Increased water colour affects freshwater plankton communities in a mesocosm study

Karen Lebret[1,3,*], Silke Langenheder[1], Noemi Colinas[1], Örjan Östman[2], Eva S. Lindström[1]

[1]Department of Ecology and Genetics/Limnology, Uppsala University, Uppsala, Sweden
[2]Department of Aquatic Resources, Swedish University of Agricultural Sciences, Öregrund, Sweden

[3]*Present address:* Centre for Ecology and Evolution in Microbial Model Systems (EEMiS), Linnaeus University, Kalmar, Sweden

ABSTRACT: Increases in water colour (brownification) have been observed in aquatic systems in the Northern Hemisphere, partly caused by increased loading of organic carbon from terrestrial origins. We investigated the effect of increase in water colour on the composition, structure and function of lake plankton communities (bacteria, phytoplankton and zooplankton) conducting a mesocosm experiment in 3 medium-coloured lakes (average absorbance at 420 nm: 0.034 cm^{-1}), with different nutrient concentrations and phytoplankton community composition. To simulate an increase in water colour, we added humic substances (HuminFeed) at 3 different concentrations. The additions significantly affected the water colour of the mesocosms, but had no measurable effect on total organic carbon concentration, thus change in light conditions was the main effect of our treatment on the plankton communities. The increase in water colour did not significantly affect the measured functions (productivity, respiration) and biomass of the plankton communities (bacteria, phytoplankton and zooplankton), but led to changes in the relative abundance of some phytoplankton taxa and, to a lesser extent, the bacterial community (differences in relative abundance). The treatments had no significant effect on zooplankton biomass or composition. Our study suggests that increases in water colour favour low-light-adapted phytoplankton species, which in turn also can affect bacterial composition, whereas the change in light climate had no clear impact on the functioning of plankton communities in weakly humic lakes.

KEY WORDS: Light climate · Plankton community · Lake · Bacteria · Phytoplankton

INTRODUCTION

In recent decades, lakes in the Northern Hemisphere have been and are affected by the brownification (or browning) phenomenon, which is an increase in water colour, mainly caused by increased concentrations of humic organic substances (Granéli 2012, Hansson et al. 2013, Solomon et al. 2015) and iron (Kritzberg & Ekström 2012, Weyhenmeyer et al. 2014). In Swedish lakes, concentrations of humic substances have more than doubled during the last 25 yr (Hansson et al. 2013), and model predictions further suggest that an increase in dissolved organic carbon (DOC) concentrations, including highly coloured humic substances, will most likely continue in the future (Larsen et al. 2011). The ultimate reasons for the increase in water colour are not fully clear (Monteith et al. 2007), but transport of organic matter and iron may have risen due to increased precipitation (Hongve et al. 2004), change in acid deposition (Monteith et al. 2007), changes in land use (Kritzberg 2017) and global warming (Evans et al. 2005, Larsen et al. 2011).

The increased carbon concentrations may lead to an increase in heterotrophic production and biomass (Blomqvist et al. 2001, Peura et al. 2014), which may

have ecosystem consequences. However, a change in light climate is another important consequence of browning in lake ecosystems (Fee et al. 1996, Thrane et al. 2014), which in turn may influence lake primary productivity and favour heterotrophic production over the light-dependent phototrophic production (Karlsson et al. 2009). Still, the impact of brownification, including the change in water colour, on ecosystems and communities may be complex through non-linear and threshold responses of brownification on ecosystem processes, and requires further investigation (Solomon et al. 2015).

Recent studies have identified a potential threshold in DOC concentrations, above which inhibition of primary production linked to light limitation becomes important, ranging approximately from 4.8 to 10 mg l^{-1} DOC (Hanson et al. 2003, Seekell et al. 2015a,b). Hence, the amplitude of the effect of brownification on ecosystems should depend on whether a lake is already above the threshold. In lakes above the threshold, which includes the most common lake type in the boreal zone (Sobek et al. 2007), further brownification should result in inhibitory effects on primary production.

In this study, we conducted an *in situ* mesocosm experiment replicated in 3 weakly coloured lakes with DOC concentrations typical of boreal lakes (10 to 20 mg l^{-1}), to investigate the effect of increased water colour on the function and composition of lake planktonic communities over a gradient of humic additions. To simulate changes in light climate due to brownification, we added 3 different levels of the highly coloured humic substance HuminFeed to each lake community. We hypothesized that bacterial activity would be enhanced with humic additions, whereas phytoplankton biomass would be reduced by the deteriorated light conditions. In addition, we expected to observe changes in the composition of the plankton communities towards low-light-adapted phytoplankton species and bacteria able to use recalcitrant organic carbon.

MATERIALS AND METHODS

Experimental lakes

In situ mesocosm experiments were performed during the summer of 2013 in 3 lakes — Ekholmssjön (59° 52′ 45″ N, 18° 32′ 11″ E), Hålsjön (59° 49′ 0″ N, 17° 13′ 46″ E) and Edasjön (59° 48′ 15″ N, 17° 54′ 8″ E) — located in central Sweden. The lakes were chosen to be representative of boreal forest lakes (e.g. relatively small, meso-humic and mesotrophic) (Table 1). Although the lakes were meso-humic, they are in the medium range of DOC concentration and water colour in the study region (Sobek et al. 2007) and are prone to be affected by brownification. These 3 lakes were slightly different in nutrient concentration (Table 1) and in the composition of the phytoplankton community (see Fig. S1 in the Supplement at www.int-res.com/articles/suppl/a081 p001_supp.pdf).

Mesocosm design

In each lake, 12 mesocosms were set up, consisting of 500-l white opaque plastic bags (150 cm deep, 60 cm diameter) floating at the surface of the lake; the bags were made buoyant with air-filled inner tubes. The mesocosms were filled with surface lake water from the respective lakes using a pump, after prefiltration of the water through an aquarium net (mesh size 125 μm) to remove larger zooplankton and small fish. The plankton communities were allowed to acclimatize to the new conditions for a week before humic substance additions (HuminFeed®) were made (Day 0). HuminFeed® has been used in previous studies as a source of humic substances to determine the impact of humic substances on aquatic organisms (Bouchnak & Steinberg 2013, 2014, Rasconi et al. 2015). HuminFeed® is extracted from Leonardite using an alkaline extraction process and is composed of 43% organic carbon, of which 82% is

Table 1. Summary of the chemical characteristics of the 3 experimental lakes. Total organic carbon (TOC) and total nitrogen were measured on 31 July

	Ekholmssjön	Hålsjön	Edasjön
Coordinates	59° 52′ 45″ N, 18° 32′ 11″ E	59° 49′ 00″ N, 17° 13′ 46″ E	59° 48′ 15″ N, 17° 54′ 08″ E
Water colour (absorbance at 420 nm cm^{-1})	0.024–0.033	0.021–0.032	0.034–0.041
TOC (mg l^{-1})	15	15.5	18.2
Total phosphorus (μg l^{-1})	25–31	31–37	37–70
Total nitrogen (mg l^{-1})	0.7	0.8	1.2

humic substances (Meinelt et al. 2007). The detailed chemical compostion of HuminFeed® has been fully characterized by Meinelt et al. (2007). In our experiment, to create a gradient in humic substances, HuminFeed® was added at 3 levels: low concentration with 3 mg l^{-1} (LH) of HuminFeed® [corresponding to approximately 1.2 mg DOC l^{-1} addition; expected DOC addition according to Meinelt et al. (2007)], medium concentration with 6 mg l^{-1} (MH) (2.4 mg DOC l^{-1} addition) and high concentration with 12 mg l^{-1} (HH) (4.8 mg DOC l^{-1} addition), all in triplicate. Three mesocosms without addition of humic substances served as controls (C). Thus, there were 12 mesocosms in each lake. With these HuminFeed® additions, we expected to observe approximately 8 to 9% increase in DOC in the LH treatment compared to the control, 16 to 18% in the MH treatment, and 33 to 37% increase in the HH treatment. The experiments were run for 32 d simultaneously in all lakes (starting on 10 June 2013). During the experiment a few mesocosms were lost or damaged due to weather conditions: in Ekholmssjön, 1 control was lost on Day 16, and 1 LH mesocosm was lost on Day 32; in Hålsjön, 2 MH mesocosms were lost on Day 16; and in Edasjön, 1 MH mesocosm was lost on Day 16.

Sampling of the mesocosm

The water was homogenized in all mesocosms before the collection of the samples by pulling a Secchi disc up and down. In each mesocosm, water samples were collected on Day 0 before addition of HuminFeed®, and after the HuminFeed® addition on Days 1, 2, 4, 8, 16 and 32 to determine water colour, chlorophyll *a* concentration and bacterial abundance. The water colour was measured on all sampling dates to control for potential bleaching and so that differences in water colour among treatments were maintained over the course of the experiment. The sampling dates were chosen to allow for the detection of rapid as well as slow responses of communities to the treatments. Water samples were also collected to measure bacterial production on Days 0, 4, 8, 16 and 32. Samples for DNA extraction to determine bacteria and phytoplankton community composition were collected on Days 0, 2, 8, 16 and 32. Plankton community respiration was measured in each mesocosm on Days 16 and 32. To determine the composition and biovolume of the zooplankton community, 10 l of water was filtered through a 100 µm net to concentrate the zooplankton on Day 32, and then the zooplankton were transfered to a bottle and immediately preserved with a few drops of Lugol's solution. On Day 1, samples were collected for nutrient analyses (total phosphorus, total nitrogen and total organic carbon) and were frozen at −20°C until analyses. In addition, water samples were collected in the water column of the 3 lakes on Days 0, 16 and 32 to compare the plankton community compositions of the mesocosms to the natural communities of each lake.

Chemical parameters

The total phosphorus concentrations were determined in triplicate for each sample using a volume of 5 ml throught persulfate oxidation and using the MRP method according to Menzel & Corwin (1965) and Murphy & Riley (1962). Total nitrogen concentations were measured in triplicate for each sample using a volume of 5 ml according to Rand et al. (1976). Total organic carbon (TOC) was analysed in triplicate in a Shimadzu TOC-L analyser using a sample volume of 50 µl. The instrument was calibrated with potassium hydrogen phthalate standards. To estimate the colour of the water, the water was filtered through GF/C glass fibre filters, and the absorbance of the filtrate was measured at 420 nm in a 5 cm quartz cuvette using a spectrophotometer (Lambda35, Perkin Elmer); the measurements were made on fresh samples within a few hours of sampling, similar to previous studies (Blindow et al. 2002, Ekvall & Hansson 2012, Urrutia-Cordero et al. 2016). A water blank (Milli-Q water) was used to zero the instrument at 420 nm prior to measuring the samples. Conductivity and pH in the mesocosms were measured on Day 1 using a pH/conductivity meter (VWR Symphony SP80PC pH/conductivity meter). The pH was also measured on Day 16.

Functions and biomass of the bacterioplankton and phytoplankton community

To quantify the abundance of bacteria in the samples, bacteria were preserved with formaldehyde to a final concentration of 2% (v/v). The samples were stored at 4°C until processing. The bacteria were then stained with 1.25 µM of Syto13 solution (New England Biolabs). Bacteria were counted with a blue laser flow cytometer (Cyflow Space, Partec). Gross bacterial production was estimated by measuring leucine incorporation into proteins according to Smith

& Azam (1992). Radiolabelled L-[4,5-³H]-leucine was diluted to 15% with unlabeled leucine using a total final leucine concentration of 100 nM. A volume of 1.7 ml of sample was then incubated for 30 min with 5 µl of the diluted leucine solution. A control sample, inactivated by the addition of 100% TCA prior to leucine addition, was also incubated for 30 min. The samples were then processed according to Langenheder et al. (2006). Respiration of the whole community was measured as the decrease in oxygen concentration over a 24 h period in darkness. Oxygen concentrations were measured in air-tight bottles filled with water from the mesocosms using a PSt1 micro-optode (Fibox 3, Precision Sensing). The phytoplankton biomass was assessed by measuring the chlorophyll *a* concentrations in the mesocosms. Up to 1 l of water samples was filtered through GF/C glass fiber filters and stored frozen until processsing. The filters were then processed according to Jespersen & Christoffersen (1987); chlorophyll *a* was extracted using 10 ml of 95% ethanol overnight, and absorbance was measured at 750 and 665 nm using a spectrophotometer (Lambda35, Perkin Elmer). The concentrations were determined according to Jespersen & Christoffersen (1987).

Zooplankton

Depending on zooplankton abundance in the sample, only a fraction of the sample was analysed, from the whole sample to one-fifth if densities were high. If only a fraction of a sample was analysed, we fractionated the samples using a centrifuge that equally divided the sample into 10 compartments. Depending on densities, 2–5 compartments were randomly chosen for analyses. Zooplankton were sedimented in a counting chamber and analysed with an inverted microscope on a computer screen. All zooplankton in the sample or subsample was identified to genus and counted. For the first 30 individuals of each genus, body length was measured on the screen. Length was not calculated for individuals that were broken, twisted or partly hidden, hindering appropriate measuring even if it was among the first 30 individuals of a genus. Biovolume of each species was calculated with genus-specific length–biovolume formulas (Botrell et al. 1976, Johansson et al. 1976, Ruttner-Kolisko 1977). In the analysis, individuals of the genus *Acroperus* were removed, as it is a littoral/benthic genus (Fryer 1968) and was assumed to be an artifact from the mesocosm conditions.

16S rRNA gene amplicon sequencing

The composition of the bacterial and phytoplankton communities was determined using amplicon Illumina sequencing of the 16S rRNA gene (variable regions 3 and 4). The 16S rRNA region is commonly used for the taxonomic identification of prokaryotes. For the eukaryote phytoplankton, the 16S rRNA region of the chloroplasts was used for taxonomic determination as previously described by Eiler et al. (2013). The plankton community was concentrated by filtration of up to 200 ml on 0.2 µm membrane filters (Pall Corporation) within 4 h of sampling; the samples were stored at *in situ* room temperature until filtration, close to the lake temperature. The filters were stored at −80°C until DNA extraction. The DNA was extracted directly from the filters using the PowerSoil DNA isolation kit (MoBio Laboratories) according to the manufacturer's instructions. The preparation of the samples for sequencing of the 16S rRNA region was performed according to Sinclair et al. (2015). A first PCR was applied to the samples to amplify the variable regions 3 and 4 of the 16S rRNA gene. The PCR was conducted using 0.5 µM of primers (forward primer: 341F, CCT ACG GGN GGC WGC AG; and reverse primer: 805R, GAC TAC HVG GGT ATC TAA TCC) (Herlemann et al. 2011), 200 µM of dNTP mix, 4 U of Q5 High-Fidelity DNA polymerase (Biolabs), and 1 µl of sample in a 20 µl final volume. The PCRs were performed with the following settings: an initial denaturation at 98°C for 30 s, followed by 20 cycles with 10 s at 98°C, 30 s at 53°C, and 30 s at 72°C, and a final extension at 72°C for 2 min. The PCR products were then purified using the Agencourt AMPure XP purification kit according to the manufacturer's instructions and diluted 50 times. A second PCR was then performed with barcoded primers (forward and reverse) to ligate distinct barcodes to each sample according to Sinclair et al. (2015). The PCR reactions consisted of 200 µM of dNTP mix, 0.5 µM of each primer including the barcodes, 4 U of Q5 High-Fidelity DNA polymerase (Biolabs), and 1 µl of sample in a 20 µl final volume. The following thermal cycle was applied to the samples: an initial denaturation step at 98°C for 30 s, followed by 15 cycles with 10 s at 98°C, 30 s at 53°C and 30 s at 72°C, and a final extension step for 2 min at 72°C. The samples were then purified using Agencourt AMPure XP purification kit according to the manufacturer's instructions. The DNA was quantified in each sample using the Quant-iT PicoGreen dsDNA quantification kit according to the manufacturer's instructions. The samples were then pooled (maxi-

mum 50 samples per pool) using 30 ng of DNA for each sample. The pooled samples were then submitted to the SciLifeLab SNP/SEQ sequencing facility hosted by Uppsala University for library preparation and pair-end sequencing using MiSeq technology.

16S rRNA sequence data processing

Sequences were processed using the illumitag pipeline as described in Sinclair et al. (2015). In short, the paired-end reads were merged using PANDAseq (Masella et al. 2012), and filtered based on their Phred scores. Multiplexing barcodes were cut off, and chimeras were removed. Chimera detection and OTU (operational taxonomic unit) clustering at 3% sequence dissimilarity were performed using UPARSE (Edgar 2013). The taxonomical annotation of the identified OTUs was performed by CREST using the SILVAmod database (Lanzen et al. 2012). After sequence processing and OTU clustering, a total of 3 571 334 reads were retained for further analyses, spread among 3749 OTUs. On average, the number of reads per sample was 20 064, with a minimum of 10 216 reads and a maximum of 61 894 reads per sample. Then, OTUs assigned to heterotrophic bacteria (*Cyanobacteria*, *Archaea* and chloroplast sequences were excluded) and phytoplankton (*Cyanobacteria* and chloroplast of autotrophic eukaryotes) were divided into 2 distinct OTU tables to be analysed separately. A total of 3225 OTUs were assigned to bacteria taxa, with a total number of reads of 3 048 847. For bacteria, the average number of reads per sample was 17 128, with a minimum of 8154 reads and a maximum of 54 473 reads. A total of 319 OTUs were assigned to phytoplankton taxa, with a total number of reads of 513 003. On average, the number of reads per sample was approximately of 2882, with a minimum of 323 reads and a maximum of 7327 reads. To avoid biases caused by different numbers of reads per sample, the 2 tables were subsampled with 100 iterations to a final number of reads per sample of 8154 for bacteria and 323 for phytoplankton. The Illumina reads have been deposited in the European Nucleotide Archive (ENA) under the accession number PRJEB11707.

Statistical analyses

All the statistical analyses were done in R using the vegan, nlme or car packages. If necessary, to meet the assumptions of each statistical test, the data were transformed as indicated below. One-way ANOVAs were performed on $\log(x+1)$ transformed data to determine whether the HuminFeed® addition had a significant effect on the concentrations of total phosphorus, total nitrogen, TOC, pH and conductivity in the samples collected on Day 1, and for pH on Day 16.

For each lake separately, repeated-measures (of sampling day) ANOVA (rmANOVA) was performed on $\log(x+1)$ transformed data to determine whether the treatments had a significant effect on water colour, bacterial abundance, bacterial production, bacterial respiration and chlorophyll *a* concentrations. To determine the general effect size of treatments in the rmANOVA, eta squared (η^2) was calculated according to Olejnik & Algina (2003) and can be interpreted as the proportion (0–1) variation of the dependent variable explained by HuminFeed® treatments after removing the effect of time. To determine the magnitude of each treatment in comparison to the control, we calculated the Cohen's *d* effect size according to Ellis (2010) using the average values of the parameter over the course of the experiment as calculated by Ekvall & Hansson (2012). Cohen's *d* is the difference between 2 treatments relative to the pooled standard deviation, and the magnitude of the effect is considered to be small if Cohen's *d* is larger than $|0.2|$, medium if larger than $|0.5|$ and large if above $|0.8|$ (Cohen 1988), where negative values indicate a negative response. For the parameters significantly affected by the treatment, a Tukey's post hoc test was applied to determine the presence of a significant difference between treatment pairs.

The zooplankton biovolume data were $\log(x+1)$ transformed prior to statistical analyses. Redundancy analyses (RDA) were performed to determine whether the treatments had an effect on the composition of the zooplankton community for each lake separately, and allowing the direct identification of the species affected by the treatments. One-way ANOVAs were done to determine the effect of the treatment on the total zooplankton biovolume for each lake separately.

To study initial bacteria and phytoplankton community composition (at Day 0) between treatments and among lakes, RDA was performed on the number of reads of subsampled data from Day 0. Species diversity was estimated for all samples by measuring the observed species richness (S_{obs}; i.e the number of OTUs per sample) using the subsampled datasets. In addition, evenness was assessed by determining the Pielou's evenness (E) index calculated by $E =$

(H'/H_{max}), where H' is the Shannon index and $H_{max} = \ln(S_{obs})$. rmANOVAs were performed to determine whether the treatments had significant effects on species richness and evenness for each lake separately. The general effect size η^2 and Cohen's d were calculated as described previously. A post hoc test was applied to determine which treatment caused changes when the rmANOVA results showed a significant effect of the factor treatment on either species richness or evenness.

To assess the general effect of time and treatment on the communities, and on individual OTUs, principal response curve (PRC) analyses were performed for each lake for bacteria and phytoplankton communities using the control samples as the baseline according to Van Den Brink & Ter Braak (1999). PRC analysis is a special case of RDA which is especially designed to analyse multivariate responses in experiments with a repeated measurement design (Van Den Brink & Ter Braak 1999). PRC allows a direct comparison of each treatment with the control by determining the deviation in community composition from the control over time. The temporal heterogeneity of the control and the treatment are taken into consideration in the analyses in order to highlight the treatment effect. The deviations of each treatment from the control can then be shown in the PRC plot using the canonical coefficient calculated using PRC. For this study, the PRC analyses were performed on reduced datasets, for which the rare OTUs (maximum number of reads <1% of total number of reads in a sample) were excluded. Rare OTUs were removed as their temporal dynamics might not be well represented due to low sequencing depth. The data (number of reads of each OTU) were $\log(x+1)$ transformed prior to analysis.

To determine whether treatment had a significant effect on the number of reads of coarser taxonomic groups, rmANOVAs were performed on $\log(x+1)$ transformed data, excluding the rare OTUs. For these analyses, the OTUs were merged according to their assignment to taxonomic groups. For bacterial communities, 14 groups based on the major or abundant taxonomic bacterial clades were formed as follows: *Alphaproteobacteria* LD12, *Alphaproteobacteria* other than LD12, *Actinobacteria* without the hgcl clade, *Actinobacteria* hgcl clade, *Betaproteobacteria*, *Bacteroidetes*, *Verrucomicrobia*, OD1, *Chloroflexi*, *Gammaproteobacteria*, *Chlorobia*, *Armatiomonadetes*, *Deltaproteobacteria* and *Acidobacteria*. These taxonomic groups vary in taxonomic resolution, but have been chosen to highlight the dynamics of abundant taxonomic groups present in the samples. The *Alphaproteobacteria* and

the *Actinobacteria* were subdivived into 2 groups due to the presence of the abundant sub-groups LD12 for the *Alphaproteobacteria* and the hgcl clade for the *Actinobacteria*. These 2 subgroups were abundant, or represented by several OTUs, and showed independent dynamics over time and among treatments from their respective taxonomical groups. For the phytoplankton communities, the OTUs were summarized into 10 main taxonomic groups: Synechoccocales, Nostocales, Oscilatoriales, Stramenopiles, Chlorophyta, Cryptophyta, Streptophyta, Haptophyta, Raphidophyta and unclassified. The taxonomic depth of these groups varies, but they have been chosen to highlight the dynamics of the abundant groups in the mesocosms. The general effect size η^2 and the Cohen's d effect size were calculated as described above.

RESULTS

Water colour and chemical characteristics

The addition of HuminFeed® had a fairly large effect on water colour in all lakes (p < 0.001 for the 3 lakes, $\eta^2 = 0.78$ for Ekholmssjön, $\eta^2 = 0.64$ for Hålsjön, and $\eta^2 = 0.82$ for Edasjön; Table S1). In comparison to the control, the water colour increased by approximately 50%, 100% and 180% in LH, MH and HH, respectively. The magnitude of the effect (Cohen's d effect size) on the water colour varied between 4.83 and 7.55 for the LH treatment in Edasjön and Ekholmssjön, respectively, and 15.63 and 23.07 for the HH treatments in Edasjön and Hålsjön, respectively (Table S2). These differences between treatments were maintained over time during the whole experiment (Fig. 1). Still, the water colour of all mesocosms decreased significantly over the course of the experiment by 4% to 16% in comparison to the water colour on Day 1 (rmANOVA: Ekholmssjön: $F_{5,30} = 27.85$, p < 0.001, Hålsjön: $F_{5,30} = 74.94$, p < 0.001; Edasjön: $F_{3,35} = 142.1$, p < 0.001).

In contrast, the HuminFeed® addition did not significantly affect the concentration of TOC on Day 1 (Fig. 2), nor did it have any significant effect on total phosphorus, total nitrogen concentration, pH or conductivity on Day 1 (ANOVA p > 0.05). On Day 16, the pH in the mesocosms was not significantly different between treatments in the 3 lakes (ANOVA: Ekholmssjön: $F_{3,6} = 0.254$, p > 0.05; Hålsjön: $F_{3,6} = 0.417$, p > 0.05; Edasjön: $F_{3,7} = 0.918$, p > 0.05). Thus, HuminFeed® additions only led to measurable effects on water colour, but not on TOC or nutrient concentrations in the mesocosms.
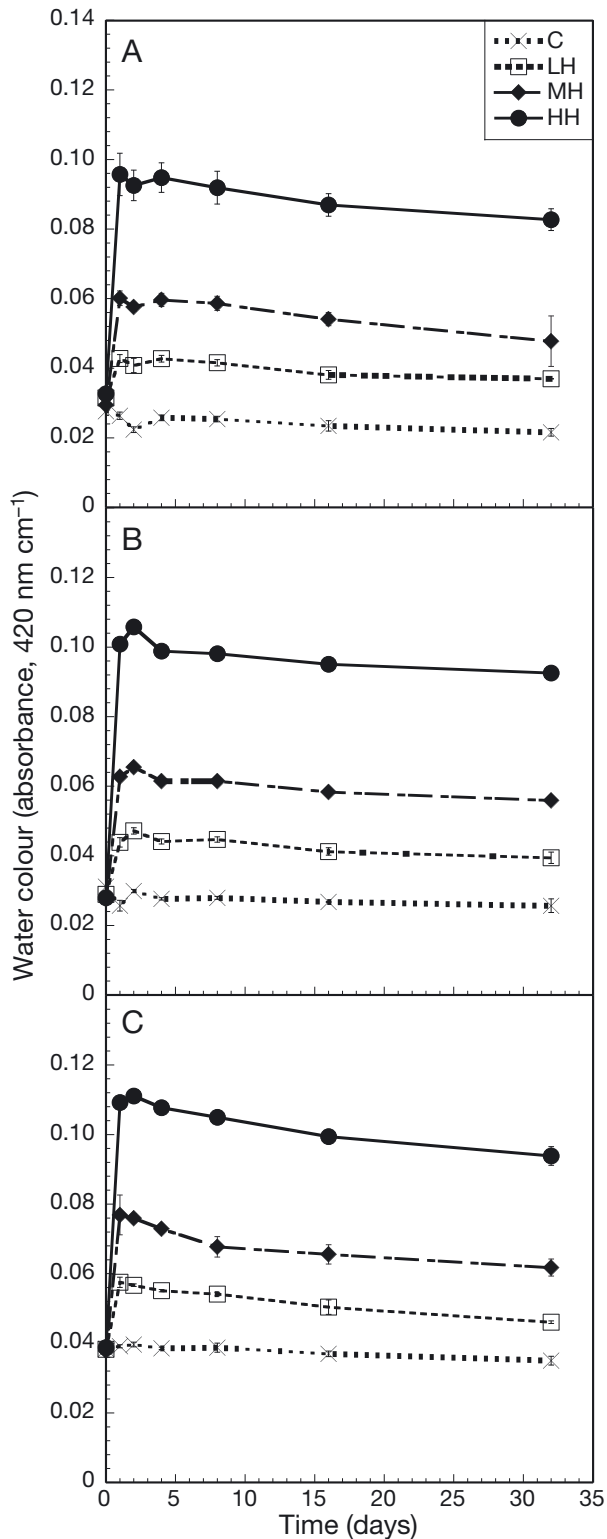
Fig. 1. Mean (±SE) water colour in the treatments over time in the 3 lakes: (A) Ekholmssjön, (B) Hålsjön and (C) Edasjön. C: control, LH: low HuminFeed®, MH: medium Humin-Feed® and HH: high HuminFeed®. In Hålsjön, after Day 16, only one mesocosm of the MH treatment remained, thus no standard deviation is available for Day 16

Abundance and function

In general, humic treatments had small and non-significant effects on the abundance and function of plankton communities. For bacterial abundance, a significant effect of the treatment was only detected in lake Edasjön (rmANOVA: $F_{3,7}$ = 5.13, p = 0.03, η^2 = 0.26; Table S1), being highest in the MH treatment and lowest in the control. Based on Cohen's d effect size, the MH and HH treatments had strong positive effects on bacterial abundance with d equal to 1.49 and 1, respectively (Table S2). While the treatments had no significant effect on the gross bacterial production in Lake Hålsjön or Lake Edasjön (p > 0.05; Table S1, Fig. S3), production was slightly lower in the HH treatment in comparison to the control in Lake Ekholmssjön (rmANOVA post hoc test: F = 4.74, p = 0.07, Cohen's d = −2.41; Tables S1 & S2, Fig. S3).

The treatments did not have significant effects on whole community respiration in any of the 3 lakes (p > 0.05; Table S1). Respiration in the mesocosms in Ekholmssjön and Hålsjön did not significantly change over time (rmANOVA: $F_{1,6}$ = 4.32, p = 0.083, and $F_{1,6}$ = 1.44, p = 0.3, respectively). In Edasjön, on the contrary, respiration decreased significantly in the mesocosms by, on average, 83% between Day 16 and Day 32 (rmANOVA, effect of time: $F_{1,7}$ = 367.26, p < 0.001) (data not shown).

Chlorophyll a concentration did not differ significantly among treatments for any of the lakes (p > 0.05; Table S1, Fig. S6). In all lakes, an initial increase in chlorophyll a concentration until Day 8 was observed, and then a decrease; this pattern was more pronounced in Lake Edasjön (Fig. S4).

Bacterial community composition and diversity

On Day 0, bacterial community composition did not differ between treatments within each lake (RDA: $F_{3,29}$ = 0.72, p = 0.65), but differed significantly among lakes (RDA: $F_{2,29}$ = 59.33, p = 0.001). In each lake, the bacterioplankton composition on Day 0 of the experiment was different (Fig. S2): 40%, 21% and 25% of lake bacterial OTUs were unique to Ekholmssjön, Hålsjön and Edasjön, respectively.

During the experiment, the treatments had only a significant effect on species richness in Edasjön (rmANOVA: $F_{3,7}$ = 5.81, p = 0.026, η^2 = 0.24) and no significant effects were observed on evenness (p > 0.05; Table S1). The observed effect of treatment on species richness in Edasjön was because of a higher richness in the HH treatment compared to the control
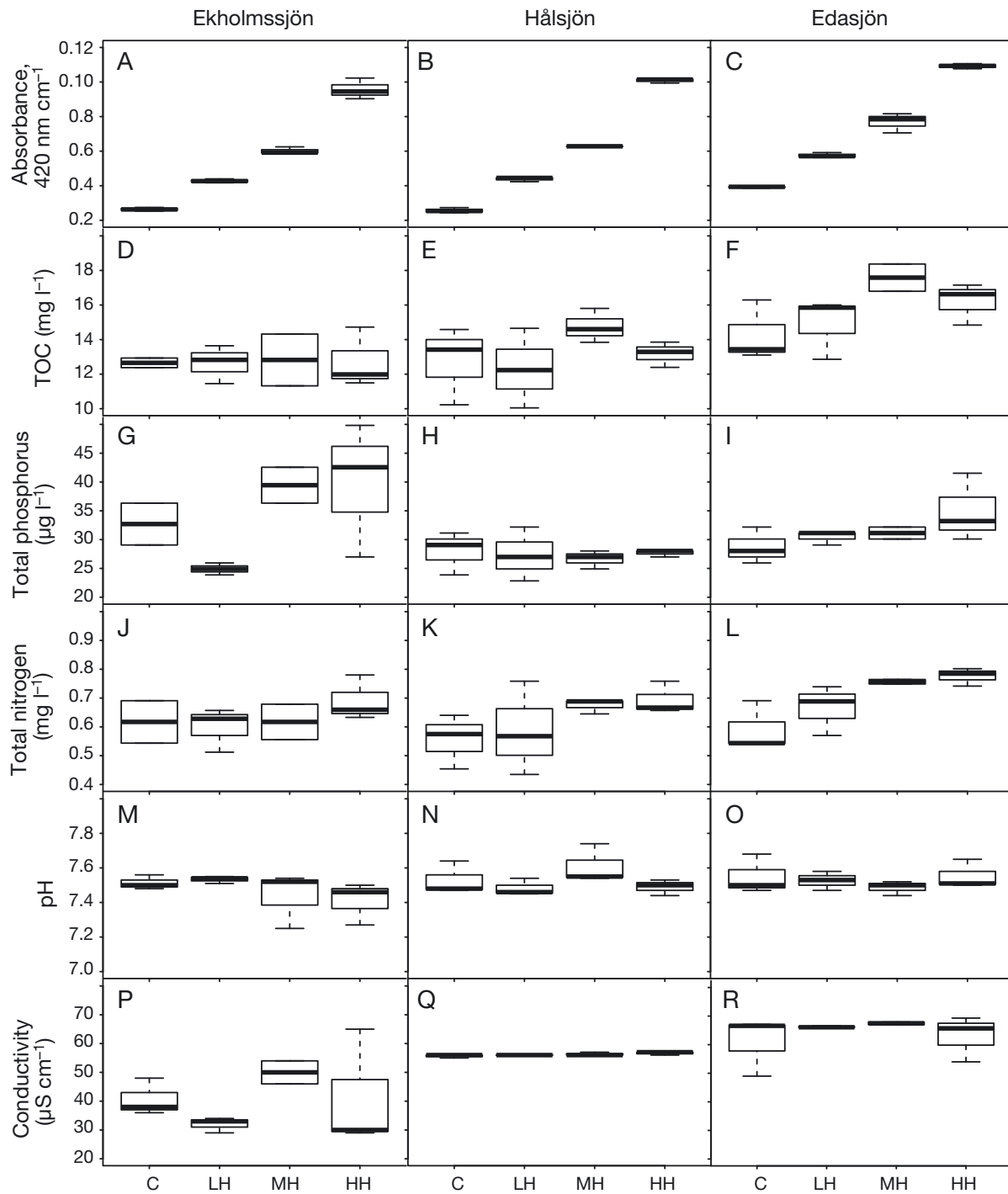
Fig. 2. Chemical characteristics of the mesocosm water on Day 1: water colour, total organic carbon (TOC), total phosphorus, total nitrogen, pH and conductivity (after the HuminFeed additions; C: control, LH: low HuminFeed®, MH: medium Humin Feed®, and HH: high HuminFeed®)

(post hoc test: $F = 3.62$, p = 0.09, Cohen's $d = 1.05$; Table S2).

The PRC analyses showed that the bacterial communities (only dominant OTUs) changed significantly for all 3 lakes (PRC: Ekholmssjön $F_{1,35} = 9.7$, p = 0.002; Hålsjön $F_{1,36} = 21$, p = 0.001; Edasjön $F_{1,38}$

= 9.8, p = 0.007) and diverged from the control community over time with increasing water colour (Fig. 3). The divergence from the control was more pronounced in the treatment with darker water (Fig. 3). According to the PRC analyses, the observed variance for the 3 lakes in bacterial communities was

mainly explained by time (51%, 66% and 70% for Ekholmssjön, Hålsjön and Edasjön, respectively) but also by treatment (20%, 17% and 13% for Ekholmssjön, Hålsjön and Edasjön, respectively). More specifically, in Ekholmssjön, a significant increase in Chlamydiae was observed in the HuminFeed® treatment in comparison to the control (Tables 2 & 3, Fig. 4). In Hålsjön, significant decreases of the relative abundances of the LD12 and hgcl clades were observed in treatments with darker water colour (Tables 2 & 3, Fig. 4). In addition, significantly higher relative abundances with large effect sizes (Cohen's $d > 0.8$) were observed in dark water treatments for the *Betaproteobacteria*, *Verrucomicrobia* and *Chloroflexi* in Hålsjön (Tables 2 & 3, Fig. 4). In Edasjön, darker water colour induced significant increases in the relative abundance of *Verrucomicrobia* and *Chloroflexi* (Cohen's $d > 0.8$), and decreased that of *Bacteroidetes* (Tables 2 & 3, Fig. 4).

Phytoplankton community composition

On Day 0 before the humic substance additions, the phytoplankton community composition differed significantly among lakes (RDA: $F_{2,29} = 78.28$, p = 0.001), but not between treatments within each lake (RDA: $F_{3,29} = 0.95$, p = 0.419). In the lakes, the phytoplankton compositions on Day 0 were different (Fig. S1): 32%, 7% and 25% of the phytoplankton OTUs in Ekholmssjön, Hålsjön and Edasjön, respectively, were not detected in any of the other lakes. In none of the 3 lakes did treatments have a significant effect on species richness or evenness (Table S1).

For Ekholmssjön and Hålsjön, the PRC analyses showed that treatments significantly affected the composition of the phytoplankton communities (PRC: Ekholmssjön $F_{1,35} = 8.2$, p = 0.001; and Hålsjön $F_{1,63} = 12.3$, p = 0.001), as the communities in the treatments diverged over time from the control community (Fig. 5). For these 2 lakes, 50 and 53% of the observed variance could be explained by time, and 20 and 22% by treatment for Ekholmssjön and Hålsjön, respectively. For Edasjön, the treatments did not significantly affect the phytoplankton community composition according to the PRC analyses (PRC: $F_{1,38} = 9.1$, p = 0.136). Hence, the phytoplankton community composition of the treatments MH and HH first diverged from the composition of the control, but after Day 16, the community tended to become more similar to the control again (Fig. 5).

In Ekholmssjön, a significant decrease in the number of Cryptophyta reads was observed with darker
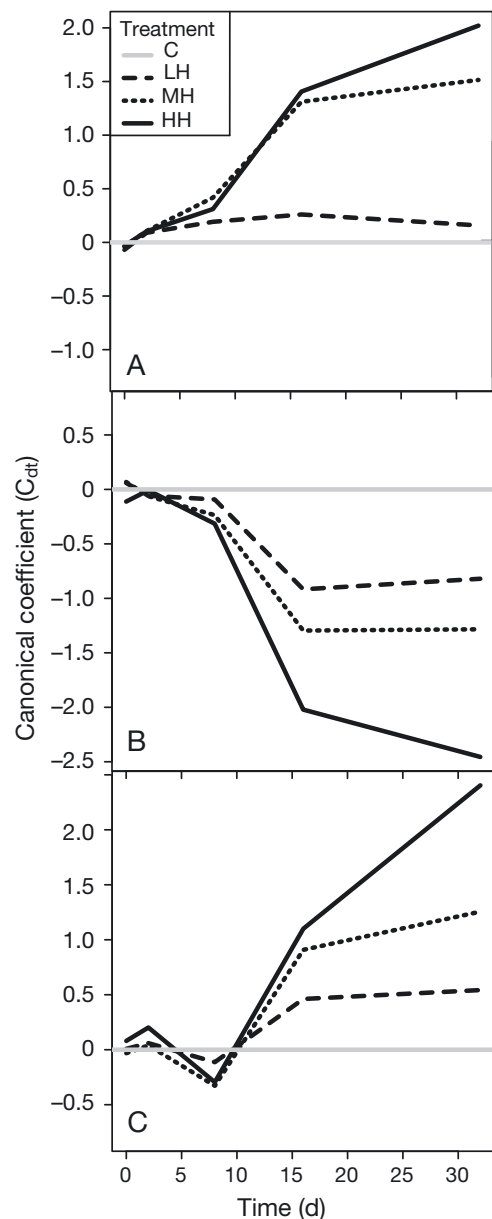

Fig. 3. Principal response curve of the bacterial community over time in the treatments in (A) Ekholmssjön, (B) Hålsjön and (C) Edasjön. C: control, LH: low HuminFeed®, MH: medium HuminFeed® and HH: high HuminFeed®

water colour, and the effect was strong for the MH and HH treatments (Tables 2 & 3, Fig. 6). For Hålsjön, increasing water colour led to a significant increase in the following phytoplankton groups: Nosctocales, Oscillatoriales and Stramenopiles (Cohen's $d > 0.8$; Tables 2 & 3, Fig. 6). However, significantly lower numbers of reads were observed for the Synechoccocales with increasing water colour (Tables 2 & 3, Fig. 6). For Edasjön, on Days 2 and 8 the relative abundance of Stramenopiles was higher in dark

Table 2. Summary of the treatment effect according to the repeated-measures ANOVA on the relative abundance of the different dominant bacteria and phytoplankton groups (–: not a dominant group). Values in **bold** indicate statistically significant values

Group	Ekholmssjön $F_{3,6}$	p	η^2	Hålsjön $F_{3,6}$	p	η^2	Edasjön $F_{3,7}$	p	η^2
Bacteria									
LD12	0.781	0.546	0.08	**1099.420**	**<0.001**	**0.99**	0.384	0.768	0.04
Actinobacteria without hgcl clade	0.54	0.671	0.04	3.649	0.083	0.23	0.804	0.530	0.03
Actinobacteria hgcl	1.97	0.220	0.10	**5.880**	**0.032**	**0.10**	2.622	0.132	0.16
Betaproetobacteria	4.10	0.067	0.08	**13.336**	**0.005**	**0.28**	2.079	0.205	0.16
Alphaproteobacteria	1.24	0.375	0.09	1.618	0.282	0.06	2.626	0.145	0.08
Bacteroidetes	3.24	0.103	0.23	3.401	0.094	0	**6.308**	**0.021**	**0.30**
Verrucomicrobia	1.42	0.326	0.03	**6.681**	**0.024**	**0.31**	**7.581**	**0.013**	**0.45**
OD1	1.47	0.315	0.11	0.766	0.553	0.02	2.064	0.194	0.04
Planktomycete	1.17	0.395	0.03	0.928	0.483	0.03	0.951	0.466	0.10
Chloroflexi	0.760	0.556	0.06	**5.724**	**0.034**	**0.16**	**16.016**	**0.002**	**0.37**
Acidobacte	–	–	–	–	–	–	1.279	0.354	0.13
Deltaproteobacteria	0.772	0.551	0.06	–	–	–	0.221	0.879	0.04
Chlorobia	–	–	–	0.684	0.593	0.04	–	–	–
Gammaproteobacteria	0.976	0.464	0.13	1.561	0.294	0.16	–	–	–
Armatimonadetes	**19.285**	**0.002**	**0.09**	–	–	–	–	–	–
Chlamydiae	**19.912**	**0.001**	**0.30**	–	–	–	–	–	–
Phytoplankton									
Synechoccocales	1.920	0.228	0.09	**29.305**	**<0.001**	**0.58**	**12.537**	**0.003**	**0.49**
Nostocales	0.571	0.686	0.04	**4.853**	**0.048**	**0.34**	1.529	0.289	0.18
Oscilatoriales	0.377	0.773	0.005	**6.051**	**0.030**	**0.15**	2.952	0.107	0.22
Stramenopiles	1.001	0.454	0.16	**5.132**	**0.043**	**0.35**	**8.985**	**0.008**	**0.28**
Chlorophyta	1.890	0.232	0.06	**10.433**	**0.009**	**0.25**	0.712	0.575	0.06
Cryptophyta	**9.314**	**0.011**	**0.46**	1.495	0.308	0.14	3.867	0.064	0.26
Streptophyta	0.843	0.518	0.10	1.083	0.425	0.05	1.137	0.398	0.11
Haptophyta	0.717	0.577	0.14	0.736	0.568	0.05	1.440	0.310	0.10
Raphidophyta	0.252	0.857	0.03	–	–	–	0.421	0.744	0.02
Unclassified	0.316	0.814	0.04	**18.046**	**0.002**	**0.59**	0.292	0.830	0.04

water treatments; however, on Days 16 and 32 the number of Stramenopiles relative reads was higher in the LH and HH treatments compared to the control and MH treatment (Fig. 6). Similarly, the relative abundance assigned to the Synechoccocales decreased with increasing water colour on Days 2 and 8; however, on Days 16 and 32 their relative abundance was lower in the LH and HH treatments compared to the control and MH treatment (Fig. 6).

Zooplankton

The zooplankton community composition did not differ significantly among treatments on Day 32 in any lake (RDA: Ekholmssjön, $F_{3,6} = 0.51$, p = 0.933; Hålsjön, $F_{3,6} = 0.88$, p = 0.298; Edasjön, $F_{3,7} = 1.38$, p = 0.215). The treatments also did not have significant effects on the total zooplankton biovolume (ANOVA: Ekholmssjön, $F_{3,6} = 0.49$, p = 0.7; Hålsjön, $F_{3,6} = 1.18$, p = 0.39; Edasjön, $F_{3,7} = 2.77$; p = 0.12).

In summary, this study showed no significant effect of increased water colour on the functions or total biomasses of plankton communities. However, we observed changes in bacterial and phytoplankton community composition and structure.

DISCUSSION

The aim of the present study was to experimentally investigate the impact of increased humic matter, coupled to increased water colour and available carbon, on aquatic ecosystems and plankton communities. Contrary to what we expected, additions of HuminFeed® did not increase carbon concentrations, but increased significantly the water colour by 50 to 180%. Thus our study only reflects the impact of changes in light climate on plankton communities occurring due to brownification. In our experiment, alteration of the light climate had no measurable functional or

Table 3. Cohen's *d* effect size for relative abundance of the different dominant bacteria and phytoplankton groups (−: not a dominant group) for the treatments LH (low HuminFeed®), MH (medium HuminFeed®) and HH (high HuminFeed®) compared to relative abundance in the control. Values in **bold** indicate medium (*d* > |0.5|) and large effect size (*d* > |0.8|)

Group	Ekholmssjön			Hålsjön			Edasjön		
	LH	MH	HH	LH	MH	HH	LH	MH	HH
Bacteria									
LD12	0.29	0.11	−0.07	−0.07	**−0.87**	−0.42	−0.02	−0.49	−0.18
Actinobacteria without hgcl clade	0.30	0.35	0.10	0.16	**−0.68**	**−0.64**	0.16	0.05	−0.09
Actinobacteria hgcl	−0.32	−0.25	**−0.59**	−0.25	−0.47	**−0.71**	**−0.74**	−0.07	−0.28
Betaproteobacteria	−0.02	0.04	0.49	0.05	**0.63**	**0.85**	**−0.71**	**−0.71**	−0.25
Alphaproteobacteria	0.05	0.34	0.37	−0.10	0.03	0.31	**−0.54**	−0.48	**−0.68**
Bacteroidetes	−0.31	**−0.72**	**−0.95**	−0.42	**−0.53**	−0.36	−0.22	−0.34	**−1.14**
Verrucomicrobia	**0.51**	0.23	0.20	**0.95**	**1.04**	**1.19**	**0.78**	**1.28**	**1.22**
OD1	0.11	−0.07	**0.51**	0.00	0.08	0.15	−0.20	−0.13	0.25
Planktomycete	−0.30	−0.25	−0.07	0.20	0.38	0.10	−0.03	0.26	0.46
Chloroflexi	**−0.53**	−0.37	−0.20	−0.17	**0.83**	**1.05**	0.32	**0.50**	**1.02**
Acidobacter	−	−	**−**	−	**−**	−	**0.62**	0.28	**0.69**
Deltaproteobacteria	**0.92**	**0.71**	**0.77**	−	**−**	−	0.21	0.08	−0.03
Chlorobia	−	−	**−**	−0.27	0.27	−0.25	−	−	−
Gammaproteobacteria	**−0.69**	**−0.52**	−0.27	**−0.60**	**−0.69**	**−0.87**	−	−	−
Armatimonadetes	−0.03	**0.51**	0.45	−	−	**−**	−	−	−
Chlamydiae	**0.82**	**0.99**	**0.84**	−	−	−	−	−	−
Phytoplankton									
Synechoccocales	−0.27	0.18	0.21	**−1.20**	**−0.90**	**−1.54**	**−1.24**	**−0.90**	**−1.52**
Nostocales	**−0.52**	−0.31	−0.10	0.18	**0.82**	**1.25**	**0.52**	0.23	0.48
Oscilatoriales	0.07	0.19	0.30	0.34	−0.07	**0.99**	0.42	0.10	**0.87**
Stramenopiles	**0.98**	**0.52**	**0.75**	**1.05**	**1.23**	**0.93**	**0.90**	**0.58**	**1.11**
Chlorophyta	0.10	−0.03	−0.26	0.45	**0.55**	**0.75**	**0.56**	0.02	0.28
Cryptophyta	−0.01	**−0.80**	**−1.47**	0.34	0.35	**0.62**	0.25	**0.86**	**0.76**
Streptophyta	0.29	**0.70**	0.35	−0.36	−0.42	0.00	**0.54**	−0.26	−0.22
Haptophyta	**0.54**	0.34	0.36	−0.37	−0.43	**−0.93**	0.29	0.11	**0.61**
Raphidophyta	−0.32	−0.39	−0.16	−	−	−	0.04	0.38	0.21
Unclassified	−0.25	0.10	−0.09	**−0.83**	**−1.72**	**−1.97**	0.04	0.01	0.40

ecosystem effects, while the composition of the plankton communities changed.

The absence of significant functional ecosystem effects is in contradiction with previous brownification studies (Solomon et al. 2015, and references therein). However, a likely explanation for this lack of functional reponse is that our HuminFeed® additions had no effect on the concentration of organic carbon in our mesocoms. Further, for the phytoplankton, the absence of significant changes in chlorophyll concentration may reflect an absence of response of the community. Alternatively, the biomass might have been reduced but the chlorophyll content per unit of biomass might have been increased in order to adapt to deteriorating light conditions (Faithfull et al. 2015). However, a previous mesocosm study has shown that primary production in the top (1 m) layer in humic lakes is not necessarily light limited, but the community is instead adapted to low light conditions so that primary production was more likely limited by nutrient concentrations (Faithfull et al. 2015). These adaptations to low light might potentially be the results of differences in the structure and composition of the phytoplankton communities observed in our lakes.

The effects of our treatments on bacterial and phytoplankton communities (according to PRC analyses) increased linearly with additions of HuminFeed® in Lakes Ekholmssjön and Hålsjön. Further, we found generally little indication that community composition recovered over the course of the experiment, which is logical given that differences in water colour remained over the course of the experiment. The only exception was found in Edasjön, where the phytoplankton community in the MH treatment partially recovered from the addition of humic substances (i.e. community composition become more similar to the control) after Day 16. However, the differential response of the MH treatment in Edasjön after Day 16 needs to be carefully interpreted as one replicate mesocosm was lost on Day 16.
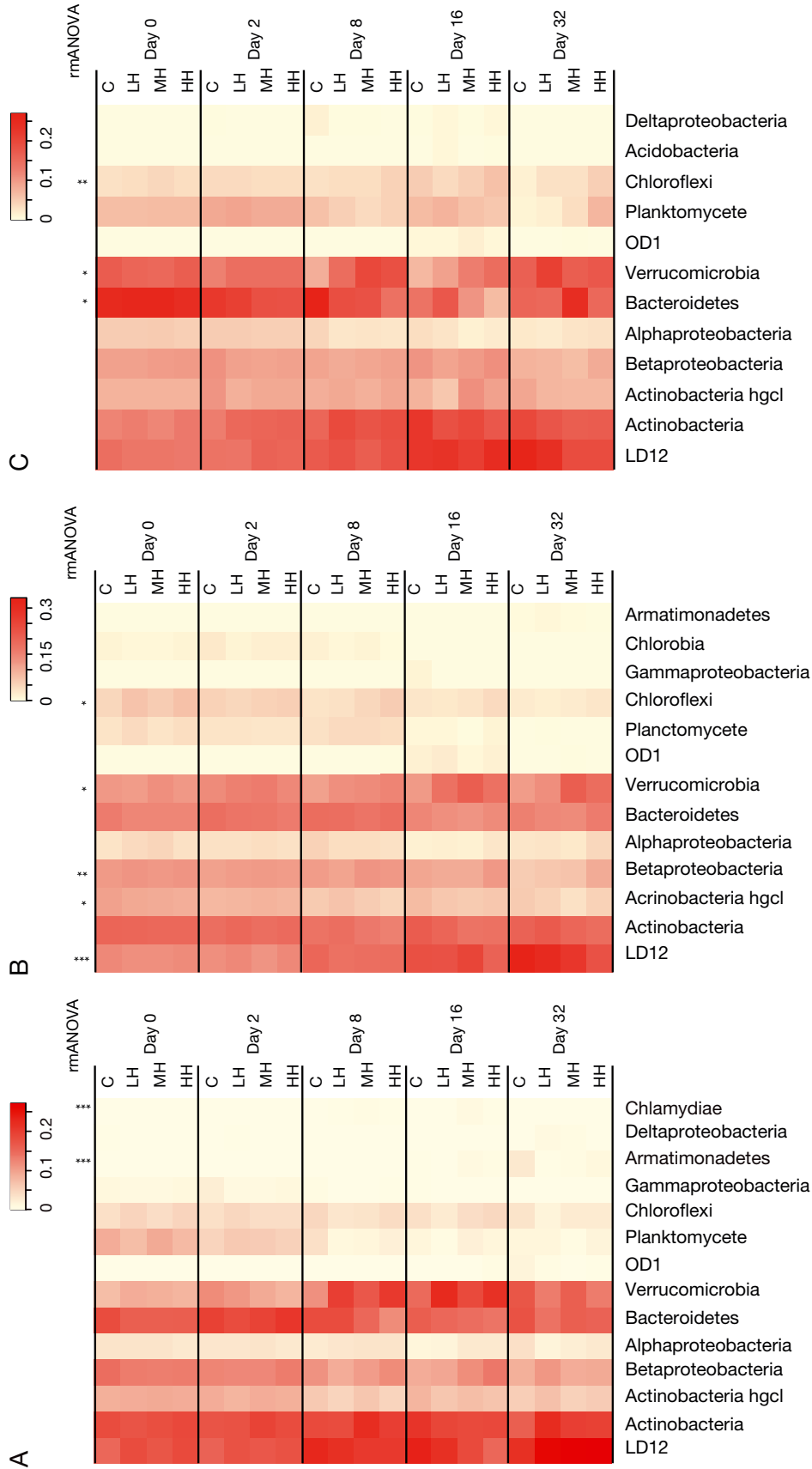
Fig. 4. Heat map of the relative number of reads for the different bacterial groups over time and among treatments in (A) Ekhomssjön, (B) Hälsjön and (C) Edasjön. C: control, LH: low HuminFeed®, MH: medium HuminFeed® and HH: high HuminFeed®. Asterisks highlight the significant effect (rmANOVA) of the treatments on the relative abundance of the bacterial group (*p < 0.05, **p < 0.01, ***p < 0.001)
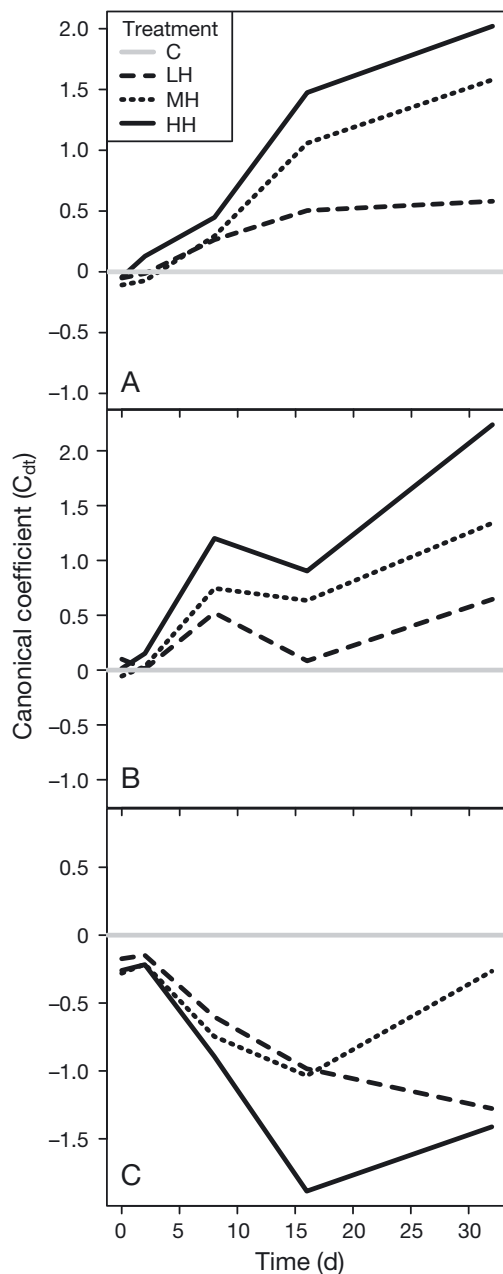
Fig. 5. Principal response curve of the phytoplankton community over time in the treatments in (A) Ekholmssjön, (B) Hålsjön and (C) Edasjön. C: control, LH: low HuminFeed®, MH: medium HuminFeed® and HH: high HuminFeed®

The phytoplankton communities in the mesocosms were dominated by cyanobacteria and diatoms, which is unexpected for brown water lakes. Instead, humic lakes often exhibit high abundances of flagellate phytoplankton such as raphidophytes (Jones 1988, Drakare et al. 2002, Willén 2003). The phytoplankton communities in the lakes outside the mesocosms were less dominated by cyanobacteria

and diatoms, with more diverse communities at the taxonomic level on Day 0 (Fig. S1). These differences are likely to be the results of a mesocosm artifact. The environmental conditions in the mesocosms are most likely slightly different, for instance, in terms of sedimentation rates, leading to the differentiation of the mesocosm communities from the *in situ* lake communities.

Darker water colour in the mesocosms led mainly to a decrease in relative abundance of the phytoplankton taxon Synechococcales in both Hålsjön and Edasjön, and of Cryptophyta in Ekholmssjön, but increased relative abundance of diatoms in Edasjön and Hålsjön, and of 2 groups of Cyanobacteria (Nostocales, Oscillatoriales) and Chlorophyta in Hålsjön. Although Cryptophyta are considered to be low light adapted species, previous studies have shown that they can increase in abundance with higher light availability under low nutrient concentration conditions (Schwaderer et al. 2011, Winder et al. 2012). These observations are consistent with the lower relative abundances of Cryptophyta with increasing water colour in Ekholmssjön. Diatoms have been observed to have competitive advantages over other taxa under low light conditions (Litchman 2000, Jäger et al. 2008). The Cyanobacteria are also considered to be low light adapted group, especially within *Anabaena* (Nostocales) and *Microcystis* (Oscillatoriales) (Richardson et al. 1983, Schwaderer et al. 2011, Maileht et al. 2013), because of efficient light harvesting systems and buoyancy regulation (Reynolds et al. 1987). The increasing abundance of these genera might induce a threat to future water quality with global climate change as many of these are toxin producers, which is especially prevalent when dark water coincides with increased temperature (Ekvall et al. 2013). Hence, a previous study showed a synergistic effect of higher temperature and brownification which stimulated the growth and toxin production of *Microcystis botrys* (Ekvall et al. 2013). However, further studies are necessary to draw clear conclusions on the potential effect of brownification on toxin production, and on the potential implications for future lake water quality.

Following our earlier conclusion that HuminFeed® additions mostly affected the light climate rather than the concentrations of organic carbon in the mesocoms, there should not have been a direct effect of the treatments on bacteria since most of them are expected to be heterotrophs. Still, in Edasjön and Hålsjön, the bacterial community composition differed among treatments, which may be an effect of the change in the phytoplankton community compo-
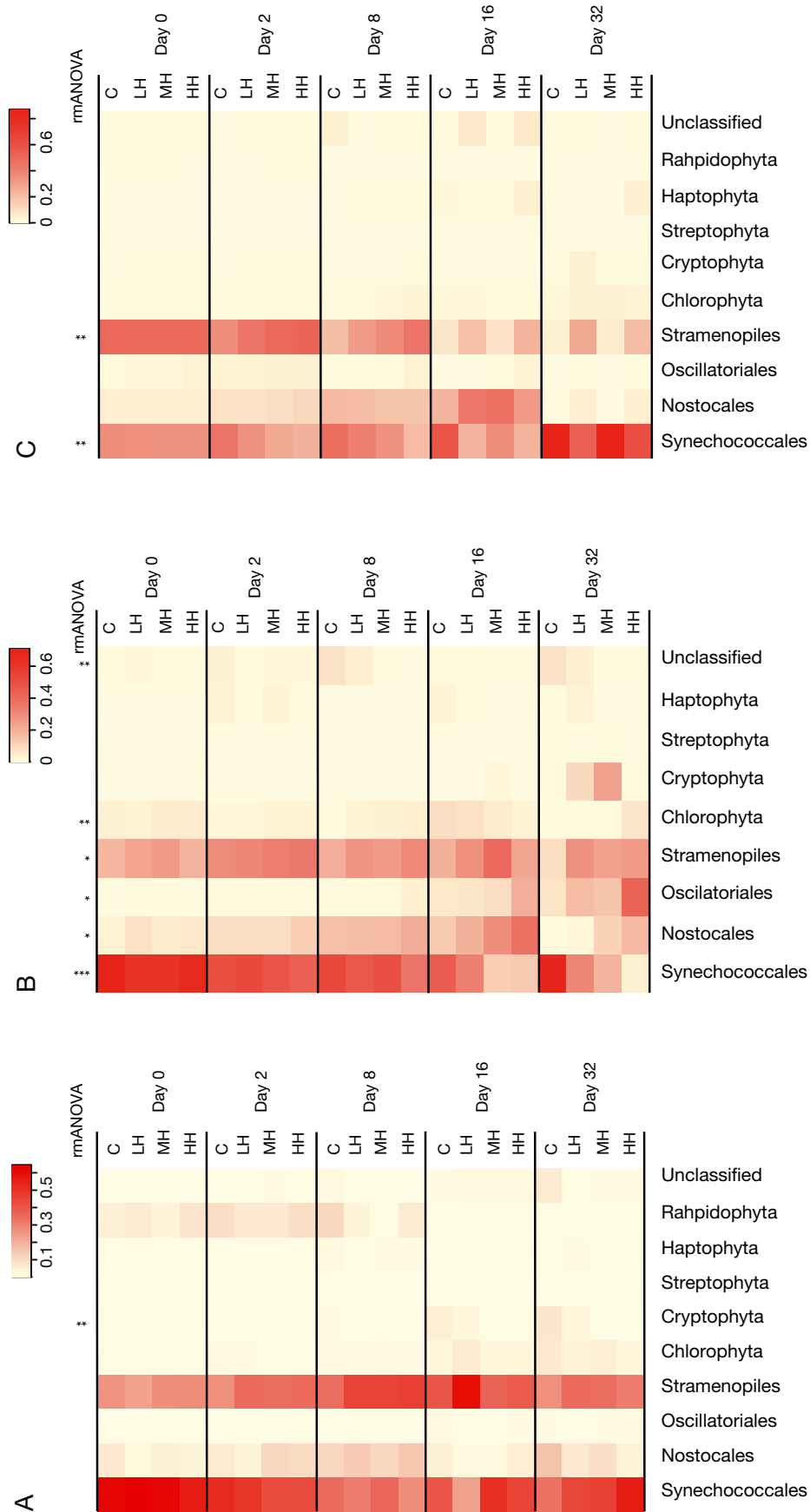
Fig. 6. Heat map of the relative number of reads for the different phytoplankton groups over time and among treatments in (A) Ekhomssjön, (B) Hålsjön and (C) Edasjön. C: control, LH: low HuminFeed®, MH: medium HuminFeed®, MH: medium HuminFeed® and HH: high HuminFeed®. Asterisks highlight the significant effect (rmANOVA) of the treatments on the relative abundance of the bacterial group (*p < 0.05, **p < 0.01, ***p < 0.001)

sition. For instance, blooms of different cyano-bacteria species have been shown to have different bacterial community composition within their phyco-sphere (Louati et al. 2015). Previous studies have shown that bacterial taxa utilize differently and specifically the organic matter produced by different phytoplankton species (Kent et al. 2007, Paver et al. 2013). Thus, the phytoplankton community can partly shape the composition of the bacterial community through differential composition of the released organic carbon. For instance, an increase in abundance of the Verrucomicrobia was previously observed during a bloom of Cyanobacteria (Kolmonen et al. 2004, Louati et al. 2015). In the present study, a similar pattern was observed with a congruent increase of the Verrucomicrobia and the 2 Cyanobacteria groups Nostocales and Oscillatoriales. Our results support the idea that a change in phytoplankton community composition had an effect on the bacterial community.

For the zooplankton communities, the treatments did not affect biomass or composition. These results are in accordance with previous studies showing a lack of effect on the abundance or rectruiment of zooplankton following the addition of humic substances (Ekvall & Hansson 2012, Nicolle et al. 2012, Robidoux et al. 2015). Thus, despite the change in the phytoplankton communities in the mesocosms, zooplankton at the community level appear to not have been affected by potential changes in food availability or quality that can occur with changes in prey species community. However, it is also possible that the zooplankton communities might have needed more time to respond to the treatment or changes in food source since the strongest differences in phytoplankton community composition were not observed until Day 16.

A previous study (Lennon et al. 2013) tested the use of Super-Hume (another Leonardite-derived humic substrate similar to HuminFeed®) as a means of manipulation in brownification experiments and found it to be a good substitute for terrestrial DOC. Still we, and also other authors (Rasconi et al. 2015), found that the additions of HuminFeed® strongly increased the water colour in the mesocosms whereas increases in the concentrations of TOC could not be detected, although we had expected an increase of up to 37%. Kritzberg et al. (2014) found that additions of highly coloured lake water to lake water in mesocosms led to flocculation of a part of the DOC, and that an observed increase in colour was the consequence of lower flocculation of highly coloured DOC coupled with increased concentrations of iron (Asmala et al.

2014, Kritzberg et al. 2014). This explanation seems plausible in our experiment, where the increase in iron concentrations caused by the addition of Humin-Feed® would have been insufficient to alone affect the water colour to the extent observed in the meso-cosms (Meinelt et al. 2007, Kritzberg & Ekström 2012). Free metal ion concentrations such as aluminium or iron (Schindler et al. 1992, Asmala et al. 2014) have an impact on flocculation, and the relatively high proportion of these elements in Humin-Feed® (Meinelt et al. 2007) could have contributed to flocculation. Currently, researchers are attempting to investigate the impact of brownification on lake ecosystems in detail; however, it is challenging to determine the best strategy to simulate brownification in experiments. Several studies have used different humic substances such as HuminFeed® (Rasconi et al. 2015) and Super-Hume (Lennon et al. 2013), but it remains unclear how these substances react relative to natural humic substances in experimental set-ups.

To summarize, since the additions of humic matter in our experiment only had measurable effects on water colour, we can only draw conclusions about the impact of changing light conditions. These changes mainly affected the phytoplankton community composition, favouring low light adapted species and toxin producers, such as some genera of Cyanobacteria. As carbon addition from this humic substance was insignificant, we conclude that the changes in bacterial communities were likely caused by different responses to the changes in the phytoplankton community. For both phytoplankton and bacteria, changes in community composition were gradual over the experimental water colour gradient. Despite the compositional changes, functional parameters such as abundance, productivity and respiration were resistant to the changes. This suggests that compositional changes seemed to buffer several ecosystem responses as water became darker in the lake systems.

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