Copper affects composition and functions of microbial communities in marine biofilms at environmentally relevant concentrations

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Abstract

Cu pollution in coastal areas is a worldwide threat for aquatic communities. This study assesses the effects of Cu exposure on microbial diversity, community structure and functions of microbial communities in marine periphyton biofilms at environmental relevant concentrations. Periphyton was exposed for 18 days to five Cu concentrations, between 0.01 and 10 µM, in a semi-static test. Diversity and community structure of prokaryotic and eukaryotic organisms were assessed by 16S and 18S amplicon sequencing, respectively. Community function was studied as impacts on algal biomass and primary production. Additionally, we studied Pollution-Induced Community Tolerance (PICT) using photosynthesis as the endpoint. Sequencing results detected an average of 9504 and 1242 OTUs for 16S and 18S, respectively, reflecting the huge biodiversity of marine periphytic biofilms. Eukaryotes represent the most Cu-sensitive kingdom, where effects were seen already at concentrations as low as 10 nM. The structure of the prokaryotic part of the community was impacted at slightly higher concentrations (60 nM), which is still in the range of the Cu concentrations observed in the area (80 nM). The current environmental quality standard for Cu of 70 nM therefore does not seem to be sufficiently protective for periphyton. Cu exposure resulted in a more Cu-tolerant community, which was accompanied by a reduced total algal biomass, increased relative abundance of diatoms and a reduction of primary production. Cu exposure changed the network of associations between taxa in the communities. A total of 23 taxa, including species within Proteobacteria, Bacteroidetes, Stramenopiles and Hacrobia, were identified as being particularly sensitive to Cu. DNA metabarcoding is presented as a sensitive tool for community-level ecotoxicological studies that allows to observe impacts simultaneously on a multitude of pro- and eukaryotic species, and therefore to identify particularly sensitive, non-cultivable species and taxa.

Key words: metabarcoding, 16S rRNA, 18S rRNA, periphyton, amplicon sequencing, metals

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INTRODUCTION

Cu pollution in coastal areas is mainly associated with domestic and industrial activities (Misson et al., 2016; Oursel et al., 2013; Parks et al., 2010), and the use of Cu-based antifouling paints on ship hulls (Thomas and Brooks, 2010; Yebra et al., 2004), especially after the ban of tributyltin (TBT) in the late 1980s in France (Alzieu, 2000) and from 2003 to the rest of Europe (Yebra et al., 2004). Elevated Cu levels can be detected in many parts of the world, especially near enclosed harbours and marinas. For instance concentrations as high as 0.33 μM have been detected in San Diego Bay, USA (Schiff et al., 2007) or up to 0.41 μM in Toulon Bay, France (Briand et al., 2017). In the west cost of Sweden, Cu levels have been detected at concentrations up to 5 μg/L (0.08 μM) (Egardt et al., 2018), exceeding the environmental quality standard (EQS) for this region, i.e. the Kattegat sea where an EQS of 4 μg/L (0.07 μM) has been established (HVMFS 2015:4). Cu is an essential element (Festa and Thiele, 2011), but becomes toxic at higher concentrations (Amara et al., 2018), depending on metal speciation, accumulation (Meylan et al., 2004; Serra et al., 2009) and exposed organism (Amara et al., 2018; Barranguet et al., 2003). In photosynthetically active cells Cu inhibits CO_2 fixation and PSII activity (Pandey et al., 1992), causes oxidative stress and ultimately inhibits cell growth (Gonçalves et al., 2018). In bacteria, Cu affects various cellular enzymes and proteins involved in energy metabolism (Waldron et al., 2009). Cu affects species composition in microbial communities, leading to a replacement of sensitive taxa with tolerant ones (Ancion et al., 2010; Gustavson et al., 1999; Serra et al., 2009). Current understanding of the responses of microbial prokaryotic and eukaryotic taxa to Cu pollution is limited (Yang et al., 2018), and even less is known about Cu effects on the interactions within a community and its potential to cause indirect effects (Barranguet et al., 2003; Yang et al., 2018).

Periphyton, also called microhytobenthos, forms biofilms of highly diverse microbial communities – including algae, bacteria, fungi and meiofauna – that live attached to submerged substrata in aquatic ecosystems (Lock, 1993; Salta et al., 2013; Sanli et al., 2015). The importance of periphyton to aquatic ecosystems is linked to its function as a primary producer and its contribution to biogeochemical cycles (Battin et al., 2003; Sundbäck et al., 2004). The use of periphyton for studies in community ecotoxicology is well established (Corcoll et al., 2015; Eriksson et al., 2009a; Sabater et al., 2007), as it allows to assess effects of contaminants across different levels of biological organization (Guasch et al., 2016). In this line, an approach commonly used for detecting long-term effects of toxicants in periphyton communities is the measurement of PICT (Pollution-Induced Community Tolerance), introduced by Blanck et al. 1988. PICT is based on the elimination of organisms sensitive to the toxicant in question and the induced inter- and intraspecific selection for organisms that are more tolerant to the toxicant. The entire community is restructured and finally displays an overall increase in its tolerance to the toxicant,

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compared to an unexposed reference community. This induced tolerance is commonly quantified as an increase of the short-term EC50 of the whole community to the toxicant in question, which is perceived as a community trait (Blanck et al., 1988; Tlili et al., 2015). The use of PICT for detecting effects from Cu on marine and freshwater periphyton has shown to be more sensitive than traditional community composition based-tools such as microscope observations, pigment-profile based approaches or PCR-DGGE fingerprints (Barranguet et al., 2003; Gustavson et al., 1999; Massieux et al., 2004; Serra et al., 2009; Tlili et al., 2010).

Recent advances in DNA sequencing represents a powerful tool to detect and quantify effects of toxic substances on ecological communities with high sample/observation throughput (Zhang et al., 2009). In particular DNA metabarcoding, a high throughput DNA-based amplicon sequencing technique, has emerged as a new molecular tool to identify a large proportion of the biological community present in an environmental (Hebert et al., 2003). In a recent study of the community responses of microbiota in freshwater sediments spiked with Cu, the diversity and structure of eukaryotic and prokaryotic communities were characterized by DNA metabarcoding, in addition to evaluating lethal and sub-lethal toxicity (Yang et al., 2018). Obtained results showed that alterations of phylogenetic biodiversity of eukaryotic communities and the structure of sediment communities were sensitive indicators for sediment contamination.

To the best of our knowledge, similar studies have not yet been performed for marine periphyton biofilms, in order to improve our understanding of how Cu, a common marine pollutant, restructures the diversity of these important elements of marine ecosystems. The present study therefore investigated (1) the effects of a range of Cu concentrations on prokaryotic and eukaryotic microbial diversity using DNA metabarcoding, (2) to assess Cu effects on ecological functions and community tolerance development using the PICT approach, and (3) to identify Cu sensitive and tolerant taxa as well as changes of the community network. For these purposes, natural marine periphyton was exposed to a range of Cu concentrations (0.1 to 10 µM) in a semi-static microcosm for 18 days. We selected the 16S rRNA (V3 region) and the 18S rRNA (V9 region) genes to describe effects on diversity and community composition of bacteria and eukaryotes, respectively. Our results provide new information of how Cu pollution affects microbial biodiversity and community composition in the marine environment, data that will aid the setting of appropriate environmental quality standards.

MATERIAL AND METHODS

Microcosm setup and experimental design

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The experiment was conducted indoors in a thermo-constant room at the facilities of the Sven Lovén Centre for marine sciences - Kristineberg by the Gullmar fjord on the west coast of Sweden, from 18th August to 6th September 2015. Eighteen independent microcosms made by rectangular glass vessels inspired by the SWIFT periphyton test described by Porsbring et al. (2007) were used for the experiment. Each microcosm contained 300 mL of natural sea water collected in the Gullmar fjord. The sea water, with its naturally occurring organisms, was filtered through a 200 µm mesh to remove large organisms and was enriched with 0.7 µM phosphate (as KH_2PO_4) and 0.8 µM nitrate (as NH_4NO_3) to avoid nutrient limitation during periphyton growth. Periphyton was allowed to colonize rectangular polyethylene therophtalate (PETG) slides (6.9 x 1.4 cm^2). Each rectangular microcosm had a glass rod attached along the long side in the middle of the bottom of the vessel and 22 PTEG slides were placed from the bottom glass rod to the side walls of the vessel, making an angle of approximately 22° between the bottom and the walls of the vessel. The sea water covered half of the surface of the slides. The water from each microcosm was renewed every day. To stimulate periphyton colonization and growth in the beginning of the experiment, marine periphyton inocula were prepared by brushing off periphyton from the upper part of 50 - 60 stones and pebbles, collected at a maximal depth of 60 cm, into seawater. The water, stones and pebbles were sampled from a nearby pristine bay (Gåseviken: 58.245373°N, 11.433628°S). The inocula was vigorously shaken and filtered through a 200 µm mesh to remove large organisms. 20 ml inocula, with an approximate chlorophyll *a* concentration of 0.3 $\mu g\ mL^{-1}$, were provided twice to each microcosm during the first week of the experiment. The microcosms were incubated in a thermo-controlled room with the temperature set to 15ºC. The daily light/dark cycle of 14h/8h was simulated with OSRAM FLUORA light tubes with a light intensity at the surface of the microcosms of approximately 120 µmol photons $m^{-2}\ s^{-1}$. The microcosms were in constant agitation by using horizontal shakes.

The experimental design included unexposed control microcosms and five Cu exposure levels, each in triplicate microcosms. The nominal Cu exposure levels were: 0.01, 0.06, 0.32, 1.78 and 10 µM . Cu stocks, one thousand times more concentrated than the nominal concentrations, were prepared from $CuCl_2{\cdot}H_2O$ (Sigma-Aldrich) with deionised water. 300 μL of the Cu stocks was added to the Cu microcosms, and the same volume of deionised water was added to the unexposed controls, to give the final volume of 300 mL. Water temperature, pH, oxygen and salinity was monitored periodically, at least 10 times through the experiment, using portables multi-probes (HANNA Instruments). Water of all microcosms was sampled before and after a water renewal for Cu analysis. For this, 50 mL of water were filtered through 0.45 µm, preserved with HNO_3 (65% suprarpure) at final concentration of 1 % and kept at 4°C until further analysis with ICP-MS.

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Periphyton sampling

After18 days, periphyton was sampled to analyse chlorophyll *a* concentration, photosynthetic pigments, photosynthetic activity, community tolerance to Cu and microbial composition of prokaryotes and eukaryotes. For each microcosm, a periphyton slurry was produced by scraping off the periphyton from the slides into 150 mL of sea water, filtered through 0.2 µm and amended with the same amount of nutrients as used in the microcosms. 5 mL of periphyton slurry were filtered through Whatman GF/filters and used immediately for chlorophyll *a* analyses. 10 mL of periphyton slurry were filtered through Whatman GF/F filters, frozen at -20°C and stored until pigments extraction. 10 mL of periphyton slurry was aliquoted in tubes, pelleted by centrifuged at 6500 g for 10 min, the supernatant was removed and the resulting pellets were snap-frozen in liquid nitrogen and stored at -80°C until DNA extraction. The remaining periphyton slurry was used to determine primary production (^{14}C-incorporation) and tolerance measurements following the PICT approach. Analyses of chlorophyll *a*, pigment profiles and microbial composition were done for all treatments. For logistic reasons, photosynthetic and community tolerance measurements were only done for the control microcosms and the microcosms with a Cu exposure of 0.32 and 1.78 μM Cu.

Chlorophyll a concentration and photosynthetic pigments

Chlorophyll *a* was extracted with 10 mL of ethanol (96%) for 24h in the dark, at room temperature, and was quantified fluorometrically (10-AU Turner flouorometer; Turner designs, Sunnyvale California, USA) according to Jespersen and Christoffersen (1987). Photosynthetic pigments were extracted in a 4 mL mixture of acetone/methanol (80%/20%, v/v) while sonicated in an ice slurry for 3 minutes. 90 µL of the extracts were filtered onto 0.45 µm filters (VWR International Syringe filters) and analysed with high performance liquid chromatography (HPLC; Shimadze Prominence HPLC Systems) following Torstensson et al. (2015). A total of 10 photosynthetic pigments were identified and were expressed as the ratio between the peak area of each identified pigment and the peak area of Chlorophyll *a*. Fucoxanthin was used as pigment marker for diatoms algal group (Roy et al., 2011).

Pollution-Induced Community Tolerance (PICT)

Pollution-Induced Community Tolerance (PICT) was measured as inhibition of photosynthesis, using incorporation of 14C-sodium bicarbonate into incorporation of ^{14}C-sodium bicarbonate into acid-stable compounds according to Eriksson et al. (2009) with some modifications. From each of the studied microcosms, triplicate unexposed control samples were prepared by mixing 1 mL of periphyton slurry and 1 mL sea water. One sample from each microcosm was exposed to 0.32, 1.35, 5.66, 23.8 and

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100 μM Cu by mixing 1 mL of periphyton slurry with 1 mL of Cu solutions. The sea water used for the controls and the Cu solutions was filtered through 0.2 µm and amended with the same amount of nutrients as used in the microcosms. The samples were mixed in scintillation vials and were incubated at 15° C and 120 µmol photons m^{-2} s^{-1}, while gently shaken during the incubation. After one hour, 50 µL ^{14}C-sodium bicarbonate solution, with a radioactivity of 80 µCi/ml, was added to each sample. After another hour, 100 μL formaldehyde was added to each sample to terminate photosynthetic activity. The samples were acidified with 100 μL of HCl to drive off unincorporated ^{14}C and 3 mL of Instagel Plus was added to each sample. Disintegrations per minute (DPM) were calculated from counts per minute (CPM) based on the correction factors for the sample quench characteristics and the machine efficiency, using a liquid scintillation spectrometer (LS 500 Beckman). The abiotic ^{14}C-incorporation was estimated by adding 100 µL formaldehyde to one sample before the incubation with ^{14}C-sodium bicarbonate, and the radioactivity of that sample was subtracted from the radioactivity of the other samples from the same microcosm. Community tolerance was determined as differences in EC_{50} values between unexposed control microcosms and microcosms exposed to 0.32 and 1.78 μM Cu. DPM values were used as estimates of periphyton primary production (photosynthetic activity).

DNA extraction, PCR amplification and sequencing

Microbial composition of prokaryotes and eukaryotes was assessed by DNA metabarcoding. Total genomic DNA was extracted using the Power Biofilm® DNAIsolation Kit (MoBio Laboratories, USA) following recommendations in Corcoll et al. (2017). DNA was precipitated with sodium acetate and ethanol prior downstream analyses. Bacterial 16S rRNA genes (V3 region) and eukaryotic 18S rRNA genes (V9 region) were amplified using V3 primers (modified primers 341F and 518R) (Klindworth et al., 2013) and V9 primers (1380F and 1510R) (Amaral-Zettler et al., 2009), respectively. Triplicate PCR reactions were performed for each sample to minimize potential PCR bias. The PCR amplicon libraries were sequenced using the Ion Torrent Proton technology according to the manufacturer's protocols.

Bioinformatics

The QIIME v.1.8.0 (Quantitative Insights Into Microbial Ecology) pipeline was used to process the raw sequences (Caporaso et al., 2010). Low quality sequences were trimmed via the "split_libraries.py" script with "-w 50 -q 20". PCR chimera filtering was performed via "parallel_identify_chimeric_seqs.py" script in QIIME with the default parameter. Operational taxonomic units (OTUs) were selected with a sequence similarity cut-off of 97% following the UPARSE pipeline (Edgar, 2013). For each OTU, a representative sequence was chosen and taxonomy was assigned using the RDP classifier (Wang et al.,

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2007) against the Greengenes database (DeSantis et al., 2006) and SILVA database (Pruesse et al., 2007) for prokaryote and eukaryote community, respectively.

All sequences obtained in this study will be deposited in NCBI under Accession Number (in progress).

Statistical analyses

One-way ANOVAs were used to assess differences between the treatments for Chlorophyll *a* concentration, photosynthetic activity and EC_{50} values using R (R Core Team, 2013). The effective concentration of Cu that had a 50% of effect (EC_{50}) was determined after fitting ^{14}C-incorporation values to a dose-response model (Weifull fit) using the package "drc" (Ritz, 2016) in R. Principal component analysis (PCA) was employed to observe difference in microbial composition of prokaryotes and eukaryotes between the Cu treatments using OTUs abundance data. Differences between Cu treatments were assessed using ANOVA with Dunnett's post hoc test. A correlation network of microbial community were generated by SparCC with 100 bootstraps to assign two-sided pseudo p values (Friedman and Alm 2012). The network was filtered by correlation magnitudes > 0.6 and < -0.6 in a Pearson correlation test, which indicates strong co-abundant and co-exclusion relationships. The response of each taxon to Cu exposure was modelled with a 3-parameter log-logistic model and the 50% effects concentration (EC50) was calculated. Beta diversity was estimated by computing weighted UniFrac distances between samples (Lozupone and Knight 2006). All samples were rarefied at the lowest sequencing depth to reduce biases resulting from differences in sequencing depth (186400 and 112730 for eukaryote and prokaryote community, respectively).

RESULTS

Periphyton responses after 18 days of exposure to five Cu concentrations, between 0.01 and 10 µM, in a semi-static test, are presented below.

Experimental conditions

Temperature, salinity and pH were constant over the entire experiment, varying by just 1 to 3% between daily water renewals. Average salinity was 20.8 PSU, water temperature was 17.9 °C and pH was 8.1 (n=59). Cu concentrations in the controls and the samples with nominal concentration 0.01 μM Cu were below the quantification limit of 0.03 μM. For the rest of the samples, the analysed Cu was between 33 % and 90 % of the nominal concentrations, being closer to the nominal concentrations at higher exposure levels (Supplementary table 1). Therefore, the nominal concentrations are used to describe the concentration response patterns.

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Effects on photosynthetic pigments and photosynthetic activity

In the unexposed controls, chl-a accounted for 52%, fucoxanthin for 12 %, diadionoxanthin + diatoxanthin for 12 %, ß-carotens for 9%, and the remaining pigments accounted for 13% of the total pigments (arithmetic mean of three replicates) (Figure 1A). Chl-a concentration decreased in a concentration-dependent manner, reaching 81% inhibition at the highest concentration of 10 μM (Figure 1B). A similar pattern was observed for the total pigment content of the periphyton (Figure 1A). In contrast, the relative abundance of fucoxanthin increased in a concentration-dependent manner, increasing to 60% of the total pigment content in the highest Cu exposure, 10 μM Cu (Figure 1B). The photosynthetic activity (^{14}C-incorporation) at concentrations of 0.32 and 1.78 μM C was inhibited up to 60% (Figure B).

Community tolerance development

Photosynthetic activity in all the short-term PICT detection tests was inhibited by increasing Cu concentrations, and EC50 values could be determined for all microcosms except one of the microcosms exposed to 1.78 μM Cu. Cu exposure of 0.32 and 1.78 μM clearly resulted in community tolerance. The short-term EC50 (AVG ± SE) of the untreated controls was 8.88 ± 0.78 μM. After pre-exposure to 0.32 μM Cu the short-term EC50 increased to 21.3 ±1.34 μM and after pre-exposure to Cu at 1.78 μM it increased further to 51.24 ±5.85 μM (Figure 1B). That is, Cu pre-exposure increased the tolerance of the community to short-term exposure by being 2.3 and 5.7, respectively, higher than in control treatment indicating an increase of community tolerance to copper exposure.

Effects on prokaryotic and eukaryotic community composition

DNA-sequencing of the 16S and 18S gene fragments yielded a total of 7,109,298 and 5,655,641 high quality reads, respectively. These reads clustered into 17,445 prokaryotic OTUs and 2,151 eukaryotic OTUs (Table 1). As shown by the Chao1 diversity index (Chao 1984; Suppl. Fig. 1) the sequencing depth was sufficient to achieve the saturation point for identifying both prokaryote and eukaryote species.

The prokaryote community in unexposed communities was dominated by Alphaproteobacteria and Flavobacteria classes, although the relevant abundances of sequences affiliated to Phycisphaerae and Saprosirae (Figure 2). The eukaryotic community was dominated by Ochrophyta and Metazoa classes, although the relevant abundances of sequences affiliated to Stramenopliles, Haptophyta and Chlorophyta (Figure 3). Cu exposure decreased the total number of OTUs (Table 1) and also significantly reduced the chao1 diversity of both, the prokaryotic and the eukaryotic part of the periphyton (Supplementary Figure S1). The impacts of Cu on prokaryotic and eukaryotic community composition were confirmed by a MANOVA on Unifrac distances (Table 2). Significant changes of bacterial community composition were

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first observed after exposure to 0.06 μM Cu, the eukaryotic part of the periphyton was already significantly impacted after exposure to 0.01 μM Cu (Table 2). These findings are also reflected in the PCA plots that visualise the effects of Cu exposure on OTU composition (Figures 2B and 3B). For prokaryotes (Fig. 2B), the first axis of the PCA explains 38% of the variance and grouped the samples from the control and 0.01, 0.06, 0.32 and 1.78 μM Cu on the right side of the axis, while samples from 10 μM Cu were grouped on the left side of the axis. The second axis of the PCA explained 37% of the variance and primarily separated the samples in three groups: i) control and low levels of Cu exposure, from ii) 1.78 μM Cu and from iii) 10 μM Cu (Figure 2B). Remarkably, in treatments exposed to high levels of Cu (1.78 and 10 μM Cu), the relative abundance of sequences affiliated to Nostocophycideae prokaryote was especially high and the relative abundance of sequences affiliated with Synechococcophycideae were especially low (Figures 2A and 4).

For Eukarya, the first axis of the PCA explained 68% of the variance and separated the samples based on an increasing gradient of Cu exposure from the left side of the axis (control treatment) to the right site of the axis (highest Cu treatment, 10 μM Cu) (Figures 3B). It should be pointed out that the relative abundance of sequences associated to Ochrophyta and Lobosa increased in treatments exposed to high levels of Cu (1.78 and 10 μM Cu). In contrast, the relative abundance of sequences associated to Haptophyta, Metazoa, Chlorophyt, Cliophora, Dinophyta and Stramenoplies classes was markedly reduced when comparing to control treatment (Figure 3A).

Cu sensitive and tolerant taxa and changes in community network

Pearson correlation analyses showed different Cu sensitives among taxa (Figures 4 and 5). As a general trend within prokaryotic taxa, the Cyanobacteria phylum had a strong positive correlation with Cu exposure but, in contrast, abundances of Planctomycetes and Proteobacteria phyla had a strong negative correlation with Cu exposure (Figure 4). For eukarya, the Stramenopiles phylum were strongly positively correlated with Cu exposure and Amoebozoa were weakly positively correlated with Cu exposure too , but all other phyla were negatively correlated to Cu exposure. In particular, Hacrobia and Alveolata phyla showed a strong negative correlation to Cu exposure (Figure 5).

A total of 23 taxa can be classified as “sensitive”, showing a clear concentration-dependent decrease (Supplementary Figure S2 and Supplementary Table S2). For prokaryotes, the most sensitive taxa were from the phyla Proteobacteria and Bacteriodetes. Most of the sensitive taxa have a relative high EC50 above 1 μM Cu, except for four taxa from the Cytophagales, Rickettsiales, Myxococcales and Oceanospirillales which had EC50 values $\leq$ 1 μM Cu (Supplementary Table S2). Five eukaryotic taxa from the Stramenopiles and Hacrobia phyla were sensitive to Cu, with EC50 values below 2 μM Cu (Supplementary Table S2).

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Cu changed the network of associations between taxa in the communities (Figure 6). The number of nodes decreased at Cu concentration of 0.32 μM and higher (Supplementary Table S3), and was lower than in the controls at 1.78 μM and 10 μM of Cu. The network density, centralization and heterogeneity increased after exposure to 10 μM Cu (Supplementary Table S3).

DISCUSSION

Our microcosm study provides new insights into the ecological effects of long-term Cu exposure on marine prokaryotic and microeukaryotic organisms within periphyton biofilms. Cu decreased bacterial and eukaryotic species richness (number of OTUs) and the number of their interactions (number of nodes). Eukaryotes were more sensitive than prokaryotic species. Despite clear changes in the community structure, which rendered the exposed periphyton communities more Cu-tolerant, Cu exposure decreased the total biomass and productivity of exposed biofilms. These effects were observed at Cu concentrations above 0.06 μM, which are known to occur in the Swedish coastal environment (Egardt et al., 2018). Furthermore, it should be emphasized that the results were recorded in an ecologically realistic setting that allowed an ecological succession and competition to shape the communities under long-term chronic Cu exposure. The current environmental quality standard for Cu of 0.07 μM (HVMFS 2015:4) therefore does not seem to be sufficiently protective for periphyton, which contain key primary producers especially in coastal areas where the euphotic zone extends to the sediment (Sundbäck et al., 2004; Wasmund, 1993).

The pollution-induced community tolerance (PICT) coincides with changes in the structure and composition of the community (Figures 1, 2 and 3), and it also coincides with a decrease in algal biomass and photosynthetic activity (Figure 1). This supports the view put forward first by Blanck et al. (1988), that community tolerance will increase as soon as sensitive species and genotypes are lost from the community. These observations are, however, in contrast to the functional redundancy hypothesis, which assumes that species loss has little impact on ecological functions (Oliver et al., 2015). Instead, they support the notion that biodiversity must be conserved fully, in order to ensure that an exposed community can continue to fulfil its ecological functions in a given ecosystem (Tilman and Downing, 1994). Although Eriksson et al. (2009a and 2009b) established a link between PICT and the genetic composition of photosynthetic microorganims in marine periphyton, this study is, to our best knowledge, the first paper that links PICT in biofilms with high-throughput DNA sequencing techniques (metabarcoding) targeting the whole prokaryotic and microeukaryotic microorganisms.

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A wide range of sensitive prokaryotic and eukaryotic species was observed (Figures 4 and 5). The highest Cu-tolerance amongst the prokaryotes was found in the Cyanobacteria phylum, especially in the Nostocophycideae and Oscilaltoriphycideae classes (Figure 4). Cyanobacteria resistance to Cu exposure has been observed previously (Barranguet et al., 2000; Serra et al., 2009) and might be attributed to their capacity to synthetize external ligands (Giner-Lamia et al., 2016) so that Cu is accumulated extracellularly (Serra et al., 2009).

Proteobacteria and Bacteroidetes phyla dominated the unexposed periphyton used in the present study, which was sampled from the Gullmar fjord on the Swedish west coast. This is in concordance with previous studies on marine bacterioplankton (Cottrell and Kirchman, 2000; Steven et al., 2012) and periphyton biofilms (Corcoll et al., 2017; Sanli et al., 2015). The tolerance of Bacteroidetes that we observed in the present study is confirmed by similar patterns observed in sediments exposed to Cu (Yang et al., 2018). In contrast, the Proteobacteria phylum was the most sensitive phylum to Cu. The abundance of thirteen of its taxa, mainly from the dominant Alpha- and Gamma-proteobacteria classes, was reduced in a concentration-dependent manner, with EC50 values as low as 0.61 and 0.91 μM Cu, respectively (Suppl. Table 2). Many species of the phylum Proteobacteria are responsible for nitrification and denitrification processes, or linked with the assimilation of carbon (Ruiz-González et al., 2012; Sanli et al., 2015; Zhao et al., 2017). Given the high sensitivity of Proteobacteria to Cu, we therefore hypothesize that Cu pollution in marine areas could lead to impaired nitrogen cycles.

Eight different eukaryotic higher taxonomic group were detected (Alveolata, Amoebozoa, Apusozoa, Archaeplastida, Hacrobia, Opisthokonta, Rhizaria and Stramenopiles; Figures 3 and 5), demonstrating once again the huge biodiversity of heterotrophic and autotrophic microeukaryotic organisms present in marine periphyton biofilms (Sanli et al., 2015). Stramenopiles and Amoebozoa were the most tolerant groups to Cu exposure, although the abundance of some fungal families within the Opisthokonta group (Figure 5). Bacillariophyta (diatoms), a family within the Stramenopiles group, was highly tolerant to Cu (Figure 5). Pigment analyses supported these results (Figure 1), since the relative abundance of fucoxanthin, a common marker for diatoms (Roy et al., 2011) also increased with increasing Cu concentrations. These findings are in agreement with previous results of (Gustavsson et al., 2017), who also reported an increase of centric Bacillariophyta in marine phytoplankton as a consequence of Cu exposure. The Cu tolerance in diatoms has been linked with their capacity to synthetize extracellular polysaccharides and frustuline (Gonçalves et al., 2018). Nevertheless, previously published mesocsom studies also provide a partly conflicting picture of diatom tolerance to Cu. For instance in the studies by Barranguet et al. (2000) and Soldo and Behra (2000), diatoms from stream periphyton were less tolerant

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than green algae or cyanobacteria to long-term Cu exposure. These different findings are likely caused by the inherent differences between freshwater and marine environments in terms of water chemistry and different initial compositions of the periphyton community, and because community composition is in itself a vital element of metal tolerance (Pérez et al., 2010).

In Fungi, the relative abundance of most classes and families was not affected by Cu exposure, which is in agreement with previous studies in sediment mesocosms (Gardham et al., 2014; Yang et al., 2018). Several resistance mechanisms in fungi to cope with Cu toxicity have been described, such as copper complexing by cell wall components, changes in membrane copper transport, synthesis of intra-cellular copper-binding metallothioneins and phytochelatins, and production of extracellular copper-complexing or -precipitating metabolites (Cervantes and Gutierrezcorona, 1994).

Five algal taxa where inhibited in a concentration-dependent manner: a member of the Pavlovaceae family (Haptophyta), a member of Erythropeltidales order (Rhodophyta) and three taxa within Stramenopiles, with EC50 values ranging from 1.2 to 2 μM Cu (Supplementary Table S2). The abundance of members of Hacrobia was strongly negatively correlated with Cu exposure (Figures 3 and 5). Within Hacrobia, the relative abundance of its Haptophyta group declined with Cu exposure. Haptophyta is an important group in the oceans, especially calcifying Haptophyta (coccolithophores) which have a strong effect on the global carbon cycles (Tsuji and Yoshida, 2017). The abundance of other algal groups (Chlorophyta, Rhodophyta and Dinophyta) was also reduced by Cu exposure, but only to a lower extent.

We conclude that Cu effects on the aforementioned algal classes caused the observed decrease of total algal biomass and photosynthetic activity (Figure 1), which goes together well with previous studies that have demonstrated Cu toxicity to photosynthesis and algal growth at low concentrations, e.g. (Pérez et al., 2010).

Ciliphora, a group of protozoa in the Alveolata superphylum, decreased in abundance under Cu exposure (Figure 5), which confirms previous results on the sensitivity of protozoa to Cu (Madoni et al., 1996; Yang et al., 2018). However, in general, little is known about the sensitivity of periphytic protozoa to Cu, metals and other pollutants, most likely because their identification is difficult using classic, microscope-based methods. The use of DNA metabarcoding appears to be able to overcome these limitations and provide a new tool to investigate protozoa in ecotoxicological studies.

The abundances of most taxa from the Metazoa class (Crustacea, Cnidaria or Rotifera) were negatively correlated to Cu exposure (Figure 5). The sensitivity of Metazoa (i.e. Nematodes) to Cu and other environmental factors is well established (Bongers and Ferris, 1999; Boyd and Williams, 2003). This group of micro-eukaryotes eats particulate organic detritus, bacteria, algae, fungi and protozoans. Hence, they act as regulators of decomposition and therefore play a key role in nutrient cycling and

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dynamics (Boyd and Williams, 2003; Stelzer, 2011). Hence, direct Cu effects on Metazoa impact broader trophic interactions in periphyton biofilms, as demonstrated in the network analyses (Figure 6). Cu changed the network of associations between various taxa in the communities (Figure 6, Supplementary Table S3) suggesting that the trophic chain interactions and the microbial loop in periphyton biofilms will be altered under Cu exposure.

Even though DNA metabarcoding has emerged as a prominent technique to detect a large number of taxa in an environmental sample (Hebert et al., 2003), the technique also has its limitations. The choice of primers affects the biodiversity assessment, and a perfectly universal primer is difficult or even impossible to design (Hugerth et al., 2014; Klindworth et al., 2013; Zhang et al., 2018). To overcome these limitations, a combination of many specific primers to target each of the eukaryotic kingdoms (e.g. ITS gene to target fungi (Nilsson et al., 2009), 23S gene to target algae (Sherwood et al., 2008) or COI to target invertebrates (Leray and Knowlton, 2015)) might provide an improved resolution and less bias.

In this study, we chose the V3 region of the 16S rRNA gene to target bacteria and the V9 region of the 18S rRNA to target eukaryotes. Both regions are widely used in DNA metabarcoding of microbial communities in various ecosystems (Amaral-Zettler et al., 2009; Corcoll et al., 2017; Klindworth et al., 2013; Yang et al., 2018). However, the region V4 of the 18S rRNA gene has been suggested as an alternative to the V9 region, in order to capture more diversity (Hugerth et al., 2014; Pernice et al., 2013). Another limitation of current metabarcoding approaches, specifically with respect to marine microbial communities is the low coverage in public sequence repositories for many natural microorganisms, and especially microeukaryotes (Bik et al., 2012; Sanli et al., 2015).

To conclude, this study allowed us to detected changes in pro- and eu-karyote community composition already at 0.01 µM and 0.06 µM Cu, respectively. These effect concentrations are environmentally realistic (Egardt et al., 2018) and are below the current environmental quality standards (EQS) for copper on the Swedish west coast (HVMFS, 2015). Hence, observed mesocosm results suggest that the current Cu EQS for the marine environment are not protective for prokaryotic and eukaryotic microbial organisms in marine biofilms. Our results provide new information of how copper pollution affects microbial biodiversity and community composition in the marine environment, data that will aid the setting of appropriate environmental quality standards. Furthermore, this work shows the robustness and the promising potential of DNA metabarcoding as a sensitive tool for community-level ecotoxicological studies that allows to observe impacts simultaneously on a multitude of pro- and eukaryotic species, and therefore to identify particularly sensitive, non-cultivable species and taxa.

AUTHOR CONTRIBUTIONS

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NC, ME and TB designed the work. NC and ME performed the experiment. NC, ME and JW processed the samples and performed the analyses. NC wrote the first draft of the paper and all authors (NC, ME, TB, JW and XZ) discussed, interpreted the results and contributed to producing the paper.

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Table 1. Sequencing information for each sample. Core OTUs means the OTU was detected by all of the three samples in the same treatment.

Cu (μM)	Prokaryote				Eukaryota			
	Raw sequences	High quality reads	OTUs	Core OTUs	Raw sequences	High quality reads	OTUs	Core OTUs
0	401492	352513	10673		298545	287308	1359	
0	421254	370057	10598	7343	314776	304546	1298	1048
0	529896	463714	10729		371406	355285	1372	
0.01	499314	437519	10842		397999	380767	1446	
0.01	481209	428187	10355	7137	367233	353131	1353	1095
0.01	485444	435963	10052		333989	321285	1429	
0.06	520732	458352	10448		351999	338684	1398	
0.06	445386	386729	10675	7354	308046	295090	1378	1211
0.06	539309	470684	10615		405241	389808	1498	
0.32	553859	493324	9830		353213	339263	1340	
0.32	594508	531872	9925	6802	409692	395912	1333	1037
0.32	354786	312126	9953		250921	241191	1334	
1.78	392169	353045	7214		321938	312893	1138	
1.78	385956	352677	6781	4537	347776	338072	1033	742
1.78	357418	326682	6934		327889	319083	1070	
10	236199	209893	8161		160531	155115	856	
10	500776	445464	7994	5137	323477	314640	891	558
10	310783	280497	7514		220522	213568	838	
Total	8010490	7109298	17445	11549	5865193	5655641	2151	1618

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721 Table 2. Mean Unifrac distance between Cu treatments. "*" means $p < 0.001$ by MANOVA test.

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	Cu (μM)	0	0.01	0.06	0.32	1.78	10
Prokaryote communities	0	0.0409					
	0.01	0.0637	0.0518				
	0.06	**0.1193***	**0.1034***	0.0330			
	0.32	**0.1281***	**0.1217***	**0.1347***	0.0525		
	1.78	**0.2107***	**0.1983***	**0.1895***	**0.196***	0.0378	
	10	**0.2127***	**0.2028***	**0.1663***	**0.2092***	**0.2413***	0.0574
Eukaryote communities	0	0.1296					
	0.01	**0.3900***	0.1758				
	0.06	**0.4496***	**0.3794***	0.1249			
	0.32	**0.4318***	**0.4134***	**0.2933***	0.1472		
	1.78	**0.7605***	**0.7346***	**0.5675***	**0.5112***	0.1637	
	10	**0.9693***	**0.9405***	**0.7708***	**0.7324***	**0.5508***	0.0968

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CAPTIONS

Fig. 1. A) Pigment profiles (HPLC analyses) presented as relative abundance of identified pigments in average per treatment and in percentage of the control treatment. B) Chlorophyll *a* (Chl-a) concentration (flurometric analyses) and photosynthetic activity in percentage of the control is showed in the left Y-axis and community tolerance to Cu exposure is showed in the right Y-axis. Note: All values represent AVG ± SE; n=3 except in Fig. 1B where the EC50 for 1.78 was AVG ± SE; n=2.

Fig. 2. Bar-plot of the species composition for bacterial community at class level recovered by amplicon-based high throughput sequencing (left panel) and Principal component analysis (PCA) based on weighted UniFrac distances of species composition for bacterial community (right panel). Note that Cu treatments: 0, 0.01, 0.06, 0.32, 1.78 and 10 are in μM Cu.

Fig. 3. Bar-plot of the species composition for eukaryotic community at class level recovered by amplicon-based high throughput sequencing (left panel) and principal component analysis (PCA) based on weighted UniFrac distances of species composition for eukaryotic community (right panel). Note that Cu treatments: 0, 0.01, 0.06, 0.32, 1.78 and 10 are in μM Cu.

Fig. 4. Prokaryotic abundance of taxa associated to 16S rRNA gene at different taxonomic levels (Phylum, Class and Family) and Pearson correlations between Cu concentration and taxa abundance. Only the taxa whose reads number more than 2000 were shown in the figure. Note: taxa abundance is presented in the bar plots to the right and include all the taxa detected in the sequence database.

Fig. 5. Eukaryotic abundance of taxa associated to 18S rRNA gene at different taxonomic levels (Phylum or Higher Taxonomic Group, Class and Family) and Pearson correlations between Cu concentration and taxa abundance. Only the taxa whose reads number more than 2000 were shown in the figure.

Fig. 6. Network between prokaryotic and eukaryotic communities in different Cu exposure treatments: A (control), B (0.01 μM Cu), C (0.06 μM Cu), D (0.32 μM Cu), E (1.78 μM Cu) and F (10 μM Cu). Associations between taxa were generated by "Pearson" correlation analysis. Only correlations with a correlation > 0.9 and a "two-tailed" P values < 0.01 were reserved. Correlation coefficients between two nodes were labelled, the positive coefficient in yellow, while negative coefficient in green. The size of node indicates the "betweenness centrality".

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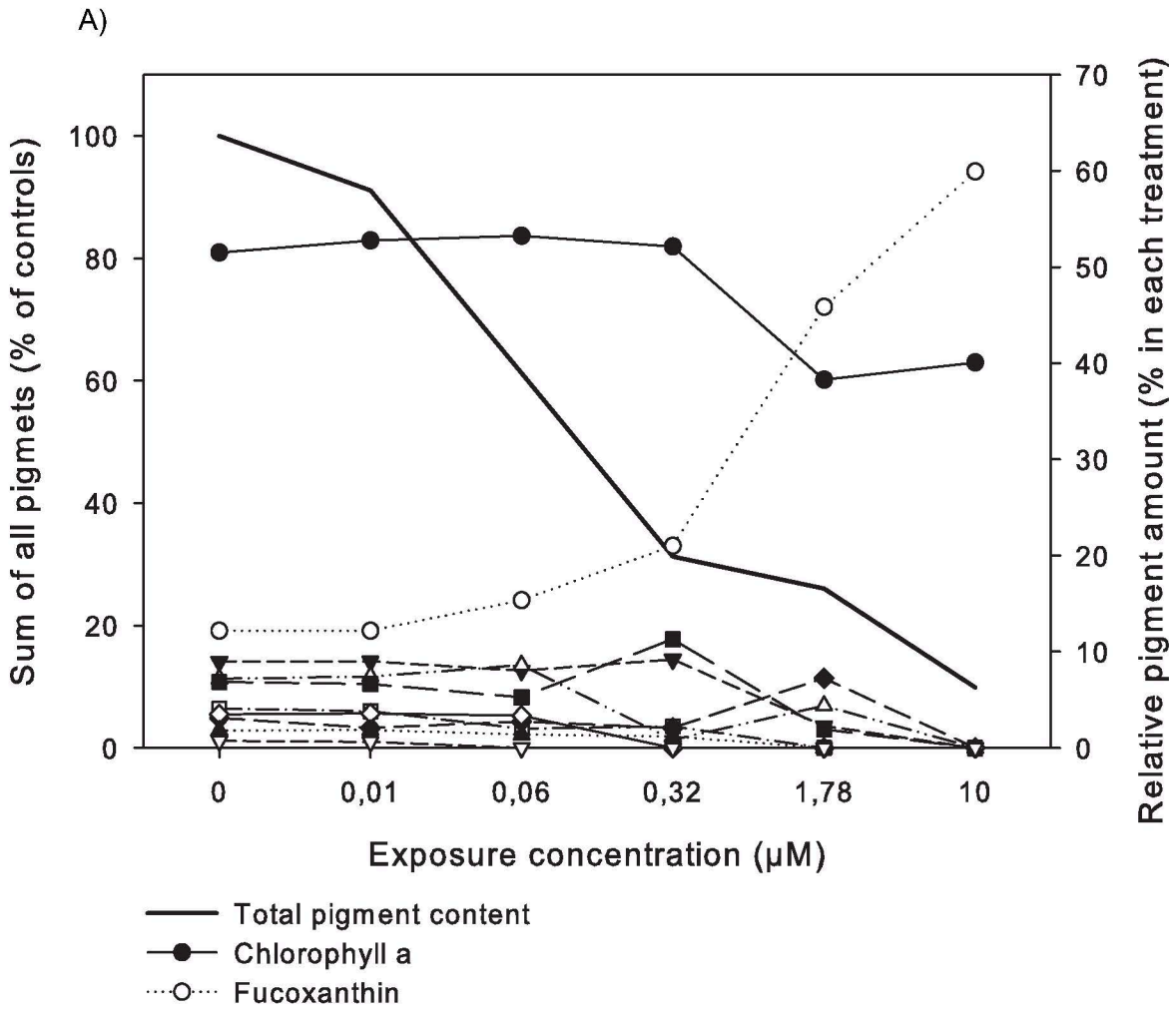
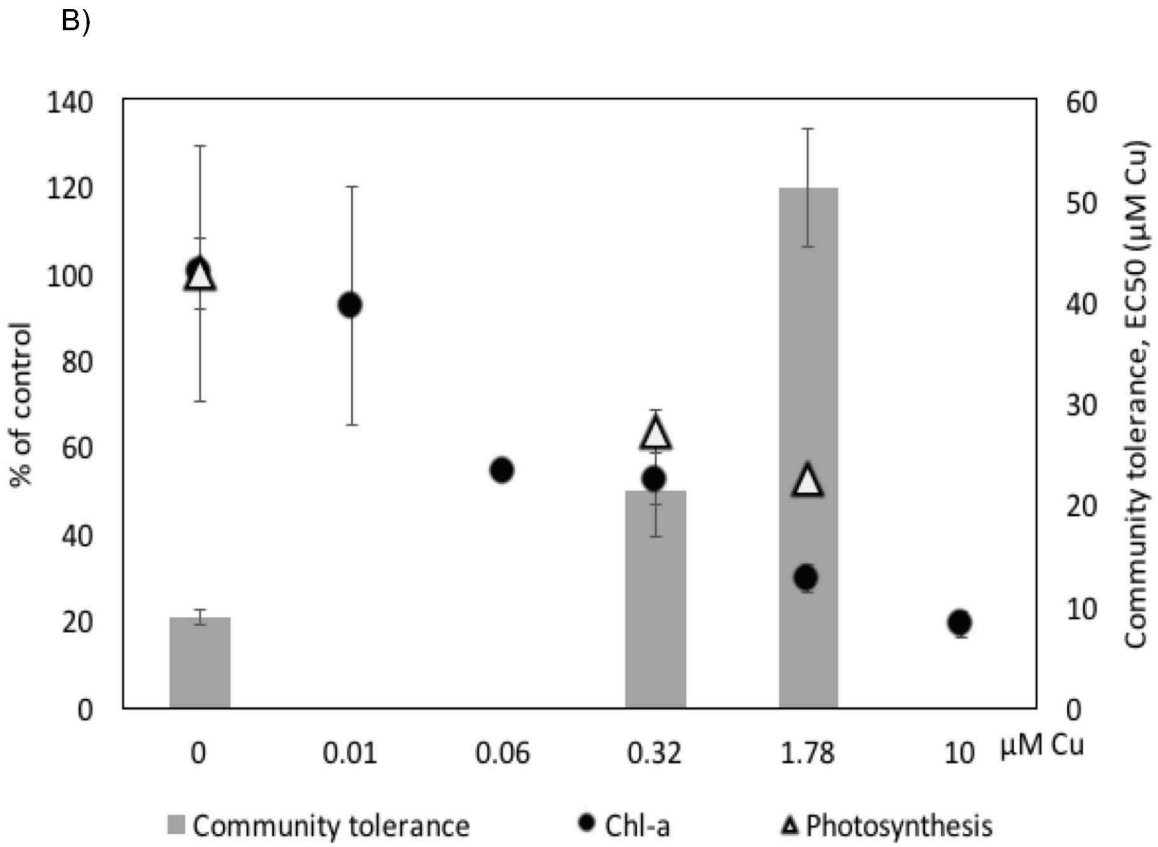
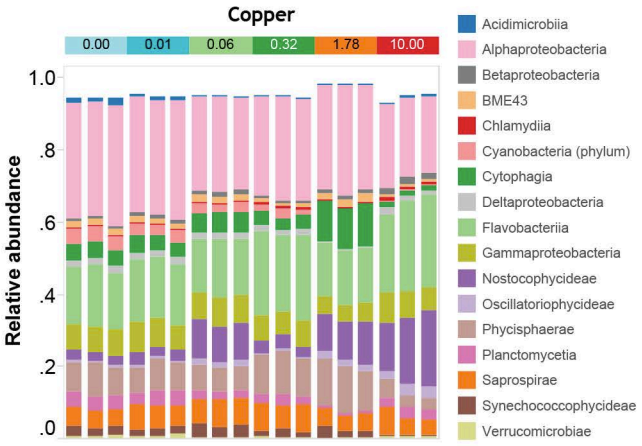
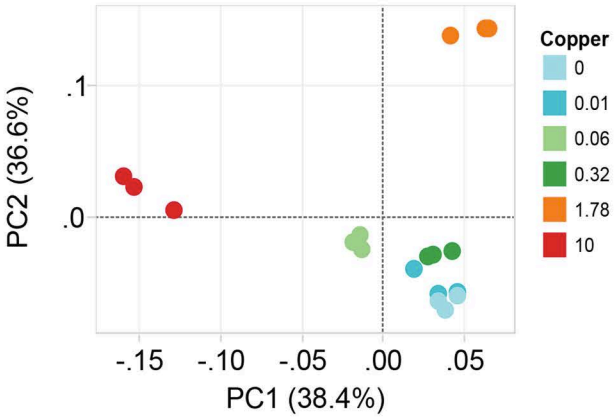
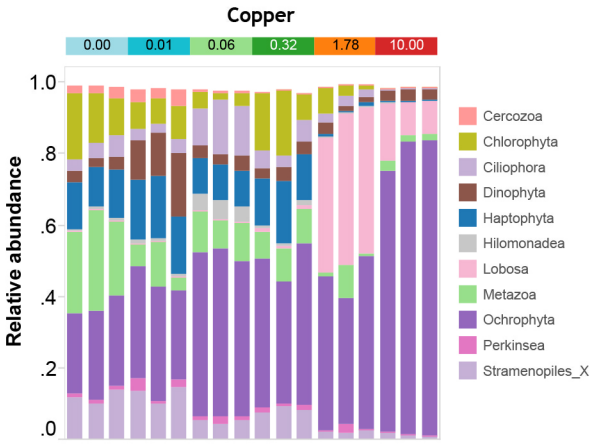
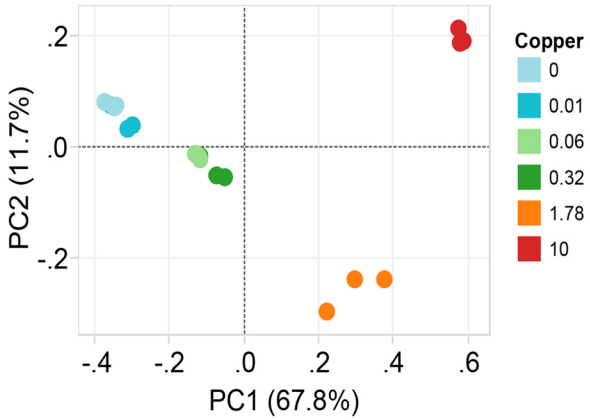

A)
Sum of all pigmets (% of controls)
Relative pigment amount (% in each treatment)
Exposure concentration (µM)
0
0,01
0,06
0,32
1,78
10
Total pigment content
Chlorophyll a
Fucoxanthin

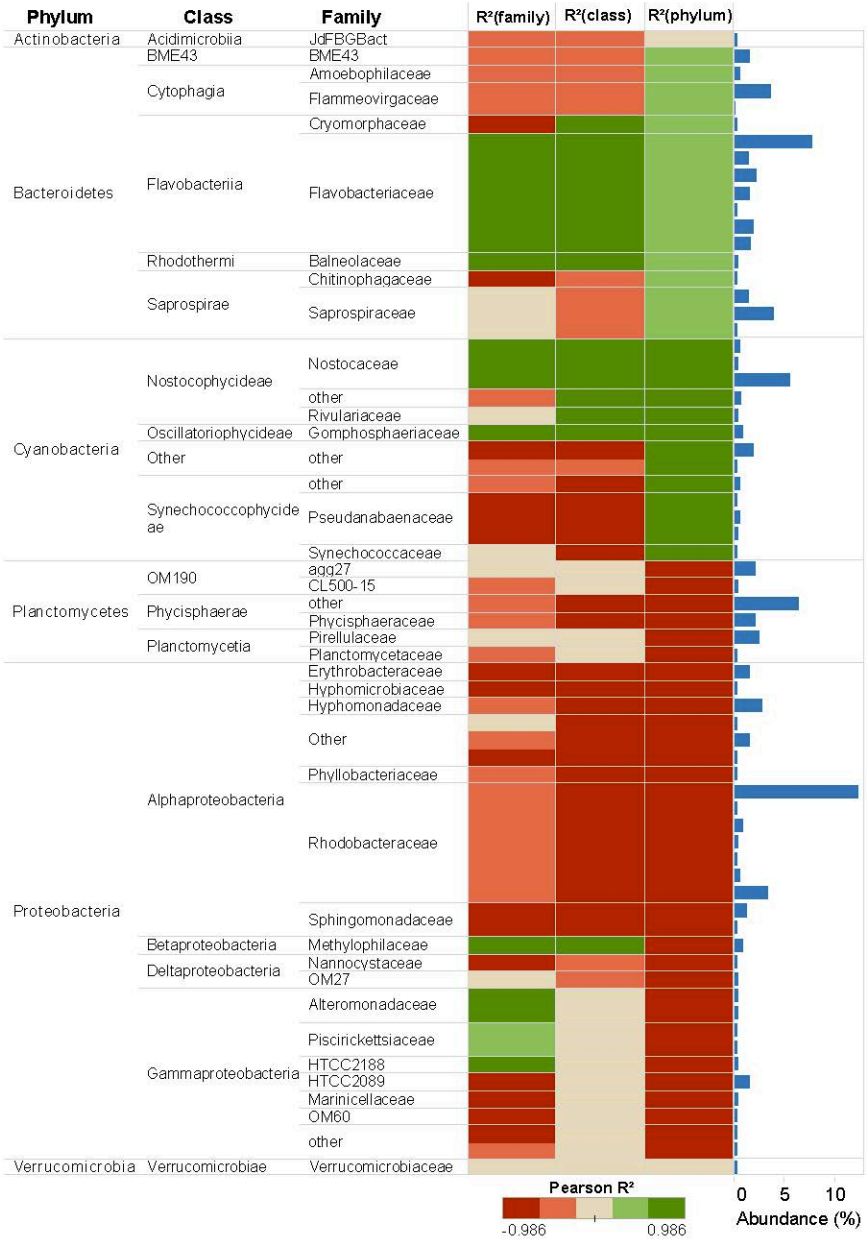

B)
% of control
Community tolerance, EC50 (µM Cu)
0
0.01
0.06
0.32
1.78
10
µM Cu
Community tolerance
Chl-a
Photosynthesis


Copper
0.00
0.01
0.06
0.32
1.78
10.00
Relative abundance
1.0
.8
.6
.4
.2
.0
Acidimicrobiia
Alphaproteobacteria
Betaproteobacteria
BME43
Chlamydiia
Cyanobacteria (phylum)
Cytophagia
Deltaproteobacteria
Flavobacteriia
Gammaproteobacteria
Nostocophycideae
Oscillatoriophycideae
Phycisphaerae
Planctomycetia
Saprospirae
Synechococcophycideae
Verrucomicrobiae


PC2 (36.6%)
.1
.0
-.15
-.10
-.05
.00
.05
PC1 (38.4%)
Copper
0
0.01
0.06
0.32
1.78
10






Phylum
Class
Family
R²(family)
R²(class)
R²(phylum)
Actinobacteria
Acidimicrobiia
JdFBGBact
BME43
BME43
Bacteroidetes
Cytophagia
Amoebophilaceae
Flammeovirgaceae
Flavobacteriia
Cryomorphaceae
Flavobacteriaceae
Rhodothermi
Balneolaceae
Saprospirae
Chitinophagaceae
Saprospiraceae
Cyanobacteria
Nostocophycideae
Nostocaceae
other
Rivulariaceae
Oscillatoriophycideae
Gomphosphaeriaceae
Other
other
Synechococcophycideae
other
Pseudanabaenaceae
Synechococcaceae
Planctomycetes
OM190
agg27
CL500-15
Phycisphaerae
other
Phycisphaeraceae
Planctomycetia
Pirellulaceae
Planctomycetaceae
Proteobacteria
Alphaproteobacteria
Erythrobacteraceae
Hyphomicrobiaceae
Hyphomonadaceae
Other
Phyllobacteriaceae
Rhodobacteraceae
Sphingomonadaceae
Betaproteobacteria
Methylophilaceae
Deltaproteobacteria
Nannocystaceae
OM27
Gammaproteobacteria
Alteromonadaceae
Piscirickettsiaceae
HTCC2188
HTCC2089
Marinicellaceae
OM60
other
Verrucomicrobia
Verrucomicrobiae
Verrucomicrobiaceae
Pearson R²
-0.986
0.986
0
5
10
Abundance (%)