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Work package:	2

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FOREWORD

The present deliverable is provided in the form of a public report. It will be transformed into a manuscript for publishing in the peer reviewed journal Environmental Microbiology. Submission is anticipated to be in October 2017.

Overview

The Baltic Sea ecosystem encounters several specific stressors due to climate change and human activity. These environmental stressors include increased input of nutrients and terrestrial derived DOM to the Baltic Sea from freshwater sources, which affect ecosystem structure and processes. The current study aims at assessing changes in ecosystem productivity and metabolism in response to environmental stressors, and to link these changes to the microbial genetic blueprint. The coupling of specific prokaryotic metabolic genes to specific stressors can potentially be applied as a useful tool for monitoring environmental conditions in the Baltic Sea, and be used to refine models of ecosystem structure and biogeochemical cycling in response to environmental changes. A large scale mesocosm experiment with Baltic Sea water was carried out to evaluate the links between environmental changes due to specific stressors such as increased riverine inputs, phytoplankton blooms and the subsequent responses in bacterial activity, community composition and gene abundance and activity. More specifically the experiments aimed at examining effects of environmental drivers/stressors on bacterial metabolism (changes in gene abundance of C, N, P transporters and other key metabolic genes, extracellular enzymatic activity, productivity and abundance) and to explore to what extend the microbial metagenome reflects specific environmental conditions.

To address these questions, we studied the response in pelagic bacterial community (production, metabolic activity, community composition and metagenome) to specific environmental stressors represented by input of inorganic nutrients and different sources of riverine allochthonous DOM to Baltic Sea water during 10 day incubation under controlled experimental conditions.

Methodological workflow

Experimental setup

The experiment was performed from May 30 – June 8 2016 at Linnaeuus University (LNU), Sweden, in 15 indoor mesocosm tanks containing 200 L. The water was collected on May 30 at the Linnaeus Microbial Observatory (LMO) site in the Baltic Sea (56°55'.51.24"N 17°3'38.52"E), from 2 m depth, using a pump, and transported in one m^3 containers to the LNU within 1 h. Salinity and temperature at the time of sampling was 7.2 and 11.7 °C (AAQ1186-H CTD, Alec Electronics co., Japan), respectively. Each mesocosm was filled with 160 L of LMO water filtered by reverse filtration using a 100 µm mesh filter with a diameter of 13 cm, and left overnight in the mesocosms (Fig. 1). Each mesocosm was covered by a transparent plexiglas plate to reduce the risk of contamination. To prevent stratification, a constant and gentle bubbling with air was applied at the bottom of each mesocosm using a Z flat aquarium air stone (diameter 8 cm, height 1.5 cm), and manually stirred approximately every 12 hours with a polypropylene stick. Light was provided on a 12 h light:dark cycle, using 250 W halogen lamps (HQI –E 250 W/D Pro E40, Powerstar), yielding a photosynthetically active radiation (PAR) light level of ~400 µmol photons m^2 s^1.

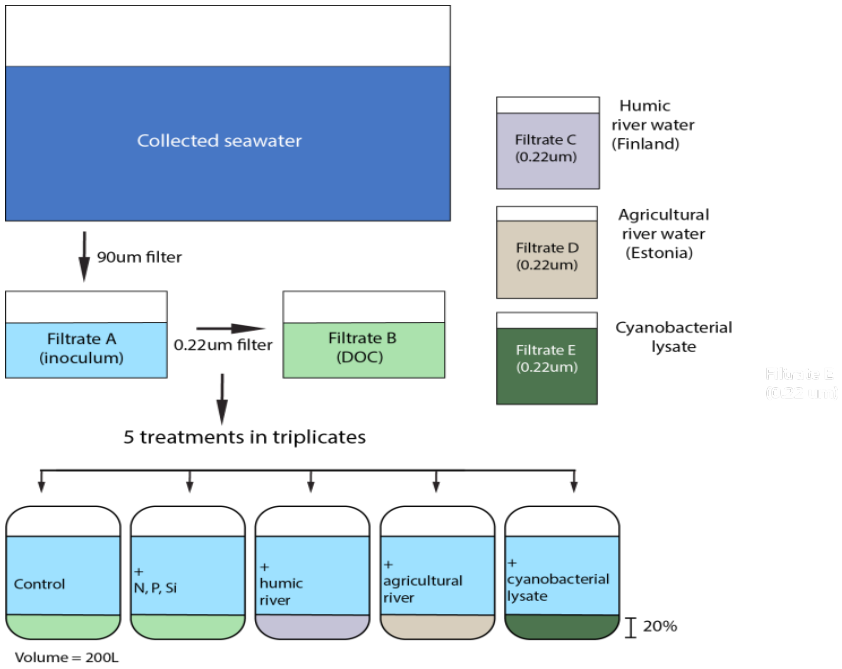

Figure 1: Schematic diagram of the preparation and design of the mesocosm experiment.

Treatments

The 15 mesocosms tanks received one of five treatments, each replicated in triplicates (Fig. 1). The Control, NP+Si and Cyanobacterial lysate mesocosms all received 40 L (20 % of final volume) of 0.22 µm filtered LMO water. In addition, the NP+Si treatment received $NaNO_3$, Na_2HPO_4, and $NaSiO_3$ in ratio and concentrations similar to *in situ* late winter conditions (final concentrations of 16 µmol L^{-1} nitrogen, 1 µmol L^{-1} phosphate, and 32 µmol L^{-1} Silicate, respectively), and the Cyanobacterial lysate mesocosms received an additional 10 mL of sonicated and centrifuged 0.22 µm filtered cyanobacterial lysate *Nodularia spumigena AV1* Chl *a* 21300 µg/L. The Humic river and Agricultural river mesocosms each received 40 L of 0.22 µm (Opticap XL filter, Millipore) filtered water from the humic river Lapväärti (Finland) or the agricultural-influenced river Lielupe (Latvia), respectively, which were collected between May 26 – 28, 2016 and stored at *in situ* temperature (10 °C) in acid rinsed polyethylene containers. Samples for water chemistry, and biological and molecular parameters were collected every ~24 h and all sampling equipment was rinsed in MQ water between samplings.

Analyzed variables

Dissolved organic carbon (DOC) and dissolved organic nitrogen (DON) were measured on a Shimazu TOC-L analyzer equipped with a TNM-L Total Nitrogen Measuring Unit. The filters for chlorophyll a (Chl a) measurement were extracted in ethanol (96%) and fluorescence was measured using a Trilogy® Laboratory fluorometer (Turner designs, USA) (Jespersen & Christoffersen, 1987). Bacterial production (BP) was assayed using ^{3}H-thymidine incorporation (Fuhrman & Azam, 1982; Lee & Fuhrman, 1987; Riemann & Bjornsen, 1987). Samples for bacterial abundance (BA) were preserved in 2 % glutaraldehyde, and counted using a CyFlow Cube 8, PartecXX according to del Giorgio et al. (1996). Extracellular enzyme activities (peptidase (PEP), beta-glucosidase (BET) and alkaline phosphatase (APA)) were assayed using fluorogenic 4-methylumbelliferone (MUF) and 7-153 amino-4-methylcoumarin (MCA) substrates (Sigma-Aldrich, St. Louis, MO, USA) according to Hoppe (1983). DNA, fixed in a 10% final volume stop-solution (5 % of phenol in 99.8 % ethanol), was isolated using the AllPrep DNA/RNA Mini Kit (Qiagen). DNA (2–10 ng) from each sample were prepared with the Rubicon ThruPlex kit (Rubicon Genomics, Ann Arbor, Michigan, USA) according to the instructions of the manufacturer. Libraries were sequenced on a HiSeq 2500 (Illumina Inc., San Diego, CA, USA) at SciLife in Stockholm, Sweden. High quality reads were mapped to the BARM database (Hugerth et al., 2015). Quantitative abundances of reads

were annotated and clustered using the database of orthologous groups and functional annotation eggNOG 4.5 (Huerta-Cepas et al., 2016). The community composition was analyzed from PCR amplicons of the 16S rRNA genes (V3-V5 region), which were then sequenced with an Illumina MiSeq paired-end multiplex platform in SciLifeLab/NGI (Solna, Sweden).

Comparative analysis of differential abundance (DA) genes.

The R bioconductor package DESeq2 was used for differential abundance analysis. The fold change (logFC) for each gene in various treatments *versus* control treatment was determined using general liner modeling (GLM) based on a negative binomial distribution. Only DA genes with logFC > 2, and adjusted p <0.05, were used in further analysis. Correspondence analysis (CA) of DA and linear fitting of measured abundance/rate/concentration variables and the distance based redundancy analysis (dbRDA) of community composition differences were analyzed using the *vegan* package in R. In addition, GLM was used to analyze the relationship of each single DA and measured abundance/activity/concentration (R package *mvabund*).

Results and Discussion

1. Environmental conditions in the manipulated mesocosms

The addition of river water represented a substantial input of DOC and inorganic nutrients. DOC concentration in both river treatments increased by ~50 % and reached DOC concentrations of approximately 6 mg C l^{-1} (Fig. 2). DOM from the Lielupe river had a higher nitrogen content compared with the Lapväärti river and the C/N ratio of the DOM decreased from >100 in the control mesocosms to 25 in the Lapväärti river treatment (humic and pristine river) and 7 in the Lielupe river treatment (polluted river in regions with intensive agriculture).

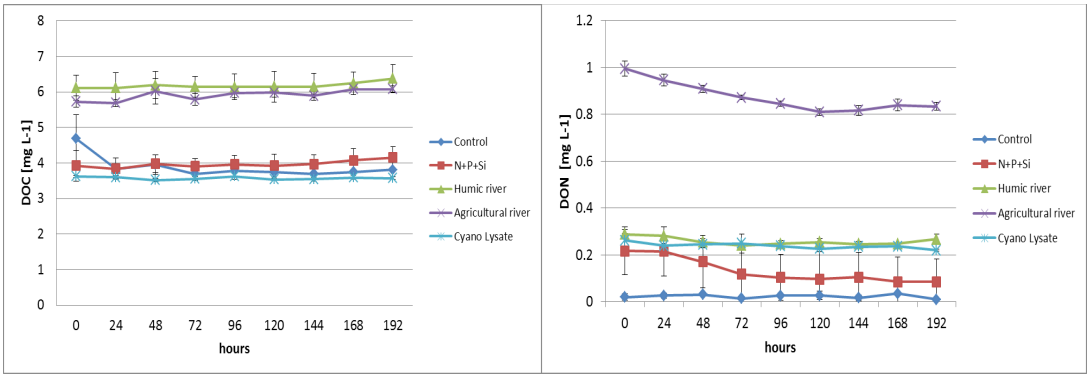

Fig. 2: Concentration of DOC and DON in the mesocosms during the experiment.

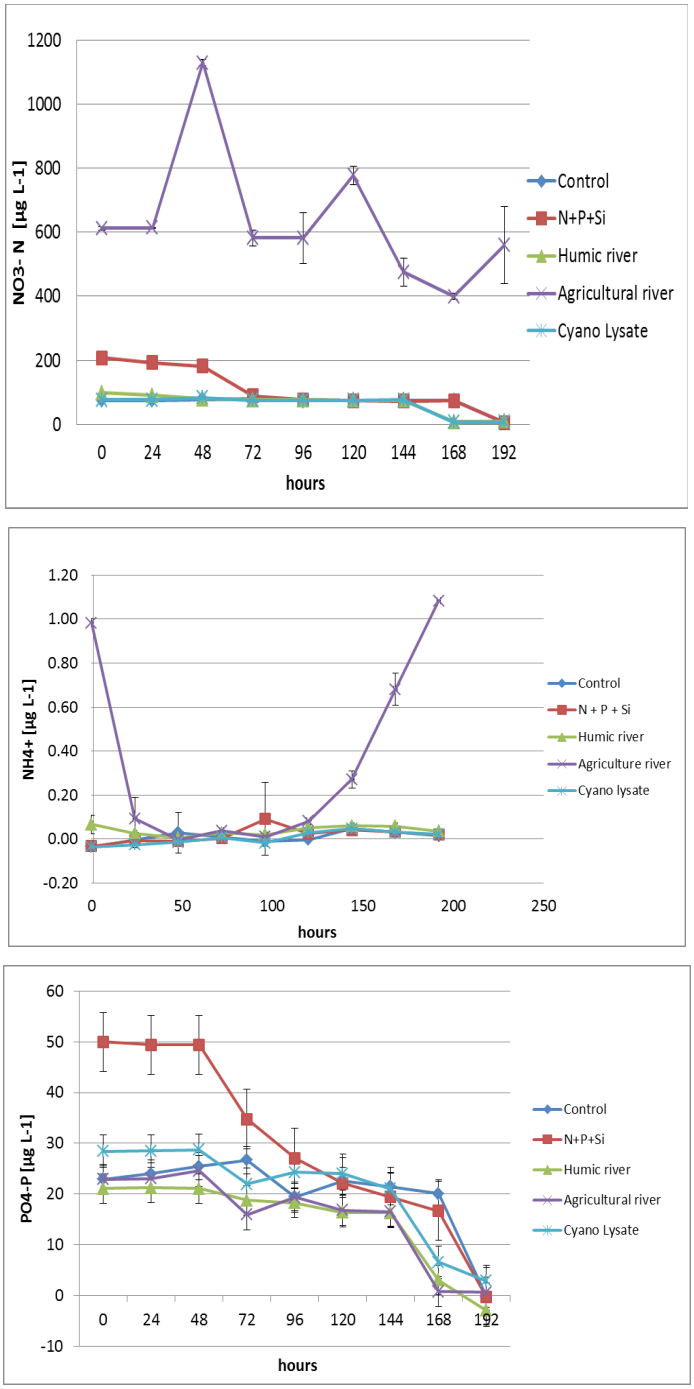

Figure 3: Concentration of nitrate, ammonium and phosphate during the experiment.

The agriculture influenced Lielupe river contained very high concentrations of inorganic nitrogen and addition of the river water increased the concentration of ammonium and nitrate substantially (Agriculture river treatment), even exceeding the inorganic nutrient-enriched treatment (+NPSi) 3 fold (Fig. 3). Ammonium was rapidly depleted in all mesocosms but an increase in ammonium concentration in the agricultural river treatment indicates increased microbial mineralization of DON during the last phase of the experiment. Phosphate concentration was not significantly elevated in the river treatments and the initial phosphate concentration was highest the +NPSi treatment (Fig. 3). Phosphate concentration decreased over time and was depleted in all treatments by the end of the experiment.

Together, the chemical composition of the water in the different treatments verified that the environmental conditions differed considerably between the treatments with respect to concentration and composition of DOM and inorganic nutrients. Compared with the control mesocosms, the agriculture river treatment was characterized by high DOC concentration, low C:N ratio and high C:P ratio, whereas the humic river treatment represented conditions with high DOC concentration, high C:N ratio, high humic content, and high C:P ratio. The treatment enriched with inorganic nutrients, on the other hand had low DOC relative to the river treatments but a lower C:P ratio than in all other treatments. These differences in initial environmental conditions imposed by the different stressors affected the responses in phytoplankton production during the experiment, thus allowing us to examine the effects of both the allochthonous inputs of different DOC sources and the phytoplankton-derived DOC on the metabolism and genetic composition of the microbial communities.

2. Development of an algal bloom under experimental condition

A bloom of algae developed in response to the increased concentrations of inorganic nutrients, as expected, primarily in the +NPSi treatment, but also in treatments receiving 20% of riverine water (Fig. 4). The plankton in LMO station was P limited and therefore inorganic P (PO_4) was depleted during the course of the experiment. The addition of agricultural river water with less inorganic P and a high N:P ratio evoked a weaker bloom, and the weakest bloom developed in treatment amended with pristine and humic rich river water (Lapväärti river) with low inorganic nutrient concentrations.

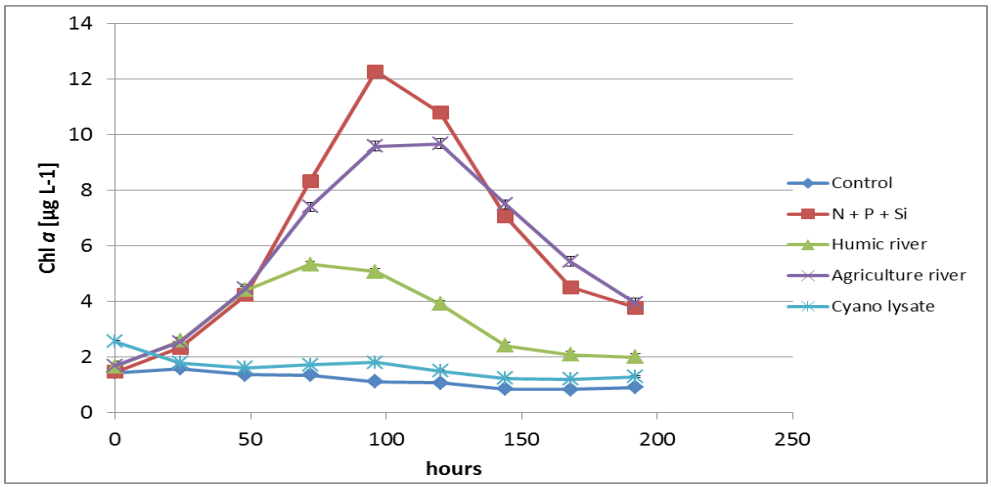

Figure 4: Chlorophyll *a* concentration in the five treatments during the course of experiments.

3. Responses of the microbial activity to the treatments

Bacterial abundance and production did not respond to treatments before the collapse of algal bloom (Fig. 5), i.e. strongest response occurred in +NPSi treatment after the collapse of algal bloom (Day 6). This strongly suggested the production of bioavailable DOM associated with the breakdown of the algal bloom in this treatment. The lack of coupling between the phytoplankton collapse and a response in the bacterial production in the river treatments, on the other hand suggested either that the phytoplankton decay was not associated with a release of DOC or, more likely, that the released DOC was not immediately available for bacterial uptake. The low concentration of inorganic P in the agricultural river treatment may suggest that the bacterial production in this experiment was P-limited. This is supported by the high phosphatase activity in this treatment indicating bacterial P demand was covered by organic sources. The humic river treatment was low in both nitrogen and phosphorus, contained DOM with a high C:N ratio and did not show elevated phosphatase activity (Fig. 6), suggesting that bacterial activity was limited by the availability of phosphorus in either organic or inorganic forms.

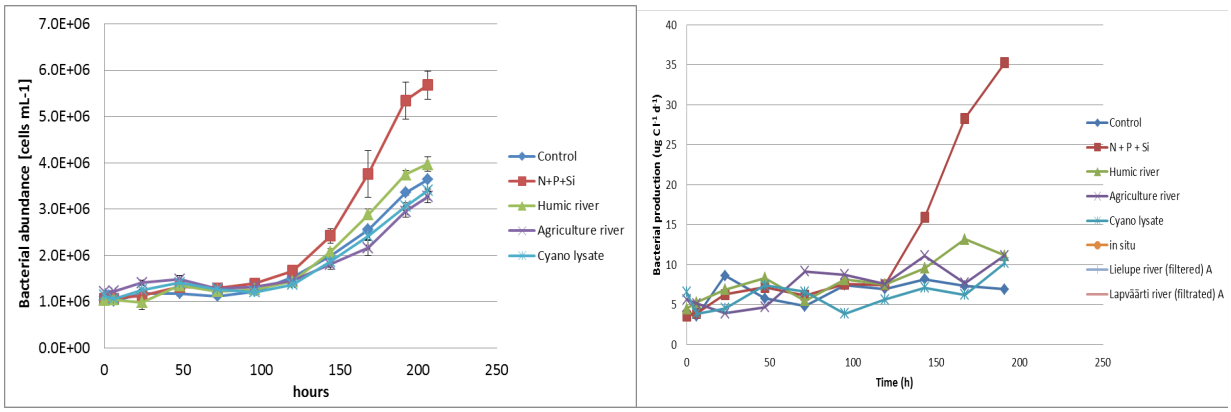

Figure 5: Dynamics of abundance and production of the bacteria in the mesocosm experiment.

The elevated beta-glucosidase (BET) and aminopeptidase (PEP) activity in the +NPSi and agricultural river treatment during the phytoplankton bloom collapse (Fig. 6) indicated intensified extracellular degradation of polysaccharides and peptides released from the phytoplankton. In the river treatment, this resulted in the accumulation of ammonium (Fig. 3), whereas the enzymatically hydrolysed nitrogen in the +NPSi treatment was apparently immediately taken up by bacteria and phytoplankton as ammonium did not accumulate. This further supports the hypothesis that bacterial activity was P-limited in the agricultural river treatment, and the P-limitation likely explains why only a moderate stimulation of microbial activity was observed in this treatment.

Together, the measurements of bacterial abundance, production and enzymatic activities underlines that the initial perturbations induced highly different environmental conditions characterized by unique compositions of organic and inorganic compounds. Despite the development of a phytoplankton bloom in all the treatments amended with inorganic nutrients alone or with the river water, the bacterial response was different in all the treatments. This indicated that despite the production of autochthonous DOC in all three nutrient enriched treatments, the differences in the initial concentration and composition of DOM resulted in different limiting factors for the bacteria, with significant effects on the metabolic characteristics of the microbial communities.

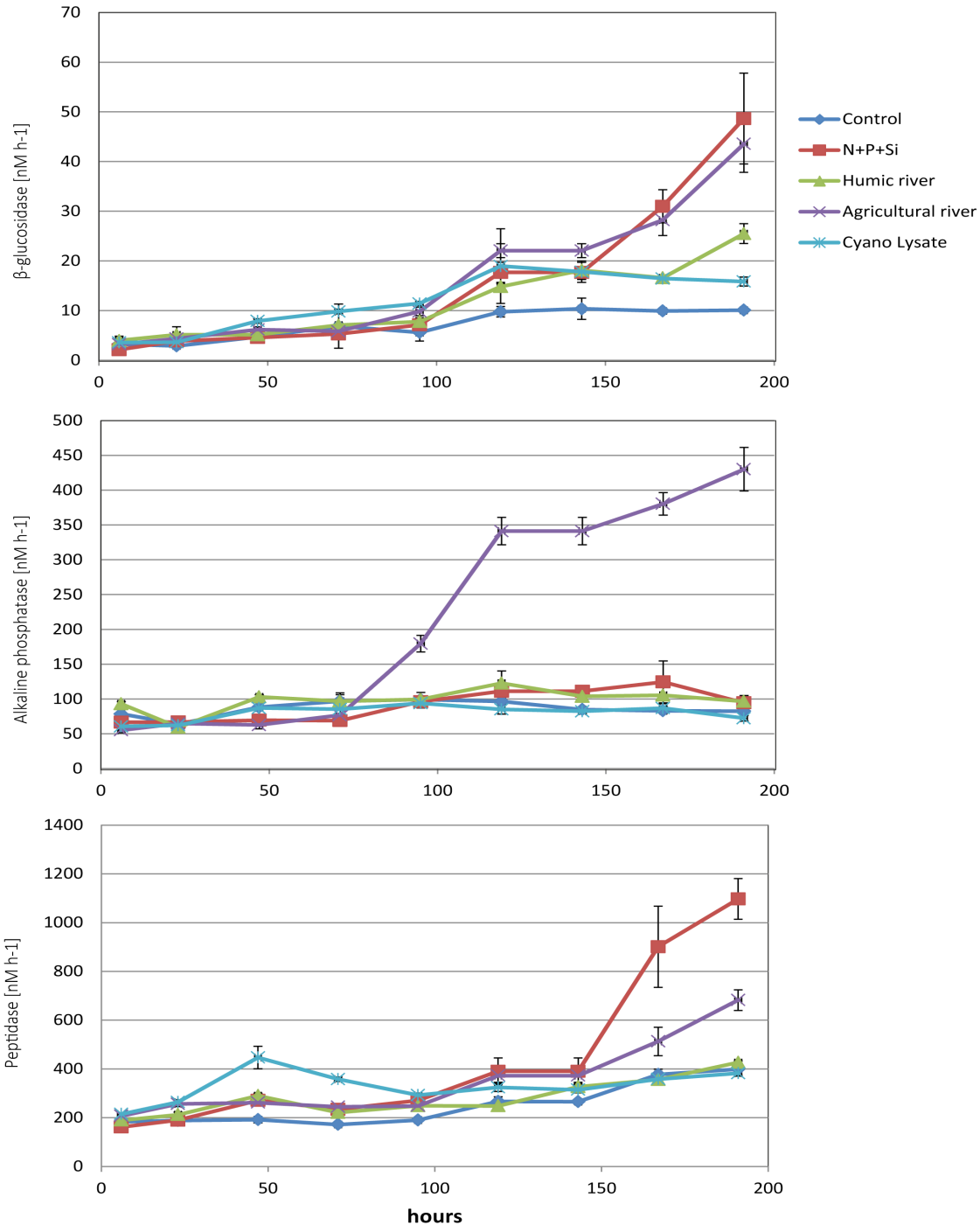

Figure 6: Aminopeptidase, beta-glucosidase and alkaline phosphatase activity, in the five treatments, average values with standard deviation (n = 3).

4. Changes in bacterial community composition.

The bacterial community composition changed significantly in all mesocosms during the course of experiment from Day0 to Day8 (dbRDA analysis, Type III Sum of Squares (SS)=10.51, pseudoF=8.91, p=0.001), while the effects of the individual treatments were significant but weaker (SS=1.19, F=2.2, p=0.002). Initially community composition was dominated by cyanobacteria (mostly Synechococcus) and some eukaryotic algae (not identifiable using 16S rRNA genes) accounting for > 50% of the total community. Over time the communities changed to more diverse communities which differed between treatments at Day7 (Fig. 7).

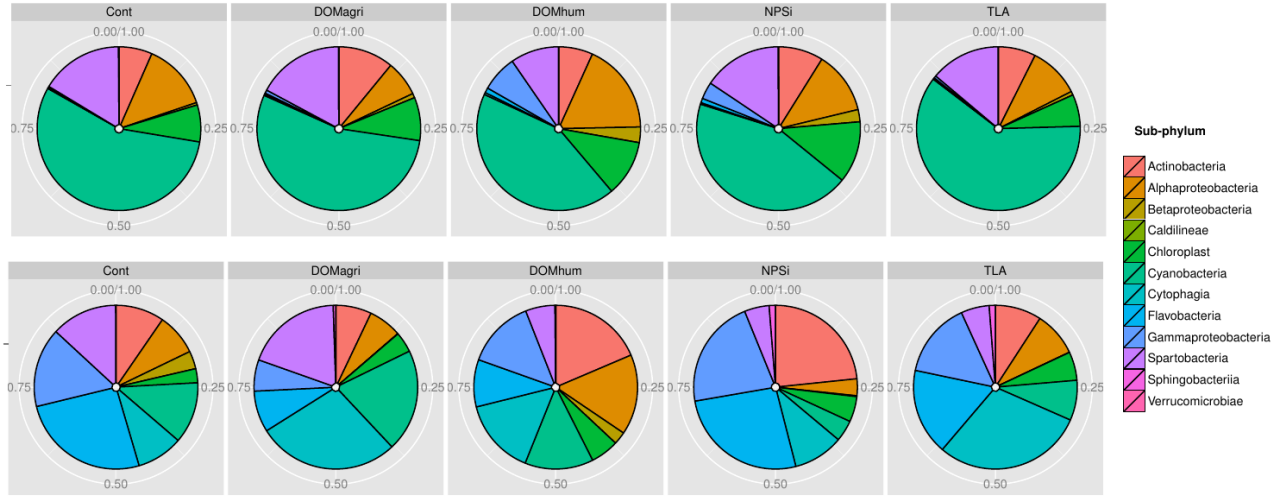

Figure 7: Average proportions of sub-phyla in the mesocosm experiment. Upper panel at Day0, lower panel at Day7.

Day7 samples were used to generate the metagenomes for analysis of metabolic profiles. In the control mesocosm, besides Cyanobacteria, dominating groups were Alphaproteobacteria (13%) and Spartobacteria (Verrucomicrobia) (16%), these groups remained important members of the community. However, Flavobacteria (26%) and Gammaproteobacteria (16%) became the most dominating sub-phyla/classes at Day7 in the control mesocosms. In +NPSi treatment the most abundant sub-phyla/classes were Flavobacteria (26%), Actinobacteria (22%) and Gammaproteobacteria (22%). The Actinobacteria (19%) together with Cytophaga (15%) and Alphaproteobacteria (16%) became abundant in the mesocosms amended with humic rich river water, which was also the treatment with the most even community composition at sub-phyla/classes abundance level. Community composition in the mesocosms amended with river

water from agricultural landscape was dominated by algae (20%), Cytophaga (28%) and Spartobacteria (19%).

The distribution of the most abundant individual operational taxonomic units (OTUs) differed considerable between treatments (Fig. 8). Three OTUs affiliated to Cytophaga/Cyclobacteriacea (closest match in GenBank AJ697704, unidentified Gammaproteobacterium (FR683233, observed in acidification experiment in coastal sea) and Candidatus Aquiluna (JN976188, observed in acidification experiment in Arctic sea) were dominant in all treatments. Single abundant OTUs which were only found in one treatment were: unidentified Flavobacteriaceae (AY794206 in control and AM279192 in DOMagri) both belonging to NS3a marine group typical to the Baltic Sea plankton community. Alphaprotebacterium OTU belonging to SAR11 brackish-water cluster (EU800476) was relatively abundant in the controls, and the humic river water and toxic cyanobacterial lysate (TLA - toxic lysate of algae) treatments. Freshwater Synechococcus (AY151246) was observed in the control and both treatments amended with river water. Prominent member in the +NPSi mesocosms was the Polaribacter (HM127504, class Flavobacteria) genus, which is also commonly found in the plankton of the Baltic Sea. An OTU similar to an uncultured betaproteobacterium found from other brackish water environments (GQ346749) was among dominant community members in the TLA and agricultural river treatments. Among Actinobacteria, an unidentified OTU similar to Actinobacteria (EU801153) isolated from surface water in other estuaries (Delaware Bay) were found in all mesocosms.

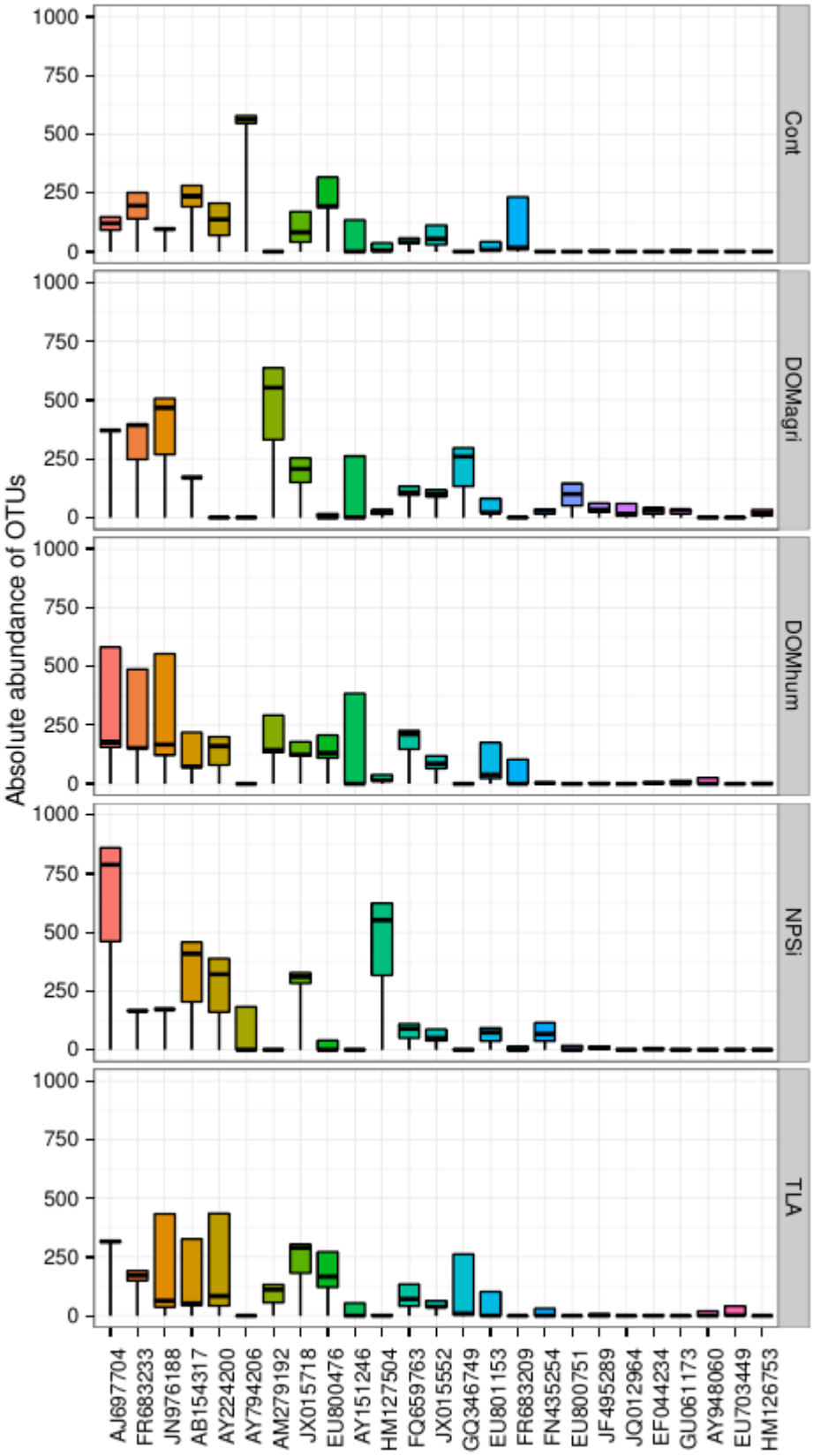

Figure 8: Most abundant OTUs in triplicate mesocoms at Day7.

5. Responses in metagenomes and its association to metabolic activity.

A subset of genes (n=1053) selected from differentially abundant (DA) analysis (increased logFC) was used to analyse the association between the metagenomes and the following variables: bacterial abundance (BA, cells ml^{-1}), bacterial production (BP, $\mu gC\ l^{-1}\ h^{-1}$), activity of ectoenzymes (alkaline phosphatase (APA), beta-glucosidase (BET), and peptidase (PEP), $nM\ h^{-1}$), amino acidas uptake (AAUp, h^{-1}), and concentrations of chlorophyll a (Chla, $\mu g\ l^{-1}$), nitrate, nitrite, ammonia (NH_4), phosphate (PO_4, $\mu g\ PO_4\text{-}P\ l^{-1}$), dissolved organic carbon (DOC, $mg\ l^{-1}$), and nitrogen (DON, $mg\ l^{-1}$).

The association of bacterial activities and concentration of nutrients indicated relationships falling into 2 major categories; The first category included BA (r2=0.96, p=0.008), BP (r2=0.46, p=0.03), PO_4 conc. (r2=0.69, p=0.002) and AAUptake (r2=0.90, p=0.001); and second APA (r2=0.90, p=0.002), various nitrogen species (r2>0.8, p<0.01) (Fig. 9), excluding nitrite concentration. Besides of these 2 major groups, Chl a (r2=0.46, p=0.04) and beta-glucosidase activity (BETA, r2=0.40, p=0.07) associated with a different set of DA genes. In addition, the relationship of predictor variables (microbial activities and nutrient concentrations) with each individual DA genes was analysed using GLM, assuming negative binomial distribution in gene count. Strongly (deviance) and significantly (adjusted p<0.05) related genes were mapped on clustering of all DA genes (Fig. 10). Such mapping reveals that BA, BP and PEP were related to the same set of genes with minor differences. Genes related to APA and DON, BETA and Chl a can be discriminated from the rest of the gene pool, while genes related to DOC concentration fell into separate clusters.

APA related metabolic enzymes are clearly related to phosphate metabolism in microbial cells, enzymes such as: EC 2.7.1.174 (DGK1; diacylglycerol kinase (CTP), phosphotransferase), EC 3.1.11.6 (Exodeoxyribonucleases producing 5'-phosphomonoesters, exonuclease VII), EC 3.1.3.48 (PGP, phosphoglycolate phosphatase). PEP and BETA related enzymes were mostly related to basic metabolic processes in metabolism. PEP – EC 3.6.3.14 (H+-transporting two-sector ATPase), EC 2.7.7.7 (DNA-directed DNA polymeras), EC 2.4.1.- (Hexosyltransferases) and BET – EC 2.3.1.48 (Histone acetyltransferase), EC 2.1.1.33 (tRNA guanine 7-methyltransferase), and EC 6.3.2.23 (E2 ubiquitin-conjugating enzyme). DON related to EC3.4.24.28 (metallopetidase) and DOC related to many (n=24) and not overlapping with genes related to any other predictor variables. Genes increased in

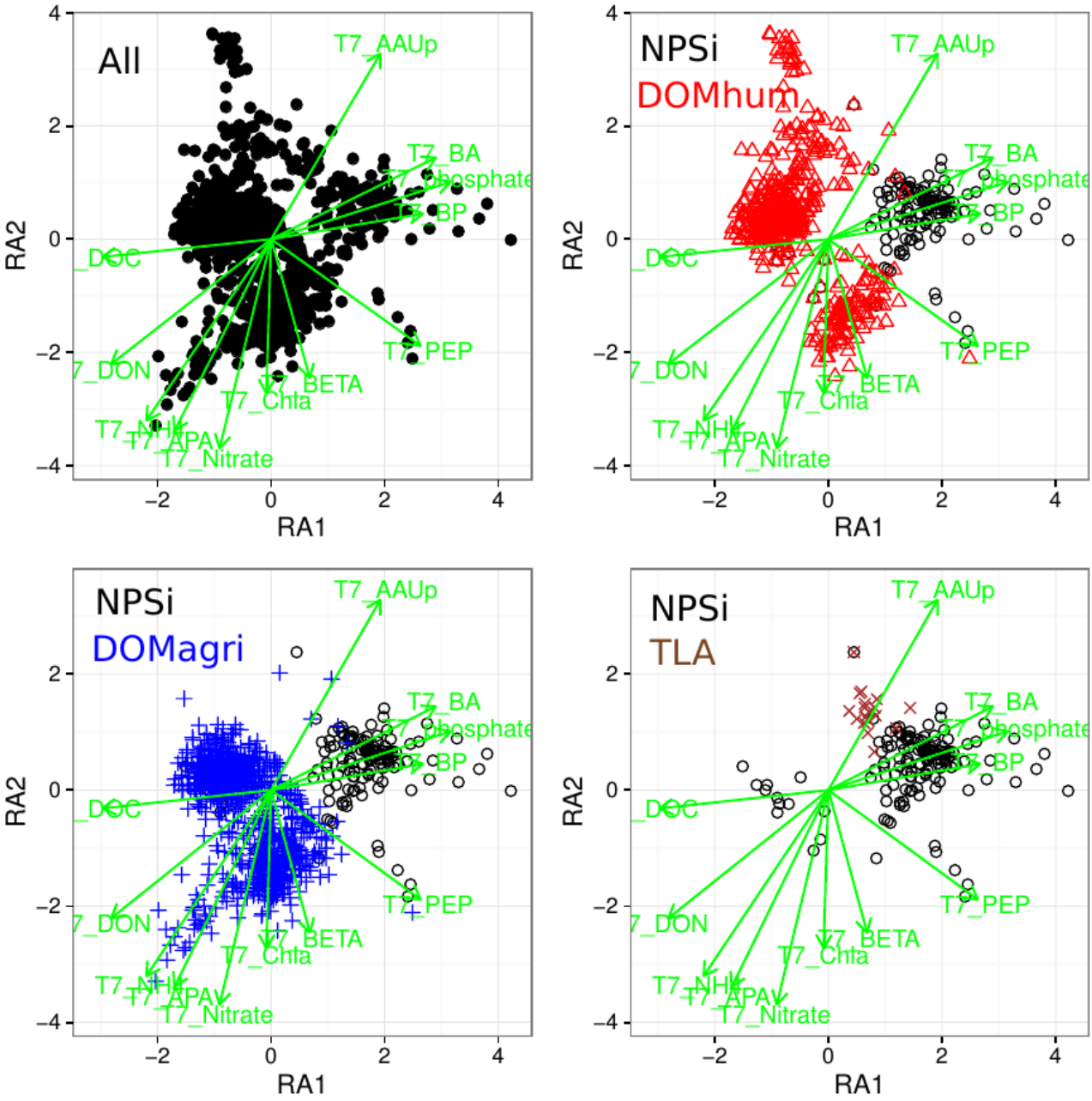

Figure 9: Association between differentially abundant (DA) genes and bacterial activity/concentration of nutrients and organic compounds analysed as linear correlation between species scores of gene abundance and measured variables (green arrows). Genes are coloured according to their origin in DA analysis on upper left and lower panels.

abundance due to higher DOC belonged mostly to basic metabolism: various peptidases (EC3.4.21.- and 3.4.24.-), hydrolases or lyases of nucleic acids (EC2.7.7.6, EC2.7.7.7, EC3.1.26.5, EC4.2.99.18), enzymes related with carbon metaboslim (EC2.8.2.11 galactosylceramide sulfotransferase, EC3.1.1.- Carboxylic-ester hydrolases, EC2.4.1.67 galactinol-raffinose galactosyltransferase).

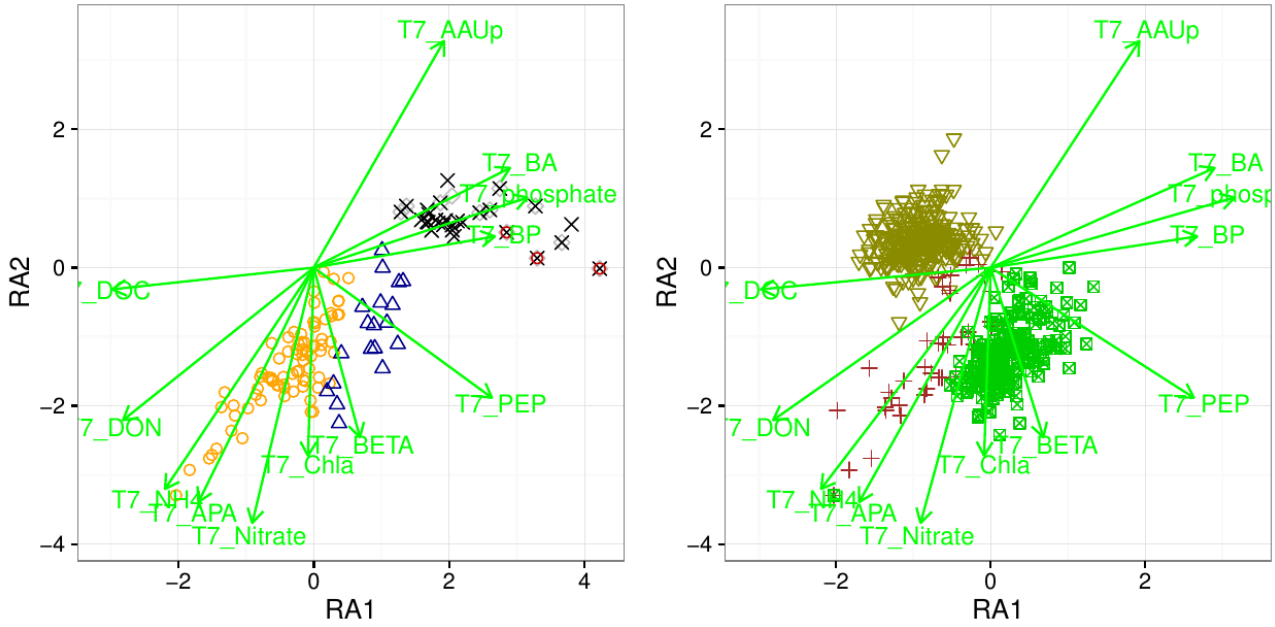

Figure 10: The same association analysis as presented in Fig. 9. Genes are coloured according to their strong and significant relationship in the GLM (using negative binomial distribution) with measured variables. Arrows indicate the same associations of linear correlation of measured variables with gene clustering in correspondence analysis. On left pane: orange rings: APA, blue triangles: BETA, black crosses: BP, grey diamonds: BA, red rings: PEP; On right panel: dark green triangles: DOC, dark red plus signs: DON and green squares with cross: Chl a.

Relationship of genes grouped according to their categories of functions is illustrated in Fig. 11. Genes related to BA, BP and PEP are affiliated to [K] Transcription; [L] Replication, recombination and repair; and [M] Cell wall/membrane/envelope biogenesis. Genes related to DOC belong to [T] Signal transduction mechanisms; [U] Intracellular trafficking, secretion, and vesicular transport; [Z] Cytoskeleton.

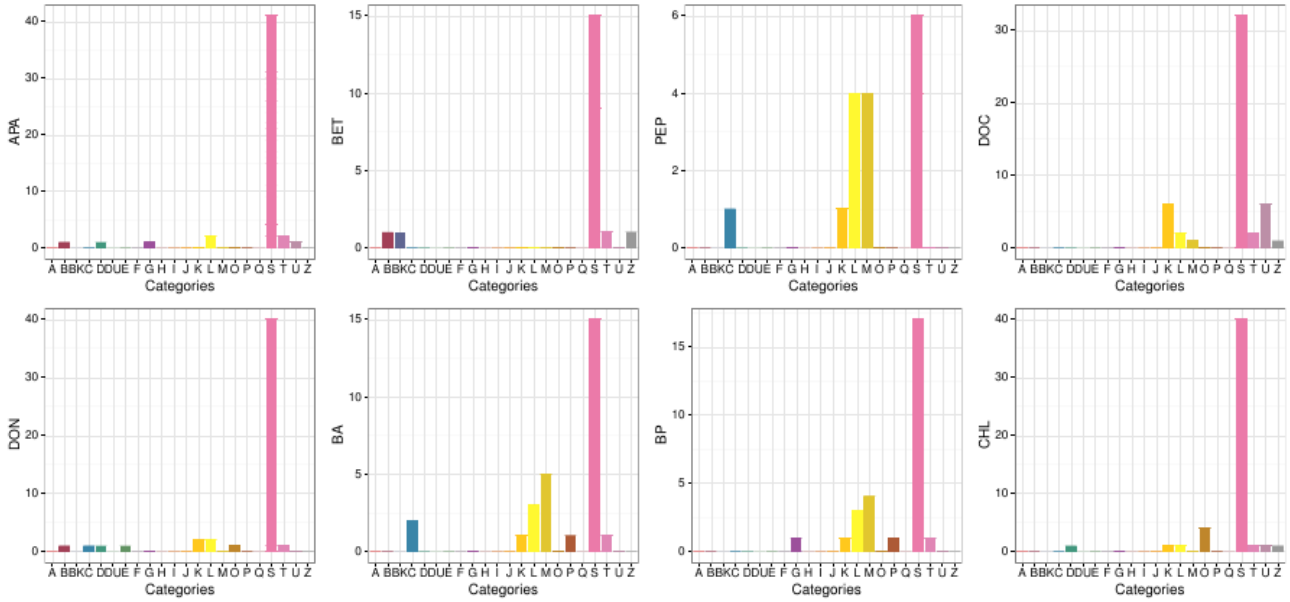

Figure 11: Count of genes associated with predictor variables (microbial activities or concentrations of nutrients, DO, or Chl a) grouped by eggNOG categories:

[J] Translation, ribosomal structure and biogenesis
[A] RNA processing and modification
[K] Transcription
[L] Replication, recombination and repair
[B] Chromatin structure and dynamics
[D] Cell cycle control, cell division, chromosome partitioning
[Y] Nuclear structure
[V] Defense mechanisms
[T] Signal transduction mechanisms
[M] Cell wall/membrane/envelope biogenesis
[N] Cell motility
[Z] Cytoskeleton
[W] Extracellular structures
[U] Intracellular trafficking, secretion, and vesicular transport
[O] Posttranslational modification, protein turnover, chaperones
[C] Energy production and conversion
[G] Carbohydrate transport and metabolism
[E] Amino acid transport and metabolism
[F] Nucleotide transport and metabolism
[H] Coenzyme transport and metabolism
[I] Lipid transport and metabolism
[P] Inorganic ion transport and metabolism
[Q] Secondary metabolites biosynthesis, transport and catabolism
[R] General function prediction only
[S] Function unknown

Conclusions

The heterotrophic bacterial community was dominated by Alphaprotebacteria and Verrucomicrobia (Spartobacteria), typical for the Baltic Sea (Herlemann et al., 2013; Herlemann et al., 2011; Riemann et al. 2008; Andresson et al. 2009). Flavobacteria and Cytophaga, both belonging to Bacteriodetes, benefited from the decaying algal bloom. Indeed, these groups of bacteria are known to degrade labile and semi-labile organic compounds (Sosa et al. 2015). The relative increase of Betaproteobacteria and Spartobacteria due to amendment with river water from humic pristine or polluted by agricultural catchment accordingly, discriminated these mesocosms from others. At the finest taxonomic level (OTUs) all abundant bacteria were typical environmental bacteria observed in the Baltic Sea communities and not the opportunistic bacteria associated to artificial conditions.

The established mesocosms reflecting different environmental stressors represented highly different environmental conditions with respect to concentration and composition of organic matter and inorganic nutrients. Despite the development of a phytoplankton bloom in most of the enriched mesocosm, the microbial metagenomes developed during the incubation into completely different genetic blueprints in the different treatments, with only a few shared genes between the different treatments at the end of the experiment (Fig. 12). Consequently, there was a clear link between the environmental conditions and the microbial blueprint, supporting the hypothesis that the specific environmental stressors are key drivers of the bacterial metagenomic composition, and thus that the bacterial blueprint can be used as an indicator of specific environmental conditions.

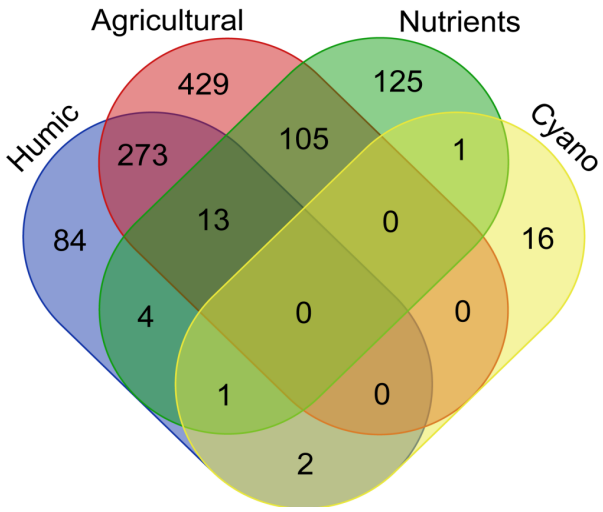

Figure 12: Venn diagram demonstrating the number of shared and unique DA genes between treatments.

Bacteria increased in number together with phytoplankton, probably scavenging carbohydrates released by growing algal cells (increase of BET and Chl a was collinear), but also benefited importantly from the collapse of algal bloom in mesocosms in the last part of the experiment. During the major bloom collapse in the +NPSi mesocosms, bacteria responded by increase of biomass, production, peptidase activity and update of amino acids. P limitation was clearly suggested by increased alkaline phosphatase activity and increased abundance of genes related to P metabolism in mesocosms amended with river water from the agricultural region. The groups of DA genes responding to various sources of allochthonous DOM, pristine humic versus polluted and nonhumic, were clearly different. However, the metabolic pathways to which these genes belong were similar. Further analysis of the metagenomes is required to provide a full overview of the links between specific metabolic traits or genes and the specific stressor conditions.

Acknowledgements

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