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Effects of wastewater treatment plant effluent inputs on planktonic metabolic rates and microbial community composition in the Baltic Sea.

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Abstract

The Baltic Sea is the largest area suffering from eutrophication-driven hypoxia. Low oxygen levels are threatening its biodiversity and ecosystem functioning. The main causes for eutrophication-driven hypoxia are high nutrient loadings and global warming. Wastewater treatment plants (WWTP) contribute to eutrophication as they are important sources of nitrogen to coastal areas. Here, we evaluated the effects of wastewater treatment plant effluent inputs on Baltic Sea planktonic communities in 4 experiments. We tested for effects of effluent inputs on chlorophyll a content, on bacterial community composition, and on metabolic rates: gross primary production (GPP), net community production (NCP), community respiration (CR) and bacterial production (BP). Nitrogen-rich dissolved organic matter (DOM) inputs from effluents increased bacterial production and decreased primary production and community respiration. Nutrient amendments and seasonally variable environmental conditions lead to lower alpha-diversity and shifts in bacterial community composition (e.g. increased abundance of a few cyanobacterial populations in the summer experiment), concomitant with changes in metabolic rates. An increase in BP and decrease in CR could be caused by high lability of the DOM that can support secondary bacterial production, without an increase in respiration. Increases in bacterial production and simultaneous decreases of primary production lead to more carbon being consumed in the microbial loop, and shifts the ecosystem towards heterotrophy.

1 Introduction

The Baltic Sea has the largest area affected by eutrophication-driven hypoxia (Conley et al., 2011). Eutrophication is expanding in the Baltic Sea; from 2007 to 2011 the entire open Baltic was found to be eutrophic (Fleming-Lehtinen et al., 2015). A 10-fold increase of the hypoxic area has been recorded for the last 115 years, mostly related to increased nutrient inputs from land (Carstensen et al., 2014). The lack of oxygen in marine waters causes death of the marine organisms and catastrophic changes in marine communities. Thus, hypoxia is emerging as a major threat to marine biodiversity (Vaquer-Sunyer and Duarte, 2008).

Municipal wastewater treatment plants (WWTPs) contribute to eutrophication because they are a substantial source of nitrogen (N) to natural waters worldwide

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(Seitzinger *et al.* 2005). To reduce the environmental impact of WWTP effluent discharge, limits on the concentration of nitrogen have been imposed. In the European Union, 'the Urban Waste Water Directive' (91/271/EEC) sets the discharge limit of effluents from urban wastewater treatment plants for total nitrogen (TN) between 10 and 15 mg N L^{-1}, depending on the number of population equivalents (pe). In other regions, such as Chesapeake Bay in the U.S., discharge limits range from 3 to 8 mg N L^{-1} (Chesapeake Bay Program 2006). Effluent from WWTPs includes both dissolved inorganic (DIN) and organic N (DON). The conventional biological treatment (secondary treatment) combines coupled nitrification/denitrification and can potentially reduce TN to around 8-12 mg N L^{-1}. Biological nutrient removal is very efficient at removing inorganic nutrients and can eliminate most of the DIN, leading to a substantial fraction of the residual N in effluent as DON (Bronk et al., 2010; Grady et al., 2011). Effluents also contribute to increase organic matter (OM) inputs to coastal areas where are discharged.

DON can play an active role in providing nutrition to both phytoplankton and bacteria (Berman and Bronk, 2003), and affects planktonic metabolism in areas receiving significant amounts of DON. Dissolved organic matter (DOM) inputs to coastal areas can also affect metabolic rates and favour bacterial processes (Berglund et al., 2007). Here, we investigated the effects of wastewater treatment plant (WWTP) effluent inputs on planktonic metabolic rates in the Baltic Sea. We did so on the basis of 4 experiments where different WWTP inputs were added to natural communities. We tested for effects of effluent inputs on metabolic rates: gross primary production (GPP), net community production (NCP), community respiration (CR) and bacterial production (BP); on chlorophyll a content; and on bacterial community composition.

2 Methods

2.1 Sampling

Natural marine planktonic communities from the Baltic Sea Proper were collected (sampling dates included in Table 1) 10 km off the east coast of Öland, Sweden, at the Linnaeus Microbial Observatory (LMO, N 56°55.851, E 17°03.640). The water was sampled from 2 m depth and filtered through a 150 μm net to remove large grazers.

Wastewater effluent was collected within 10 days prior to experiment (sampling dates

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92 included in Table 2) from the wastewater treatment plant (WWTP) in Kalmar for
93 effluent enrichment. Samples from WWTP were filtered using pre-combusted (450ºC,
94 4 h) glass-fiber (GF/F Whatman) filters and 0.2 μm membrane filters and frozen until
95 the start of the experiment. All equipment used for handling the samples was acid
96 washed.

97 **2.2 Treatments**

98 Four experiments were performed to cover all seasons: spring, summer, autumn and
99 winter. Each experiment consisted of 5 different treatments: One with WWTP
100 addition in a proportion of 1:10 vol:vol in seawater (1:10), a second with WWTP
101 addition in a proportion of 1:5 (1:5); a treatment with addition of inorganic nutrients
102 (nitrate, nitrite and phosphate) equivalent to that contained in the DON 1:5 treatment
103 (IN). There was a control (C) treatment with only seawater, and a diluted control (CD)
104 consisting of seawater diluted with autoclaved milli-Q water to have the same portion
105 of community that the 1:10, 1:5 and IN treatments. To keep salinity constant in all
106 treatments, a salt solution was added with the amendments/dilutions.

107 **2.3 Chlorophyll a and nutrient measurements**

108 Chlorophyll a was measured following recommendations by Jespersen and
109 Christoffersen (1987) on a Turner TD-700 fluorometer.

110 Total dissolved nitrogen (TDN) was measured in duplicate after persulfate oxidation.
111 The method of persulfate oxidation was chosen instead of high temperature combustion
112 (HTC), as it has been demonstrated to be more appropriate for eutrophic waters, such as
113 the Baltic Sea, as well as coastal areas (Bronk et al., 2000). Inorganic nutrient analyses
114 (nitrate (NO_3^-), nitrite (NO_2^-) and phosphate (PO_4^{3-})) were analysed in duplicate on an
115 automated nutrient analyser SmartChem® 200. Concentration of ammonium (NH_4^+)
116 was measured in duplicate on a spectrophotometer following the manual phenol
117 hypochlorite method by (Koroleff, 1983). The concentration of DON was calculated by
118 difference after subtracting the concentration of NH_4^+, NO_3^-, and NO_2^- from the TDN
119 concentration. Dissolved primary amines (DPA) concentrations were measured in
120 triplicate on a spectrofluorometer following the OPA (*o*-phthaldialdehyde) method
121 (Parsons et al., 1984).

122 **2.4 Metabolic rates**

123 Changes in dissolved oxygen (DO) in closed bottles were assumed to result from

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biological metabolic processes and to represent net community production (NCP = GPP – CR). Water from the respective treatments was siphoned carefully to avoid bubble formation into 2.3 L glass bottles sealed with gas tight stoppers. Bottles were incubated at the in situ temperature in a temperature-controlled chamber during one week. Oxygen was measured every minute using optical oxygen sensors (optodes) and a 10-channel fiber optic oxygen transmitter (oxy-10, PreSens®).

Incubations were illuminated by artificial light (OSRAM L36W/865 Lumilux Daylight), with a mean PAR intensity of 1373.2 $\mu W/cm^2$. Light hours ranged from 8h 30m on the winter experiment performed on January 2013 to 16h 30m on the summer experiment on July 2013.

Individual estimates of GPP, NCP and CR resolved at one-minute intervals were accumulated over each 24-h period during experiments and reported in mmol O_2 m^{-3} day^{-1}, detailed description of calculation of metabolic rates can be found at Vaquer-Sunyer et al. (2015).

2.4.1 Bacterial Production

BP was estimated by measuring incorporation of ^{3}H-leucine following the method established by Smith and Azam (1992) on days 0, 1, 3, 5 and 7. Water samples (1.5 ml, 3 replicates and 1 killed control) were incubated 60 minutes with 98.8 nM of ^{3}H-leucine (13.4 Ci $mmol^{-1}$). The incubation was terminated by adding trichloroacetic acid (TCA, 5% final concentration). The samples were then centrifuged at 16000g for 10 minutes and the bacterial pellet was washed once with 5% TCA and once with 80% ethanol. After the supernatant was discarded, 0.5 ml of scintillation cocktail (Ecoscint A, Kimberly Research) was added and ^{3}H -activity measured on a Beckman LS 6500 scintillation counter. BP was calculated according to Smith and Azam (1992) assuming a leucine to carbon conversion factor of 1.5 kg C mol^{-1} leucine (Kirchman, 2001).

2.5 Bacterial Diversity

Bacterial 16S rRNA gene fragments was amplified with bacterial primers 341F and 805R (Herlemann et al., 2011) following the PCR protocol of Hugerth et al. (2014) with some modifications. Amplification was carried out in duplicates for each biological replicate using an annealing temperature of 58°C in the first PCR and 12 cycles in the second PCR. The resulting purified amplicons were sequenced on the

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Illumina Miseq (Illumina, USA) platform using the 300 bp paired-end setting at the Science for Life Laboratory, Sweden (www.scilifelab.se). Raw sequence data generated from Illumina Miseq were processed using the UPARSE pipeline (Edgar, 2013). Taxonomy was determined against the SINA/SILVA database (SILVA 115; Quast et al., 2013). After quality control, our data consisted of a total of 3.8 million reads, with an average of 68218.61±33048.86 reads per sample. These sequences resulted in a final OTU table consisting of 3420 OTUs (excluding singletons) delineated at 97% 16S rRNA gene identity. DNA sequences have been deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive under accession number SRP059501.

2.6 Statistics

Metabolic rates data from the four experiments were combined to test the relationship between the given metabolic rates and water properties by a mixed effects model. To account for pseudo-replication we used season (i.e. experiment) as a random factor. The pseudo-R^2 of the models was calculated following Xu (2003).

Differences in community composition between microcosms were tested using permutational analysis of variance (PERMANOVA) on Bray-Curtis distances. For testing the correlation between absolute changes in environmental conditions, metabolic rates and absolute shifts in bacterioplankton community composition we performed MANTEL tests. For alpha-diversity measures we subsampled each sample to 10 000 sequences. Analyses performed at the OTU level were based on selecting the top 200 most abundant OTUs. For OTU level analyses on Cyanobacteria we selected OTUs affiliated with Cyanobacteria among the top 200 most abundant OTUs. Taxonomic annotation from SINA/SILVA database was limited for cyanobacterial OTUs and we therefore extended the annotation by using BLASTn (NCBI). All, statistical tests were performed in R 3.0.2 (R Core Team, 2014) and using the package Vegan (Oksanen et al., 2010). Graphical outputs were made using the package ggplot2 (Wickham, 2009). Phylogenetic analyses using maximum likelihood trees were performed with MEGA 6.0.6 and the Tamura-Nei model (Tamura et al., 2011).

3 Results

Treated wastewater nutrient content differed between seasons (Table 2). The highest TDN values were measured in winter (600.1 ± 6.6 µM), whereas the lowest values

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were measured in summer (518.4 ± 2.4 µM). DON content in wastewater effluent varied between 75.2 ± 4.4 µM in autumn and 503.3 ± 2.9 µM during winter. The DOC:DON ratio was low (2.1 – 9.4), indicating nitrogen rich dissolved organic matter (DOM).

Nutrient content in the seawater also differed between seasons (Table 1), with the highest TDN value in autumn (21.0 ± 0.30 µM), and the lowest values were measured in spring (16.4 ± 0.6 µM). DON content in coastal water ranged between 11.4 ± 0.9 µM and 17.9 ± 0.5 µM, measured in winter and autumn respectively.

3.1 Chlorophyll a

Coastal waters showed a typical seasonal pattern (Vahtera et al., 2007), with low chlorophyll a (*Chl.a*), and high nutrient content in winter; in spring, with the increase in sunlight radiation, *Chl.a* increased, and inorganic nutrients started to decrease. In summer with high temperature and high sunlight radiation, *Chl.a* values increased to the maximum measured, and inorganic nutrients were depleted (Table 1). During autumn, *Chl.a* content decreased to the second lowest values and nutrient concentration started to replenish as a consequence of re-mineralization (Table 1).

Chlorophyll a content strongly depended on light availability ($p < 0.0001$, $R^2 = 0.60$) and on temperature ($p < 0.0001$, $R^2 = 0.41$), with the summer experiment having the highest values (Mean ± SE = 7.59 ± 0.41 µg L^{-1}), with 16.5 light hours and a mean temperature of 18.4 ºC (Fig.1, Supplementary Information (SI) Table S1). 66% of *Chl.a* variation could be explained by changes in light exposure time and NO_3^- concentration ($p < 0.0001$).

3.2 Metabolic Rates

3.2.1 Gross Primary Production

Gross primary production (GPP) for natural communities in the experiments varied from 2.03 ± 2.00 to 54.16 ± 5.31 mmol O_2 m^{-3} d^{-1}, both extremes measured on the 5th day of the experiment, for experiments conducted in winter and summer, respectively. In the amended treatments, GPP also varied greatly between days of experiment and seasons, with the lowest measured GPP being 0.14 ± 1.91 mmol O_2 m^{-3} d^{-1} for the 5th day of the 1:10 treatment in the experiment conducted in winter; and

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the highest measured GPP was 85.67 ± 7.13 mmol O_2 m^{-3} d^{-1} on the final day (day 7) of the inorganic nutrient addition treatment in summer (fig. 2).

GPP variability was explained by changes in Chl *a* content and DOC concentration (Table 3), with these 2 variables explaining 78% of its variability (fig 3a). Whereas GPP increased with Chl *a* content, it decreased with DOC concentration (Table 3). Neither organic nor inorganic nutrient concentrations affected GPP values. Consequently, the different treatments did not affect GPP values, with GPP values not significantly different between the different treatments.

3.2.2 Community Respiration

Community respiration (CR) for natural waters in the experiments varied between 5.30 ± 0.99 and 34.89 ± 1.35 mmol O_2 m^{-3} d^{-1} (Table S1).
CR varied from 0.95 ± 1.32 mmol O_2 m^{-3} d^{-1} for the day 1 on the IN treatment from the winter experiment to 54.16 ± 55.59 mmol O_2 m^{-3} d^{-1} for the final day on the 1:5 treatment during the fall experiment (fig. 4). The high SD associated to these measures is due to differences between incubation bottles.

CR depended on DOC concentration, *Chl.a* content and temperature. These 3 variables explained the 74% of CR variability (Table 3, fig. 3b). CR increased with temperature and *Chl.a* content and decreased with DOC concentration.

3.2.3 Net Community Production

Net community production (NCP) for natural communities in the experiments varied between -8.83 and 20.17 ± 5.78 mmol O_2 m^{-3} d^{-1} measured on fall and on summer, respectively. The range of variability in the treatments with nutrient additions was wider ranging from -16.64 ± 17.69 to 36.69 ± 1.49 mmol O_2 m^{-3} d^{-1} measured in the day 1 on the 1:10 treatment in the winter experiment and in day 7 on the IN treatment during the summer experiment, respectively (fig. 5).

NCP was dependent on DOC concentration and Chl *a* content, with these 2 variables explaining the 62 % of its variability (Table 3, fig. 3c).

3.2.4 Bacterial Production

Bacterial production (BP) tended to increase in the treatment with the higher addition of effluent (fig. 6). Repeated measures MANOVA showed significant differences in BP for different sampling days, for treatments and for the interaction between

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sampling day and treatment for experiments conducted in summer and fall ($p < 0.0001$ for both cases). Conversely, BP was not significantly different between treatments for experiments conducted in spring and winter. For those experiments there were significant differences in BP between sampling days and in the interaction between treatment and sampling day.

BP depended on DOC content in spring, summer and winter ($p < 0.003$, $p < 0.005$ and $p < 0.05$, respectively), but it was independent of DOC concentration in fall ($p > 0.05$).

The variables that best explained BP variability were temperature, DOC and NO_3^- concentration ($R^2 = 0.85$, Table 3, fig. 3d). BP increased with the increase of these three variables.

3.3 Bacterial diversity and community composition

Bacterial community structure showed two distinct clusters with summer communities separated from spring and winter across all experiments (fig. S1, Supplementary Information). Community composition in each experiment exhibited, in general, a temporal succession and an additional response to different treatments. We carried out MANTEL tests to elucidate the influence of environmental factors on community composition and metabolic rates. Changes in temperature significantly explained absolute shifts in bacterioplankton community composition across all experiments (Pearson $r > 0.5$; Table 4). Changes in GPP, CR, BP, Chl *a*, NO_2^- and PO_4^{3-} were significantly correlated with absolute shifts in bacterioplankton community composition, with the highest correlation observed for PO_4^{3-} (Pearson $r = 0.30$; Table 4).

Alpha diversity estimated from Shannon index was relatively similar between treatments in each experiment (fig. 7a). Nevertheless, when samples were grouped into all nutrient amendments collectively, a lower Shannon index was observed for these treatments compared to the controls in all experiments except April (fig. 7b).

Betaproteobacteria, Bacteroidetes and Alphaproteobacteria dominated the April experiment where Betaproteobacteria displayed a marked increase in relative abundance from T0 to T7 (Fig. 8). In general, few differences in community composition between treatments were observed. Nevertheless, at T7, Betaproteobacteria exhibited much lower relative abundance in the control treatment

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compared to the other treatments and time-points. For the January experiment differences between treatments were more pronounced (fig. 8). Bacterial groups other than the 8 major phyla/class ("Others") had higher relative abundance in the 1:5 treatment compared to the other treatments and the controls. At T3 Cyanobacteria had considerably higher relative abundance in the 1:10 and IN treatments compared to the controls and 1:5 treatment. The July experiment showed a higher relative abundance of Cyanobacteria and Verrucomicrobia, with the relative abundance of Cyanobacteria increasing over time in the amended treatments. In contrast, the relative abundance of Verrucomicrobia increased in the control treatments and was highest in the diluted control (CD) (fig. 8). Hence, Cyanobacteria had higher relative abundance in treatments with additions of nutrients (both DON and IN; fig. 8). For the November experiment there was an overall greater variation in community composition. Still, relative abundances of Gammaproteobacteria increased in the IN treatments at T3 and T7 compared to the other treatments and control.

3.4 Population dynamics

Patterns in community composition indicated that nutrient amendments had an effect on bacterial population dynamics in our experiment coupled with the concomitant changes in metabolic rates. Hence, we performed Pearson correlation tests to determine links between environmental factors, metabolic rates and shifts in relative abundances at finer phylogenetic scales when communities responded to experimental treatments. Cyanobacteria, Planctomycetes and Verrucomicrobia were positively correlated with temperature (fig. 9). In contrast, Alphaproteobacteria, Bacteroidetes and Betaproteobacteria were negatively correlated with temperature. Cyanobacteria, Planctomycetes and Verrucomicrobia displayed a strong negative correlation with community respiration but a positive correlation with bacterial production. These three groups of bacteria were also negatively correlated with PO_4^{3-} while Alphaproteobacteria, Bacteroidetes and Betaproteobacteria were positively correlated with PO_4^{3-}. In particular, PO_4^{3-} explained > 50 % of the variance for Bacteroidetes (fig. 9). In addition, Verrucomicrobia had a strong correlation with NO_2^-. Actinobacteria, Gammaproteobacteria and bacterial groups other than the 8 major phyla/class ("Others") showed only weak correlations with environmental parameters and metabolic rates.

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Changes in relative abundance of particular bacterial populations typically followed the overall pattern within each major phyla/class. For example *Chtoniobacterales* OTUs within Verucomicrobia exhibited positive correlations with temperature and bacterial production but negative correlations with PO_4^{3-} (fig. S2). Although Gammaproteobacteria showed overall weak correlations with metabolic rates and environmental factors, specific OTUs in this taxon, such as OTU 001410 and two *Halioglobus* OTUs (OTU 001149 and OTU 000045), displayed strong correlations with e.g. temperature, bacterial production and community respiration. Betaproteobacteria OTUs showed overall weak correlations with metabolic rates and environmental factors except for two MWH-UniP1 OTUs (OTU 002372 and OTU 000041). Betaproteobacteria affiliated with BAL58 showed in some cases a substantial correlation with DOC (OTU 001633, OTU 001481, OTU 000008 and OTU 001907) (fig. S2). Within Alphaproteobacteria most OTUs had weak correlations. However, one particular alphaproteobacterial OTU affiliated with Rhodobacteraceae (OTU 000044) exhibited strong correlations with metabolic activities and environmental variables, both negative (e.g. PO4 and community respiration) and positive (e.g. temperature and bacterial production). Moreover, 10 *Rhodobacteraceae* OTUs were positively correlated with DOC. *Synechococcus* OTUs were positively correlated with temperature, NCP, GPP, bacterial production and Chl *a* (fig. S2).

To extend the analysis of the strong Cyanobacteria population dynamics observed in the July experiment, we investigated particular OTUs and plotted relative abundances of this group across all experiments (fig. S3). For the other experiments, cyanobacterial populations had, in general, low relative abundance but were still more abundant in treatments with nutrient amendments than without (except for the April experiment). Six OTUs showed particularly high relative abundance in the July experiment (fig. S3). These cyanobacterial populations increased with time and at T7 both *Synechococcus* and *Cyanobium* populations had higher relative abundance in treatments of 1:10, 1:5 and IN compared to controls.

4 Discussion

Nitrogen-rich dissolved organic matter (DOM) from WWTP effluents had significant impacts on Baltic Sea planktonic metabolic rates: DOM significantly increased

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bacterial production, whereas it decreased gross and net primary production and community respiration rates. A parallel increase in BP and decrease in bacterial respiration rates results in an increase in bacterial growth efficiency (BGE = (BP)/(BP + BR), (del Giorgio and Cole, 1998)). Literature values for BGE in the Baltic Sea vary substantially from 0.06 to 0.6 (Donali et al., 1999). Here we did not measure bacterial respiration separately, but as a part of total community respiration. Assuming that bacterial respiration contributes 50% of community respiration we can estimate BGE. As BR is known to be higher than 50% of CR (Williams, 1981), this approach will result in an underestimation of bacterial growth efficiency but will suffice to validate our hypothesis that DOM additions increased BGE. Estimated BGE for our experiments varied between 0.06 and 0.59, consistent with previous reported values (Donali et al., 1999; Zweifel et al., 1993). Estimated BGE increased with temperature, nitrate and DOC concentration, whereas it deceased with chlorophyll a content (linear model, $R^2 = 0.51$, $p < 0.0001$). An increase of BGE with nutrient addition was reported for communities from the Bothnian Bay, increasing from a range of 0.11 - 0.54 to 0.14 - 0.58 for treatments with nutrient amendment (Zweifel et al., 1993). Our estimation of BGE shows a positive effect of N-rich DOM on bacterial growth efficiency, suggesting high lability of N-rich WWTP effluent DOM, where most of the carbon can be used for secondary bacterial production and a low portion is respired.

Wastewater treatment plant effluent inputs to the Baltic Sea raised bacterial carbon demand at the same time as it reduced primary production, leading to more carbon being used by the microbial loop. This increase in bacterial carbon demand parallel with a decrease in primary production moves the ecosystem towards heterotrophy. Increased flow of organic matter through the microbial loop could result in a reduction of the transfer of carbon to higher trophic levels and of the efficiency of the biological carbon pump in sequestering carbon (Berglund et al., 2007; Wohlers et al., 2009). Whereas some studies suggest that an increased flow of carbon through the microbial loop would result in a reduction of the biological carbon pump efficiency in sequestering carbon, a recent study suggests the opposite: marine bacteria can produce refractory exometabolites that would result in carbon sequestration (Lechtenfeld et al., 2015). Other studies suggest that a future reduction of marine primary production due to higher stratification would result in less refractory marine DOC (Koch et al., 2014).

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381 A change in the planktonic community towards more heterotrophic communities will
382 result in a reduction of photosynthetic rates, decreasing oxygen production in the
383 photic layer. The Baltic Sea is already the largest eutrophication-driven hypoxic area
384 in the world (Conley et al., 2011), and a decrease of biological oxygen production
385 could further aggravate hypoxic conditions in this already affected area. The lack of
386 oxygen is an important environmental problem is this area, it produces a reduction of
387 marine benthic diversity as a result of the death of sensitive marine organisms and it
388 affects biogeochemical cycles (Conley et al., 2009) by increasing phosphorus fluxes
389 from sediments into overlaying waters, changing redox conditions in the water
390 column and reducing the ecosystem capacity of removing nitrogen. Inputs of WWTP
391 effluent in summer further stimulated bacterial production, when it was already high
392 due to elevated temperatures. Summer was the period of the year that responded
393 sharply to nutrient additions.

394 Links between metabolic activity and compositional changes of bacterial communities
395 are frequently observed in aquatic ecosystems (Bell et al., 2005; Allison and Martiny,
396 2008; Logue et al., 2016). Yet, in other cases, such linkages are relatively weak and
397 possibly confounded by environmental complexity (Comte and Del Giorgio, 2011;
398 Comte et al., 2013; Langenheder et al., 2005; Langenheder et al., 2010). Our results
399 on the effect of effluent inputs showed that disturbances caused simultaneous shifts in
400 community composition coupled with changes in metabolic rates. Changes in
401 temperature were the major driver of community structure but also phosphate
402 significantly explained variations in the relative abundance of particular groups and
403 taxa. This emphasizes that changes in temperature and nutrient availability can affect
404 bacterioplankton community dynamics. Similarly, differences in temperature and
405 nutrient conditions lead to shifts in community structure in for example mesocosm
406 experiments with Mediterranean and Baltic Sea microbial assemblages (Degerman et
407 al., 2013; Gomez-Consarnau et al., 2012; Pinhassi et al., 2006; von Scheibner et al.,
408 2014). More importantly, in these studies, compositional shifts occurred with
409 concomitant responses in community metabolic activity. It is noteworthy that
410 community composition is closely linked with community functioning and it would
411 therefore be relevant to investigate how changes such relationships are linked with
412 bacterial growth efficiency. In addition, alpha-diversity was lower in effluent input
413 treatments. The observed effect of species loss, i.e. lower alpha-diversity, may be

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414 closely linked with the functioning of microbial communities and could potentially
415 render the whole community more sensitive to environmental perturbations (Allison
416 and Martiny, 2008; Bell et al., 2005; Loreau, 2000, 2004; Shade et al., 2012). Hence,
417 our findings suggest that linked alterations in bacterial community composition and
418 metabolic activity from anthropogenic changes could potentially affect
419 biogeochemical cycling of elements in the coastal Baltic Sea.

420 Although several microbial taxa showed weak correlations with contemporary
421 changes in environmental conditions and/or metabolic activity, specific opportunistic
422 populations proliferated in effluent input treatments. In particular, effluent inputs
423 caused responses among verrucomicrobial and cyanobacterial populations. In fact,
424 nutrient additions in summer increased the relative abundance of a few specific
425 cyanobacterial populations. The Baltic Sea suffers from huge Cyanobacteria blooms
426 in summer that can easily be observed from space, mainly caused by eutrophication
427 (Vahtera et al., 2007). The death and sedimentation of the Cyanobacteria blooms, and
428 the subsequent decay of this organic material is a contributing mechanism for oxygen
429 depletion in bottom waters. Consequently, Cyanobacteria blooms have been linked to
430 hypoxia development and expansion in the Baltic Sea. Warming could further
431 increase cyanobacteria blooms in the Baltic Sea (Paerl and Huisman, 2008; Paerl and
432 Paul, 2012). Warming could also increase respiration rates to a larger degree than
433 primary production, moving the system towards heterotrophy (Vaquer-Sunyer et al.
434 2015). Simultaneous warming and inputs from wastewater treatment plant effluents
435 increased planktonic respiration rates and bacterial production faster than it increased
436 planktonic primary production in the Baltic Sea (Vaquer-Sunyer et al., 2015), leading
437 to higher biological oxygen consumption than production, which may lead to the
438 depletion of the oxygen pool, further aggravating hypoxia in the Baltic Sea.

439

440 ## 5 Conclusions

441 DOM from WWTP effluents is nitrogen-rich. The current study showed that inputs of
442 such DOM caused increased bacterial production and decreased primary production
443 and community respiration, which leads to an increase in BGE. The increase in BP
444 and decrease in CR could be caused by high lability of the OM that supported
445 secondary bacterial production, without an increase in respiration. Seasonal changes

in temperature were the most important factor for structuring community composition but also phosphate concentrations significantly explained variations in the relative abundance of particular groups and taxa. In summer, effluent inputs lead to an increase in Cyanobacteria abundance. Cyanobacteria have been linked to hypoxia in the Baltic Sea, and an increase in their abundance could result in oxygen depletion of the Baltic bottom waters. Inputs from wastewater treatment plant effluent could further worsen hypoxic conditions in the Baltic Sea.

Reductions of the OM content in wastewater treatment plant effluents are needed to reduce the potential negative consequences of changes in the planktonic community towards more heterotrophic communities that would result in a reduction of photosynthetic rates, decreasing oxygen production in the photic layer in the Baltic Sea.

Effluent inputs shifted the community towards heterotrophy, increasing BP and decreasing primary production. DOM inputs significantly enhanced BP at the same time that inhibited GPP, CR and NCP. High BP and low CR lead to high BGE, pointing to high lability of the effluent OM.

Authors contributions

RVS designed research and performed experiments. ML, JP and SDM analysed bacterial diversity samples and data. HER wrote the code for metabolic rates calculations. All authors were involved in the writing stage of the manuscript and collaborated on the analysis, interpretation, and discussion of the results.

Acknowledgements

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stressors in the Baltic Sea" project, funded by FORMAS, grant number 217-2010-126.

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Tables

Table 1. Nutrient content in coastal seawater for the different sampled seasons.

	Winter	Spring	Summer	Autum
Date	23/01/2013	03/04/2013	18/07/2013	04/11/2013
TDN (± SE) (µM)	17.01 (±0.87)	16.40 (±0.63)	16.51 (±0.08)	20.99 (±0.34)
NO2 (± SE) (µM)	0.35 (±0.02)	0.14 (±0.00)	0.09 (±0.01)	0.31 (±0.21)
NO3 (± SE) (µM)	4.93 (±0.39)	3.69 (±0.14)	0.50 (±0.09)	2.64 (±0.32)
NH4 (± SE) (µM)	0.35 (±0.01)	0.01 (±0.01)	0.24 (±0.00)	0.23 (±0.03)
PO4 (± SE) (µM)	0.55 (±0.03)	0.63 (±0.03)	0.03 (±0.01)	0.39 (±0.02)
DON (± SE) (µM)	11.44 (±0.95)	12.56 (±0.64)	15.76 (±0.12)	17.91 (±0.47)
DPA (± SE) (µM)	0.09 (±0.01)	0.31 (±0.01)	0.17 (±0.01)	0.24 (± 0.03)
DOC (± SE) (mg/l)	5.80 (±0.82)	3.57 (±0.04)	5.7	3.82 (±0.11)
DON % of TDN	67.03	76.58	95.48	85.33
Temperature (ºC)	3	4	18	7
Salinity (psu)	6.30	6.10	6.3	7.3
Chlorophyll a (µg/l)	0.30 (±0.00)	2.34 (±0.27)	6.49 (±0.01)	1.76 (±0.04)

Table 2. Wastewater effluent nutrient content for the different seasons sampled.

	Winter	Spring	Summer	Autum
Date	23/01/2013	03/04/2013	16/07/2013	25/10/2013
TDN (± SE) (µM)	600.12 (6.56)	576.20 (3.20)	518.39 (2.39)	498.20 (9.77)
NO2 (± SE) (µM)	8.00	32.74	29.44 (0.04)	29.29
NO3 (± SE) (µM)	81.00	113.64 (2.17)	192.00 (6.38)	228.57
NH4 (± SE) (µM)	7.76		117.93 (1.20)	165.15 (1.21)
PO4 (± SE) (µM)	0.02			0.19
DON (± SE) (µM)	503.35 (2.93)	429.83*	179.02 (7.95)	75.20 (4.39)
DPA (± SE) (µM)		18.71 (2.64)	2.64 (0.17)	
TOC (± SE) (mg/l)	16.19 (2.47)	11.10 (± 0.08)	13.00 (± 0.03)	8.49 (± 0.12)
DON % of TDN	83.88	74.60*	34.53	15.09
C/N ratio	2.68	2.15	6.05	9.40

*Calucated without NH4 concentration (overestimation)

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628 Table 3. Statistics for the fitted models for the different metabolic rates and the
629 variables that explain its variability, to account for pseudo-replication experiment was
630 included as random factor. SE: standard error, DF: degrees of freedom; N: number of
631 observations.

	Estimate	SE	t Ratio	p	R2	N
GPP					0.78	73
Intercept	16.31	4.15	3.93			
Mean *Chl.a* (µg/l)	-2.18	0.53	-4.09	< 0.0001		
DOC (mg/L)	4.80	0.49	9.80	< 0.0001		
CR					0.74	69
Intercept	14.94	3.97	3.76			
DOC (mg/L)	-1.44	0.38	-3.74	< 0.0001		
Mean *Chl.a* (µg/l)	2.05	0.37	5.59	< 0.0001		
Temperature	0.55	0.32	1.71	< 0.0001		
NCP					0.62	77
Intercept	-3.34	2.99	-1.12			
Mean *Chl.a* (µg/l)	2.60	0.33	7.79	< 0.0001		
DOC (mg/L)	-0.61	0.34	-1.79	< 0.0001		
BP					0.85	73
Intercept	-1.864536	0.404777	-4.606			
DOC (mg/L)	0.105028	0.0416	2.525	<0.0002		
Temperature	0.295275	0.026891	10.98	< 0.0001		
Mean nitrate (µM)	0.024114	0.004919	4.903	< 0.0001		

632

633 Table 4. Results of MANTEL tests to examine if absolute shifts in bacterioplankton
634 community composition were correlated to specific environmental variables and
635 metabolic rates

Variable	**Pearson r**	**p-value**
Temperature	0.5118	0.001*
NCP	0.05345	0.149
GPP	0.2095	0.004*

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CR	0.2651	0.001*
BP	0.3208	0.001*
Chl *a*	0.2147	0.001*
DOC	0.03064	0.273
TDN	0.01526	0.346
NO_x	0.05468	0.122
NO_2	0.1558	0.003*
NO_3	0.05622	0.111
NH_4	0.02908	0.311
DON	0.0004315	0.391
DPA	-0.01335	0.529
PO_4	0.2982	0.001*

636

637 **Figures captions**

638 Figure 1. Chlorophyll a content for the different incubation days and different
639 treatments for the four experiments.

640 Figure 2. Gross primary production (GPP) in mmol O_2 m^{-3} d^{-1} measured the seven
641 incubation days for the different treatments and experiments.

642 Figure 3. Whole models plots for the metabolic rates (a) gross primary production
643 (GPP), (b) community respiration (CR), (c) net community production (NCP) and (d)
644 bacterial diversity, and the variables that explain its variability, and show how the
645 data fit the model (table 3). The graph represents the actual value in front of the
646 predicted value by the model. Black solid line represents the 1:1 line.

647 Figure 4. Community respiration (CR) in mmol O_2 m^{-3} d^{-1} measured the seven
648 incubation days for the different treatments and experiments.

649 Figure 5. Net community production (NCP) in mmol O_2 m^{-3} d^{-1} measured the seven
650 incubation days for the different treatments and experiments.

651 Figure 6. Bacterial production in µg C L^{-1} h^{-1} for the different measured days for the
652 different treatments and experiments.

653 Figure 7. Variation in alpha-diversity, estimated from Shannon index, across
654 individual treatments within each experiment and over time (A), and differences in

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655 alpha-diversity between controls and nutrient amendment, i.e. all nutrient amended
656 treatments were binned and compared against all controls (B). Circles in (B) denote
657 variation in alpha-diversity within the binned samples where colour corresponds to
658 different treatments.

659 Figure 8. Relative abundances (i.e. percentage of total sequences) of major bacterial
660 groups at phyla/class level in the different treatments and experiments. Colour denote
661 specific groups.

662 Figure 9. Correlations between shifts in relative abundances of major bacterial groups
663 at phyla/class level and environmental factors and metabolic activity. The level of
664 correlation is estimated from Pearson r where blue and red colour indicate negative
665 and positive correlations, respectively.
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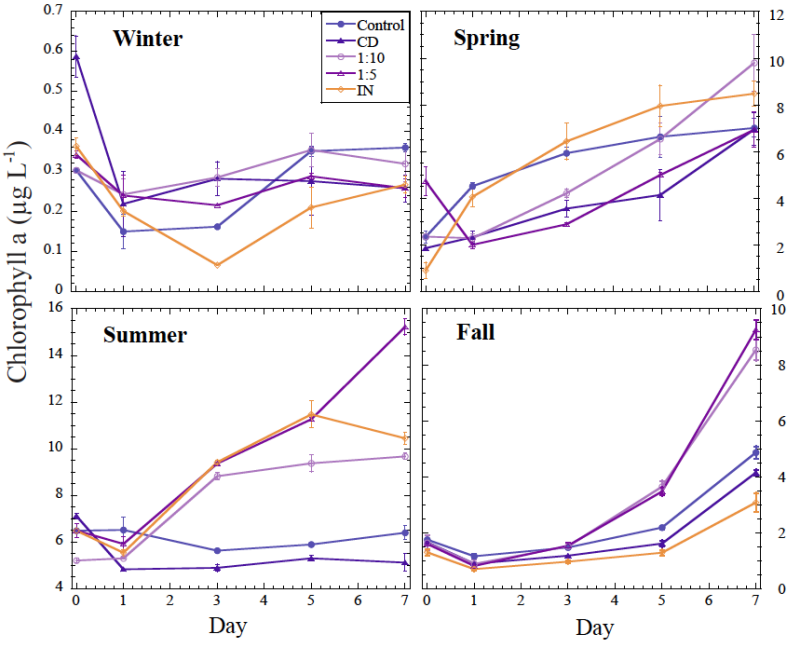

667
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669 Figure 1
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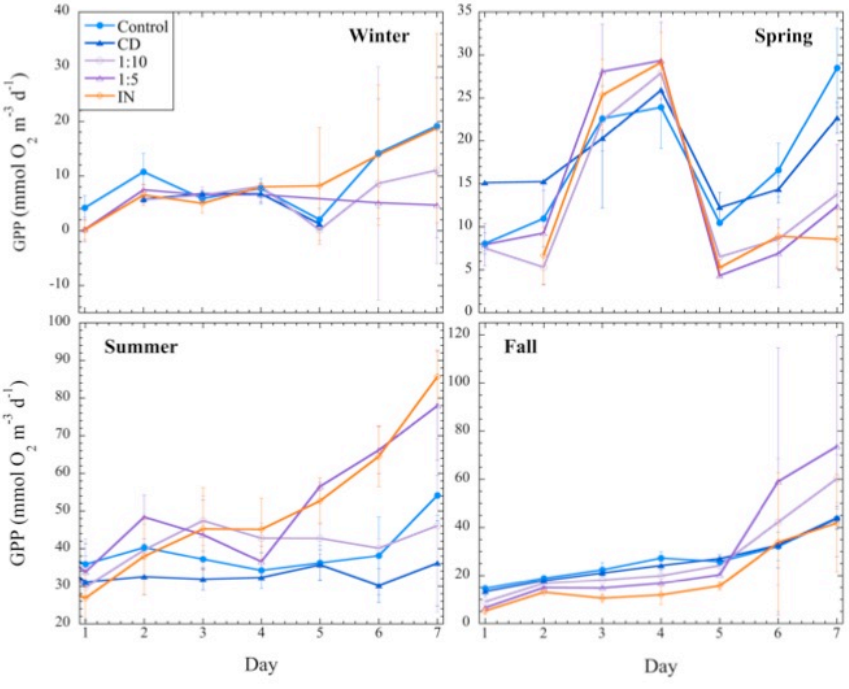

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674 Figure 2

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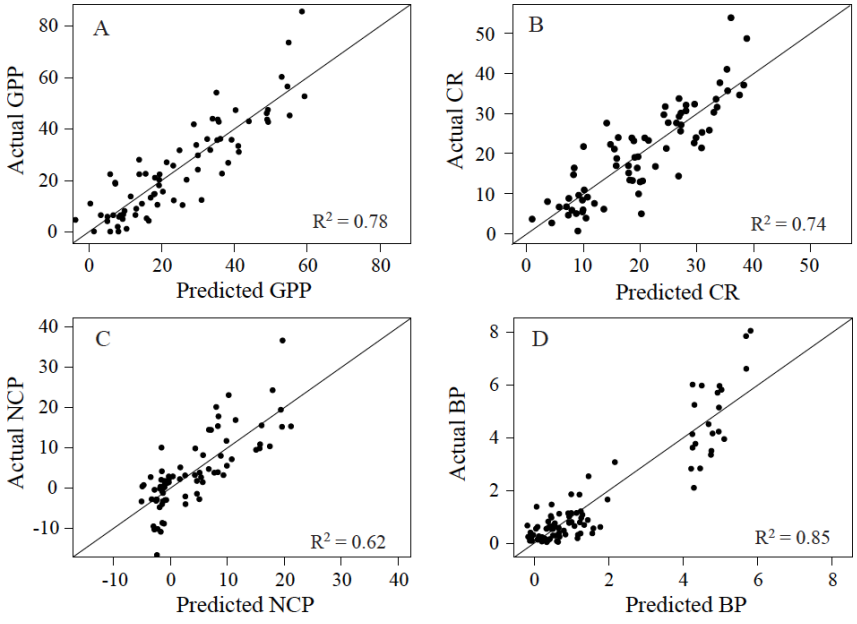

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677 Figure 3

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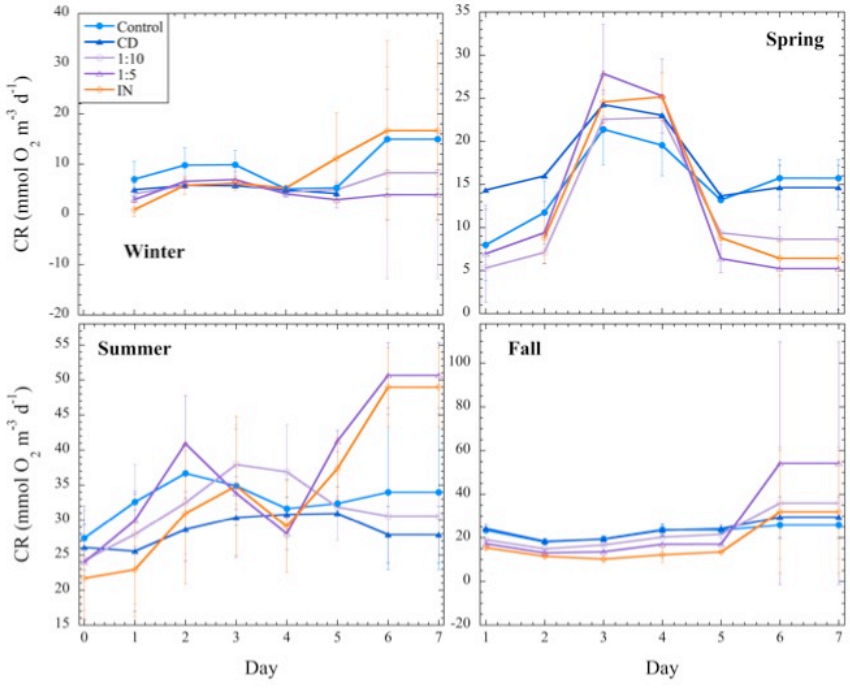

Figure 4

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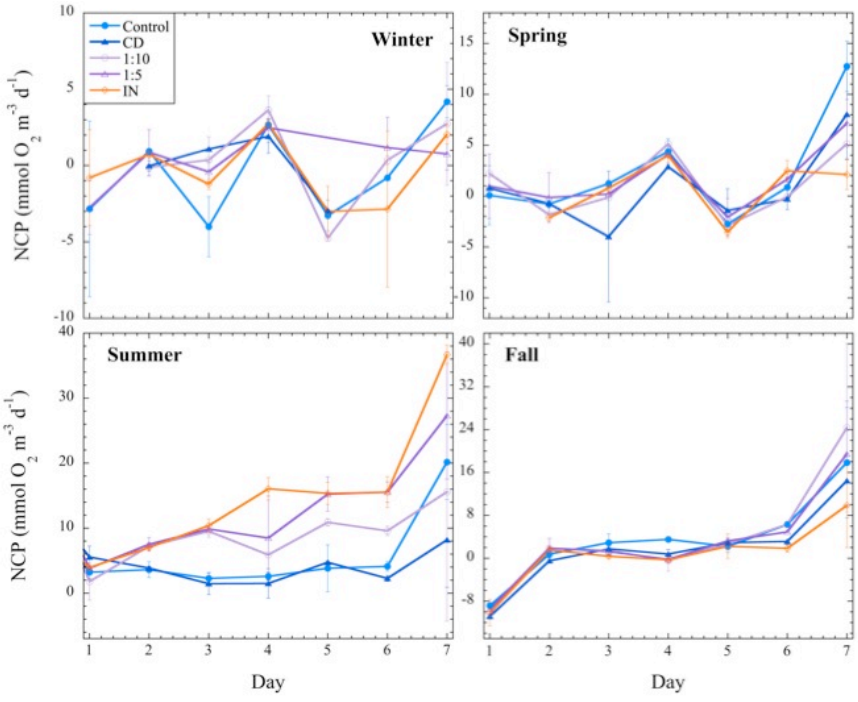

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683 Figure 5

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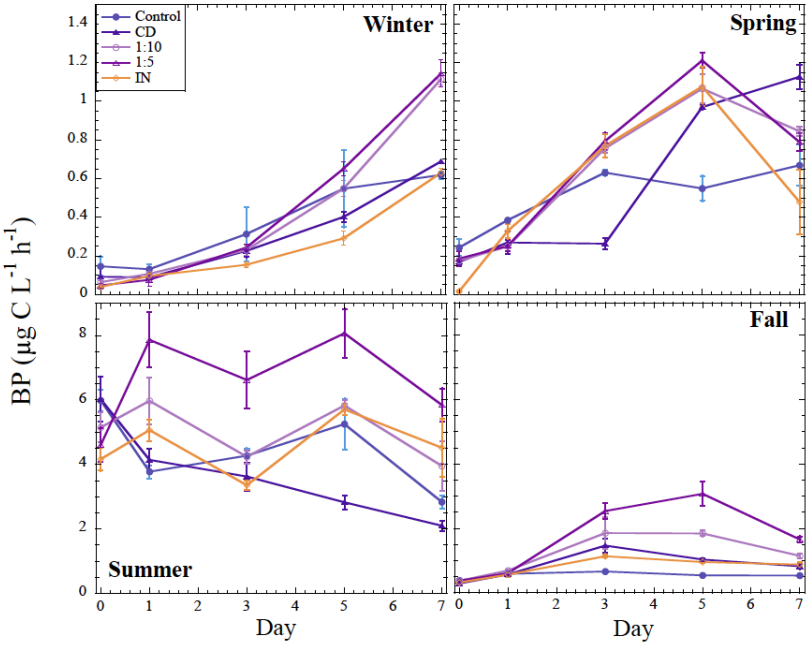

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688 Figure 6

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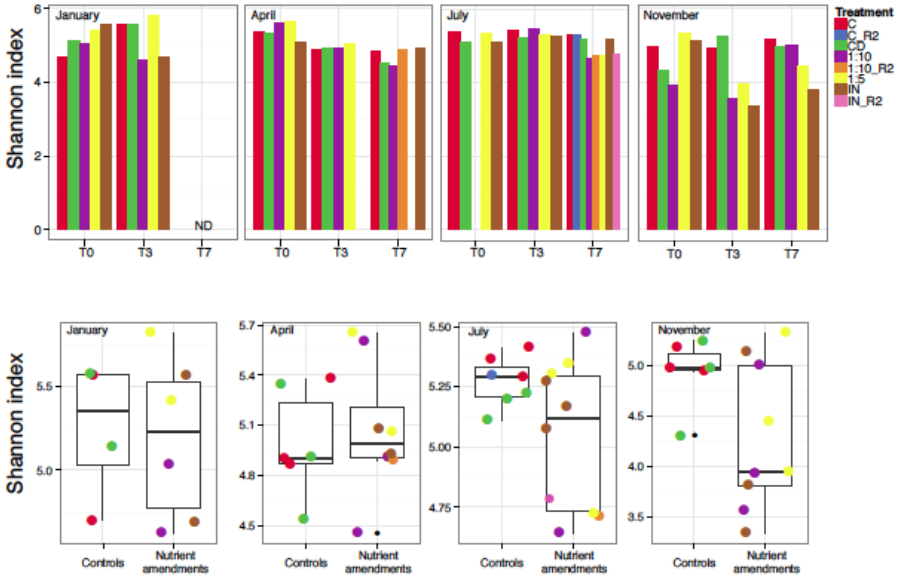

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692 Figure 7

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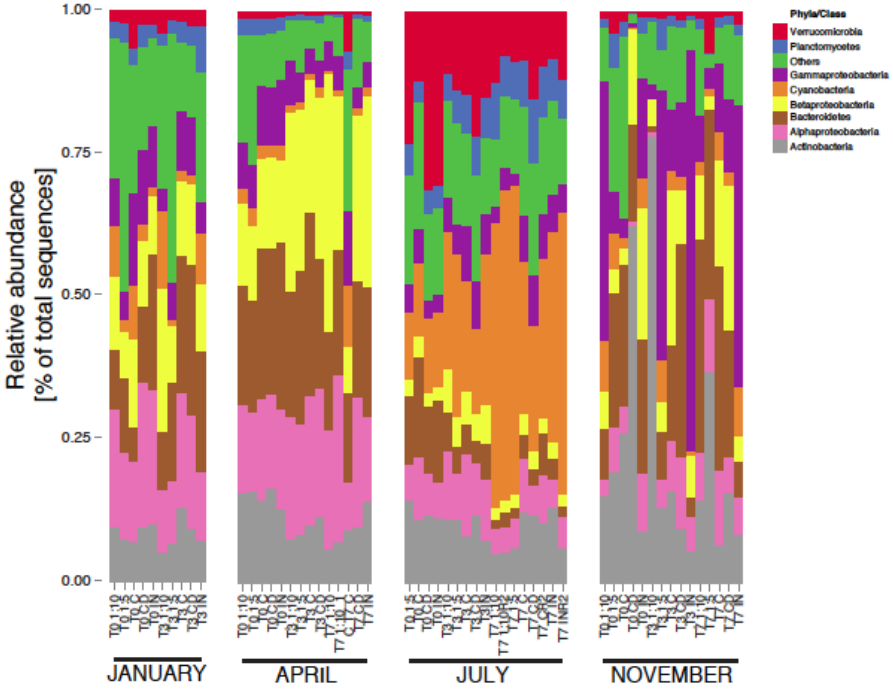

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696 Figure 8

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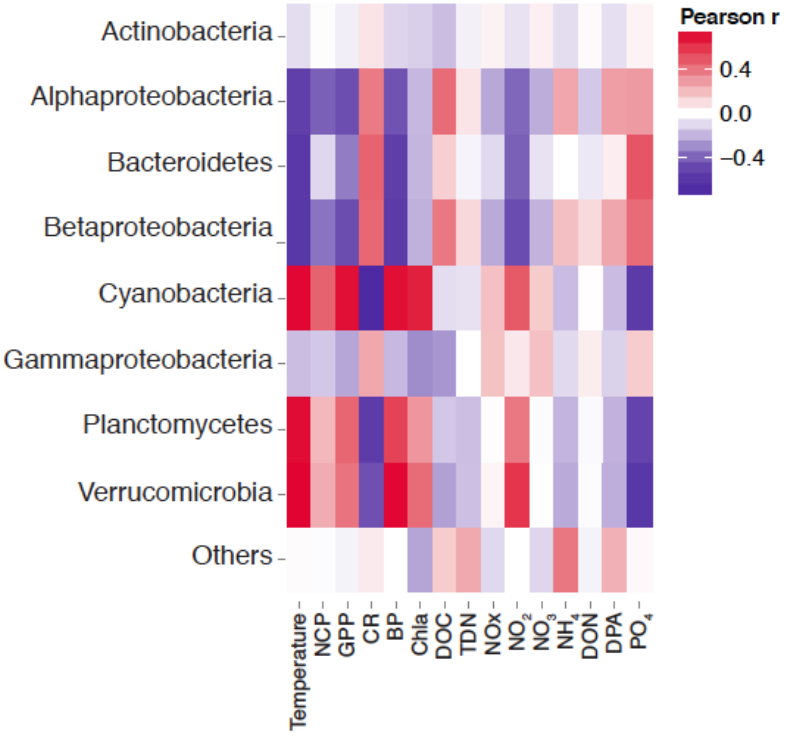

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699 Figure 9