Effects of excess phosphate on a coastal plankton community

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Abstract

Eutrophication in the Baltic Sea has caused an imbalance in the inorganic nitrogen (N) to phosphorus (P) ratio, leaving excess phosphate (PO_4) remaining after the phytoplankton spring bloom that terminates after N depletion. Here we investigated the fate of such excess phosphate in a two-week mesocosm (1.2 m^3) experiment. Treatments included a non-treated control (control), nitrate addition (N-add; 3.6 µM), glucose addition (C-add; 25 µM) and combined nitrate and glucose addition (N+C-add). All treatments had 0.66 µM PO_4 at the start of the experiment and were done in triplicates. Addition of N (N-add and N+C-add) stimulated nano- and microphytoplankton, with the picophytoplankton abundance increasing after N depletion. N-fixing cyanobacteria were present but in low abundance. Copepod biomass was positively affected by the N-addition at the end of the experiment. Carbon addition increased heterotrophic bacterial production but without enhancing PO_4 uptake, nor affecting the phyto- or zooplankton community composition. The PO_4 concentration was reduced to ~0.4 µM in the control and C-add treatments and to 0.16 µM in the two N-amended treatments, with an inorganic NP uptake ratio of 7.2. The results underscore the role of picophytoplankton in reducing the excess phosphate pool after the spring bloom, a function traditionally ascribed to bloom-forming diazotrophic cyanobacteria in the Baltic Sea.

Key words: biogeochemical cycles, coastal seas, eutrophication, nutrient stoichiometry, *Nodularia spumigena* and *Aphanizomenon flos-aquae*, Baltic Sea

Introduction

Loss of oxygen, i.e. deoxygenation, in the ocean is an increasing global problem (Keeling et al. 2010) that has implications not only directly for organisms using oxygen in their metabolism but also effects on biogeochemical cycles. There are two primary causes of the deoxygenation of the ocean: global warming and human input of nutrients to marine ecosystems, i.e. eutrophication. Warming of surface waters happens on a global scale and reduces gas solubility including oxygen. Eutrophication is a local or regional environmental problem that causes algal blooms that typically aggravate oxygen consumption in deeper water layers following termination of the bloom, when the biomass is sinking and being decomposed.

In relatively shallow coastal seas, elevated oxygen consumption may cause hypoxic and anoxic conditions on the seafloor, and sediment microbial processes typically reduce inorganic nitrogen through denitrification and anammox (Hannig et al. 2007). At the same time, anoxia increases the release of phosphate through the chemical reduction of iron-bound phosphate. The net result of anoxic bottoms is a decreased bioavailable nitrogen and an increased phosphate concentration, causing the inorganic NP ratio to decrease in several parts of the world (Conley et al. 2002, Hauss et al. 2012, Kalvelage et al. 2013, Meyer et al. 2016).

The Baltic Sea has been affected by eutrophication for several decades, which has increased the area of anoxic seafloor and the release of phosphate (Conley et al. 2002). In the Baltic Proper and Gulf of Finland, the most productive period with accompanying flux of organic material to the sediment is during the spring bloom, which terminates when dissolved inorganic Nitrogen (DIN) has been depleted. There was a regime-like shift in the mid-1990s when the dissolved

inorganic phosphorus (DIP) pools started to be at elevated levels after the spring bloom. Low phosphate concentrations were generally detected during early summer before the mid-1990s, although N-limitation of the spring bloom was demonstrated even then (Tamminen 1995).

Much of the Baltic Sea is nitrogen-limited and there has been a trend of decreasing inorganic NP ratios in several sub-basins caused primarily by a massive release of phosphate from the seafloor after turning anoxic (Conley et al. 2002). This has led to a surplus of phosphate after the spring bloom that may range from 0.1 up to 0.6 µmol PO_4 L^{-1} in the Gulf of Finland and Baltic Proper (Spilling et al. 2018). This excess phosphate is believed to directly benefit N-fixing cyanobacteria that often create toxic or nuisance blooms during the summer (Larsson et al. 2001, Kangro et al. 2007).

Nodularia spumigena and *Aphanizomenon flos-aquae* are amongst the typical dominating filamentous cyanobacteria creating blooms in July and August in large parts of the Baltic Sea. Although these species can take up and store P in the form of polyphosphate, they grow relatively slowly in water temperatures below 15°C (although *A. flos-aquae* is less sensitive to cold water than *N. spumigena*), and typically start growing after phosphate has been already depleted. An alternative pathway of P is going through other primary producers or heterotrophic bacteria (Nausch and Nausch 2004), reducing the available P for the filamentous cyanobacteria. However, the prevailing N-limitation restricts the growth of other phytoplankton and consequently limits their luxury uptake of P. Heterotrophic bacteria may also take up P, but these are typically limited by carbon (Lignell et al. 1992). At present it remains unclear which organisms take up the excess phosphate, as most of it seems to settle out of the euphotic zone before cyanobacterial blooms develop (Nausch et al. 2008) and other P sources must exist for these cyanobacteria (Raateoja et al. 2011), most likely through upwelling (Wasmund et al. 2012).

Here we addressed the fate of this excess phosphate during a mesocosm early summer experiment via additions of bioavailable nitrogen (N), carbon (C) and both (N+C), to stimulate primary producers and heterotrophic bacteria. We hypothesized that picophytoplankton would become relatively more important after N depletion taking up part of the excess phosphate, but that heterotrophic bacteria can also exploit the inorganic P pool when they have access to a labile carbon source.

Materials and methods

The experiment was set up in 1.2 m^3 mesocosm bags that were moored outside the Tvärminne Zoological Station, Gulf of Finland (59° 50’ 40” N, 23° 14’ 57” E). The bags were placed in a row next to a floating platform (Spilling et al. 2022a). In total, 14 bags were installed but the two outermost bags were not used. These ‘dummy’ bags were used to create the same light conditions for all the experimental units. All units were covered to prevent rain or bird droppings to fall into the bags. The experimental setup consisted of an untreated control (control), a treatment with added nitrate (N-add; 3.6 µmol N L^{-1}), a treatment with added carbon in the form of glucose (C-add, 36 µmol C L^{-1}), and a treatment with both nitrate and glucose addition (N+C-add).

The bags were filled by gentle pumping in surface water on 7th June 2021, which was day -1 of the experiment. This filling method had visually (microscope) been tested and found not to affect zooplankton but prevents small fish from entering the mesocosms. The following day (day 0),

nutrients and carbon sources were added in the morning and the first full sampling took place right after the addition. The temperature was approximately 18 and the salinity was 5.5 at the start of the experiment. Water samples were taken daily with a Limnos water sampler (Hydro-Bios, Germany) from 1.5 m depth from the middle of the bags. CTD profiles were taken daily from the bags and outside the experimental units, and we had temperature and dissolved oxygen loggers (HOBO U26; Onset Inc. USA) mounted inside each mesocosm bag. In addition to minor sampling every day (e.g. CTD profile), the regular sampling was carried out on days 0, 1, 3, 6, 8,10, 13 and 15.

Total chlorophyll a (Chla) fluorescence was determined from each mesocosm bag by daily use of a handheld fluorometer (AquaPen, Photon Systems Instruments, Czech Republic), and direct Chla measurements were carried out by filtration in duplicates on regular sampling days. For Chla GF/F filters (Whatman) were used and the Chla was extracted in EtOH, stored at -20°C and in the dark for up to two months, and measured with a spectrofluorometer (Varian Cary Eclipse) with excitation and emission wavelengths of 430 and 670 nm, respectively (Jespersen and Christoffersen 1987).

We used standard colorimetric methods (Grasshoff et al. 1999) using a photometric analyzer (Aquakem 250, Thermo Scientific, USA) to measure dissolved inorganic nutrients: nitrite + nitrate (NO_2 + NO_3), phosphate (PO_4) and dissolved silicate (Dsi). Ammonium (NH_4) was measured separately using a spectrophotometer (Hitachi U-1100).

Particulate organic carbon (POC), nitrogen (PON) and phosphorus (POP) were determined from duplicate water samples filtered onto acid-washed (2M HCl for 15 min, then rinsed carefully with ultrapure water) and pre-combusted (450°C, 4 h) GF/F filters (Whatman, UK). POC and

PON were measured with an element analyzer coupled with a mass spectrometer (Europa Scientific ANCA-MS 20-20 15N/13C). POP was determined according to (Solórzano and Sharp 1980) and modified by Koistinen et al. (2017). Samples for biogenic silicate (Bsi) were filtered on polycarbonate membrane filters (0.8 µm, GVS, Italy) and measured according to Koistinen et al. (2018).

Flow cytometer counts were done daily with a Sysmex (Japan), Partec – Cube 8 equipped with two lasers (488 and 561 nm), two scattering (forward and side) and three fluorescence detectors (610/30; 661/16; and 670/40, corresponding to the detection of phycoerythrin, phycocyanin, and Chla, respectively). The trigger was set on Chla fluorescence (670 nm). On each sampling day, we made several measurement-runs with beads and blanks to ensure correct particle counts. Size fractionations (0.8, 1, 2, 5, 10, 20 µm filters) were also used to identify the approximate size of different phytoplankton groups. The scattering and fluorescence properties were used to gate different phytoplankton groups using the FCS Express 6 software. Five groups were identified: picoeucaryotes: size <2 µm with only Chla, *Synechococcus*-like cells <2 µm with phycocyanin fluorescence, Cryptophyte-like >2 µm with Chla and phycoerythrin fluorescence, nanophytoplankton size 2–20 µm and microphytoplankton: size >20µm with only Chla fluorescence.

Zooplankton was collected three times, at the start, middle and end of the experiment. Samples were taken with a plankton net (25 cm diameter, 50 µm mesh size) carefully dragged from the bottom of the mesocosm bags to the surface. Samples were immediately preserved with acid Lugol's solution and stored at 4°C. Zooplankton was identified to genus level from images created using a flatbed scanner and analyzed at 20 x magnification.

Data analysis

A one-way analysis of variance (ANOVA) was performed to test whether significant effects could be revealed by the treatments. Tukey's Honest Significant Difference (HSD) post hoc test was performed to compare different treatments and assess the significance of differences between them. All statistical tests were performed using the SigmaPlot 15 software.

Results

There was a clear effect of N addition, but no effect of carbon addition, on the phosphate concentration (Fig 1). The phosphate concentration decreased from ~0.66 µmol L^{-1} at the start of the experiment to ~0.16 µmol L^{-1} in the two treatments with N added on Day 8, and in the control and C-addition, the PO_4 concentration was 0.33 and 0.36 µmol PO_4 L^{-1}, respectively, at the end of the experiment. There was residual ammonium with a concentration in the range of 0.15 to 0.35 µmol L^{-1} throughout the entire experiment. Dissolved silicate was constant in the control and C-add but decreased slightly towards the end of the experiment in the N-add and N+C add treatments, with an uptake of approximately 1.9 µmol L^{-1} (Fig 1).

The phytoplankton biomass was declining from the onset of the experiment in the control and C-add treatments but with a delay of two days in the two treatments with added N (Fig 1). The initial Chla concentration was 3.5 µg L^{-1} that decreased to 1–2 µg Chla L^{-1} and within two days in both control and C-add treatments (Fig 2). After reaching a minimum of <1 µg L^{-1}, the Chla concentration increased again at the end of the experiment to ~2 µg Chla L^{-1}. The nitrate added to the N-add and N+C-add treatments was depleted already on day 3.

Heterotrophic bacteria had the same dynamics in all treatments (Fig 2); their abundance increased after day 1, with a peak at day 8. The overall bacterial abundance was higher in the N-add treatment compared to the control and C-add treatments (Tukey: $p = 0.001$ and $p = 0.003$ respectively), the rest of the treatments were similar ($p > 0.14$).

All particulate nutrients decreased in the experimental units during the first week, but the decrease was more rapid in treatments without any nitrate addition (control and C-add), except for biogenic silicate (BSi) where the decrease was similar in all treatments (Fig 3). Stoichiometric ratios were relatively stable with an average C:N:P ratio of 140:14:1, and without any differences between treatments ($p > 0.1$).

The temperature and salinity throughout the experiment is presented in Supplementary Figs S1 and S2. There was a drop in temperature after day 6 from ~18□ to ~14□ due to upwelling, but this increased again towards the end of the experiment to ~20□. Salinity was constant in the bags and there was no sign of mixing with the outside water.

Community

Nitrate additions benefited micro- and nanophytoplankton that increased in abundance in the N-add and N+C add treatments (Fig 4), contributing to the high Chla concentration over the first three days. This was also evident from the FlowCam images, where, *e.g.* the cell concentration and total biovolume of dinoflagellates >10 µm increased in these two treatments until nitrate had been depleted (Fig 5). The green algae *Monoraphidium* sp. (nanophytoplankton) increased also in the N-amended treatments, but kept growing after N depletion, reaching a maximum biovolume at experiment day 8 (Fig 5).

After nitrate depletion, picoeukaryotes started to increase in abundance, especially in the control and N-add treatment, but also, to a lesser extent, in the N+C-add and C-add treatments (Fig 4). *Synechococcus*-like cells decreased in all treatments after the start of the experiment and stayed at a low abundance until the last few days when it started to increase in all treatments, with the highest increase in the N-add treatment (Fig 3). *Planktothrix* sp., *Pseudoanabaena* sp. and *Cyanobium* sp. were the main component of colonial and filamentous cyanobacteria. They peaked in the N-add and N+C-add treatments on day 3 and 6, respectively, with the highest peak in biovolume in the N+C-add treatment (Fig 6).

Microzooplankton consisted of heterotrophic dinoflagellates and ciliates (Fig 5). Heterotrophic dinoflagellates increased in the N-add and N+C-add treatments with a peak on day 8 before declining to reach similar biovolume as in the control and the C-add treatments at the end of the experiment (Fig 6). The biovolume of ciliates was highly variable between replicates and - although the average biovolume was higher in the two N add treatments, there was no consistent difference between treatments (ANOVA, $p = 0.767$).

The mesozooplankton community was dominated by copepods, cladocerans and pelagic stages of *Amphibalanus* sp. (Fig 7). The number of copepods was highest in N-add and N+C-add treatments towards the end of the experiment including both copepodite and nauplii stages ($p = 0.024$ and $p = 0.017$, respectively). Among cladocerans, the abundance of *Bosmina* sp. was positively affected by the nitrate addition ($p = 0.04$), whereas *Podon* sp. (Fig 6) and *Evadne* sp. (data not shown) both decreased over time and had completely disappeared in all treatments at the end of the experiment. The difference in *Amphibalanus* nauplii abundance between treatments was not clear ($p = 0.06$), but cypris larvae only developed in the two N-amended treatments at the end of the experiment (Fig 7)

Discussion

Inorganic nutrients initially stimulated nano- and microphytoplankton. This was expected, as they have a larger volume for internal storage, i.e. luxury uptake. Nonetheless, this effect only delayed the gradual decline of phytoplankton biomass. The main driver for the differences between treatments was the addition of nitrate, whereas the carbon addition did not affect the community of the larger phytoplankton or zooplankton. After nitrate had been depleted, there was a clear increase in picophytoplankton abundance, which was also observed in previous mesocosm experiments in this area (Crawfurd et al 2017, Spilling et al. 2022b). Smaller cells have a larger surface-to-volume area and are better adapted to low inorganic nutrient concentrations, which is the likely reason for this group to be dominating during periods when easily available nutrient sources have been depleted by larger cells. Often, a surplus of P would benefit nitrogen-fixing cyanobacteria, especially if they have been able to increase their biomass during the nitrate depletion phase. Although cyanobacteria were present in all treatments, there was no indication that the typical bloom-forming diazotrophic species proliferated. Rather, the additional P uptake after N depletion seems to have been driven by the increase in picoeukaryotes.

Interestingly, carbon addition had a negative effect on the abundance of picoeukaryotes. This could have been due to competition for nutrients with heterotrophic bacteria, which likely got a boost due to the sudden, easily available carbon sources. This, however, is not evidenced by the abundance of heterotrophic bacteria that remained similar between control and C-add treatment.

This could be different for particle-attached heterotrophic bacteria, but unfortunately no data is available for this group. Alternatively, grazing pressure could have played a role, but no noticeable difference in microzooplankton abundance was observed.

The addition of glucose did stimulate bacterial productivity and extracellular activity (Vanharanta ref), but there was no indication of any bacterial drawdown of phosphate when compared to the control. Also, in this respect, the combined N+C addition was similar to the N addition. This indicates that the majority of phosphate uptake was due to picoeukaryotes with little to no contribution of heterotrophic bacteria. In the N-add and N+C-add treatments, ~75% of the phosphate was removed, whereas in the control and C-add treatments ~ 40% was removed within a week. The decrease in the phosphate pool was not seen as an increase in the particulate phosphorus fraction although the N-amended treatments had a higher concentration of particulate organic elements. This was likely due to export of particulate organic matter to the bottom of the mesocosm bags.

The mesocosm bags prevent effective wind-driven mixing, although there was some temperature driven advection. There was particulate organic matter sedimentation during the experiment, but unfortunately, we could not sample the bottom part of the mesocosms to quantify it. This export loss might have had some consequences for the phytoplankton community composition as non-motile groups, such as diatoms, likely sedimented out quicker than motile flagellates. This could be deduced from the decrease in BSi over time, and diatoms were a minor group in terms of biovolume. The bags were not very deep and the available organic material in the bottom likely supported the community of mesozooplankton that could easily move vertically between different water depths, especially since higher predators were absent in the mesocosms. This

could be a likely reason for a treatment effect on mesozooplankton even though the available, suspended food items were similar in all bags after the drawdown of nitrate.

In a recent study, using smaller 20 L tanks, we found that the excess phosphate had been depleted completely under N-deficiency similar to this experiment, even without N addition (Vanharanta and Spilling 2023). This previous experiment had a longer duration (35 d) than the one presented here, with drawdown of phosphate in the control units of 20% to 50 % (from a starting concentration of 0.55 µM) after 15 days, depending on the temperature (Vanharanta and Spilling 2023), and it is likely that we would have also seen a complete drawdown of phosphate, if the experiment would have lasted a few weeks more. Likely, phytoplankton and most notably picoeukaryotes and cyanobacteria were taking up phosphate and storing part of the excess phosphorus as polyphosphate (Jentsch et al. 2023). This was also seen under the microscope, but we have no quantitative data on polyphosphate storage. Approximately 15% of the phosphate pool, or 20–30% of the PO_4 taken up was released into the dissolved organic phosphorus (DOP) pool, but DOP concentration decreased again during the last week of the experiment suggesting the added DOP was relatively labile and microbially available.

One of the most prominent effects of eutrophication in the Baltic Sea is the intensifying occurrence of diazotrophic cyanobacterial blooms during summer. The prevailing N-limitation leads to a low inorganic N:P ratio, which favors the N-fixing cyanobacteria during warm, calm summer months (Niemi 1979, Wasmund et al. 2005). These cyanobacterial blooms amplify the eutrophication problem as the amount of cyanobacterial fixed N is comparable to the anthropogenic N-loading in parts of the Baltic Sea (Savchuk 2005, Wasmund et al. 2005). The cyanobacterial input of N might thus have a self-enforcing nature through a positive feedback

loop: N-fixation increases the biomass load reaching the sediment, increasing oxygen consumption, and adding more P to the ecosystem (Vahtera et al. 2007a, Spilling et al 2018).

The two main cyanobacterial species: *Aphanizomenon flos-aquae* and *Nodularia spumigena* have different P uptake strategies. *A. flos-aquae* requires inorganic phosphate and can take up and store excess P for later growth, whereas *N. spumigena* has a higher affinity for phosphate and, thus, relies more on recycled P (Hagström et al. 2001, Vahtera et al. 2007). Both species were present in the experiment but at exceptionally low concentrations. It was other filamentous cyanobacteria (*Planktothrix* sp, and *Pseudoanabaena* sp) that made up the bulk of the measured cyanobacterial biovolume. On the one hand, this shift in cyanobacterial community composition could be due to an enclosure effect, as there are examples of different primary producers being negatively affected in a mesocosm setup. On the other hand, both. *A. flos-aquae* and *N. spumigena* have relatively low growth rates, and it is likely that they did not grow fast enough in the experiment to increase much biomass in the relatively short time span of the experiment.

A decreasing inorganic N:P ratio resulting from reduced O_2 concentrations can be observed in different ecosystems. Short-term shifts are to some extent dependent on the plankton community composition (Spilling et al. 2019, Bach et al. 2020). However, potential long-term consequences of this phenomenon are little known. A decreasing NP ratio in marine ecosystems is not an universal process, however, as there are examples of other processes that can increase this ratio, for example, more effective P-removal from wastewater, increased use of N-rich fertilizer, or increased atmospheric N-deposition from anthropogenic sources (McQuatters-Gollop et al. 2007, Kim et al. 2014).

In conclusion, we found that N-addition stimulated both nano- and microphytoplankton, and the picophytoplankton abundance increased following nitrate depletion. Based on these results, , we

conclude that it was the picophytoplankton that incorporated substantial parts of the excess P, confirming our initial hypothesis. Yet, it was unable to completely deplete the inorganic P-pool, and most of the organic P formed was exported. At this point, we are missing direct evidence and measurements of excess phosphate uptake, e.g. using radiolabeled P. Carbon addition did stimulate heterotrophic bacteria production, but did not increase excess phosphate uptake, suggesting there is available organic phosphorus that is available for heterotrophic bacteria. Heterotrophic bacterial uptake of excess phosphate could still take place under specific environmental conditions. Though the typical bloom-forming cyanobacteria in the Baltic Sea, which are considered to greatly benefit from excess phosphate, were not abundant in our mesocosms, our results demonstrate that other groups, most notably picophytoplankton, are also active in the uptake and processing of this bioavailable P-pool. Luxury uptake of P early in the season can fuel the N-fixing cyanobacteria blooms later in summer. Yet, it is likely not as prevalent as often thought, suggesting other sources of P is available for these blooms. The results here underscore the role of picophytoplankton in reducing the excess phosphate pool after the spring bloom, a function traditionally ascribed to bloom-forming diazotrophic cyanobacteria in the Baltic Sea.

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Figure legend

Fig 1. The concentration of nitrite + nitrate (NO2 + NO3), phosphate (PO4), ammonium (NH4) and dissolved silicate (DSi) plotted against time. The error bares represent the standard error (SE, n = 3).

Fig 2. The concentration of Chlorophyll a (Chla) and abundance of heterotrophic bacteria plotted against time. The error bares represent the standard error (SE, n = 3).

Fig 3. The concentration of particulate organic carbon (POC), nitrogen (PON), phosphate (POP), and biogenic silicate (BSi) plotted against time. The error bares represent the standard error (SE, n = 3).

Fig 4. The abundance of *Synechoccoccus*-like cells, picophyto(?)eukaryotes, nano- and microphytoplankton plotted against time. The error bares represent the standard error (SE, n = 3).

Fig 5. The sum of all biovolume measurements throughout the experiment of different nano- and microplankton groups. The presented p-value is an ANOVA statistical test comparing the different treatments. The temporal development in biovolume is presented in Fig 6.

Fig 6. The biovolume of different nano- and microplankton groups plotted against time. The error bars represent the standard error (SE, n = 3).

Fig 7. The abundance of different mesozooplankton groups plotted against time. The error bares represent the standard error (SE, n = 3).

Supplementary Figures

FigS1. The temperature in the surface water (10 cm depth) throughout the experiment.

Fig S2. Salinity measurements taken from individual mesocosms and outside the mesocosms.

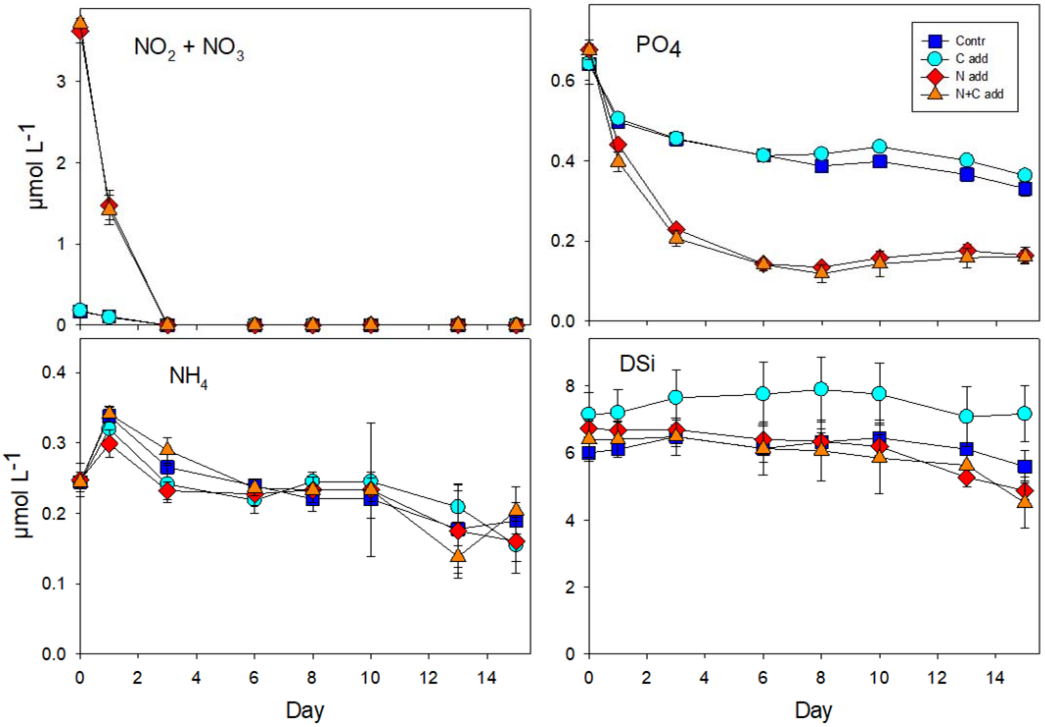


Fig 1

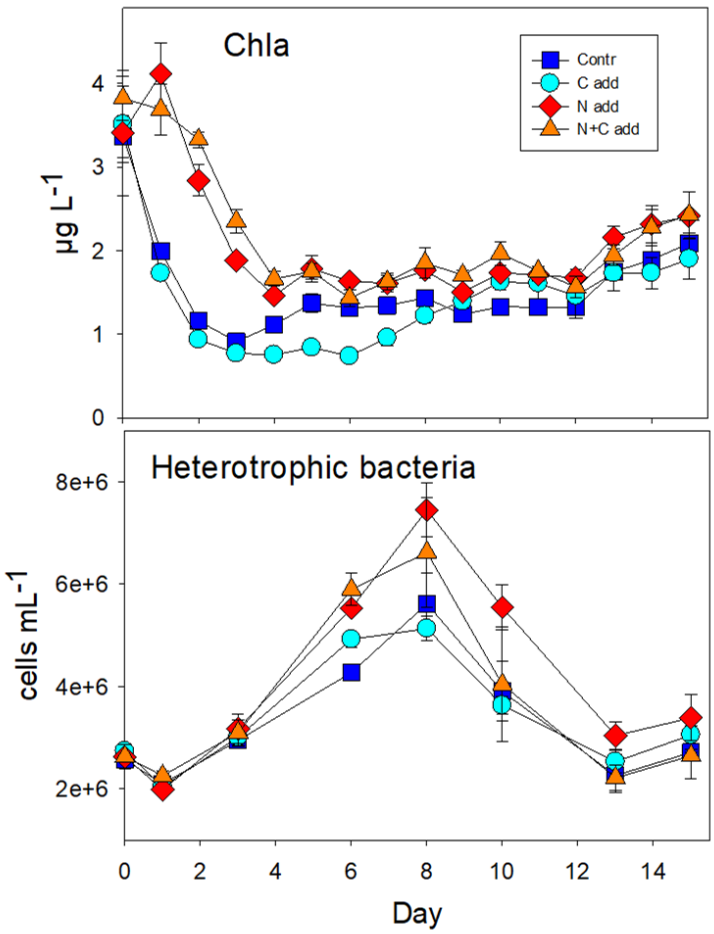

Chla
Contr
C add
N add
N+C add
µg L-1
0
1
2
3
4
Heterotrophic bacteria
cells mL-1
2e+6
4e+6
6e+6
8e+6
0
2
4
6
8
10
12
14
Day

Fig 2

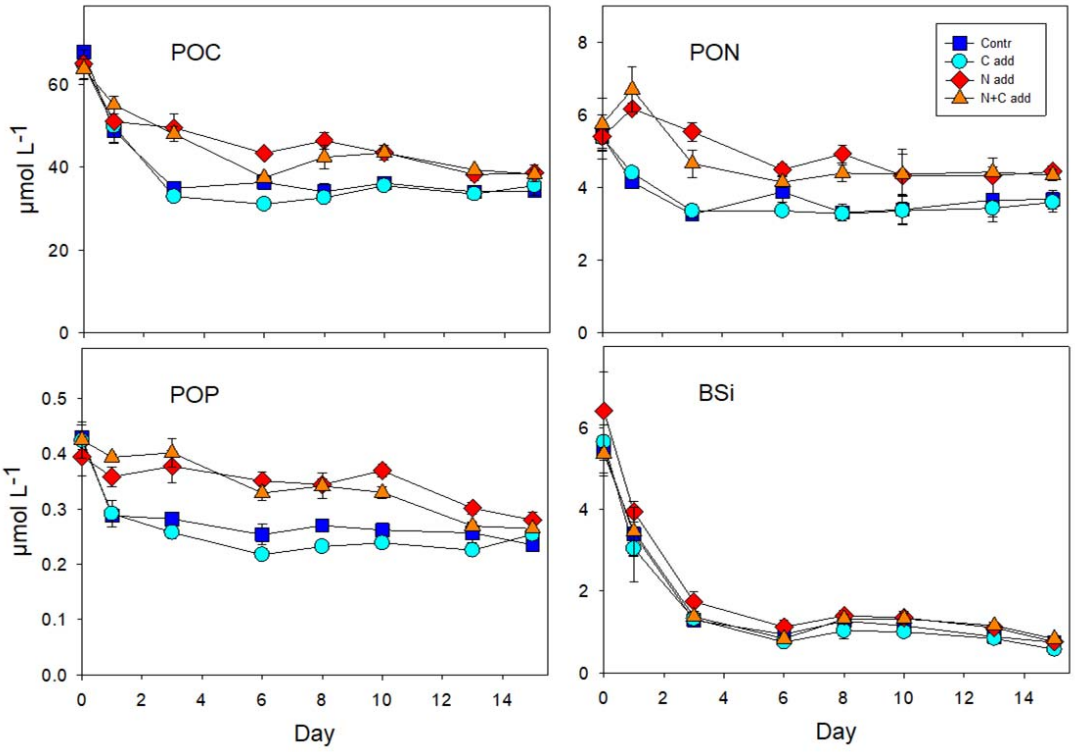


FIG 3

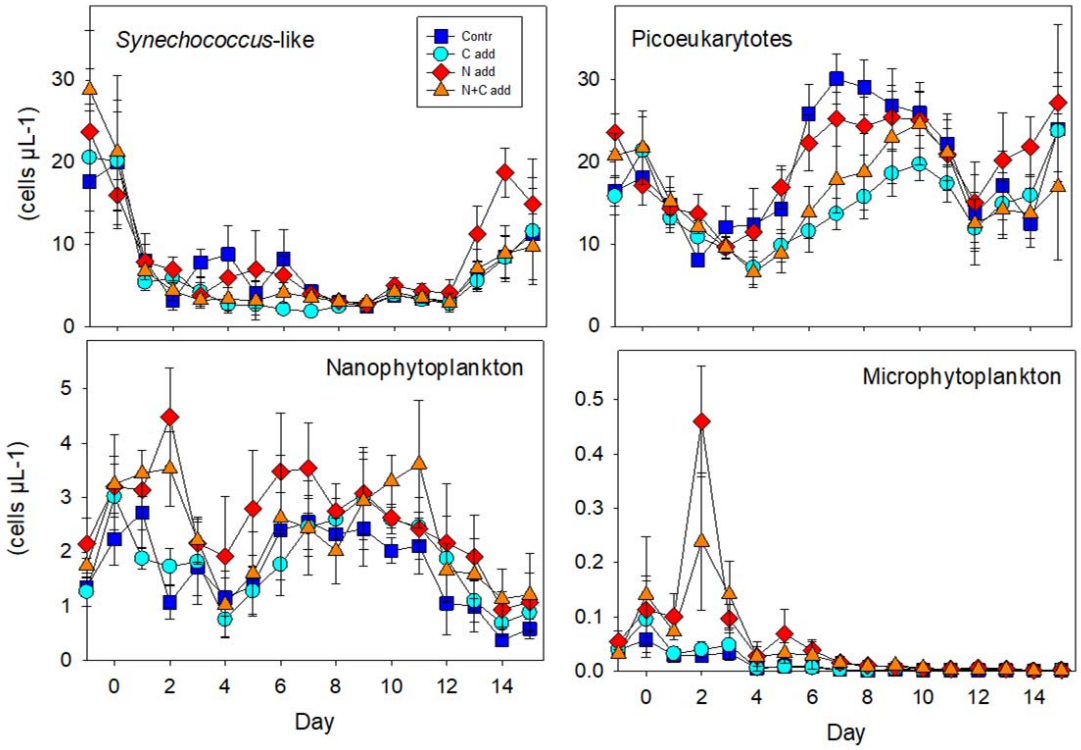

Synechococcus-like
Contr
C add
N add
N+C add
Picoeukarytotes
Nanophytoplankton
Microphytoplankton
(cells µL-1)
(cells µL-1)
Day
Day

FIG 4

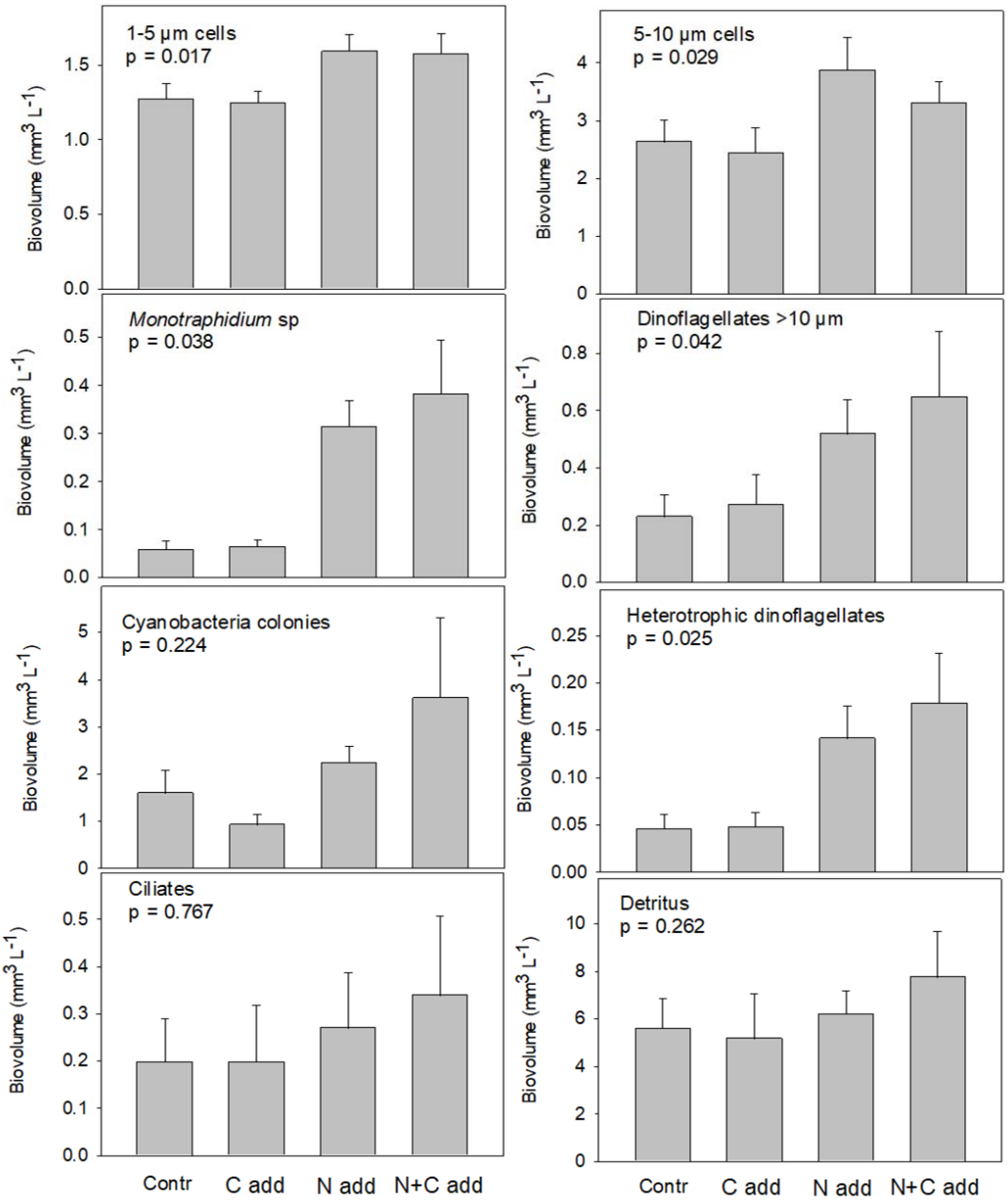
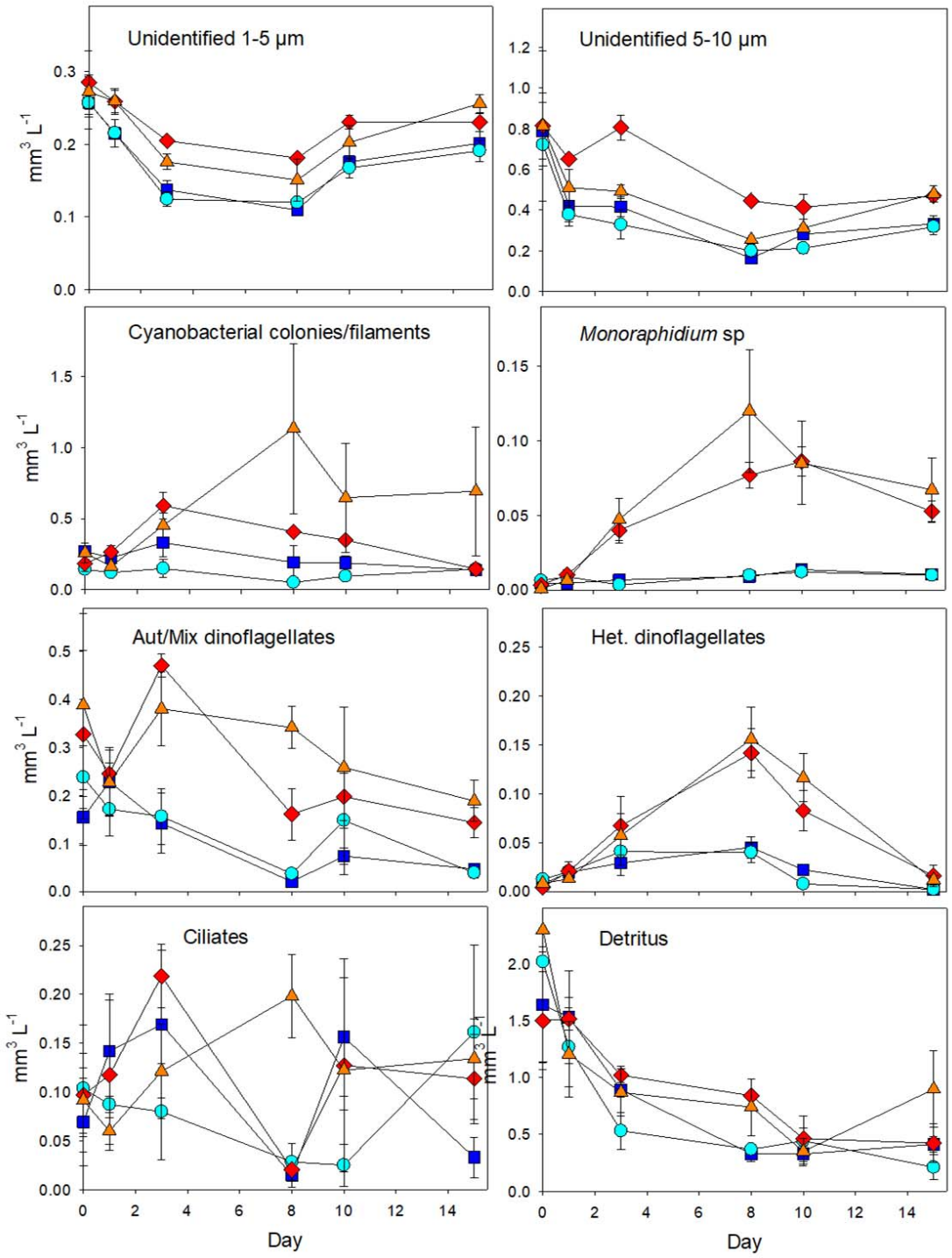

1-5 µm cells
p = 0.017
5-10 µm cells
p = 0.029
Monotraphidium sp
p = 0.038
Dinoflagellates >10 µm
p = 0.042
Cyanobacteria colonies
p = 0.224
Heterotrophic dinoflagellates
p = 0.025
Ciliates
p = 0.767
Detritus
p = 0.262
Biovolume (mm3 L-1)
Contr
C add
N add
N+C add

FIG 5



508

509 Fig 6

510

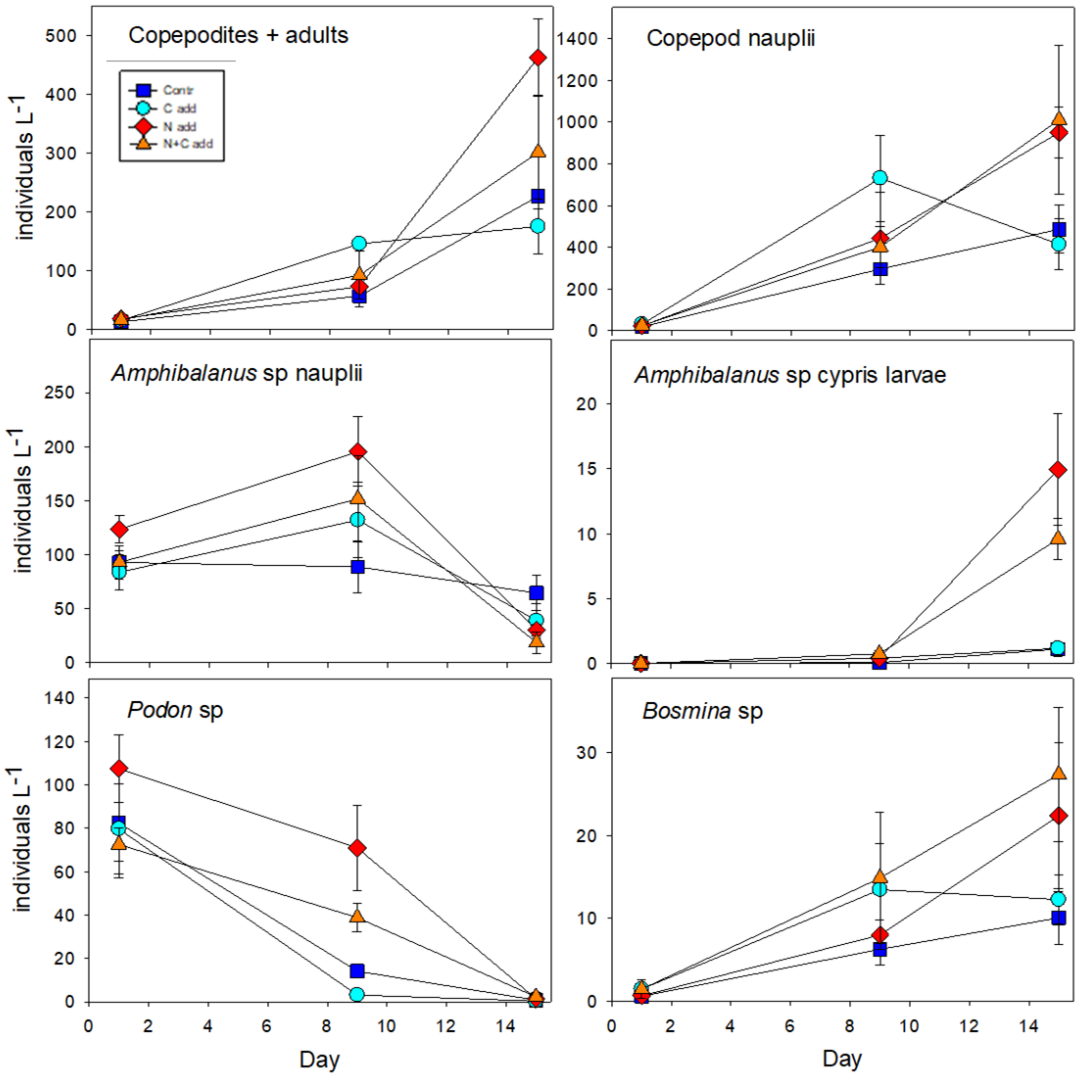
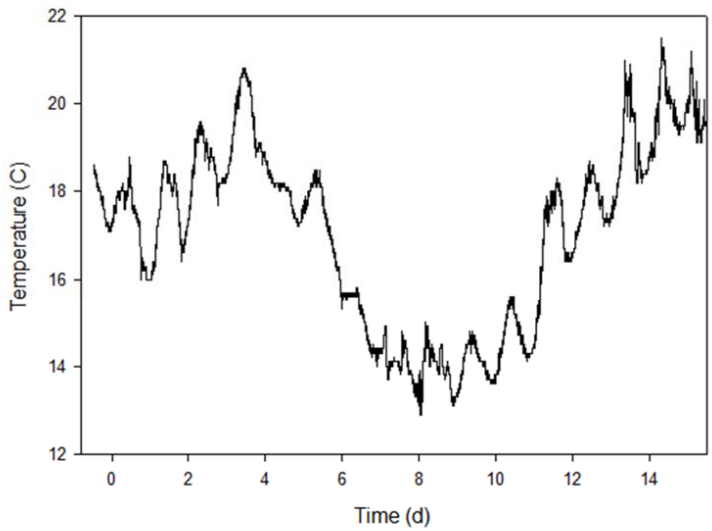

Copepodites + adults
Contr
C add
N add
N+C add
Copepod nauplii
Amphibalanus sp nauplii
Amphibalanus sp cypris larvae
Podon sp
Bosmina sp
individuals L-1
Day

FIG 7



SUPPLEMENTARY FIG S1

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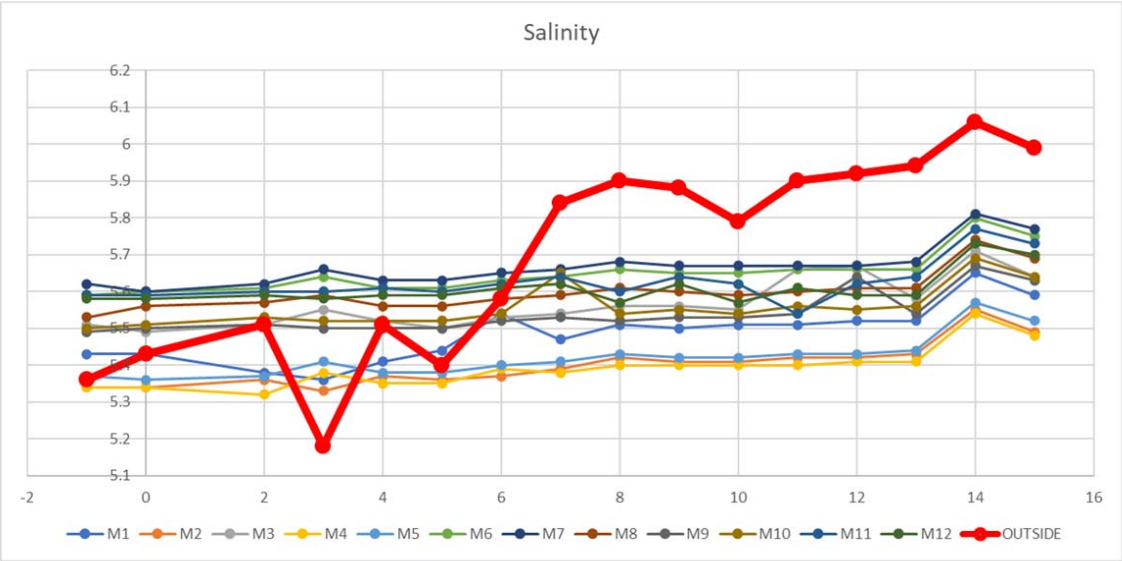

Supplementary Fig S2