\ flux_x = (hourly\ dark\ flux \times h_D) + (hourly\ light\ flux \times h_L)$$

where $h_D$ and $h_L$ are the number of dark and light hours during incubation, respectively.

At the end of the incubation, all the individuals were recovered and characterised for the flesh wet weight (WW) and for the flesh dry weight (DW), after drying the soft tissue at 60 °C to a constant weight.

Net and gross $O_2$ fluxes measured in the light were converted into theoretical net and gross nutrient uptake by benthic algae. Rates were multiplied by a photosynthetic quotient of 1.2 to convert $O_2$ production into C-fixation (Sundbäck, Linares, Larson, Wulff, & Engelsen, 2004). We used oxygen data instead of measured DIC fluxes as microbial processes (i.e. nitrification) may results in overestimation of C-fixation rates by benthic algae. Calculated net and gross C-fixation were divided by the Redfield ratio 106:16:15:1 and converted into theoretical inorganic N, Si and P uptake (Redfield, 1958).

**Experiment 2: respiration, excretion and filtration rates by native and invasive mussels**

Plexiglass liners identical to those described for experiment 1, but devoid of sediment, were used to measure rates of $O_2$ consumption and nutrients ($NH_4^+$, $SiO_2$ and $PO_4^{3-}$) excretion by mussels. Chlorophyll *a* (chl *a*) fluxes were also measured and considered as proxies of filtration rates. Three treatments, each with three replicates, were considered: *in situ* water with a dreissenid clump (D), *in situ* water with a single unionid (U) and *in situ* water with a single unionid fouled by dreissenids (D+U). The biomass of mussels in each replicate reproduced approximately the biomass in the intact cores of experiment 1. An additional set of three cores, containing *in situ* water without mussels, was used as control. All treatments underwent a two hours dark incubation. At the beginning and at the end of the incubation, dissolved $O_2$ concentration was measured with a microelectrode, a water subsample was collected and filtered (GF/F) into plastic vials for dissolved

inorganic nutrient analyses ($NH_4^+$ and $SiO_2$) and another aliquot was collected into glass vials to measure $PO_4^{3-}$. Additionally, 300 ml of water from the incubation tank (at the start of the incubation, n=3) and 300 ml of water from each core (at the end of the incubation) were collected and filtered (GF/F) to measure chl *a* fluxes. At the end of the incubation, all the individuals were recovered and characterised for the flesh wet weight (WW) and for the flesh dry weight (DW), after drying the soft tissue at 60 °C to a constant weight. Fluxes of chl *a*, normalised by the dry mussel flesh (µg $g_{dw}^{-1}$ $hr^{-1}$), were converted into µmol of particulate carbon (PC) removed from the water column by mussel filtration. To this purpose, we multiplied chl *a* fluxes by the factors of 2.5 and 3.3, that include most chl *a*: C conversion factors (µg : µmol) reported in the literature (Banse, 1977). The PC flux (µmol C $g_{dw}^{-1}$ $hr^{-1}$) was then converted into particulate N (PN), particulate P (PP) and particulate Si (PSi) removed from the water column as phytoplankton. Due to large errors associated to such estimates, we used a range of 5-20 for the C:N stoichiometry, 25-400 for C:P stoichiometry and 0-10 for C:Si stoichiometry (all mol : mol). Oxygen fluxes were converted into DIC production assuming a DIC : $O_2$ = 1 : 1 stoichiometry. Rates measured in control cores were subtracted from treatments.

**Experiment 3: phytoplankton growth assay**

To measure phytoplankton growth in presence and absence of mussels, dialysis bags (n=16) were incubated for 3 days in a 10 L tank containing frequently renewed *in situ* water with and without mussels. The dialysis bags were filled each with ca. 600 ml of *in situ* water, filtered with a 50 µm-mesh to remove zooplankton but not phytoplankton. The water used to fill the dialysis bags was analysed at the beginning of the incubation for the concentration of chl *a* and dissolved inorganic nutrients ($NH_4^+$, $PO_4^{3-}$ and $SiO_2$). The dialysis bags were made from Spectra/por 1 dialysis membrane consisting of regenerated cellulose with a molecular weight cut off of 6-8 kDa (Mura et al., 1996). Each bag was cylindrical, with a diameter of 6 cm, a length of 15 cm and closed at the extremes with plastic ties. A single dialysis bag was put in each tank. Four treatments were tested, each with 4 replicate tanks: a dialysis bag submersed in water (C), in water with a dreissenid clump (D), in water with a single unionid (U) and in water with a unionid fouled with dreissenids (D+U) (Figure 1b). At the end of the incubation, the dialysis bags were opened and, from each one, 300 ml of water were collected and filtered (GF/F) to analyse chl *a* and dissolved inorganic nutrients. Phytoplankton growth rate (µ) was calculated via the equation:

$$B_f = B_0 * e^{\mu t}$$

$$\mu = \frac{(ln(B_f) - ln(B_0))}{t}$$

where $B_f$ and $B_0$ are the concentrations of chl *a* at the end and at the beginning of the incubation (µg $l^{-1}$) and t is the incubation time (day).

**Analytical methods**

Dissolved $O_2$ was measured by means of polarography with a microelectrode (90 % response time in <5 s, 50 µm tip; Unisense, Denmark). Dissolved $N_2$ and $CH_4$ were analysed by membrane inlet mass spectrometer (MIMS, Bay instruments, USA) – Schlüter & Gentz (2008). Dissolved $N_2$ concentrations were calculated from obtained $N_2$:Ar ratio and theoretical Ar concentration derived from Weiss (1970). DIC was measured via six end points 0.1 N HCl microtitration (Anderson et al., 1986). Dissolved nutrients ($PO_4^{3-}$ and $SiO_2$) from incubations were measured with a continuous flow analyser ($San^{++}$, Skalar, sensitivity 0.3 µM) using standard colorimetric methods (Grasshoff, Ehrhardt, & Kremling, 1983). $NH_4^+$ was analysed spectrophotometrically using salicylate and hypochlorite, with nitroprussiate as catalyst (Bower & Holm-Hansen, 1980). Lipophilic pigments from water samples were extracted in 5 ml of 90 % acetone during 24 h at 4 °C. The extracts were centrifuged and chl *a* was measured by spectrophotometry according to Lorenzen (1967). Absorption was measured before and after adding 1 M HCl to separate between chl *a* and phaeopigments.

**Statistical analysis**

Two-way analysis of variance (ANOVA) was used to test the effects of illumination (light/dark measurements), treatments (without or with different combination of mussels) and their interaction on benthic gas and nutrient fluxes. One-way ANOVA was used to test the effect of mussels on phytoplankton growth. The significance (p-value) was set at <0.05 and pairwise multiple comparison was performed with the post-hoc Holm-Sidak test. All the statistical analyses were performed with Sigma Plot 11.0.

## 3 RESULTS

Densities of native mussels at the study site averaged 4 ± 5 ind $m^{-2}$ while density of invasive dreissenids averaged 92 ± 13 ind $m^{-2}$. Oxygen bubbles at the sediment surface indicated photosynthetic activity of benthic algae. During the sampling period, the discharge of the Nemunas River was nearly 300 $m^3$ $s^{-1}$, which corresponds to nearly 40 % of the annual average discharge and suggesting relatively low transport of nutrients and particulate matter at the study site and slow water turnover.

**Experiment 1: benthic respiration and nutrient fluxes**

Benthic $O_2$ fluxes were affected by the presence of mussels, which shifted, the benthic system from net autotrophic to net heterotrophic (Figure 2a). Fluxes were more negative in sediments in presence of both mussels (D+U), with rates of -4.3 ± 0.7 mmol $m^{-2}$ $hr^{-1}$ and -12.8 ± 1.6 mmol $m^{-2}$ $hr^{-1}$ (mean ± standard error), measured in the light and in the dark, respectively (Figure 2a), and significantly more negative than the other treatments (Holm-Sidak test, $p<0.05$). Both factors illumination and treatment produced a significant effect on $O_2$ fluxes (two-way ANOVA, Table 1). Gross primary production (GPP, obtained combining light and dark fluxes) was not different among treatments suggesting that there were active microalgae growing on the sediment and mussel surface in all treatments. The highest rate of DIC production was found in the dark in D+U treatment, with a rate of 6.3 ± 1.1 mmol $m^{-2}$ $hr^{-1}$ (Figure 2b), which was significantly higher than in the other three treatments (Holm-Sidak test, $p<0.05$). Similar to $O_2$, both illumination and treatment factors produced a significant effect on the fluxes of dissolved DIC (two-way ANOVA, Table 1). The simultaneous presence of mussels (D+U) turned the benthic system from net DIC sink to a net DIC source (Figure 2b). Bare sediments (C) displayed negative $N_2$ fluxes in the light and in the dark (Figure 2c). In the light, the presence of mussels set to zero the net $N_2$ uptake while in the dark it reversed the flux measured in C. The two-way ANOVA on $N_2$ fluxes revealed that both factors illumination and treatment, but not their interaction, were very close to the 5 % significant level (Table 1). Fluxes of $CH_4$ were generally low and variable among replicates and treatments. In the light, they were generally not measurable while in the dark $CH_4$ tended to be released from sediments, with higher rates measured in D and D+U (Figure 2d). Differences between light and dark rates were significant (Holm-Sidak test, $p<0.05$), but not differences among treatments (two-way ANOVA, Table 1).

Dissolved inorganic nutrients measured during light and dark incubations were reported in figure 3. There was a statistically significant interaction between illumination and treatment factors in $NH_4^+$ fluxes (two-way ANOVA, Table 1), suggesting that the effect of illumination depended on the treatment. The presence of mussels always resulted in a net $NH_4^+$ regeneration to the water column during the dark incubation (Figure 3a), with rates that were significantly different from bare sediment (Holm-Sidak test, $p<0.05$). In the light, uptake processes measured in C were significantly reduced or reversed (D+U) by the presence of mussels. Only bare sediment (C) acted as a net $NH_4^+$ sink while in the presence of mussels $NH_4^+$ was always regenerated. The factor illumination had no effect on $PO_4^{3-}$ fluxes, that were strongly influenced by the presence of *D. polymorpha* clumps (Figure 3b)(two-way ANOVA, Table 1). Bare sediments and sediments with unionids were $PO_4^{3-}$ sinks, with negative fluxes measured in light and dark incubations. Sediments with *D. polymorpha*

were on the contrary always $PO_4^{3-}$ sources, regardless the illumination condition, with rates exceeding 30 µmol $m^{-2}$ $hr^{-1}$ measured in the dark in D+U (Figure 3b). Fluxes of $SiO_2$ were negative in all treatments and illumination conditions (Figure 3c); two-way ANOVA revealed significant differences only in the treatment factor, with the lowest $SiO_2$ uptake measured in D+U (Table 1). Mussels affected differentially the ecological stoichiometry of regenerated nutrients: both dreissenids and unionids, alone or in combination, alleviated N limitation by recycling large $NH_4^+$ amounts. Only dreissenids alleviated P limitation by recycling $PO_4^{3-}$, whereas both mussels had no appreciable effects on Si, which was never recycled to the water column.

**Experiment 2: respiration, excretion and filtration rates by native and invasive mussels**

The dry flesh normalised respiration of mussels incubated in water (DIC fluxes, µmol C $g_{dw}^{-1}$ $hr^{-1}$) was double in dreissenids compared to unionids (Figure 4; one-way ANOVA, p=0.05). Community respiration rates calculated for the D+U treatment displayed intermediate values between D and U. The mussels excretion rate of $NH_4^+$ did not differ among treatments, whereas $PO_4^{3-}$ excretion measured in D was nearly seven time higher than that measured in the presence of unionids (U and D+U; one-way ANOVA, p=0.03). Silica was never excreted to the water column; fluxes were, on the contrary, small and negative (Figure 4). On a molar basis, the DIC : $NH_4^+$ ratio of inorganic nutrients excreted by mussels were 9.8, 6.0 and 9.9 in the D, U and D+U treatments, respectively; whereas the $NH_4^+$ : $PO_4^{3-}$ ratio were 3.5, 20.4 and 17.0 in the D, U and D+U treatments, respectively.

Figure 4 reports also conservative ranges of particulate nutrient fluxes, all derived from chl *a* filtration rates. Biomass-normalised rates of chl *a* removal and calculated particulate nutrients were not significantly different among treatments. The comparison of particulate fluxes (to the mussels) and dissolved nutrients excretion (from the mussels) reveals that: i) DIC production was always in excess to PC fluxes; ii) $NH_4^+$ excretion was always within the calculated range but close to the highest value of PN fluxes and iii) $PO_4^{3-}$ excretion was within the range (in U and D+U) or higher (in D) than PP fluxes. Unmeasurable $SiO_2$ excretion supports the hypothesis that algal material filtered by mussels was not siliceous. Feces and pseudofeces were not collected at the end of the incubation.

**Experiment 3: phytoplankton growth assay**

Phytoplankton growth rates measured in the four treatments are reported in figure 5a. Phytoplankton growth was significantly higher in presence of mussels and in particular in presence of *D. polymorpha* (Holm-Sidak test, $p<0.05$). Along the incubation period, $NH_4^+$ concentration decreased in all treatments as compared to initial value ($t_0$)(Figure 5b). Reactive phosphorus

concentration also decreased as compared to the initial value, except for D+U treatment (Figure 5c). At the end of the experiment, $SiO_2$ displayed a marked decrease in presence of *D. polymorpha* by a factor of nearly three, whereas it was similar to initial value in U and C treatments (Figure 5d).

## 4 DISCUSSION

### Mussel beds as biogeochemical hot spots in sediments

This study contributes to our understanding of how invasions by non-native mussels may alter benthic biogeochemistry and nutrient availability in the pelagic environment, stimulating primary production (Heath, Fahnenstiel, Gardner, Cavaletto, & Hwang, 1995; Strayer, 1999; Conroy et al., 2005; Zhang, Culver, & Boegman, 2011; Ruginis et al., 2014). Results from this study confirm that filter-feeding mussels increase the net heterotrophy of the benthic system, augmenting oxygen uptake, total dissolved inorganic carbon, nitrogen and phosphorus release and nitrogen loss via denitrification (Ruginis et al., 2014; Welsh, Nizzoli, Fano, & Viaroli, 2015; Benelli et al., 2017; Smyth et al., 2017). Light measurements highlight also the importance of benthic primary production for nutrient cycling in the absence and even more in the presence of filter feeders, with high and comparable rates of gross primary production in all the considered treatments. Mussels displace pelagic primary production at the sediment level and provide surface for algal growth (Atkinson & Vaughn, 2015; Ozersky, Evans, & Ginn, 2015; Vaughn, 2017). Gross primary production and nutrient uptake in sediments with or without mussels largely attenuated the benthic regeneration or even reversed nutrient effluxes (Atkinson, Sansom, Vaughn, & Forshay, 2018).

### Dreissenids vary the ecological stoichiometry of regenerated nutrients

Through filtration, mussels remove particulate materials from the water column. A fraction of such algal material is assimilated by the mussels and stored for their growth, a fraction is excreted to the water column as dissolved inorganic nutrients, and a fraction is egested as biodeposits (feces and pseudofeces) to the sediment (Strayer, 2014; Smyth et al., 2017; Vaughn, 2017). In this study, we did not measure the quality and quantity of biodeposits. However, we found large interspecific metabolic differences between the two families of mussels, in terms of excreted nutrients, which afterwards may differentially stimulate pelagic primary production. These outcomes align with those reported by Vanni and McIntyre (2016), dealing with species-specific rates of nutrient excretion by aquatic animals and by Atkinson et al. (2013), dealing with the role of mussel aggregates as nutrient sources to the water column. They suggest also that while widely documented $NH_4^+$ excretion by some mussels (e.g., Unionidae) may balance N limitation, other mussels (e.g., Dreissenidae) may further unbalance the ecological stoichiometry of nutrients, due to large $PO_4^{3-}$ production (Heath, Fahnenstiel, Gardner, Cavaletto, & Hwang, 1995; Conroy et al.,

2005; Ruginis et al., 2014). Results from this study show that mussels may reverse the sink role of sediments through the excretion of high rates of dissolved inorganic N and P to the water column, as reported for many other filter-feeding animals (Nalepa, Gardner, & Malczyk, 1991; Ruginis, Zilius, Vybernaite-Lubiene, Petkuviene, & Bartoli, 2017; Benelli et al., 2018). We demonstrated that this regeneration may support completely, or in part, the theoretical nutrient demand by benthic primary producers (Table 2). In particular, *D. polymorpha* regenerated proportionally more P than N, and such regeneration entirely supported inorganic P but not N requirements by benthic primary production. This may result in competition for N between algae and bacteria and may stimulate N uptake from the water column or from pore water or N-fixation. Nitrogen and P excretion by unionid mussels was on the contrary in excess to nutrient requirements by algae (Table 2). The excretion rates that we measured are among the values that Vanni et al. (2017) reported reviewing the literature in a range of temperature between 17-19 °C. The amount of nutrients excreted is generally proportional to the composition of particulate matter ingested (Vanni, 2002). As *D. polymorpha* excreted high rates of inorganic $PO_4^{3-}$ to the water column, we assume that its biodeposits are depleted in phosphorus (Gergs, Rinke, & Rothhaupt, 2009). These biodeposits may remain photosynthetically active and enhance benthic primary production (Roditi, Caraco, Cole, & Strayer, 1996; Newell, 2004) and benthic secondary production from pelagic resources (Gergs, Rinke, & Rothhaupt, 2009). The molar ratio of excreted inorganic nutrients ($NH_4^+$ : $PO_4^{3-}$) was 3.50 ± 0.87 for *D. polymorpha* and 20.43 ± 8.12 for unionids. These ratios are in line with the mean value found by Vanni et al. (2017) in the studies on dreissenids and unionids in the range of temperature between 17 and 19 °C. Changes in the ratios of available nutrients may alter the community of primary producers and may vary the background nutrient conditions of the system (Atkinson, Vaughn, Forshay, & Cooper, 2013).

**Benthic buffers of excreted nutrients**

Mussels increase sediment heterotrophy and nutrient recycling but our results suggest that a large fraction of regenerated nutrients is assimilated and retained by primary producers within the benthic compartment. Flux measurements performed in the dark in sediment with mussels or the upscaling of mussel excretion rates provide a partial picture of the effects of an invasive species, overestimating its impact. A similar line of reasoning is reported by Caraco et al. (2000) analysing the effects of dreissenids on dissolved oxygen decline in the Hudson River. The large discrepancy between *in situ* oxygen decline and that, much higher, predicted by mussel respiration rates was explained in terms of increased photosynthesis by macrophytes. The latter, due to dreissenid-mediated increase of light penetration, moderated the impact of the mussels.

Combining biomass-specific excretion rates (Figure 4) and the biomass of mussels recovered in the incubated sediment (Table 3) it was possible to calculate the theoretical nutrient release by mussels in the different experimental conditions (4$^{th}$ column of Table 2). Heterotrophic measurements of nutrient fluxes reported in figure 3 are always much lower than those expected from mussel excretion, suggesting different retention mechanisms, operating also in the dark and including precipitation and co-precipitation, coupled nitrification and denitrification and uptake. Large differences between nutrient recycling via excretion measured with mussels alone and net nutrient regeneration in intact cores with mussels were generally found (Benelli et al., 2017; Ruginis, Zilius, Vybernaite-Lubiene, Petkuviene, & Bartoli, 2017; Murphy et al., 2018). Such differences can be due to higher mussel metabolism when incubated without sediments and to a diversified community of microbes and algae, which are growing in sediments with mussels and may promote net uptake of the excreted solutes.

**Dreissenids may favour cyanobacteria blooms**

Our data allow direct comparison of the metabolism of a native and an invasive species in the delta area of the Nemunas River. The rates of phytoplankton removal by the two families of mussels were comparable on a mussel flesh dry weight basis, but nutrient regeneration was different, with significantly larger reactive P excretion by *D. polymorpha* (Heath, Fahnenstiel, Gardner, Cavaletto, & Hwang, 1995; Ruginis et al., 2014; Vanderploeg et al., 2017). This suggests different metabolic pathways between mussels, as the nutrient source (i.e. the algal community and its nutrient stoichiometry) was the same during the incubations. The invasive species, singly or in association with the unionids, determined an unbalanced stoichiometry of regenerated nutrients, with a P excess that may favour the growth of N-fixing algae. Cyanobacteria blooms are regularly occurring in the Curonian Lagoon, which is a large freshwater estuary (Gasiūnaitė, Daunys, Olenin, & Razinkovas, 2008). The large nutrient inputs of the Nemunas River to the Curonian Lagoon (44,208 ± 12,677 tons total N yr$^{-1}$, 59,048 ± 10,770 tons total Si yr$^{-1}$ and 1,547 ± 266 tons total P yr$^{-1}$) largely contribute to the hypertrophic status of this estuary (Vybernaite-Lubiene et al., 2018). Besides dissolved nutrients, the Nemunas River delivers large amounts of phytoplankton, with an estimated annual load of ~350 tons chl *a* y$^{-1}$ (Vybernaite-Lubiene et al., 2017). Such loads undergo profound seasonal variations, with summer minima for inorganic nutrients and summer peaks for particulate forms. Interestingly, the ecological stoichiometry of dissolved inorganic N, Si and P displays a pronounced and steep drop in the critical period between the spring and the summer, when N and Si limitation establishes for 4-5 months (Vybernaite-Lubiene et al., 2018). Such limitation was generally considered to be the main reason favouring cyanobacteria blooms in the Curonian Lagoon. Our results suggest that other factors besides external loads, among which macrofauna

activity and sediment recycling, may foster cyanobacteria blooms, by further unbalancing $NH_4^+$ : $PO_4^{3-}$ ratios. We believe that nutrient regeneration by mussels is diluted within the huge loads delivered from September to May by the Nemunas River, but that internal recycling might represent an important fraction during summer, when river discharge and nutrient concentrations are at their minimum. In particular, large areas facing the Nemunas River Delta and invaded by dreissenids may represent benthic reactors turning fluvial chl *a* into reactive nutrients characterised by P excess. As such, mussels may be co-drivers of cyanobacteria blooms, as demonstrated in experimental or theoretical works by Conroy et al. (2005); Bykova et al. (2006); De Stasio et al. (2008); Zhang et al. (2008, 2011). The peak of dissolved inorganic P regeneration measured in D+U (30 µmol P $m^{-2}$ $hr^{-1}$) overlaps the highest P flux reported for the Curonian Lagoon benthic system. Such dramatic P regeneration was measured with the same batch incubation of intact sediments without mussels in a single occasion, during the occurrence of a large cyanobacteria bloom and was interpreted as a positive biogeochemical feedback to blooms (Zilius et al., 2014).

In the present study, the benthic system of the Nemunas River deltaic area did not release any dissolved reactive Si, regardless the presence of mussels. This suggests either a strong Si limitation of the benthic primary producers and a retention/translocation of Si at the sediment-water interface or the dominance in late summer of not siliceous algae feeding the mussels (Vybernaite-Lubiene et al., 2017 and 2018).

The shallow depth (<1 m) of the study area, the minimum discharge of the Nemunas River and the absence of wind in the study period, combined with the relatively large population of mussels, resulted in transparent water with low chlorophyll concentrations. This might suggest top-down control of pelagic primary production by the filter-feeding community, at least under the specific hydrographic and meteorological conditions of the sampling period (Prins, Smaal, & Dame, 1998; Caraco, Cole, & Strayer, 2006). However, this is not the rule in the Curonian Lagoon, as for most of the year the water is turbid due to wind-wave action resuspending sediments, high river discharge, elevated phytoplankton biomass and limited capacity of benthic and pelagic grazers to control algal growth (Vybernaite-Lubiene et al., 2018). Elsewhere, dreissenid mussels were demonstrated to exert top-down control of phytoplankton, increase water transparency and promote benthic primary production (Caraco et al., 2000; Cha, Stow, & Bernhardt, 2012). Such outcome is likely true for well-mixed lakes with moderate nutrient loads, but it cannot be generalised to all the invaded aquatic ecosystems. Mussels have limited access to phytoplankton in stratified lakes, where the main effect of mussels is to fasten nutrient recycling (Yu & Culver., 1999; Zhang, Culver, & Boegman, 2008). Caraco et al. (1997) suggested also that phytoplankton may compensate the grazing pressure of dreissenids in various ways, depending upon factors as turbidity or available nutrients, and resulting in sometimes opposite scenarios (e.g., much lower or higher phytoplankton

concentrations). The dialysis bag experiment revealed strong enhancement of phytoplankton growth in presence of mussels, larger with *D. polymorpha* as compared to unionids. This result is robust in terms of comparison between the effects of native and invasive species on potential growth of phytoplankton but it should be carefully considered due to the specific and controlled laboratory conditions (e.g., removal of grazing, absence of sediments, etc.).

In conclusion, this study suggests that the replacement of native with invasive mussels may produce large changes in benthic nutrient cycling and phytoplankton growth. In particular, different ecophysiology of dreissenids results in different stoichiometry of benthic nutrient regeneration as compared to sediments with native mussels. Dreissenids, when incubated in intact sediment cores or in the water column without sediments, increased the recycling of reactive P; as such, they may favour or sustain the growth of cyanobacteria.

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**CONFLICT OF INTEREST**

The authors declare no conflicting interests.

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## TABLES

**TABLE 1** Results of two-way ANOVA testing the effects of the factors incubation condition (illumination: light/dark) and treatment (control, D, U, D+U) on gas ($O_2$, DIC, $N_2$, $CH_4$) and nutrient ($NH_4^+$, $PO_4^{3-}$ and $SiO_2$) fluxes. *df*, degree of freedom; SS, sum of squares; MS, mean of squares; F, F-statistics; *p*, *p*-value. Significant values are printed in bold.

| | Source of variation | *df* | SS | MS | F | *p* |
|---|---|---|---|---|---|---|
| **$O_2$** | | | | | | |
| | Illumination | 1 | 472 | 472 | 84.4 | **<0.001** |
| | Treatment | 3 | 492 | 164 | 29.3 | **<0.001** |
| | Interaction | 3 | 20 | 7 | 1.2 | 0.341 |
| | Residual | 24 | 134 | 6 | | |
| | Total | 31 | 1123 | 36 | | |
| **DIC** | | | | | | |
| | Illumination | 1 | 542 | 542 | 131.6 | **<0.001** |
| | Treatment | 3 | 228 | 76 | 18.3 | **<0.001** |
| | Interaction | 3 | 23 | 8 | 1.8 | 0.166 |
| | Residual | 24 | 100 | 4 | | |
| | Total | 31 | 888 | 29 | | |
| **$N_2$** | | | | | | |
| | Illumination | 1 | 4 | 4 | 3.8 | 0.064 |
| | Treatment | 3 | 9 | 3 | 2.7 | 0.072 |
| | Interaction | 3 | 2 | 1 | 0.5 | 0.718 |
| | Residual | 24 | 28 | 1 | | |
| | Total | 31 | 43 | 1 | | |
| **$CH_4$** | | | | | | |
| | Illumination | 1 | 225 | 225 | 4.3 | **0.048** |
| | Treatment | 3 | 149 | 50 | 1.0 | 0.429 |
| | Interaction | 3 | 132 | 44 | 0.8 | 0.482 |
| | Residual | 24 | 1243 | 52 | | |
| | Total | 31 | 1775 | 57 | | |
| **$NH_4^+$** | | | | | | |
| | Illumination | 1 | 261818 | 261818 | 28.9 | **<0.001** |
| | Treatment | 3 | 361429 | 12476 | 13.3 | **<0.001** |
| | Interaction | 3 | 99582 | 33194 | 3.7 | **0.026** |
| | Residual | 24 | 217175 | 9049 | | |
| | Total | 31 | 946477 | 31499 | | |
| **$PO_4^{3-}$** | | | | | | |
| | Illumination | 1 | 19 | 19 | 0.3 | 0.561 |
| | Treatment | 3 | 9294 | 3098 | 58.0 | **<0.001** |
| | Interaction | 3 | 139 | 46 | 0.9 | 0.470 |

| | | | | | | |
|---|---|---|---|---|---|---|
| | Residual | 24 | 1282 | 53 | | |
| | Total | 31 | 10729 | 346 | | |
| **$SiO_2$** | | | | | | |
| | Illumination | 1 | 12058 | 12058 | 0.3 | 0.581 |
| | Treatment | 3 | 384698 | 128233 | 3.3 | **0.037** |
| | Interaction | 3 | 196426 | 65475 | 1.7 | 0.194 |
| | Residual | 24 | 924804 | 38533 | | |
| | Total | 31 | 1510298 | 48719 | | |

**TABLE 2** Contribution of mussels N and P excretion to theoretical gross benthic primary production. Biomass-specific excretion rates measured in experiment 2 were multiplied by the biomass of mussels in experiment 1 and compared to the theoretical nutrient demand to sustain gross primary production. The latter was calculated form oxygen fluxes measured in the light and from algal nutrient stoichiometry. Mean ± standard error are reported. GPP=gross primary production, TNU=theoretical nitrogen uptake by benthic algae, TPU=theoretical phosphorus uptake by benthic algae.

| | **GPP** | **TNU** | **TPU** | **Mussel excretion** | | **Contribution to uptake** | |
|---|---|---|---|---|---|---|---|
| | ($mmol\ m^{-2}\ hr^{-1}$) | ($\mu mol\ m^{-2}\ hr^{-1}$) | ($\mu mol\ m^{-2}\ hr^{-1}$) | ($\mu mol\ m^{-2}\ hr^{-1}$) | | (%) | |
| | | | | $NH_4^+$ | $PO_4^{3-}$ | N | P |
| **D** | 9.91 ± 0.63 | 1794 ± 114 | 112 ± 7 | 499 ± 110 | 143 ± 60 | 28 ± 8 | 128 ± 62 |
| **U** | 5.38 ± 2.09 | 974 ± 379 | 61 ± 24 | 1135 ± 392 | 56 ± 29 | 117 ± 340 | 92 ± 84 |
| **D+U** | 8.47 ± 2.20 | 1534 ± 399 | 96 ± 25 | 2393 ± 954 | 320 ± 167 | 156 ± 662 | 333 ± 261 |

**TABLE 3** Biomass of mussels in the three treatments (D, U and D+U) incubated in experiment 1. Mean ± standard error are reported.

| | **Biomass of mussels** | | |
|---|---|---|---|
| | ($g_{dw}\ m^{-2}$) | | |
| | Dreissenids | Unionids | Total |
| **D** | 82 ± 9 | 0 | 82 ± 9 |
| **U** | 0 | 225 ± 21 | 225 ± 21 |
| **D+U** | 140 ± 45 | 304 ± 97 | 444 ± 107 |

**FIGURE LEGENDS**

**FIGURE 1** Setup of the experiment targeting light and dark fluxes in intact sediments with and without mussels (a) and phytoplankton growth in dialysis bags submersed in tanks with different combination of mussels (b). See the text for more details.

**FIGURE 2** Benthic fluxes of a) dissolved oxygen ($O_2$), measured in light (NPP= Net Primary Production; white bars) and in dark (R= Respiration; black bars) incubations of C, D, U and D+U treatments (n=4). b) Total dissolved inorganic carbon (DIC); c) molecular nitrogen ($N_2$) and d) methane ($CH_4$) measured in light (white bars) and dark (black bars) incubations of C, D, U and D+U treatments (n=4). Grey bars in $O_2$ and DIC graphs represent Gross Primary Production (GPP). Mean ± standard error are reported. All fluxes are expressed in µmol $m^{-2}$ $hr^{-1}$ or mmol $m^{-2}$ $hr^{-1}$. Dots represent daily mean ± standard error expressed in mmol $m^{-2}$ $day^{-1}$.

**FIGURE 3** Light and dark benthic fluxes of: a) ammonium ($NH_4^+$), b) soluble reactive phosphorus ($PO_4^{3-}$) and c) dissolved reactive silica ($SiO_2$) measured in C, D, U and D+U treatments (n=4). Mean ± standard error are reported. All fluxes are expressed in µmol $m^{-2}$ $hr^{-1}$. Dots represent daily mean ± standard error of nutrient fluxes expressed in mmol $m^{-2}$ $day^{-1}$.

**FIGURE 4** Respiration and excretion rates measured in presence of *D. polymorpha* clumps (a), unionids (b) and both (c) are indicated by red arrows. Measured chl *a* fluxes filtrated by mussels are expressed in µg $g_{dw}^{-1}$ $hr^{-1}$ and indicated by red arrows. Ranges of particulate nutrients (carbon, PC; nitrogen, PN; phosphorus, PP and silica, PSi) fluxes derived from chl *a* are indicated by blue arrows in presence of *D. polymorpha* clumps (a), unionids (b) and both (c). Mean ± standard error are reported. All units are expressed in µmol $g_{dw}^{-1}$ $hr^{-1}$.

**FIGURE 5** Phytoplankton growth rates in the dialysis bags incubated for three days in control treatment (only water, C) and in presence of mussels (D, U and D+U) expressed in µ $day^{-1}$ (a). Concentrations of dissolved inorganic nutrients b) $NH_4^+$; c) $PO_4^{3-}$ and d) $SiO_2$), expressed in µM, in control treatment (only water, C) and in treatments with mussels at the end of experiment. The dash line is the reference value of nutrient concentrations at the beginning of the experiment (t0).

**Figure 1**

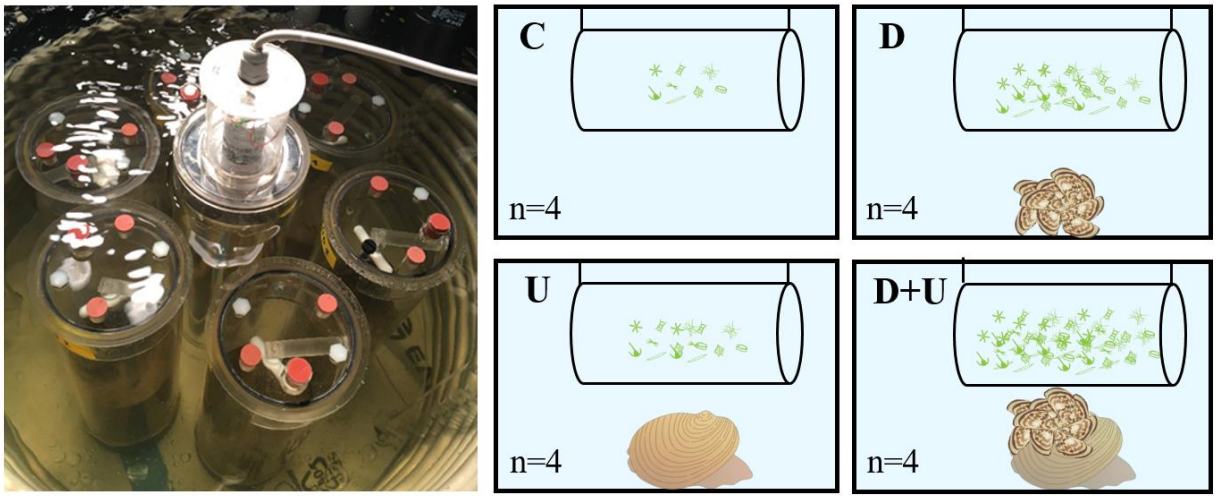



**Figure 2**

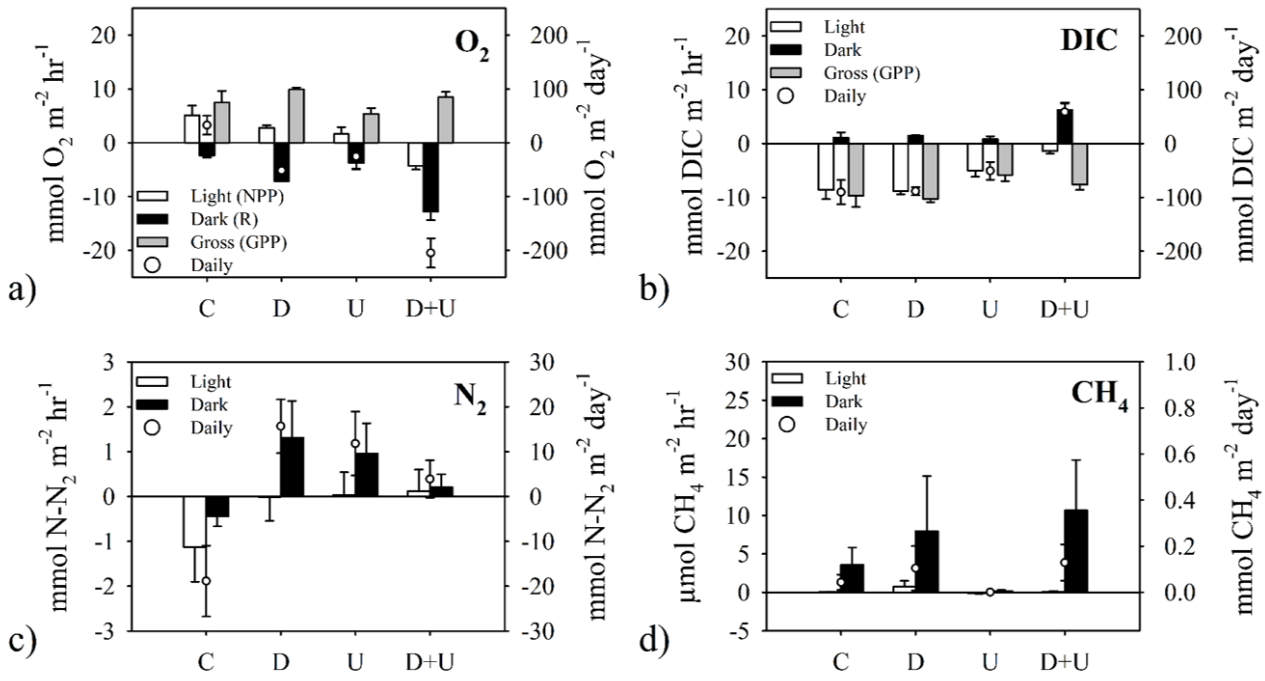


**Figure 3**

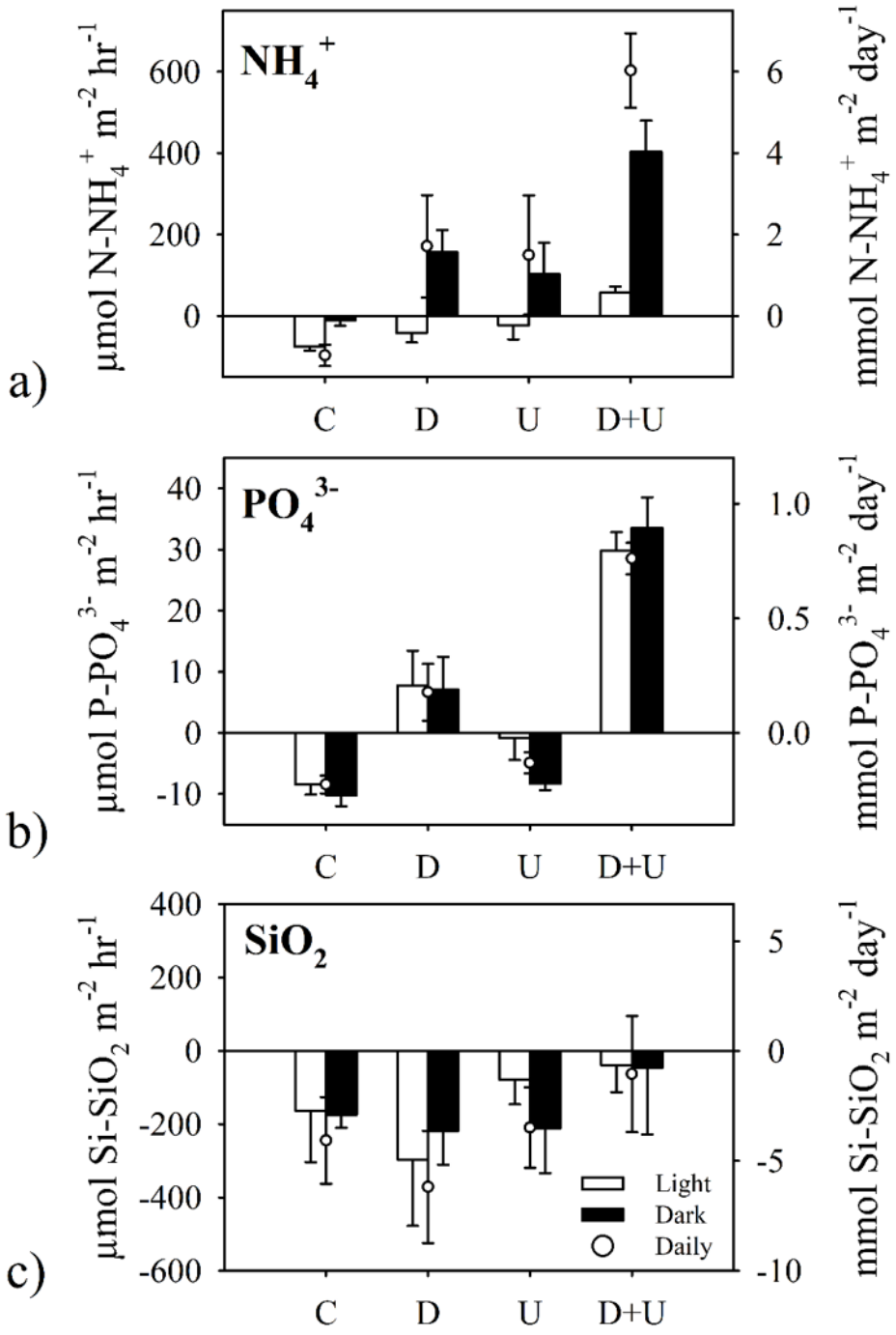

a)
NH4+
µmol N-NH4+ m-2 hr-1
mmol N-NH4+ m-2 day-1
600
400
200
0
6
4
2
0
C
D
U
D+U
b)
PO4 3-
µmol P-PO4 3- m-2 hr-1
mmol P-PO4 3- m-2 day-1
40
30
20
10
0
-10
1.0
0.5
0.0
C
D
U
D+U
c)
SiO2
µmol Si-SiO2 m-2 hr-1
mmol Si-SiO2 m-2 day-1
400
200
0
-200
-400
-600
5
0
-5
-10
Light
Dark
Daily
C
D
U
D+U

**Figure 4**

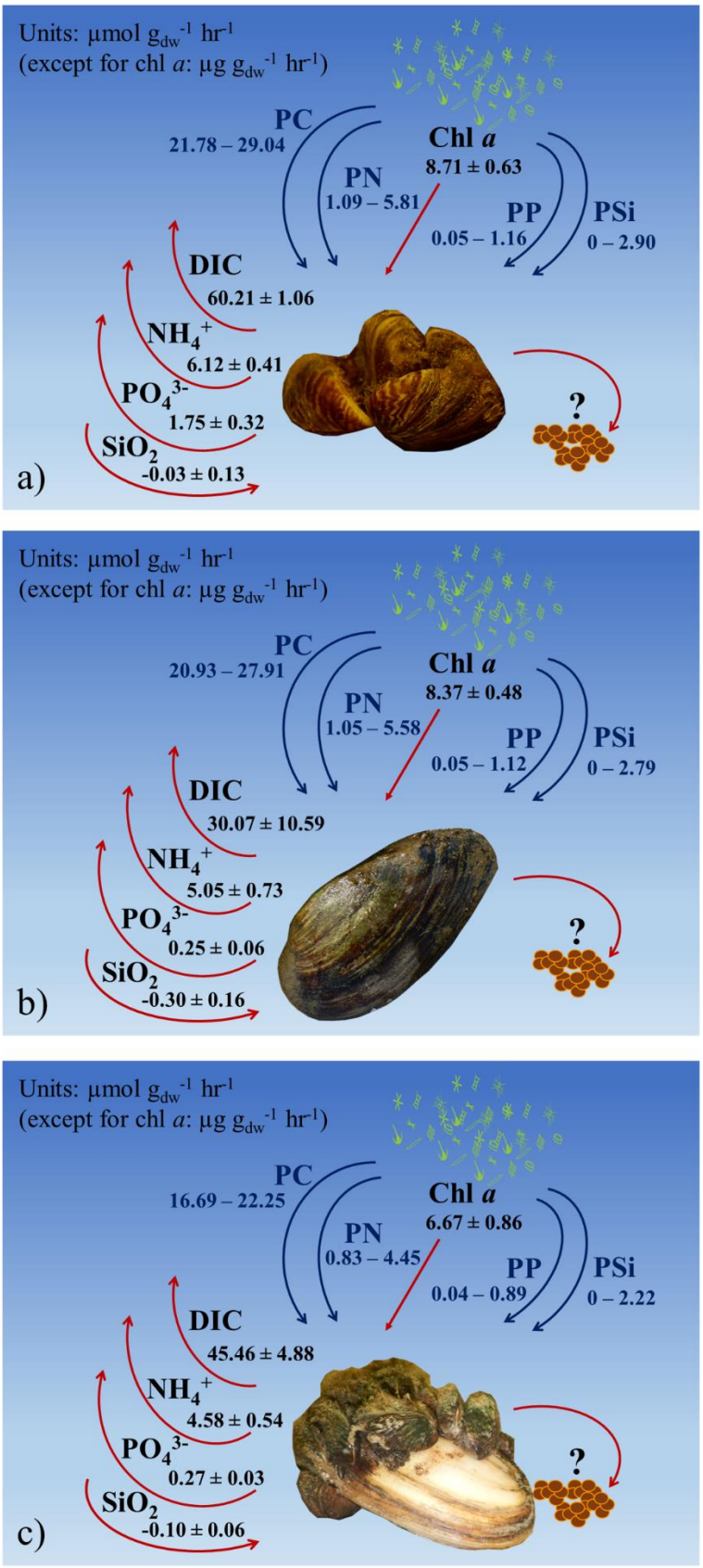
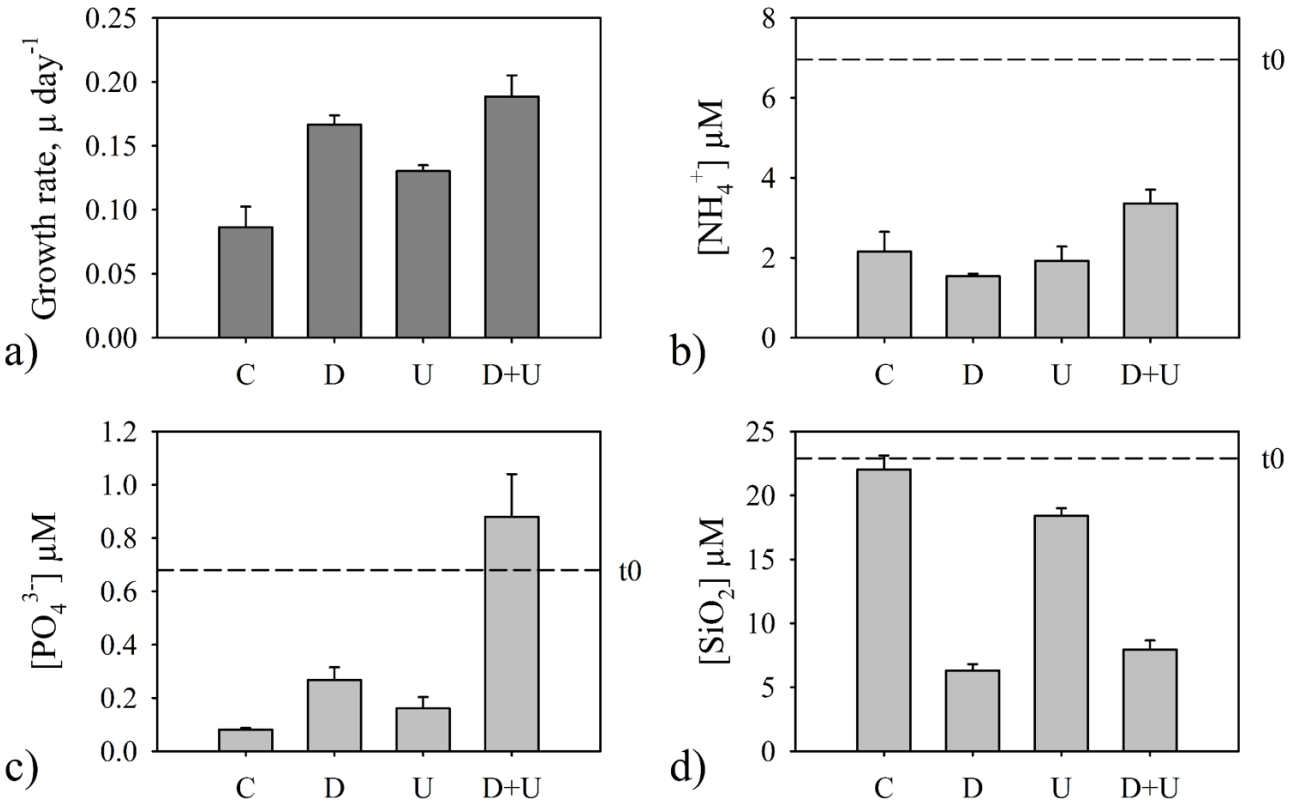

Units: µmol $g_{dw}^{-1}$ $hr^{-1}$
(except for chl *a*: µg $g_{dw}^{-1}$ $hr^{-1}$)
PC
21.78 – 29.04
PN
1.09 – 5.81
Chl *a*
8.71 ± 0.63
PP
0.05 – 1.16
PSi
0 – 2.90
DIC
60.21 ± 1.06
$NH_4^+$
6.12 ± 0.41
$PO_4^{3-}$
1.75 ± 0.32
$SiO_2$
-0.03 ± 0.13
?
a)
Units: µmol $g_{dw}^{-1}$ $hr^{-1}$
(except for chl *a*: µg $g_{dw}^{-1}$ $hr^{-1}$)
PC
20.93 – 27.91
PN
1.05 – 5.58
Chl *a*
8.37 ± 0.48
PP
0.05 – 1.12
PSi
0 – 2.79
DIC
30.07 ± 10.59
$NH_4^+$
5.05 ± 0.73
$PO_4^{3-}$
0.25 ± 0.06
$SiO_2$
-0.30 ± 0.16
?
b)
Units: µmol $g_{dw}^{-1}$ $hr^{-1}$
(except for chl *a*: µg $g_{dw}^{-1}$ $hr^{-1}$)
PC
16.69 – 22.25
PN
0.83 – 4.45
Chl *a*
6.67 ± 0.86
PP
0.04 – 0.89
PSi
0 – 2.22
DIC
45.46 ± 4.88
$NH_4^+$
4.58 ± 0.54
$PO_4^{3-}$
0.27 ± 0.03
$SiO_2$
-0.10 ± 0.06
?
c)

**Figure 5**


a)
Growth rate, μ day-1
0.25
0.20
0.15
0.10
0.05
0.00
C
D
U
D+U
b)
[NH4+] μM
8
6
4
2
0
t0
C
D
U
D+U
c)
[PO43-] μM
1.2
1.0
0.8
0.6
0.4
0.2
0.0
t0
C
D
U
D+U
d)
[SiO2] μM
25
20
15
10
5
0
t0
C
D
U
D+U