Inland Waters

Shallow water phytoplankton responses to nitrate and salinity enrichment may be modified by benthic processes

--Manuscript Draft--

Full Title:	Shallow water phytoplankton responses to nitrate and salinity enrichment may be modified by benthic processes
Manuscript Number:	TINW-2018-0054R2
Article Type:	Special Issue
Keywords:	Phytoplankton; shallow lakes; chlorophyll and carotenoid pigments; mesocosm experiments
Abstract:	The Instuctions to authors indicate that "A non-structured abstract of no more than 250 words." is required. The box here indicates that is should be 200 words. Ours is currently 248 words- we can edit if necessary, but some unambiguous guidance is required
Order of Authors:	Suzanne McGowan
	Peter Leavitt
	Tom B Barker
	Brian Moss
Response to Reviewers:	Authors' Responses Thank you for spotting the issues below. We have amended the manuscript as suggested with the details of the revision documented below: I thank the authors for improving their manuscript by taking the reviewers' comments into account. There are some minor details that need to be attended to before the manuscript can be accepted but these should be quick and easy to do. 1.You responded to Reviewer 1 comment 4 about allocating the different pigment concentrations to different phytoplankton groups. Can you please add some of this text to the revised manuscript. > We added a paragraph in the methods starting line 196: We selected five of the most common pigments as biomarkers of the main phytoplankton taxa: siliceous algae (fucoxanthin), chlorophytes (Chl b), cryptophytes (alloxanthin), total cyanobacteria (echinenone) and all phytoplankton (Chl a; an estimate of total phytoplankton; algae + cyanobacteria). Given the history of Prymnesium parvum blooms at this site, we specifically searched chromatograms for the diagnostic pigment 19'-butanoyloxyfucoxanthin, which should have been a marker for P. parvum blooms. This pigment was not detected and so, we infer that siliceous algae (i.e. diatoms + synurophytes) and not haptophytes were the primary algae producing fucoxanthin here. We assigned Chl b as a biomarker of chlorophytes because, although it may be produced by euglenophytes, they are rare in Hickling Broad (Bales et al. 1993). We also designated echinenone as a biomarker of cyanobacteria because it is produced in only trace amounts in other taxa (chlorophytes and euglenophytes). 2.L27 Spell out RM-ANOVA.- done 3.L34 Insert 'an' before 'elevated'- done 4.L38 'Together,…'- added a comma 5.L47 What is the distinction between 'lotic' and 'surface'?- we have deleted surface 6.L51- 54 I think it would be easier to read if you started with nitrogen then go on to salinity. The order has been reversed 7.L83- 'influence the bioavailability…' added THE 8.L84 Delete comma after 'sediments'. done 9.L134 There is still a problem with the volume and/or diameter. A 3 m diameter cylinder with 1.2 m depth would have a volume of over 8 m3 and even if only 1 m of water it would be 7 m3. Moran et al 2010 quote a dimeter of 2 m (unlike Barker which gives 3 m). A 2 m diameter cylinder with 1m m of water would equate to 3 m3, so I suspect this is correct. Please check.

	Tom has double-checked and we discovered that the confusion arises from a mistake in Barker et al 08a where the diameter is wrongly given as 3m. In fact, the diameter is 2m as previously stated and, while they were 1.2 m deep when empty, there was 27 cm of sediment (when settled) so assuming 0.93 m of water (water level varied a bit), that would make each tank water volume about 2.92 m3. So we are suggesting we leave the rounded up number at 3m3 (given some seasonal variability), and amend the tank diameter to 2m. The text is now: “48 tanks of 2m diameter and 1.2 m depth containing 3 m3 of water” 10.L134 and throughout- please check there is always a space between a number and its unit. Done 11.L162 add space between P and L-1. 12.L170 Apart from here the growing season is defined as April to August. Here is it March to August- if this is correct simply give these months and stick to growing season April to August elsewhere. We have been consistent with April-August throughout for the growth season definition. 13.L176 ‘…extending through the…’- amended 14.L187 Give the name of the filer and manufacturer- e.g. What GF/C. You may also want to acknowledge that with glass-fibre filters the pore sizes are only nominal. Amended to: Whatman GF/C (ca. 1.2-µm pore size) glass-fibre filters 15.L267 Insert ‘mg’ between ‘1’ and ‘NO3’. Done 16.L311 Suggest you delete the values in parentheses as these are already given in the Table. Done 17.L379 Delete ‘the’. Done 18.L384 Instead of ‘manipulation’ can you be more specific- e.g. ‘increases’? Changed to increases in salinity 19.L415 Is this VPA or RDA or maybe Table 4 instead of Figure 4? Yes sorry it was RDA 20.L430- Be more specific about what you mean by ‘prefer’. Changed to- each of which grows more efficiently at NH4 concentrations… 21.L458 Brian would have wanted me to ask you to unsplit your infinitive! Yes he would! I got rid of it entirely…changed to….”Although further work is required to identify the direct (planktonic) and indirect (via benthos) mechanisms of nitrate and salinity on phytoplankton assemblages” 22.Table 3. Add that significant p values are shown in bold. Done 23.Fig 2 legend. Salinity (S) treatments and later Nitrate (N) treatments…Done 24.Fig. 3 legend I suggest you repeat the description of the S and N treatments. Done 25.Fig. 2 Can the dashed vertical line be edited so it does not interfere with the panel labels? Figure 2 has been amended

Author bio for McGowan et al: Shallow water phytoplankton responses to nitrate and salinity enrichment are modified by benthic processes

Suzanne McGowan is a Professor of Freshwater Sciences at the University of Nottingham and is fortunate to have been given the best start in her career as a PhD student of Brian Moss. She studies human and climate impacts on freshwater ecosystems and specialises in the use of algal pigment biomarkers.

Peter Leavitt is a Canada Research Chair in Environmental Change and Society in Canada, and a Professor at the Institute of Global Food Security in Northern Ireland. His research investigates the direct, reciprocal and interactive effects of climate and humans on surface waters.

Tom Barker an ecologist specialising in lake restoration at the University of Liverpool and elsewhere. He was Head of Education at the Centre for Alternative Technology Graduate School, Wales, and has taught and published in ecology and sustainability.

Brian Moss, inspirational freshwater ecologist. We are very proud to make this posthumous contribution on his behalf. Amongst many other achievements, Brian pioneered the use of experimental pond mesocosms to revolutionise understanding of shallow lake ecosystems.

Shallow water phytoplankton responses to nitrate and salinity enrichment may be modified by benthic processes

Suzanne M[c]Gowan[1], Peter R. Leavitt[2,3], Tom Barker[4] & Brian Moss[4]*

1. School of Geography, University of Nottingham, University Park, Nottingham, NG7 2RD, UK. Suzanne.mcgowan@nottingham.ac.uk Tel: +44 (0)1159 951 5451; Fax: +44 (0)1159 951 5249
2. Institute of Environmental Change and Society, University of Regina, Regina, Saskatchewan, S4S 0A2, Canada.
3. Institute of Global Food Security, Queen's University Belfast, BT9 7BL, UK
4. School of Environmental Sciences, University of Liverpool, L69 3BX, UK.

*Corresponding author

Keywords: phytoplankton, shallow lakes, chlorophyll and carotenoid pigments, mesocosm experiments

Abstract

The effects of salinity (600, 1000, 1600 and 2500 mg Cl L^{-1}) and nitrate (loading rates of 1, 2, 5 and 10 mg N L^{-1}) additions on phytoplankton communities (as chlorophyll and carotenoid pigments) were determined using a fully factorial 3 m^3 mesocosm pond experiment. Redundancy analysis followed by variance partitioning analysis (VPA) statistically compared phytoplankton with water chemistry, zooplankton, phytobenthos (aquatic plants and periphyton) and zoobenthos to understand relationships among benthic and pelagic components. Repeated measures analysis of variance (RM-ANOVA) indicated no interactive effects of the two treatments. VPA indicated that physicochemical variables explained the greatest amount of variance (33.6%) in the phytoplankton pigment dataset, relative to benthic primary producers (0.4%) and invertebrates (2.3%). Salinization led to an increase in biomass of planktonic siliceous algae (≥ 1600 mg Cl L^{-1}) and chlorophytes and cyanobacteria (≥ 2500 mg Cl L^{-1}), which we infer was caused by increased phosphorus release from sediments whilst aquatic plants and periphyton declined. Nitrate additions modified phytoplankton in a non-linear manner, leading to an elevated biomass of cryptophytes and chlorophytes at intermediate loading rates of 5 mg N L^{-1} (associated with greater NH_4-N availability and shifts in aquatic plant composition). These findings support the hypothesis that the relative availability of reduced versus oxidised nitrogen forms is an important driver of phytoplankton composition. Together, these results suggest that pelagic biota are highly sensitive to salinity and nitrate increases and that the phytoplankton compositional shifts are driven by indirect effects on water chemistry (bioavailable P mobilization, changes in nitrogen forms), which are mediated by benthic processes.

Introduction

Lowland lakes close to coastal areas are highly vulnerable to multiple environmental stressors (Moss et al. 1996). Located towards the terminus of watersheds, such water bodies are typically shallow with high pollutant loads, including nitrogen, delivered via lotic, atmospheric and groundwater pathways (Jansson et al. 1994; Galloway et al. 2008). Coastal areas are also under threat from rising sea levels, which may lead to saline incursions into freshwaters and aquifers (Schallenberg et al. 2003) due to overland flooding, subsurface intrusion, or increased aerial transport during storms (Lantz et al. 2015). In particular, agricultural practices can influence the prevalence of both stressors, with nitrogenous fertilizers being applied to land and making their way into water courses, and extensive pumping for land drainage drawing saline waters further inland (Carpenter et al. 1998; Steinich et al. 1998; Nielsen et al. 2003). Such stressors are widespread for many lowland and coastal wetlands, which are often important conservation sites (Moss et al. 1991; Jeppesen et al. 1994). It is now recognized that the biogeochemical transformations occurring in freshwater-marine transition zones are critical determinants of coastal water quality, requiring enhanced understanding of processes in these complex wetlands.

Phytoplankton are a key indicator of ecosystem state in shallow lakes (Scheffer et al. 1993). Regime shifts from clear to turbid water can be triggered by increases in both salinity and nitrogen (Jeppesen et al. 2007; Barker et al. 2008a; 2008b). Elevated phytoplankton biomass and algal blooms are well-documented characteristics of turbid lakes, but the impact of state changes on phytoplankton composition is less well studied.

Effects of nitrogen on phytoplankton communities appear to depend on the form in which it is supplied (Bronk et al. 2007; Donald et al. 2013). Overturning previous assumptions that algae and phototrophic bacteria prefer to assimilate N as NH_4, it is now known that phytoplankton groups differ in their ability to utilise different nitrogen sources: diatoms are better at assimilating oxidised nitrogen (NO_3), whereas cryptophytes, cyanobacteria and dinoflagellates appear more suited to utilisation of reduced and organic forms (NH_4, urea, amino acids) (Glibert et al. 2016). Seawater pulses in culture experiments can reduce phytoplankton diversity, although chlorophytes tend to survive because the taxonomic diversity within this group allows switching among species with different salinity tolerances (Flöder and Burns 2004). However, because salinity and nitrogen pollution causes ecosystem state changes underpinned by complex benthic-pelagic interactions (Moss et al. 1991; Jeppesen et al. 2007), understanding phytoplankton community responses in shallow lakes requires consideration of such processes (Vadeboncoeur et al. 2002).

There are well-established mechanisms which suggest that salinity and nitrate might alter water chemistry through interactions with the benthos. Both may affect the chemical properties of sediment P binding, and so influence the bioavailability of P in the water column. In saline environments, marine sulphates in anoxic sediments sequester iron and inhibit the capacity for PO_4 binding (Blomqvist et al. 2004). Therefore, P is generally bioavailable in saline coastal waters and N, rather than P, is more likely to limit primary production (Howarth and Marino 2006). Nitrate additions are also used in lake management to oxidise sediments and bind phosphates, thereby reducing bioavailable P

supply for phytoplankton growth (Ripl 1976). Each of these processes is mostly relevant in deeper waters where anoxic sediments predominate. In contrast, shallow lakes and their sediments are often well oxidised, creating conditions that enhance P retention in sediments (Ripl 1986; but see Orhiel et al. 2015). Deviations from this general rule occur seasonally and spatially when variability in benthic periphyton, aquatic plants and bacterial communities can also influence sediment P release (van Donk et al. 1993; Spears et al. 2007; Shinohara et al. 2017). Therefore, the potential for complex interactions and responses to salinity and nitrogen enrichment in shallow lakes is high.

Mesocosm 'pond' experiments are able to simulate conditions in shallow lakes including benthic components, whilst providing the necessary controls to manipulate treatments (Stewart et al. 2013). This study presents new data from a previously-published fully factorial mesocosm experiment which investigated the effects of salinity and nitrogen (as nitrate) additions to surface water biota sourced from Hickling Broad, Eastern England (Moss and Leah 1982; Moss et al. 1991; Bales et al. 1993; Irvine et al. 1993). The effects of the experimental treatments on total phytoplankton biomass (as Chlorophyll *a;* Chl *a*), zooplankton, phytobenthos, zoobenthos and aquatic plants have been previously reported in Barker et al. (2008a; 2008b) and are briefly summarised here.

Previously published results

Salinity enhancement led to increases in phytoplankton abundance (as Chl *a*) and total P, and declines in macrophyte and periphyton biomass and richness (Barker et al 2008a). Zooplankton responses to salinity were dependent on the fish biomass. In the

presence of high fish biomass (up to 18 g fresh weight m^{-2}) after April 2005, salinity increases led to lower biomass of cladocera (including daphnids) and higher copepod biomass. Nitrogen additions resulted in significantly lower TP values in the treatments of 1 mg N L^{-1} and 10 mg N L^{-1}, with higher planktonic Chl *a* in the intermediate nitrogen levels (2 mg N L^{-1} and 5 mg N L^{-1}; Barker et al 2008b). Soluble P and NH_4^+ rose significantly in the 5 mg N L^{-1} treatment. Macrophyte % PVI (percentage volume infested) and species richness declined and periphyton growth increased above the lowest (1 mg N L^{-1}) level of N. *Elodea canadensis* cover increased at the two intermediate N treatments. Previous results from this experiment therefore demonstrate pronounced and independent salinity effects on the pelagic food web (dependent on fish density) and negative influence on aquatic plant cover and richness. By contrast, nitrogen had non-linear effects on aquatic macrophytes and water chemistry, with marked shifts in both under intermediate nitrogen loading rates. These results show that phytoplankton abundance is highly sensitive to nitrogen and salinity additions. Here, we explore whether these stressors also cause shifts in phytoplankton composition either independently (e.g. Saros and Fritz 2002; Donald et al. 2011; 2013) or in interaction in these shallow mesocoms.

Methods

Experimental design

Phytoplankton biomass and community composition were estimated using biomarker chlorophyll and carotenoid pigments in the waters of a mesososm experiment published by Barker et al. (2008a; 2008b). The experiment was set up early in 2004 and ran until

September 2005 in 48 tanks of 2 m diameter and 1.2 m depth containing 3 m^3 of water, located in the botanical gardens of the University of Liverpool at Ness, UK (53° 16´ N, 3° 02´ W) (Barker 2008a; 2008b) (Figure 1). The results presented here are from the final year of the experimental operation (August 2004-05) after the tank ecosystems had become established. The experimental set up involved sediments (20cm deep), water, zooplankton and plants being translocated from Hickling Broad, a shallow brackish lake in Norfolk, UK (1° 35´ E, 52° 44´ N) into each tank, together with two male sticklebacks (*Gasterosteteus aculeatus* L.; 'low' fish densities). In March 2005 a further two male and two female sticklebacks were introduced to each mesocosm to allow the fish populations to rise to carrying capacity during the growth season ('high' fish densities). The final fish biomass in the mesocosms of 9-18 gm^{-2} exceeds historical densities in Hickling Broad (conservative estimate of 1.29-6 g m^{-2} in 1989, but probably excluding smaller fish <8cm; Irvine et al 1993).

The experiment was a fully-factorial randomised block design with four levels of salinity and four levels of nitrogen. Each treatment was replicated three times, with replicates separated into three blocks along a slight elevation gradient to isolate the effects of tank location. Salinity was adjusted by the addition of sea salt (commercial brand for domestic use) or dilution with deionised water on at least three occasions to achieve stable mean chloride concentrations of ~600, 1000, 1600 and 2500 mg Cl L^{-1} (S1-S4; Table 1a). As described in Barker et al (2008), salinity is expressed here as chloride (Cl^- ion) concentrations which was monitored in the field as conductivity and converted to chloride concentrations using a regression relationship (chloride mg L^{-1} =

0:359) (conductivity (mS cm-^{1}))–275) (r^2 = 0:964; p<0:0001; n = 48) to allow adjustments to be made. Cl^- measurements during water chemistry monitoring were conducted using Mohr titrations. For the nitrogen treatments (N1-N4), $NaNO_3$ was added approximately monthly at a dose of 1, 2, 5 and 10 mg N L^{-1} to increase the concentrations by 3-30 fold above that of the Hickling Broad waters (inorganic lake water N concentrations were < 2 mg L^{-1}). Phosphate was added to all tanks as KH_2PO_4 to increase the mean lake-water concentration by 50 µg P L^{-1} (Table 1), and attempt to ensure a replete supply of this element. Further details of the experimental set up are given in Barker et al. (2008a; 2008b).

Sampling and analysis

Sampling for phytoplankton pigments and physicochemical parameters (Table 2) occurred at least monthly during September2004-February 2005 and biweekly during March-August 2005 using a plastic tube which spanned the water column. Sampling of zooplankton, benthic invertebrates and periphyton occurred at monthly intervals and was conducted only during the growth season (April-August 2005). Zooplankton was estimated using10 L of bulk samples from the entire water column taken with a tube, passed through a 64 µm mesh net and preserved in ethanol. Periphyton (as Chl *a*) and macroinvertebrates were sampled from standardized substrates which were strips of doubled plastic netting (2 cm wide), of mesh size 1 cm, extending through the full depth of the water column. The strips were suspended from a rod placed diagonally across the tank and removed monthly during the course of the experiment. Aquatic plant coverage (as % volume infested; PVI) was estimated visually at biweekly intervals during the

growth season, and estimates were converted to biomass after calibration with post-experiment harvests. Fish densities were measured at the end of the experiment (Barker et al. 2008a; 2008b).

Changes in phytoplankton abundance and gross taxonomic composition were estimated using high performance liquid chromatographic (HPLC) analysis of diagnostic chlorophyll and carotenoid pigments following standard protocols (Leavitt and Hodgson 2001). Measured volumes of mesocosm water were filtered through Whatman GF/C (ca. 1.2-µm pore size) glass-fibre filters. Filter papers were extracted overnight in a mixture of acetone: methanol: water (80:15:5) at -15 °C, filtered and dried under nitrogen gas and quantitatively re-dissolved before injection into the HPLC system. The system comprised an Agilent 1100 series separation module with Quaternary pump, a C-18 column for reversed-phase separation and an on-line photo-diode array detector (Mantoura and Llewellyn 1983). The HPLC was calibrated using commercial pigment standards (DHI Denmark) and pigment concentrations were expressed in nanomoles pigment L^{-1}.

We selected five of the most common pigments as biomarkers of the main phytoplankton taxa: siliceous algae (fucoxanthin), chlorophytes (Chl *b*), cryptophytes (alloxanthin), total cyanobacteria (echinenone) and all phytoplankton (Chl *a*; an estimate of total phytoplankton; algae + cyanobacteria). Given the history of *Prymnesium parvum* blooms at this site, we specifically searched chromatograms for the diagnostic pigment 19'-butanoyloxyfucoxanthin, which should have been a marker for *P. parvum* blooms. This pigment was not detected and so, we infer that siliceous algae (i.e. diatoms +

synurophytes) and not haptophytes were the primary algae producing fucoxanthin here. We assigned Chl *b* as a biomarker of chlorophytes because, although it may be produced by euglenophytes, they are rare in Hickling Broad (Bales et al. 1993). We also designated echinenone as a biomarker of cyanobacteria because it is produced in only trace amounts in other taxa (chlorophytes and euglenophytes).

Numerical analyses

Statistical analysis of phytoplankton response to nitrate and salinity were based on time-repeated measurements of the biomarker phytoplankton pigments. Counts of zooplankton were classified as total *Daphnia*, other Cladocera, total copepods, and rotifers whereas benthic invertebrate taxa were amalgamated into the groups detailed in Table 2. All variables were checked for normality using a combination of Kolmogorov-Smirnov tests and visual inspection of histograms. All pigment data were log (x+1) transformed before analysis, while the transformations applied to other physicochemical and biological variables are given in Table 2.

Log (x+1)-transformed concentrations of each phytoplankton pigment were analysed using a two-way repeated-measures analysis of variance (RM-ANOVA) with sampling occasion (time) as the repeated measure, nitrogen and salinity as factors, and block included as a covariate. This analysis was applied over the entire year (August 2004-05). Data homogeneity of covariance was tested using Mauchly's test of sphericity. Because this test indicated significant non-homogeneity in some instances, we also applied the more conservative Greenhouse–Geisser tests to evaluate significance of responses to treatments. *Post-hoc* testing was only possible by running the analysis

without block as a covariate. Because block effects were not significant (Table 3; except for alloxanthin) we ran a further RM-ANOVA with only nitrogen and salinity as factors and applied Bonferroni tests to identify which treatment pairs were significantly different when block effects were not taken into account (significance level of $p < 0.01$ applied as Bonferroni correction for multiple tests). Where results with and without block effects differed, the most conservative result was used for interpretation. Analyses were conducted in SPSS 24.0 for Windows.

To investigate relationships in the broader mesocosm ecosystem and assist in understanding the potential mechanisms by which nitrate and salinity might be influencing phytoplankton communities, we conducted multivariate analyses to quantify statistical relationships between phytoplankton pigment assemblages and associated physicochemical and biological parameters (Table 2). Because of the reduced sampling frequency of invertebrates, these analyses were conducted on monthly mean pigment values harmonized to common sampling dates during the "growth season" (April-August 2005) when invertebrates were collected. All variables were sampled on the same day, with the exception of PVI, and in this case, values from the preceding week were used in statistical analyses. Detrended correspondence analysis (DCA) of the pigment data gave a short axis 1 length of 2.182, indicating that the linear technique of redundancy analysis (RDA) should be used to relate pigments to environmental variables.

Twenty-seven parameters were included in the initial RDA (Table 2); however, final analysis included only variables which were correlated significantly ($p < 0.05$) with pigment assemblages when using forward selection and Monte Carlo analysis with 999 permutations. Variables eliminated included NO_3, temperature, *Daphnia* spp., copepods,

rotifers, Odonata, Oligochaetae, Coleoptera, Gastropoda, Nematocera, Ostracoda and other rare invertebrates. Conductivity was subsequently removed from the dataset because of redundancy with Cl^- concentration, as indicated by variance inflation factors > 20. Consequently, the final analysis was based on 14 predictor variables including TP, Cl, Alkalinity, pH, TN, O_2, SRP, NH_4, Cladocera excluding *Daphnia*, Malacostraca, Diptera, Hirudinea, periphyton Chl *a*, PVI. All multivariate analyses were conducted on CANOCO v. 4.0.

Variance partitioning analysis (VPA) was conducted to determine the relationships between physicochemical parameters, invertebrate assemblages and benthic primary producer communities and changes in phytoplankton community composition. Variables were assigned to each predictor category (Table 2) and a series of constrained and partially constrained RDAs were performed following (Hall et al. 1997) to determine the relationships between pigments and each variable category.

Results

RM-ANOVA revealed that salinity and nitrate had significant effects on phytoplankton groups, but that there were no significant interactions between factors (Figure 2, Table 3). Salinity additions increased the concentration of pigments from siliceous algae (fucoxanthin), chlorophytes (Chl *b*), cyanobacteria (echinenone), and total phytoplankton (Chl *a*). Nitrate amendments also increased cryptophytes (alloxanthin), chlorophytes (Chl *b*) and total algae (Chl *a*), with maximum pigment concentrations occurring at intermediate levels of N fertilization. Bonferroni tests showed that the highest salinity treatment S4 (2500 mg Cl L^{-1}) resulted in significantly higher abundance

of chlorophytes (Chl *b*), cyanobacteria (echinenone) and total phytoplankton (Chl *a*) than the three lower treatments (Figure 2 and Table 3). Salinity treatments led to significant and progressive increases in siliceous algal pigments (fucoxanthin) at S3 (1600 mg Cl L^{-1}) and S4 levels (2500 mg Cl L^{-1}). Nitrate treatment N3 (5 mg NO_3-N L^{-1}) had significantly higher concentrations of pigments from cryptophytes (alloxanthin), chlorophytes (Chl *b*) and total algae (Chl *a*) than the lowest nitrate treatment (1 mg NO_3-N L^{-1}) (Figure 2 and Table 2). Siliceous algae (fucoxanthin) pigment concentrations were significantly higher than all other levels at treatment N2 (2 mg NO_3-N L^{-1}), but the significance level ($p = 0.036$) was marginal when a Bonferroni correction was applied, and so this result was rejected.

Time series plots demonstrated the seasonal nature of phytoplankton responses, which differed among taxonomic groups (Figure 3). Pigments from siliceous algae (fucoxanthin, Figure 3a) showed two maxima during the winter of 2004-05 (with peaks offset among treatments) and during the following growth period which spanned from April-June 2005 for this group. Pigments from chlorophytes (Chl *b*, Figure 3c) were abundant throughout the year of sampling. In contrast, pigments from cryptophytes (alloxanthin; Figure 3b) and cyanobacteria (echinenone; Figure 3d) were much more prevalent later in the experiment, and after the increase in stickleback biomass (dashed line). Cryptophyte pigments increased markedly after June 2005, whereas maximum concentrations of cyanobacterial pigments developed for a shorter period between April-June 2005. Responses of Chl *a* (Figure 3e) to treatments integrated the patterns in the individual algal pigments, consequently, timing of total phytoplankton responses was variable among treatments, reflecting the unique responses of individual phytoplankton

groups. Because fish were added in the spring of the second year (dashed line), changes in biomass of *Daphnia* spp. are presented to assess any shifts in grazing potential which could have influenced phytoplankton biomass. As reported in Barker et al (2008a), *Daphnia* biomass was significantly suppressed by higher salinities, but only during the 'high fish' period (Figure 3). In contrast, there were no significant effects of nitrogen treatments on *Daphnia* biomass. Across the experiment, mean *Daphnia* biomass was more regularly recorded as zero after fish biomass had increased.

RDA axis 1 explained 50.4% ($p < 0.05$) of the variance in the dataset, and was correlated positively and strongly with total phytoplankton (Chl *a*), cyanobacteria (echinenone), chlorophytes (Chl *b*) and TP and more weakly with TN and NH_4 (Figure 4). Axis 2 explained only 5.2 % ($p < 0.05$) of total variance, and was correlated negatively with oxygen concentration. Other variables including pH, PVI, Cladocera (excluding *Daphnia*), chloride concentration, and Malacostraca had a strong influence on the environmental dataset, contributed to RDA axes equally, but were correlated weakly with most phytoplankton pigments. In fact, only fucoxanthin from siliceous algae was associated with these variables, being correlated positively with salinity (chloride concentration) and Malacostraca density, and negatively with pH, PVI and Cladocera (excluding *Daphnia*) abundance. Unexpectedly, alloxanthin from cryptophytes was correlated positively with periphyton abundance and SRP concentrations, and negatively with oxygen concentrations.

VPA showed that physicochemical, invertebrate and benthic primary producers (algae and macrophytes) together explained 57.6 % of the variance in the experimental pigment assemblage during the "growth season" (Table 4). Physicochemical (C) variables

were correlated more strongly with pigment assemblages than were invertebrate variables (I) or benthic primary producers (P). Additional variance in phytoplankton composition was correlated with combinations of C and P, C and I, and C with both I and P.

Discussion

The strong and independent effects of both nitrate and salinity on phytoplankton seen here (Fig. 2; Table 3) were consistent with findings from studies of the unique effects of different nitrogen compounds (Donald et al. 2011, 2013) and salinity (Flöder and Burns 2004). In short-duration microcosm (reviewed in Erratt et al. 2018) and mesocosm experiments (Finlay et al. 2009, Donald et al. 2011, Bogard et al. 2017), addition of N and NO_3^-, NH_4^+ or urea stimulates growth of most phytoplankton over similar gradients of fertilization, while elevated salinity is associated with phytoplankton compositional change (Medvedeva 2001). However, most experiments to date have focused exclusively on plankton, excluding benthos and limiting the insights for shallow lake systems. While we currently see little evidence for interactive effects of nitrate and salinity at these ranges, further research is needed to identify if interactions exist under different environmental conditions (e.g, ionic composition, dissolved organic matter composition, lake depth, climatic conditions).

Factor interactions between salinity and nutrients have been observed in other experiments (Jeppesen et al. 2007) and may reflect the relatively lower salinity of treatments (all < 5 ppt) and enhanced nutrient supply in our experiment relative to other trials. There, phytoplankton abundance increased above threshold salinities of 6-8 ppt,

but only when nutrient concentrations exceeded 50 µg PL^{-1} and 0.5 mg N L^{-1} (Jeppesen et al. 2007). Interestingly, these Danish experiments were conducted at low fish densities (1 stickleback m^{-2}), comparable to our overwintering densities (of 0.67 m^{-2}), but lower than during the final 6 months of our experiment when mean fish density was 18 (adult + juvenile) sticklebacks m^{-2}. High fish densities should have optimized the potential for trophic cascades, and we did observe that higher salinities (above S1 levels) inhibited daphnids at higher fish densities (Figure 3; Brooks and Dodson 1965). Jeppesen et al (2004, 2007) also noted a (lower) threshold salinity of 2 ppt for daphnid elimination in Danish lagoons with higher fish densities. Therefore, fish may be important in structuring brackish lake ecosystems and nutrient and salinity effects may be mediated, in part via changes in food web linkages. However, as discussed below, the effects of changes in fish densities on phytoplankton in our experiment appear to be rather limited, suggesting that phytoplankton changes are driven primarily by 'bottom-up' (chemical) effects than by 'top-down' processes (VPA, Figure 4, Table 4).

Responses to salinity enrichment

The phytoplankton pigment analysis suggests that salinity increases of 2500 mg Cl L^{-1} (S4) led to ~10-fold increases in abundance of chlorophytes, siliceous algae and cyanobacteria, with a significant increase in siliceous algae also occurring at the S3 salinity level (1600 mg Cl L^{-1}). Together, these changes, combined with the lack of response of cryptophytes, resulted in a progressive decline in the latter taxa as salinity increased. Increases in siliceous algae and chlorophyte groups persisted during low and high fish periods (Figure 3), despite higher daphnid abundances during the low fish period (Figure 3). The maintenance of a significant salinity effect at S4 suggests that,

although periods of higher *Daphnia* biomass might have temporarily reduced phytoplankton abundance via grazing (August-October 2004), the effect was not sustained and did not alter the overall experimental effect of salinity enhancement on chlorophyte and siliceous algal taxa. Similarly, changes in densities of the effective grazer, *Daphnia* spp., were uncorrelated to any metric of pigment assemblages (Figure 4). In contrast, cyanobacterial biomass peaked predominantly during the period of higher fish density in the highest salinity treatment. If grazing was a dominant driver, this trend suggests that *Daphnia* spp selectively removed cyanobacteria rather than chlorophytes and siliceous algae during the low fish periods, which seems unlikely (Lampert 1987). More feasibly, cyanobacterial peak abundance also corresponds to the warmest water temperatures, when cyanobacteria are known to proliferate (Paerl and Huisman 2008) and also to low N:P ratios induced by the S4 treatment (Pick and Lean 1987).

Variance partitioning analysis suggested that physicochemical factors rather than biological processes were correlated most strongly with the increase in phytoplankton biomass at the highest salinity treatments (Table 4). For example, phytoplankton biomass in this experiment was correlated strongly with TP concentrations (Figure 4) which in turn increased significantly at the two highest salinities (Barker et al. 2008a) (Figure 5). These patterns may reflect changes in the P binding capacity of sediments with salinization (Blomqvist et al. 2004), as observed in low Fe freshwater lakes (Orihel et al. 2015). In such a scenario, released P is assimilated into phytoplankton and results in elevated TP and Chl *a* in the water column. Enhancing this effect, these highly productive mesocosms accumulate sedimentary organic matter, which should lower sedimentary redox and further increase sedimentary P release (Shinohara et al. 2017).

In contrast with observed changes in phytoplankton in the source lake Hickling Broad (Moss et al. 1991), this experiment provided little support for the hypothesis that salinity increases favour blooms of toxic algae, such as *Prymnesium parvum* (Moss et al. 1991) because we detected no 19'-butanoyloxyfucoxanthin marker pigment. Further microscopic counts to verify this are desirable, but unfortunately not possible. However, our analyses show that when phytoplankton reached very high densities characteristic of a regime shift to the turbid state (100-230 µg L^{-1} Chl *a*) (Barker et al. 2008a), increases in salinity favoured populations of cyanobacteria that can include potent toxin producing species (e.g., Finlay et al. 2009; Donald et al. 2011). The presence of cyanobacteria, which are scarce in the source lake (Moss et al. 1991), demonstrates that the mesocosms have exceeded natural conditions in the lake. This steady increase in prokaryotes over time may also reflect the mixotrophic nature of some cyanobacteria as organic matter accumulates (Burkholder et al. 2008), as well as the development of reduced N sources favoured by these taxa (see later) (Glibert et al. 2016).

Responses to nitrate enrichment

The most obvious response to nitrogen fertilization was a significant increase in cryptophyte (as alloxanthin) and chlorophyte densities (as Chl *b*) at N3 level (5 mg NO_3-N L^{-1}), as well as a marginally significant increase in siliceous algae (fucoxanthin) at N2 level (2 mg NO_3-N L^{-1}). These patterns agree with evidence from some lake mesocosms which suggest that moderate N concentrations (2-4 mg N L^{-1}) stimulate cryptophyte growth, but that greatly elevated nutrient concentrations (>10 mg N L^{-1}; >100 µg P L^{-1}) favour cyanobacteria and chlorophytes (Gonzalez Sagrario et al. 2005, Donald et al. 2012; Bogard et al. 2017). Similarly, the intermediate effect of nitrate on diatoms has

been recorded elsewhere (Donald et al. 2013) despite predictions from physiological literature that diatoms should be better at assimilating oxidised N forms (nitrate) than cryptophytes or chlorophytes and should increase linearly with nitrate concentrations (Glibert et al. 2014; Glibert et al. 2016). Instead, cryptophytes and chlorophytes increased in a non-linear manner, peaking at intermediate nitrate enrichment levels. Such a pattern may be partly driven by the very high N:P ratios at the highest salinity levels which periodically induce P limitation (Figure 5). The observation is also consistent with a recent hypothesis suggesting that moderate nitrate concentrations may supress primary production over broad regional scales (Filstrup and Downing 2018), and also agrees with species-level analyses which show a wide range of algal responses, even among closely related taxa (Donald et al. 2013). The controlled conditions in the mesocosms allow us to elucidate some of the potential mechanisms for these observations.

Several lines of evidence suggest that the benthic-pelagic interactions within the mesocosms may be important modifiers of phytoplankton responses to nitrate enrichment. First, unlike salinity amendments, addition of nitrate was not selected as a significant correlate of temporal changes in phytoplankton assemblages in the RDA (Figure 4). Instead, we found that concentrations of chemically-reduced N (NH_4^+) and total N were weakly but significantly correlated with changes in pigment composition. Second, nitrate amendments were associated with significant shifts in the phytobenthos: both % PVI and macrophyte species richness declined, while periphyton abundance increased above 1.5± 0.4 mg total N L^{-1}, and *Elodea canadensis* was predominant in the intermediate nitrogen treatments (N2, N3) (Barker et al. 2008b), as seen elsewhere (James

et al. 2005). Together, these observations suggest that the quantity and composition of phytobenthos may influence nitrogen cycling within mesocosms.

Growth and decomposition of organic matter from aquatic plants, periphyton and sediments likely affects the relative availability of different chemical forms of N during the experiment (van Donk et al. 1993). For example, ammonium concentrations are highest in the N3 level treatment, whereas NO_3 was proportionally lower in the N1 and N2 treatments (Figure 5). Changes in the relative supplies of NH_4 versus NO_3 are known to differentially affect the production of cryptophytes, chlorophytes and cyanobacteria, each of which grows more efficiently at NH_4 concentrations similar to those seen in the N3 trials (Glibert et al. 2016). Here, aquatic plant communities with fast-growing *Elodea canadensis* and high periphyton biomass appear to be associated with enhanced NH_4 availability (Figure 5).

Mesocosm conditions and benthic-pelagic interactions

Although the closed conditions in the mesocosms differ from the natural field environment, small-scale experiments provide useful insights into the role of solutes in regulating phytoplankton abundance and community composition (Spivak et al. 2011). Many mesocosm experiments do not include sediments, and so our results elucidate possible mechanisms in shallow lakes (c.f. Donald et al. 2013). In addition, long-term trials such as conducted here provide unique insights into the role of seasonality in modifying algal and cyanobacteria response to uniform treatments. For example, much higher abundances of cyanobacteria and cryptophytes in the final growth season relative to the previous year (Figure 3) might be linked to changes in fish abundance and *Daphnia*

grazing (Figure 3), but are also consistent with changes in accumulation and recycling of organic matter in the mesocosms. Both cryptophytes and cyanobacteria are potentially mixotrophic (Katechakis et al. 2005; Subashchandrabose et al. 2013) and can take nutritional advantage of organic matter provision (Tranvik et al. 1989; Burkholder et al. 2008) to outcompete other taxa, such as diatoms. In particular, the role of benthic processes in altering the availability of chemically-reduced N may be an important control of phytoplankton seasonality, because organic N builds up in microbial material as the growth season progresses, strengthening the microbial loop (Donald et al. 2011; Glibert et al. 2016). Cyanobacteria and cryptophytes can be dominant in the phytoplankton of macrophyte-dominated lakes, ceding to siliceous algae or chlorophytes under more turbid conditions (Jensen et al. 1994; Cross et al. 2014). In addition, cycling and long-term accumulation of P in this mesocosm situation is likely to reduce N:P ratios in the water column, such as seen in treatment S4, resulting in enhanced cyanobacterial growth (Figure 5). Although further work is required to identify the direct (planktonic) and indirect (via benthos) mechanisms of nitrate and salinity on phytoplankton assemblages, findings from this study suggest that modification of benthic communities in mesocosms and shallow lakes has the potential to greatly alter the proportions and effects of bioavailable nutrients in the water column.

Conclusions

The results from this large mesocosm experiment illustrate the difficulty in predicting phytoplankton response to multiple stressors in shallow lakes (Jeppesen et al. 1997; Lee et al. 2015). Whilst potential for interactive effects of salinity and nitrogen

enrichment on phytoplankton exists (Jeppesen et al. 2007), our findings suggest that pelagic responses are dependent on the scale of changes in nutrient and/or salt content, as well as potential indirect interactions via the benthos or food web. Whilst viable 'top-down' and 'bottom-up' mechanisms exist to modify phytoplankton communities, evidence from our mesocosm experiments suggests that 'bottom-up' effects might be more important within this range of conditions. Salinization of shallow lakes can mobilise phosphorus release from sediments to increase biomass of siliceous algae, chlorophytes and cyanobacteria, while the effects of nitrate additions on phytoplankton are mediated via in-lake nitrogen cycling and N:P ratios that is, in turn, influenced by benthic community structure. Modifications of chemical forms of nitrogen in shallow lakes (reduced versus oxidised) and availability of organic nutrients (via the microbial loop) help explain the patterns in the phytoplankton composition observed. As many mesocosm experiments exclude benthos, we argue that further research is needed to establish the role of littoral and demersal sedimentary processes in regulating phytoplankton production and composition in shallow lakes (Moss et al. 2013).

Acknowledgements

This research was supported by a UK NERC Grant # NER/A/S/2002/00759 to BM. Research by PRL and SM was supported by NSERC Canada, Canadian Foundation for Innovation and Sask Learning. Natural England, the Norfolk Wildlife Trust and Broads Authority provided logistical support in the experimental set up and Samantha Pham

provided assistance with pigment analyses. We are grateful to two anonymous reviewers for their insights.

References

Bales M, Moss B, Phillips G, Irvine K, Stansfield J. 1993. The changing ecosystem of a shallow brackish lake, Hickling broad, Norfolk, UK II. Long-term trends in water chemistry and ecology and their implications for restoration of the lake. Freshwat Biol. 29:141-165.

Barker T, Hatton K, O'Connor M, Connor L, Bagnell L, Moss B. 2008a. Control of ecosystem state in a shallow, brackish lake: implications for the conservation of stonewort species. Aquatic Conservation: Marine and Freshwat Ecos. 18(3):221-240.

Barker T, Hatton K, O'Connor M, Connor L, Moss B. 2008b. Effects of nitrate load on submerged plant biomass and species richness: results of a mesocosm experiment. Fund Appl Limnol/ Archiv für Hydrobiol. 173(2):89-100.

Blomqvist S, Gunnars A, Elmgren R. 2004. Why the limiting nutrient differs between temperate coastal seas and freshwater lakes: a matter of salt. Limnol. Oceanogr. 49(6):2236-2241.

Bogard, M.J., K. Finlay, M.J. Waiser, V.P. Tumber, D.B. Donald, E. Wiik, G.L. Simpson, P.A. del Giorgio, and P.R. Leavitt. 2017. Effects of experimental nitrogen fertilization on planktonic metabolism and CO2 flux in a hypereutrophic hardwater lake. PLoS One 12(12): e0188652.

Bronk D, See J, Bradley P, Killberg L. 2007. DON as a source of bioavailable nitrogen for phytoplankton. Biogeosci. 4(3):283-296.

Burkholder JM, Glibert PM, Skelton HM. 2008. Mixotrophy, a major mode of nutrition for harmful algal species in eutrophic waters. Harmful Algae. 8(1):77-93.

Carpenter SR, Caraco NF, Correll DL, Howarth RW, Sgarpley AN, Smith VH. 1998. Nonpoint pollution of surface waters with phosphorus and nitrogen. Ecol Appl. 8(3):559-568.

Cross I, McGowan S, Needham T, Pointer C. 2014. The effects of hydrological extremes on former gravel pit lake ecology: management implications. Fund Appl Limnol/ Archiv für Hydrobiol. 185(1):71-90.

Donald DB, Bogard MJ, Finlay K, Bunting L, Leavitt PR. 2013. Phytoplankton-specific response to enrichment of phosphorus-rich surface waters with ammonium, nitrate, and urea. PloS one. 8(1):e53277.

Donald DB, Bogard MJ, Finlay K, Leavitt PR. 2011. Comparative effects of urea, ammonium, and nitrate on phytoplankton abundance, community composition, and toxicity in hypereutrophic freshwaters. Limnol Oceanogr. 56(6):2161-2175.

Dugdale R, Goering J. 1967. Uptake of new and regenerated forms of nitrogen in primary productivity. Limnol Oceanogr. 12(2):196-206.

Erratt, KJ, Creed IF, and Trick CG. 2018. Comparative effects of ammonium, nitrate and urea on growth and photosynthetic efficiency of three bloom-forming cyanobacteria. Freshwat Biol. 63: 626-638.

Finlay, K., A. Patoine, D.B. Donald, M. Bogard, and P.R. Leavitt. 2010. Experimental evidence that pollution with urea can degrade water quality in phosphorus-rich lakes of the northern Great Plains. Limnol Oceanogr. 55: 1213-1230.

Flöder S, Burns CW. 2004. Phytoplankton diversity of shallow tidal lakes: influence of periodic salinity changes on diversity and species number of a natural assemblage. J Phycol. 40:54-61.

Galloway JN, Townsend AR, Erisman JW, Bekunda M, Cai Z, Freney JR, Martinelli LA, Seitzinger SP, Sutton MA. 2008. Transformation of the nitrogen cycle: recent trends, questions, and potential solutions. Science. 320(5878):889-892.

Glibert PM, Dugdale RC, Wilkerson F, Parker AE, Alexander J, Antell E, Blaser S, Johnson A, Lee J, Lee T. 2014. Major–but rare–spring blooms in 2014 in San Francisco Bay Delta, California, a result of the long-term drought, increased residence time, and altered nutrient loads and forms. J Exp Mar Biol Ecol. 460:8-18.

Glibert PM, Wilkerson FP, Dugdale RC, Raven JA, Dupont CL, Leavitt PR, Parker AE, Burkholder JM, Kana TM. 2016. Pluses and minuses of ammonium and nitrate uptake and assimilation by phytoplankton and implications for productivity and community composition, with emphasis on nitrogen-enriched conditions. Limnol Oceanogr. 61(1):165-197.

Golterman HL, Clymo RS, Ohnstad MAM 1978. Methods for Physical and Chemical Analysis of Fresh Waters. Second edition. Oxford: Blackwell.

Gonzalez Sagrario MA, Jeppesen E, Goma J, Søndergaard M, Jensen JP, Lauridsen T, Landkildehus F. 2005. Does high nitrogen loading prevent clear-water conditions in shallow lakes at moderately high phosphorus concentrations? Freshwat Biol. 50:27-41.

Hall RI, Leavitt PR, Smol JP, Zirnhelts N. 1997. Comparison of diatoms, fossil pigments and historical records as measures of lake eutrophication. Freshwat Biol. 38:401-417.

Howarth RW, Marino R. 2006. Nitrogen as the limiting nutrient for eutrophication in coastal marine ecosystems: evolving views over three decades. Limnol Oceanogr. 51:364-376.

Irvine K, Moss B, Bales M, Snook D. 1993. The changing ecosystem of a shallow, brackish lake, Hickling Broad, Norfolk, U.K. I. trophic relationships with special reference to the role of *Neomysis integer*. Freshwat Biol. 29:119-139.

James C, Fisher J, Russell V, Collings S, Moss B. 2005. Nitrate availability and hydrophyte species richness in shallow lakes. Freshwat Biol. 50(6):1049-1063.

Jansson M, Andersson R, Berggren H, Leonardson L. 1994. Wetlands and lakes as nitrogen traps. Ambio.320-325.

Jensen JP, Jeppesen E, Olrik K, Kristensen P. 1994. Impact of nutrients and physical factors on the shift from cyanobacterial to chlorophyte dominance in shallow Danish lakes. Can J Fish Aq Sci. 51:1992-1699.

Jeppesen E, Erlandsen M, Søndergaard M. 1997. Can simple empirical equations describe the seasonal dynamics of bacterioplankton in lakes: an eight-year study in shallow hypertrophic and bioloogically highly dynamic Lake Sobygaard, Denmark. Microbial Ecology. 34:11-26.

Jeppesen E, Søndergaard M, Kanstrup E, Petersen B, Eriksen RB, Hammershoj M, Mortensen E, Jensen JP, Have A. 1994. Does the impact of nutrients on the biological structure and function of brackish and freshwater lakes differ? Hydrobiol. 275/276:15-30.

Jeppesen E, Søndergaard M, Pedersen AR, Jurgens K, Strzelczak A, Lauridsen TL, Johanssen LS. 2007. Salinity induced regime shift in shallow brackish lagoons. Ecosystems. 10:47-57.

Johnes PJ, Heathwaite AL. 1992 A procedure for the simultaneous determination of total nitrogen and total phosphorus in freshwater samples using persulphate microwave digestion. Wat. Res. 26(10): 1281-1287.

Katechakis A, Haseneder T, Kling R, Stibor H. 2005 Mixotrophic versus photoautotrophic specialist algae as food for zooplankton: The light: nutrient hypothesis might not hold for mixotrophs. Limnol Oceanogr 50 (4): 1290-1299.

Kraemer EO, Stamm AJ. 1924 Mohr's Method for the Determination of Silver and Halogens in other than Neutral Solutions, J. Am. Chem. Soc.; 46(12): 2707- 2709.

Lampert W (1987) Laboratory studies on zooplankton-cyanobacteria interactions. New Zealand J. Mar. Freshwat. Res. 21(3): 483-90.

Lantz TC, Kokelj SV, Fraser RH. 2015. Ecological recovery in an Arctic delta following widespread saline incursion. Ecol Appl. 25(1):172-185.

Lee TA, Rollwagen-Bollens G, Bollens SM. 2015. The influence of water quality variables on cyanobacterial blooms and phytoplankton community composition in a shallow temperate lake. Env Monit Assess. 187(6):315.

Mackereth F J H, Heron J, Talling J F. 1989. Water Analysis: Some Revised Methods for Limnologists. Freshwater Biological Association Scientific Publication No. 36. Second edition. Kendal, UK: Titus Wilson and Son.

Mantoura RFC, Llewellyn CA. 1983. The rapid determination of algal chlorophyll and carotenoid pigments and their breakdown products in natural waters by reverse-phase high-performance liquid chromatography. Anal Chim Acta. 151:297-314.

Medvedeva LA. 2001. Biodiversity of aquatic algal communities in the Sikhote-Alin biosphere reserve (Russia). Cryptogam Algol 22 (1): 65-100

Moss B, Bales M, Irvine K. 1991. Food web and water quality relationships in the saline broads of the River Thurne, Norfolk. Mem Ist Ital Idrobiol. 48:345-362.

Moss B, Jeppesen E, Søndergaard M, Lauridsen TL, Liu Z. 2013. Nitrogen, macrophytes, shallow lakes and nutrient limitation: resolution of a current controversy? Hydrobiol. 710(1):3-21.

Moss B, Leah RT. 1982. Changes in the ecosystem of a guanotrophic and brackish shallow lake in Eastern England: potential problems in its restoration. Int Revue ges Hydrobiol. 67:625-659.

Moss B, Madgwick J, Phillips G. 1996. Guide to the restoration of nutrient-enriched shallow lakes. Broads Authority.

Nielsen D, Brock M, Rees G, Baldwin DS. 2003. Effects of increasing salinity on freshwater ecosystems in Australia. Aust J Bot. 51(6):655-665.

Orihel DM, Schindler DW, Ballard NC, Graham MD, O'Connell DW, Wilson LR, Vinebrooke RD. 2015. The ‘nutrient pump:’ Iron-poor sediments fuel low nitrogen-to-phosphorus ratios and cyanobacterial blooms in polymictic lakes. Limnology Oceanogr. 60(3):856-871.

Paerl W, Huisman J. 2008 Blooms like it hot. Science 320, (5872): 57-58.

Pick FR, Lean DR. 1987 The role of macronutrients (C, N, P) in controlling cyanobacterial dominance in temperate lakes. New Zealand J. Mar. Freshwat. Res 21(3): 425-434.

Ripl W. 1976. Biochemical oxidation of polluted lake sediment with nitrate: a new lake restoration method. Ambio.132-135.

Ripl W. 1986. Internal phosphorus recycling mechanisms in shallow lakes. Lake Res Mgmt. 2(1):138-142.

Romo S, Villena M-J, García-Murcia A. 2007. Epiphyton, phytoplankton and macrophyte ecology in a shallow lake under in situ experimental conditions. Fund Appl Limnol/ Archiv für Hydrobiol. 170(3):197-209.

Saros JE, Fritz SC. 2000. Changes in the growth rates of saline-lake diatoms in response to variation in salinity, brine type and nitrogen form. J Plankton Res 22: 1071-1083.

Schallenberg M, Hall CJ, Burns CW. 2003. Consequences of climate-induced salinity increases on zooplankton abundance and diversity in coastal lakes. Mar Ecol Prog Ser. 251:181-189.

Scheffer M, Hosper SH, Meijer M-L, Moss B, Jeppesen E. 1993. Alternative equilibria in shallow lakes. TREE. 8:275-279.

Shinohara R, Hiroki M, Kohzu A, Imai A, Inoue T, Furusato E, Komatsu K, Satou T, Tomioka N, Shimotori K. 2017. Role of organic phosphorus in sediment in a shallow eutrophic lake. Wat Resources Res. 53(8):7175-7189.

Spears BM, Carvalho L, Perkins R, Kirika A, Paterson DM. 2007. Sediment phosphorus cycling in a large shallow lake: spatio-temporal variation in phosphorus pools and release. Hydrobiol. 584:37-48.

Spivak, AC, Vannie MJ, Mette EM. 2011. Moving on up: can results from simple aquatic mesocosm experiments be applied across broad spatial scales? Freshwat Biol, 56: 279-291.

Steinich B, Escolero O, Marin LE. 1998. Salt-water intrusion and nitrate contamination in the Valley of Hermosillo and El Sahuaral coastal aquifers, Sonora, Mexico. Hydrogeol J. 6: 518–526

Stewart RI, Dossena M, Bohan DA, Jeppesen E, Kordas RL, Ledger ME, Meerhoff M, Moss B, Mulder C, Shurin JB. 2013. Mesocosm experiments as a tool for ecological climate-change research. Advances in Ecological Research. Elsevier; p. 71-181.

Subashchandrabose SR, Ramakrishnan B, Megharaj M, Venkateswarlu K, Naidu R. 2013. Mixotrophic cyanobacteria and microalgae as distinctive biological agents for organic pollutant degradation. Environ Int. 51: 59-72.

Tranvik LJ, Porter KG, Sieburth JM. 1989. Occurrence of bacterivory in Cryptomonas, a common freshwater phytoplankter. Oecologia. 78(4):473-476.

Vadeboncoeur Y, Vander Zanden MJ, Lodge DM. 2002. Putting the lake back together: reintegrating benthic pathways into lake food web models. Biosci. 52(1):44-54.

van Donk E, Gulati RD, Iedema A, Meulemans JT. 1993. Macrophyte-related shifts in the nitrogen and phosphorus contents of the different trophic levels in a biomanipulated shallow lake. Nutrient Dynamics and Retention in Land/Water Ecotones of Lowland, Temperate Lakes and Rivers. Springer; p. 19-26.

Table 1: Mean concentrations ± standard deviations of chemical variables in the tanks subjected to (a) salinity and (b) nitrogen treatments between August 2004-05. Note that the loading of nitrate to each tank, rather than the final concentration was manipulated using monthly doses of $NaNO_3$ at of 1, 2, 5 and 10 mg N L^{-1}, for levels 1-4 respectively.

	Level 1 (S1, N1)	Level 2 (S2, N2)	Level 3 (S3, N3)	Level 4 (S4, N4)
(a) Salinity treatments				
Chloride (mg L^{-1})	596 ± 185	1190 ± 233	1732 ± 313	2687 ± 462
SRP (µg L^{-1})	7.1 ± 16.8	5.4 ± 10.4	3.2 ± 4.4	4.4 ± 6.0
TP (µg L^{-1})	57.6 ± 72.9	53.2 ± 62.8	62.4 ± 57.4	125.5 ± 98.5
(b) Nitrogen treatments				
Nitrate-N (mg L^{-1})	0.12 ± 0.32	0.32 ± 0.58	1.94 ± 1.88	11.76 ± 6.45
TN (mg L^{-1})	1.59 ± 0.71	2.50 ± 1.50	5.02 ± 2.94	14.18 ± 6.38
SRP (µg L^{-1})	3.9 ± 7.8	4.7 ± 5.8	8.1 ± 17.8	3.5 ± 5.3
TP (µg L^{-1})	52.1 ± 55.7	84.2 ±75.5	102.6 ± 102.4	59.8 ± 69.8

Table 2: Variables included in the RDA with the dominant taxa summed into groups. Analytical methods are described in [1]Mackereth et al. (1989), [2]Johnes and Heathwaite (1992), by [3]Dionex DX120 ion chromatography, [4]Golterman et al (1978), [5]Kraemer and Stam (1924) (Mohr titration), [6]Barker et al (2008a, b).

Category	Parameter	Units	Dominant taxa in groups	Transformation
Physico-chemical	Temperature	º C		None
	Oxygen (O_2)	mg L^{-1}		None
	Conductivity	µS cm^{-1}		None
	Total phosphorus (TP)[1]	µg L^{-1}		Log (x+1)
	Soluble reactive phosphorus[1]	µg L^{-1}		None
	Total nitrogen (TN)[2]	mg L^{-1}		Log (x+1)
	Nitrate-nitrogen (NO_3-N)[3]	mg L^{-1}		Log (x+1)
	Ammonium-nitrogen (NH_4-N) [1]	mg L^{-1}		Log (x+1)
	Alkalinity[4]	Mequiv L^{-1}		None
	pH	pH units		None
	Cl^- ions[5]	mg L^{-1}		None
Invertebrates[6] (zooplankton)	Sum *Daphnia* spp.	Individuals L^{-1}	*D. hyalina, D. magna, D. pulex, D. cucullata*	Log (x+1)
	Cladocera except *Daphnia*	Individuals L^{-1}	*Chydorus sphaericus, Alona rectangularis*	Log (x+1)
	Sum copepods	Individuals L^{-1}	Cyclopoid and calanoid copepods	Log (x+1)
	Sum rotifers	Individuals L^{-1}		Log (x+1)
Invertebrates[6] (benthic)	Malacostraca	Individuals standard area $^{-1}$	*Gammarus duebeni, G. pulex, Asellus aquaticus, Sphaeroma* spp.	Log (x+1)
	Diptera	Individuals standard area $^{-1}$	Chironomid, Tanypus, Cyclorrapha & Ceratopogonids	Log (x+1)
	Coleoptera	Individuals standard area $^{-1}$	Dytiscid, Elmid & other beetle larvae	Log (x+1)
	Nematocera	Individuals standard area $^{-1}$		Log (x+1)
	Odonata	Individuals standard area $^{-1}$		Log (x+1)
	Oligochaeta	Individuals standard area $^{-1}$	Tubificid, Oligochaetae	Log (x+1)
	Hirudinea	Individuals standard area $^{-1}$	*Helobdella, Erpobdella* spp.	Log (x+1)
	Ostracoda	Individuals standard area $^{-1}$		Log (x+1)
	Gastropoda	Individuals standard area $^{-1}$	*Potamopygrus jenkinsi*, *Physa fontinalis*, *Planorbis* spp., *Lymnaea* sp	Log (x+1)
	Sum rare invertebrates	Individuals standard area $^{-1}$	Arachnida, Bivalva, Ephemeroptera, Hemiptera, Thysanoptera	Log (x+1)
Phytobenthos[6]	Aquatic macrophytes	% volume infested (PVI)		Arcsine
	Periphyton Chl *a*	µg standard area $^{-1}$		Log (x+1)

Table 3: Two-way RM-ANOVA with block as a covariable and 21 repeated measures to assess for nitrogen and salinity effects on five log (x+1) transformed biomarker pigments. The reported values are df (degrees of freedom), MS (mean square), F ratio and *p* value, which are Greenhouse-Geisser adjusted. Significant *p* values ($p < 0.05$) are shown in bold.

		Within subjects effects						**Between subjects effects**					
		Time	Time x block	Time x salinity	Time x nitrogen	Time x salinity x nitrogen	Error	Intercept	Block	Salinity	Nitrogen	Salinity x nitrogen	Error
Fucoxanthin (Siliceous algae)	df	5.318	5.318	15.955	15.955	47.866	154.234	1	1	3	3	9	29
	MS	0.670	0.247	1.331	0.473	0.295	0.392	18.282	1.183	14.124	1.383	0.798	0.425
	F	1.710	0.631	3.397	1.207	0.752		42.968	2.780	33.195	3.251	1.875	
	p	0.131	0.686	**<0.001**	0.268	0.873		<0.001	0.106	**<0.001**	**0.036**	0.097	
Alloxanthin (Cryptophytes)	df	5.159	5.159	15.476	15.476	46.428	149.600	1	1	3	3	9	29
	MS	0.342	0.676	0.631	0.514	0.287	0.282	14.754	0.226	0.270	2.895	0.388	0.565
	F	1.216	2.400	2.240	1.825	1.020		26.107	0.400	0.478	5.122	0.686	
	p	0.304	**0.038**	**0.007**	**0.034**	0.451		<0.001	0.532	0.700	**0.006**	0.715	
Chl *b* (Chlorophytes)	df	6.972	6.972	20.916	20.916	62.748	202.189	1	1	3	3	9	29
	MS	0.337	0.096	0.487	0.189	0.170	0.170	5.059	0.025	6.055	1.550	0.580	0.300
	F	1.982	0.563	2.870	1.114	0.999		16.846	0.084	20.164	5.162	1.930	
	p	0.059	0.785	**<0.001**	0.336	0.488		<0.001	0.774	**<0.001**	**0.006**	0.087	
Echinenone (Cyanobacteria)	df	3.858	3.858	11.573	11.573	34.719	111.873	1	1	3	3	9	29
	MS	0.049	0.034	0.151	0.048	0.060	0.089	0.071	0.001	0.300	0.024	0.016	0.048
	F	0.548	0.385	1.695	0.544	0.668		1.469	0.016	6.222	0.501	0.341	
	p	0.694	0.812	0.080	0.876	0.913		0.235	0.901	**0.002**	0.685	0.953	
Chl *a* (all phototrophs)	df	7.898	7.898	23.695	23.695	71.085	229.053	1	1	3	3	9	29
	MS	0.747	0.458	1.279	0.736	0.459	0.373	74.688	0.783	13.385	6.389	1.232	1.440
	F	2.006	1.228	3.433	1.975	1.233		51.865	0.544	9.295	4.437	0.855	
	p	0.048	0.284	**<0.001**	**0.006**	0.127		<0.001	0.467	**<0.001**	**0.011**	0.574	

Table 4: Variance partitioning analysis on the relationships between categories of physicochemical (C), benthic primary producers (macrophyte PVI and periphyton Chl *a*; P) and invertebrate (I) variables on the phytoplankton pigment assemblages during the growth season (April-August 2005) period.

Component	% variance
Total	57.6
C	33.6
P	0.4
I	2.3
C + P	5.1
C + I	8.1
P + I	0.1
P + C + I	8.0
Unidentified	42.4

Figure legends

Figure 1: Brian Moss stylishly sampling aquatic plants from the mesocosm experiment. The arrangement and dimensions of the mesocosms located at Ness Botanical Gardens on Wirral, UK is shown in the photo (credit Tom Barker).

Figure 2: Mean concentrations (± standard error) of (a) fucoxanthin (siliceous algae), (b) alloxanthin (cryptophytes), (c) Chl *b* (chlorophytes), (d) echinenone (cyanobacteria) and (e) Chl *a* (all phytoplankton) between August 2004-05 within each treatment of the experiment. Salinity (S) treatments are denoted by the shading of the bars: S1 (600 mg Cl L^{-1}), S2 (1000 mg Cl L^{-1}), S3 (1600 mg Cl L^{-1}) and S4 (2500 mg Cl L^{-1}). Nitrate (N) treatments are arranged along the horizontal axis: N1 (1 mg N L^{-1}), N2 (2 mg N L^{-1}), N3 (5 mg N L^{-1}), N4 (10mg N L^{-1}). Significant differences ($p < 0.05$) or no significant differences (n.s.d.) are indicated among treatments pairs as assessed by Bonferroni tests.

Figure 3: Temporal changes in phytoplankton pigments fucoxanthin, alloxanthin, Chl *b*, echinenone and Chl *a* and biomass of the sum of *Daphnia* spp. (the latter uses data previously published in Barker et al 2008a) sampled between August 2004-05 and arranged according to salinity (S) treatments on the left: S1 (600 mg Cl L^{-1}), S2 (1000 mg Cl L^{-1}), S3 (1600 mg Cl L^{-1}) and S4 (2500 mg Cl L^{-1}) and nitrate (N) treatments on the right: N1 (1 mg N L^{-1}), N2 (2 mg N L^{-1}), N3 (5 mg N L^{-1}), N4 (10mg N L^{-1}). The plotted values are means ± standard errors of each treatment with levels indicated by shading as indicated in the legend. The vertical dashed line indicates the timing of stickleback introductions when fish density was increased from 'low' levels 0.67

stickleback m^{-2} (before April 2005) to 'high' densities of between 9 and 18 m^{-2} afterwards.

Figure 4: RDA showing the relationship between phytoplankton pigments (bold lines and italics; and significantly correlated environmental variables (thin lines and plain text; see Table 2 for further descriptions).

Figure 5: Box and whisker plots to summarise patterns in water chemistry variables across the salinity (left panel) and nitrate (right panel) treatments. The middle horizontal line represents the median of samples measured between August 2004-2005. Atomic N:P ratios were calculated from mesocosm total N and P measurements. Significant differences among levels are indicated with letters, where different letters indicate significant differences ($p<0.05$) between levels.

Figure 1

Figure 2

Figure 2

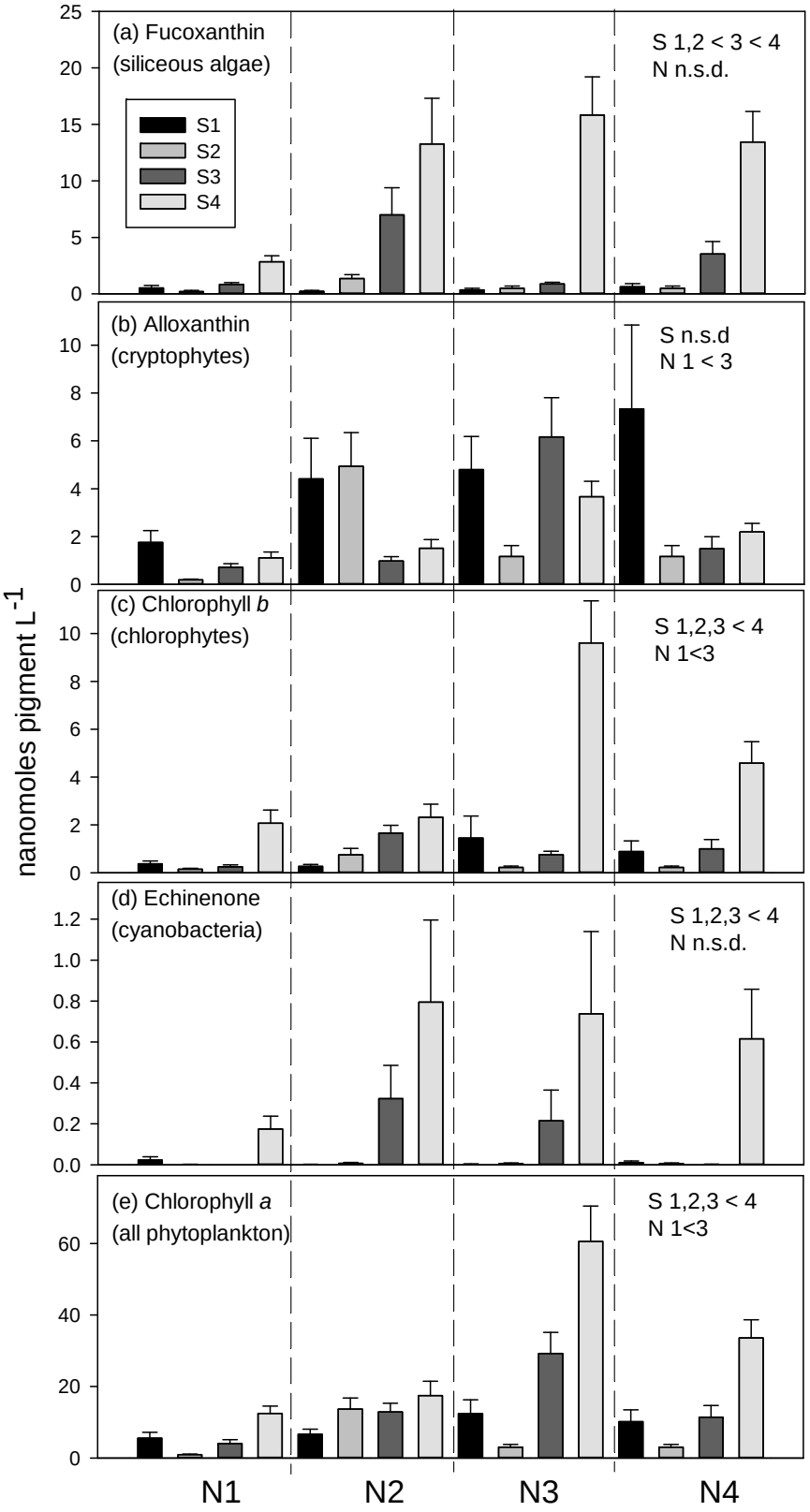


Figure 3

Figure 3

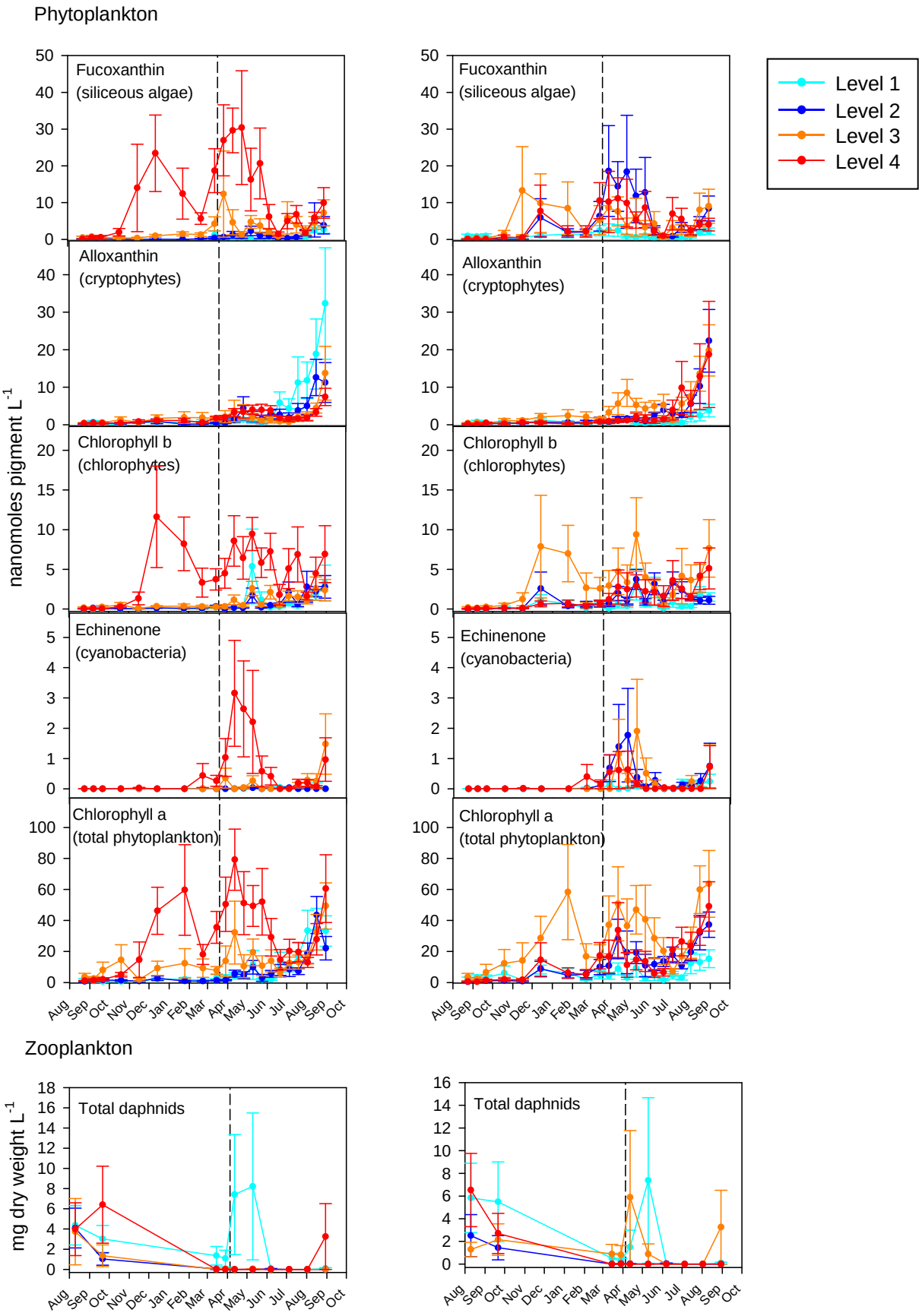


Figure 4

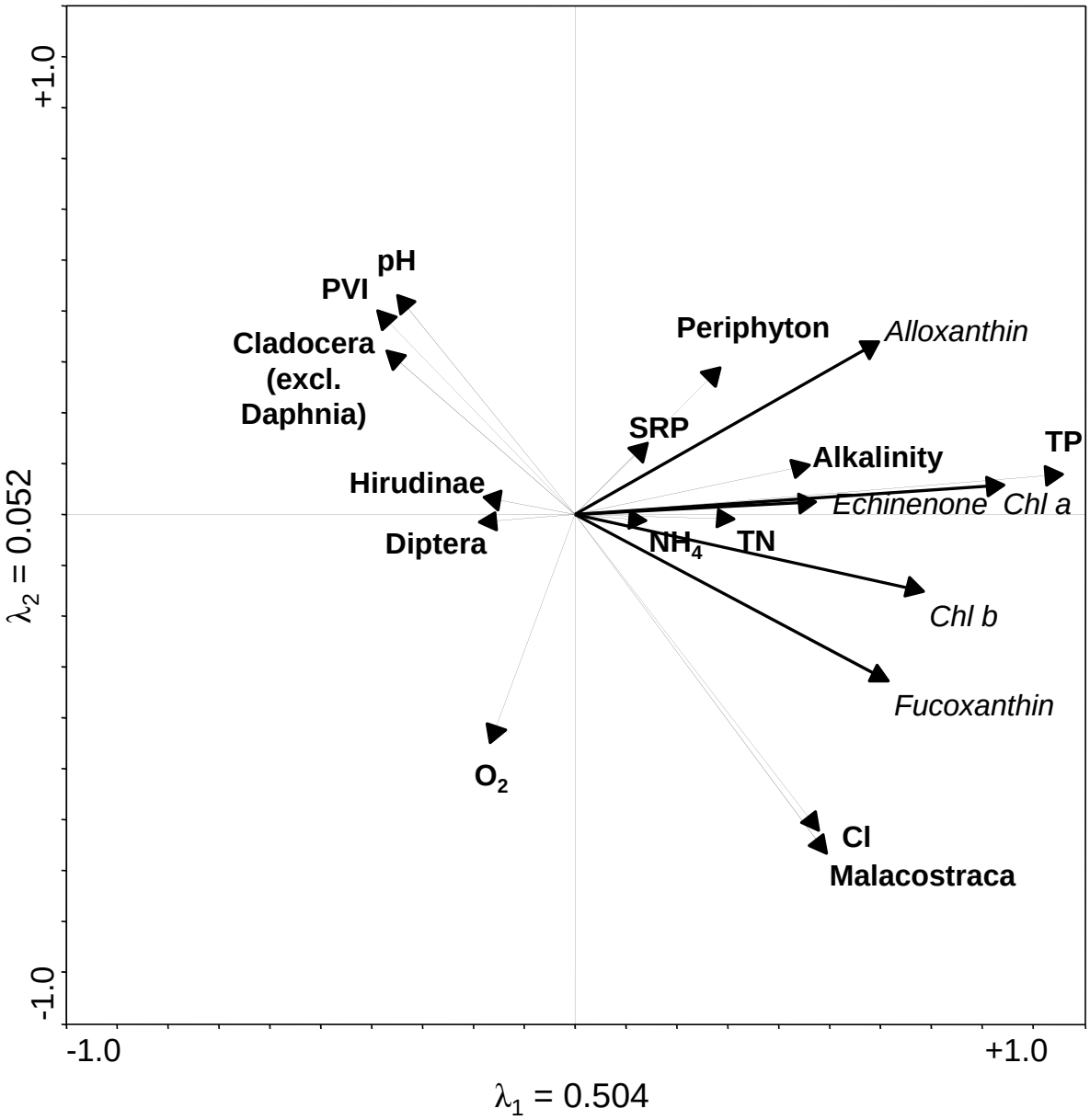
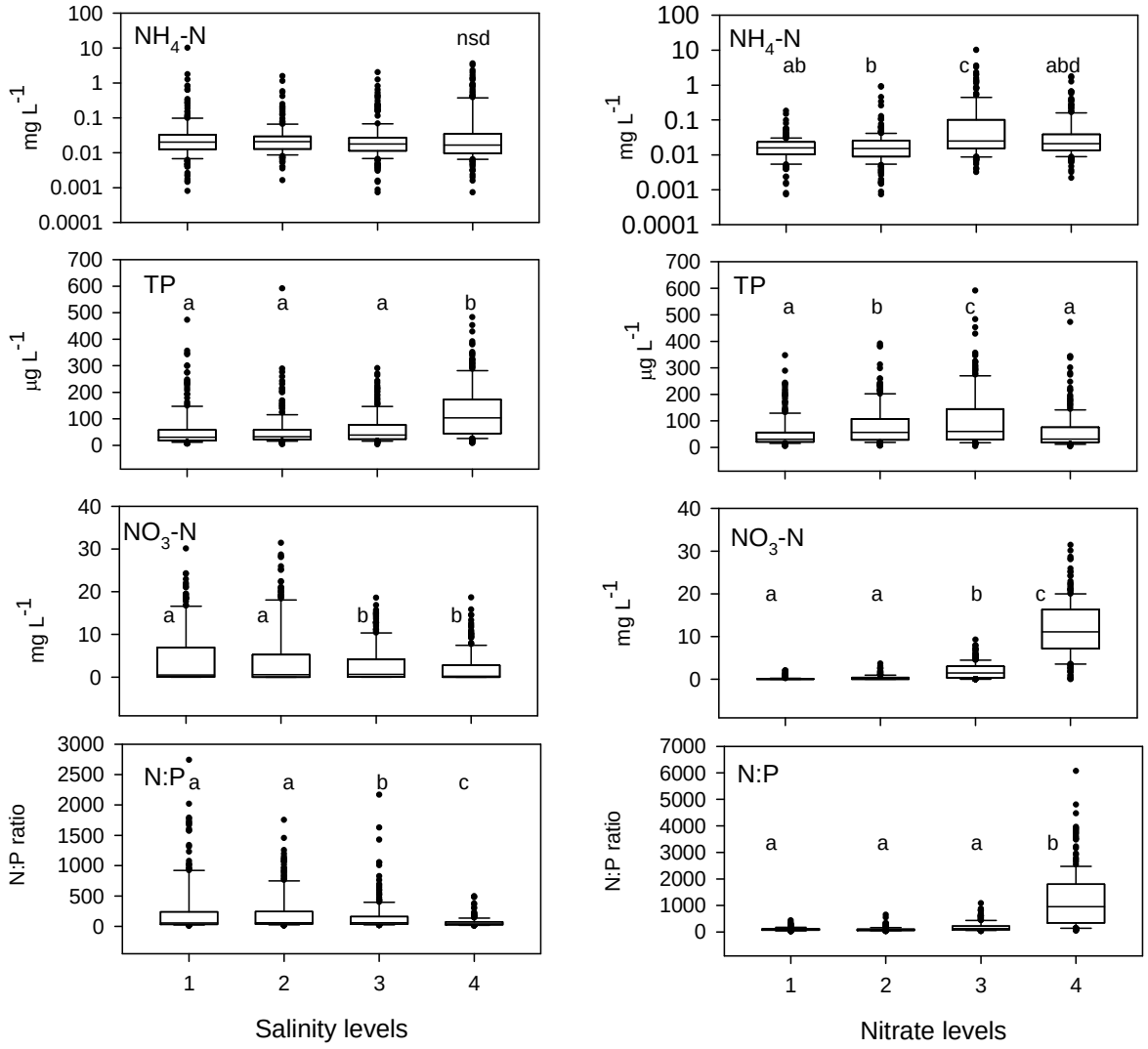

+1.0
-1.0
λ_2 = 0.052
-1.0
+1.0
λ_1 = 0.504
pH
PVI
Cladocera (excl. Daphnia)
Hirudinae
Diptera
O_2
Periphyton
SRP
Alloxanthin
Alkalinity
TP
Echinenone
Chl a
NH_4
TN
Chl b
Fucoxanthin
Cl
Malacostraca

Figure 5


NH4-N
nsd
mg L-1
TP
a a a b
µg L-1
NO3-N
a a b b
mg L-1
N:P
a a b c
N:P ratio
1 2 3 4
Salinity levels
NH4-N
ab b c abd
mg L-1
TP
a b c a
µg L-1
NO3-N
a a b c
mg L-1
N:P
a a a b
N:P ratio
1 2 3 4
Nitrate levels