Limited influence of primary treated sewage waters on bacterial abundance, production and community composition in coastal seawaters

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Abstract

The response of bacteria in terms of abundance, production and community structure to changes induced by the discharge of primary treated sewage waters was investigated combining microbiological, chemical and molecular tools. The primary treatment did not affect substantially the bacterial community structure in wastewaters and did not reduce the concentrations of fecal indicators. The spatial distribution of the sewage plume was governed by vertical stratification and currents. Bacterial abundance and production in the sea receiving waste waters depended predominantly on environmental conditions. In the waters with the highest concentration of fecal pollution indicators the bacterial community was characterized by allochthonous bacteria belonging to *Epsilonproteobacteria*, *Firmicutes, Gammaproteobacteria* and *Bacteroidetes*. The latter two taxa were also present in unpolluted waters but had a different structure, typical for oligotrophic environments. Although the impact of primary treated sewage waters was limited, a sanitary risk persisted due to the relevant presence of potentially pathogenic bacteria.

Key words: sewage waters, coprostanol, fecal indicator bacteria, bacterial community structure, next-generation sequencing, potentially pathogenic bacteria

1. Introduction

Untreated sewage waters consisting of high nutrient loads, chemicals, pharmaceuticals and fecal waste present severe challenges to coastal ecosystems. Among these pollutants, fecal waste poses the most acute risk to human health due to its high content of pathogens. In urban environments, both sewage and storm waters serve as common delivery routes for fecal matter to aquatic ecosystems (Aragonés et al. 2016; Al Aukidy and Verlicchi 2017). Sanitary quality of waters is generally assessed by using fecal indicator bacteria (FIB) such as fecal coliforms and fecal streptococci. This approach has unfortunately some serious limitations as it depends on the survival and growth of those bacteria. Thus, a complementary set of chemical indicators based on the concentrations of fecal sterols has been introduced in the last decades (Isobe et al., 2002; Isobe et al., 2004; Carreira et al. 2004; Mudge and Duce 2005). Coprostanol (COP), the main fecal sterol, is produced by the microbial degradation of cholesterol in the human intestine (Martins et al., 2007) and comprises 40%-60% of total sterols present in human waste (Leeming and Nichols, 1996). COP has persistence in the marine environment longer than FIB (Leeming and Nichols, 1996), with a half-life of approximately 10 days at 20 °C under aerobic conditions (Isobe et al., 2002). Generally, the presence of COP in aquatic environments is taken as an indication of relatively fresh fecal pollution (Savichtcheva and Okabe, 2006).

More recently, DNA based molecular methods have been increasingly employed for the profiling of the microbial community composition in waste water treatment plants (McLellan et al., 2010) and the source tracking of sewage in the environment (Sauer et al., 2011; Newton et al., 2013). To date, most studies have focused on sludge and pilot scale bioreactors (Sanapareddy et al., 2009; Xia et al., 2010; Wang et al., 2012) or treated effluents (Ye and Zhang, 2011), while others have provided important clues about the composition of untreated sewage microbial communities (Shanks et al., 2013). A phylogenetic microarray analysis of marine water and sewage samples collected during a sewage spill indicated that sewage communities differ significantly from marine water, even when the marine water is mixed with small amounts of sewage (Dubinsky et al., 2012). Pyrosequencing of samples from wastewater influent revealed that the microbial community consists of microorganisms coming from human feces, soil, and ambient water (introduced through gray water, rainwater, and stormwater). Some of these microbes can be considered as typical residents of sewage systems (McLellan et al., 2010; VandeWalle et al., 2012). Published reports have been mainly focused on describing complex communities in sludge and within bioreactors while only

marginally focusing on the effect of sewage output on the structure of autochthonous microbial communities and their temporal dynamics.

The majority of sewage waters released in the Mediterranean are generally untreated or subjected only to primary processing, i.e. mechanical removal of solids, fats and sand (EC, 2006). Our study site (town of Rovinj, northeastern Adriatic coast) represents a typical urban Mediterranean area characterized by intense tourism in summer months when the population triples. Around 80% of urban waste waters, that include domestic sewage and storm runoff, are discharged in a coastal bay, very close to the most important marine recreational areas. The aim of this study was to assess the response of bacteria in terms of abundance, production and community structure to the changes induced by the discharge of primary treated sewage waters. To achieve this purpose, molecular methods were combined with microbiological and chemical indicators. To our knowledge this is the first report where the temporal response of the marine bacterial community exposed to primary treated wastewaters has been investigated.

2. Materials and methods

2.1. Study site and samplings

Cuvi bay occupies an area of 2 km^2 with an average depth of 27 m. The sewage treatment plant (STP), installed in 1984 accepts sewage and storm water runoff from the major part of Rovinj's urban area. Sewage and storm water runoff arrived combined to the STP. The waste water treatment includes the removal of solids, fats and sand. Afterwards, the treated waters are temporarily stored in a retention basin, exposed to air but without mechanical mixing or air bubbling. Depending on the quantity of the waste waters arriving to the system the retention in the basin lasts between 15 minutes to one hour before flushing the basin content through an 800 m long submarine pipe in the sea at a depth of 27 m. In the investigated period (2010/2011; municipal service of Rovinj, pers. comm.) the bay received the highest amount of urban waste waters in August (189,216 m^3) and October (127,925 m^3) when the population was 44000 and 20000, respectively (touristic office of Rovinj, pers. comm.), the lowest in February (73,533 m^3) and March (81,105 m^3) when the population was 14000 (only local residents), while May (17000 residents) was close to the monthly average (104,878 m^3).

The sampling stations were located at the sewage outfall (C0) and around it along four directions (NW, NE, SW, and SE) at a distance of 50 m (1), 150 m (3) and 300 m (4) (Fig. 1). Seawater samples were taken with 5 L horizontal Niskin bottles at three depths (5 m, 10 m

and 20 m). The bacterial community structure was determined at the stations C0, SE1 and SE3. All sampling was done in August and October 2010 and in February, March and May 2011, except for the STP where samples were taken in February 2016. The purpose of the STP sampling was to have an insight into the bacterial community structure before and after the treatment.

2.2. Environmental parameters

Temperature (T) and salinity (S) were measured continuously throughout the water column during the downcast of a SEABIRD SBE 25 CTD probe. Water samples were collected with 5 l PVC Niskin samplers. Inorganic nutrients; nitrate (NO_3), nitrite (NO_2), ammonia (NH_4) and orthophosphate (PO_4) were analyzed in unfiltered water immediately after collection (Parsons et al., 1984; Ivančić and Degobbis, 1984). Dissolved inorganic nitrogen (DIN) was calculated as the sum of nitrate, nitrite and ammonia. Total chlorophyll a concentrations (Chl *a*) were determined by filtration of 500 ml on Whatmann GF/C filters. Filters were frozen (−18 °C) and analyzed within a few days by fluorometric procedure after Parsons et al. (1984).

2.3. Heterotrophic bacteria (HB) abundance and production

For determining HB abundance, 2 ml of each sample was stained with 4,6-diamidino-2-phenylindol (DAPI; 1 μg ml^{-1} final conc.) for 10 min, and then passed through 0.2 μm black polycarbonate filters (Nuclepore, Whatman, UK). HB abundance was determined by epifluorescence microscopy (Leitz Laborlux D) according to Porter and Feig (1980). From the total number of counted prokaryotes the number of cyanobacteria was subtracted in order to obtain the number of HB.

Prokaryotic bulk production was estimated by measuring incorporation of two different substrates: ^{3}H-thymidine (TdR; specific activity: > 70 Ci $mmol^{-1}$; 20nM final concentration) and L- [3,4,5-^{3}H] leucine (Leu; specific activity > 100 Ci $mmol^{-1}$; 20 nM final concentration) according to Fuhrman and Azam (1982) and Smith and Azam (1992), respectively. Radioactivity was measured with a liquid scintillation counter (Canberra Packard Tricarb 2900 TR, Perkin Elmer Packard, USA). Specific leucine (Leu $cell^{-1}$) and thymidine (TdR $cell^{-1}$) incorporation rates were obtained by dividing the rates per liter by bacterial abundance.

2.4. Fecal indicator bacteria (FIB)

Fecal coliforms (FC) and fecal streptococci (FS) were quantified using the membrane filtration method (WHO, 1994). Sample aliquots of 100 ml, 10 ml, 1 ml and 0.1 ml were filtered through 0.45 μm pore size membrane filters (47 mm). Samples were diluted with phosphate buffer. For FC counts membrane filters were placed on the surface of mFC agar in Petri dishes and incubated at 44.5 °C for 24 hours. The colonies that displayed a characteristic blue color were counted and the result was expressed as the number of colony forming units (CFU) in 100 ml of water.

FS were determined by placing membrane filters on the surface of Slanetz-Bartley agar in Petri dishes and incubated at 36 °C for 48 hours. The filters that had red centered colonies were further tested by placing them on the surface of bile aesculin agar in Petri dishes and incubated at 44.5 °C for 2 hours. The colonies that displayed a brown color around them were considered to be fecal streptococci. The final result was expressed as CFU in 100 ml of water.

2.5. Bacterial community structure

One liter of seawater was filtered onto 0.2 μm Nucleopore polycarbonate membrane filters (Whatman, UK) with a peristaltic pump. Filters were stored in 1 ml sucrose buffer (40 mM EDTA, 50 mM Tris-HCl and 0.75 M sucrose), frozen in liquid nitrogen and afterwards stored at -80 °C. The DNA was extracted according to Massana et al. (1997). The bacterial V3-V4 16S rRNA region was amplified using bacterial primers S-D-Bact-0341-b-S-17 (5'-CCTACGGGNGGCWGCAG-3') and S-D-Bact-0785-a-A-21 (5'-GACTACHVGGGTATCTAATCC-3') (Klindworth et al., 2013) in four parallel reactions. Each 25 μL PCR reaction contained: 1× DreamTaq Green PCR Master Mix (Thermo Fisher Scientific, USA), 0.5 μM of forward and reverse primers and 10 ng of DNA template. The PCR amplification conditions were: 5 min initial denaturation at 95 °C, 30 cycles of 40 s denaturation at 95 °C, 2 min annealing at 55 °C and 1 min elongation at 72 °C, finalized by 10 min at 72 °C. After pooling of the replicate reactions, PCR products were purified using the Wizard SV Gel and PCR Clean-Up System (Promega, USA) and sent for sequencing on the Illumina MiSeq platform (2 x 250 bp paired-end) at IMGM Laboratories (Martinsried, Germany).

The forward and reverse sequences contained in fastq files were assembled using mothur's command make.contigs and split into sample specific fasta files using mothur's command

split.groups (Schloss et al., 2009). Multifasta files were processed by the SILVAngs 1.3 pipeline (https://www.arb-silva.de/ngs) (Quast et al., 2013) as described in Ionescu et al. (2012). Briefly, sequences were aligned against the SILVA SSU rRNA SEED using the SILVA Incremental Aligner (SINA) (Pruesse et al., 2012). Sequences shorter than 50 aligned nucleotides, with more than 2% of ambiguities or 2% of homopolymers were removed. Putative contaminations and artefacts, reads with a low alignment quality (50 alignment identity, 40 alignment score reported by SINA), were excluded from downstream analysis. Identical sequences were identified (dereplication) and the unique sequences were clustered (Operational Taxonomic Units [OTU]) at 97% sequence identity using cd-hit-est (version 3.1.2; http://www.bioinformatics.org/cd-hit) (Li and Godzik, 2006) running in accurate mode and ignoring overhangs. The representative OTU sequence was classified against the SILVA SSU Ref dataset (release 123.1; http://www.arb-silva.de) using blastn (version 2.2.30+; http://blast.ncbi.nlm.nih.gov/Blast.cgi) with standard settings (Camacho et al., 2009). Statistical data regarding the SILVAngs pipeline analysis are given in the supplementary materials (Table S1). The sequencing effort applied was insufficient to determine the whole bacterial richness as could be observed in the rarefaction curves that did not level off even for the samples with the greatest number of sequences (Fig. S1).

2.6. Sterol analysis

Sterol standards, including coprostanol (COP, 5β-cholestan-3β-ol), 5α cholestane and perylene (IS) and N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TMCS) were provided by Sigma-Aldrich Chemical Company (Stenheim, Germany).

The extraction and purification procedure was performed according to Isobe et al. (2002). A filter sample containing suspended particles was placed in a 50 mL glass tube, spiked with 5α cholestane and ultrasonically extracted by 30 mL each of methanol (MeOH), MeOH/DCM (1:1, v/v), and dichloromethane (DCM), consecutively for 1 hr for each solvent. The extracts were combined, concentrated to dryness by rotary evaporator, redissolved into 1 mL of hexane/DCM (3:1, v/v) and separated into fractions by silica gel column (100-200 mesh, Sigma-Aldrich). The fractions eluted with 40 mL of DCM and 30 mL of acetone/DCM, 3:7 v/v were combined, evaporated; perylene (IS) was added and after derivatization with BSTFA-TMCS at 60°C for 1 h analyzed for sterols by GC/MSD.

Sterols were analyzed by Agilent gas-liquid chromatography (GLC) 6890N GC System (Agilent Technologies, USA) equipped with a 5973 Network Mass Selective Detector, Zebron ZB-5MSi capillary column (30 m × 0.25 mm × 0.25 μm; 5% Phenyl-95% Dimethylpolysiloxane) and ultra-high purity helium as the carrier gas. The GLC settings were: programmed column temperature rise from 150 °C (1 min) by 20 °C/min up to 310 °C (5 min), at a constant column pressure of 2.17 kPa. Retention times, peak areas and mass spectra were recorded with Chemstation software. Data were acquired in the full scan mode between ions of m/z 50 and 550.

2.7. Data analysis

The correlations among parameters were tested using Pearson's correlation coefficient (r). The level of statistical significance was $p < 0.05$. Differences in fecal indicators, HB abundance and production among sampling months and depths were tested by one-way analysis of variance (ANOVA; Systat 12). The normality and homogeneity of variances were tested by the Lilliefors and Levene tests, respectively. Results found to be significant by ANOVA ($p < 0.05$) were then analyzed by post hoc Tukey's honestly significant difference (HSD) multiple-comparison tests to investigate which of the means were different.

Principal component analysis (PCA) was used to identify the most important variables that explain the most variation in dataset. The analyses were based on correlation matrices (constructed using the S, T, COP, FC, FS, NH_4, DIN, PO_4, HB, Leu and TdR) involving the normalization of all variables due to their different scales. Only the principal components with eigenvalues > 1 were considered to account for much of the parameter variability. PCA was performed using the software Primer 6.

3. Results

3.1. Environmental conditions

In August the water column was completely stratified while the mixing process began in October. In February and March isothermal and isohaline conditions characterized the whole study area. The water column stratification started to appear again in May (Fig. 2). The overall temperature and salinity values in the investigated period were typical for the coastal waters of Rovinj during all measurements (Ivančić et al., 2010).

194 High concentrations of DIN (up to 28.96 μmol L^{-1}) with the highest contribution of NH_4 (up
195 to 96%) were recorded along the 20 m layer in August in a radius of 300 m and in October
196 only in the proximity of the outfall (C0). In February and March DIN decreased with respect
197 to the warmer months, and was highest at 5 m and 10 m. In May, DIN concentrations were
198 similar to the winter months but showed a distribution similar to the summer months with
199 relative increase at 20 m (Fig. 2). During the summer the concentrations of phosphates (PO_4)
200 were increased only in the proximity of the outfall (up to 0.36 μmol L^{-1}), while all the other
201 values (during all samplings) were comparable to the ones typical for the northern Adriatic
202 coastal waters (0.01 to 0.08 μmol L^{-1}, data not shown). The average monthly Chl *a* in the area
203 of Cuvi were within the long-term measurements for the northeastern Adriatic waters. The
204 lowest values of Chl *a* were detected in August (0.28 μg L^{-1}) and October (0.31 μg L^{-1})
205 followed by an increase in February (0.48 μg L^{-1}) and the maximum values in May (0.55 μg
206 L^{-1}).

207

208 3.2. Fecal pollution indicators; FIB and COP

209 All fecal pollution indicators were strongly mutually correlated (FC vs FS: n=195, r=0.964, p
210 <0.001; FC vs COP: n=195, r=0.808, p <0.001; FS vs COP: n=195, r=0.788, p <0.001),
211 displaying a very similar distribution throughout the year (Fig. 2). In general, FC were by an
212 order of magnitude greater than FS. In October elevated concentrations of fecal indicators
213 were found only in the vicinity of the outfall. In August, however, higher concentrations of
214 indicators were present in the broader area spreading towards the western quadrant. During
215 February and March elevated concentrations of indicators were present in the whole water
216 column, reaching highest values at 5 m. In February, the peak of FIB concentrations was
217 found at SE1, while in March it was localized at C0. In May, the distribution of indicators was
218 similar to August, but the concentrations remained close to the winter months (Fig. 2). The
219 monthly differences for all the indicators (one-way ANOVA) were not statistically
220 significant. On the other hand the concentrations of all indicators were significantly higher at
221 20 m (factor: depth) and at C0 (factor: distance, Table 1).

222 At the STP the concentrations of fecal pollution indicators were very high and displayed no
223 relevant differences before (FC: $8.3 \cdot 10^8$ CFU/100 ml, FS: $6.1 \cdot 10^7$ CFU/100 ml, COP: 1256 μg
224 L^{-1}) and after (FC: $7.9 \cdot 10^8$ CFU/100ml, FS: $5.4 \cdot 10^7$ CFU/100 ml, COP: 1162 μg L^{-1}) the
225 primary treatment.

3.3. Heterotrophic bacteria in the receiving waters; abundance and production

The abundances of heterotrophic bacteria (HB) significantly differed between the months but not between the depths and distance from the outfall (Table 1). In August (2.1-7.6 10^8 cell L^{-1}) and October (2.8-13.7 10^8 cell L^{-1}) HB abundance was similar and significantly higher than in February (1.9-4.2 10^8 cell L^{-1}), March (1.1-5.3 10^8 cell L^{-1}) and May (1.3-3.9 10^8 cell L^{-1}). During August and October when the waste water input was at maximum the abundance of HB was increased C0 at the depth of 20 m while during the other months this effect was not evident (Fig. 3).

Significant temporal variations were also observed in the cell specific leucine (Leu) and thymidine (TdR) incorporation rates and their ratio Leu/TdR (Table 1). Leu was significantly higher during February (96.1±56.8 zmol cell^{-1} h^{-1}) than in March (42.7±19.5 zmol cell^{-1} h^{-1}), May (29.4±19.6 zmol cell^{-1} h^{-1}), August (31.5±25.9 zmol cell^{-1} h^{-1}) and October (17.4±15.0 zmol cell^{-1} h^{-1}).The differences in Leu between the depths and distances were not significant. TdR was significantly higher during May (9.8±6.9 zmol cell^{-1} h^{-1}) than in the other months when the values were comparable: August (5.0±9.9 zmol cell^{-1} h^{-1}), October (3.2±2.6 zmol cell^{-1} h^{-1}), February (2.3±1.7 zmol cell^{-1} h^{-1}) and March (1.9±3.0 zmol cell^{-1} h^{-1}). Also, TdR was significantly higher at 20 m of depth, while the differences with distance were not significant (Table 1). Leu/TdR ratios in March (86.6±146.8) were significantly higher than in May (5.4±4.7) and October (6.3±5.6), and not significantly different from the ratios in February (74.7±86.5) and August (27.2±23.4). The differences in Leu/TdR ratios between the depths and distance were not significant (Table 1).

The relationship among environmental variables, FIB, COP, HB abundance and production is shown on the principal component analysis plot (Fig. 4) where PC1 and PC2 explained 44.77% and 24.32% of total variance, respectively. The highest loadings for negative relationship were obtained for NH_4 (-0.447), FC (-0.436), FS (-0.431) and COP (-0.412) on PC1 while for S (-0.523) and Leu (-0.444) on PC2. For the positive relationship the highest loadings showed T (0.501) and HB (0.401) on PC2. The pollution variables (FC, FS, and COP) were simultaneously strongly related mutually and linked with NH_4 and DIN. This relationship indicated that NH_4 and DIN mainly resulted from the input of waste waters. In contrast, the input of waste waters affected less HB, PO_4 and TdR. The positive relation between HB and T and negative with S indicated that higher HB numbers occurred in warmer and less salty layers. The positive relation between PO_4 and S and negative with T indicated that increased PO_4 concentrations occurred in the saltier and colder layers. Leu appeared to be completely independent of waste water input and governed by the natural fluctuations in T

and S, being increased at higher salinity and lower temperature. On PC1 the majority of samples were scattered around zero implying no significant influence of the waste waters on these layers. A portion of the samples, particularly from August and October was shifted following the increased concentration of FC, FS, COP and NH_4 with an appreciable number of outliers positioned in colder and saltier layers (20 m). The separation along the PC2 was evident between the August-October and the clustered February, May and March samples due to the seasonal differences in temperature and salinity (Fig. 4).

3.4. Bacterial community structure

The bacterial communities of sewage waters before and after the primary treatment were very similar (Fig. 5). In untreated waters the community consisted of *Epsilonproteobacteria* (29.9%, with prevalence of the genus *Arcobacter* [29.1%]), *Firmicutes* (28.6%, with prevalence of the family *Lachnopiraceae* and *Ruminococcaceae* comprising 24% and 22% of the phylum, respectively), *Gammaproteobacteria* (21.6%), *Bacteroidetes* (8.8%), *Betaproteobacteria* (5.9%) and *Fusobacteria* (1.2%). After the primary treatment the only noticeable changes were evidenced in the decrease of *Firmicutes* (20.8%) and a comparable increase of *Arcobacter* (39.2%). For other phyla and classes changes were less than 2%. In wastewaters the most represented genera of *Gammaproteobacteria* were *Acinetobacter* (up to 7.15%), *Aeromonas* (6.62%), *Escherichia/Shigella* (2.37%) and *Shewanella* (1%). In the marine environment those genera rarely reached 1%, mainly in the mostly polluted layers, while in the remaining waters the clades SAR86 and OM60 (NOR5) replaced them. *Bacteroidetes* in wastewaters were mainly represented by the genus *Bacteroides*, while the marine samples were primarily dominated by NS4 and NS5 marine groups (Fig.6). The genus *Acidovorax* appeared to be the main representative of *Betaproteobacteria.* *Alphaproteobacteria* were only a very small share of the waste water community.

The alpha diversity (OTU richness) of bacterial communities in recipient waters evidenced March as having a significantly higher number of OTUs than the rest of the year while the differences between the stations were not significant (Table S2 and Table S3). Among the allochthonous bacteria in the receiving waters, the presence of *Firmicutes* and the epsilonproteobacterial genus *Arcobacter* were recognized. In August and October those bacteria represented an important share of the community exclusively below the thermocline (20 m). *Firmicutes* were relevant only at the outfall (C0) and rapidly decreased with distance, while in August *Arcobacter* was consistently present up to SE3. In October, *Firmicutes* and

293 *Arcobacter* were concentrated only around the outfall, while in February allochthonous
294 bacteria were present in the whole water column especially near the surface (5 and 10 m) at
295 SE1 and SE3. Interestingly, at C0 their contribution was negligible. In March, *Arcobacter* and
296 *Firmicutes* were detected at C0 (5 m and 10 m) while a major share of *Firmicutes* was present
297 at SE1 (20 m). In May, a smaller portion of allochthonous bacteria was present at C0 and SE1
298 only at 20 m (Fig. 6).

299 In August and October, *Gammaproteobacteria* dominated the community at 20 m increasing
300 with distance, but were much less present in the upper layer where the gammaproteobacterial
301 clade SAR86 became quite recognizable. During February, March and May the share of
302 *Gammaproteobacteria* remained in general constant (13.9%-42.2%) in the entire water
303 column. The presence of the SAR86 clade was remarkably high during the colder months (up
304 to 18.4%), while it decreased in May when a higher contribution of the gammaproteobacterial
305 clade OM60 (NOR5) was observed. *Alphaproteobacteria* were also abundant in marine
306 samples throughout the year (up to 56%). In warmer months the upper layers were much
307 enriched with this class; however in the polluted layers a consistent decrease was observed.
308 From February to May *Alphaproteobacteria* were uniformly distributed in all layers.
309 However, different relative proportions of alphaproteobacterial members, the SAR11 clade
310 and AEGEAN-169 marine group, appeared in various sampling months. The AEGEAN-169
311 marine group was important in August; the SAR11 clade in October, February and March,
312 while in May both of them became marginal (Fig. 6). *Betaproteobacteria* were a minor
313 component of the community until March and May when they became relevant, with the
314 prevalence of the genus *Limnobacter.* They showed a different spatial distribution between
315 the two months being present in polluted superficial waters in March, while in May they were
316 characteristic for the other layers.

317 Regarding *Bacteroidetes*, the genus *Bacteroides* was found in marine samples at the most
318 polluted sites. The share of other members of *Bacteroidetes* (NS4 and NS5 marine groups)
319 decreased with depth during warm months, while during the rest of the year they remained
320 fairly homogenous. In August and May, these marine groups were quite relevant in the
321 community. *Actinobacteria*, consisting principally of the genus "*Candidatus* Actinomarina",
322 were mainly present in October and February with a similar abundance at all depths, except at
323 C0 (20 m) (Fig. 6). Among the *Cyanobacteria*, the genus *Synechococcus* was recorded in
324 August in the upper layers and only marginally in October, March and May. *Prochlorococcus*
325 was less abundant following the same distribution as *Synechococcus* (Table_dataset).

3.5. Potentially pathogenic bacteria (PPB)

The diversity and abundance of PPB were analyzed at the genus level according to the reference pathogenic bacteria list made up of 32 genera (Ye and Zhang, 2011). A total of 173580 sequences were attributed to the PPB genera which comprised 7.41% of all the sequences. The results showed that in the STP before and after the primary treatment the relative abundances were 38.0% and 49.7%, respectively. In the sea, the most polluted layers in August, October and February had high relative abundances of PPB (19.37%-32.37%), while in the rest of the year they were relatively low (<10%). In general, the most abundant and common genera of PPB were *Aeromonas*, *Arcobacter*, *Pseudomonas*, *Vibrio* and *Escherichia/Shigella*, while the genera *Borrelia*, *Chlamydophila*, *Leptospira* and *Listeria* were not detected (Fig. 7).

4. Discussion

Our results revealed that the primary treatment plant in Cuvi bay had a negligible effect on both the reduction of fecal indicators and the alteration of the sewage bacterial community structure. The concentrations of FIB in the retention basin were within the reported ranges for other untreated sewage waters which normally contain between 10^7-10^9 CFU/100 mL of FC and between 10^6-10^8 CFU/100 mL of FS (George et al., 2002).

The bacterial community in the STP influent consisted of a complex array of taxa dominated by *Epsilonproteobacteria*, *Firmicutes*, *Gammaproteobacteria*, *Bacteroidetes* and *Betaproteobacteria*. *Arcobacter*, the most abundant epsilonproteobacterial genus, has generally a low prevalence in human feces but it is typical for sewer systems (Collado and Figueras, 2011). Its high abundance was hypothesized to be a consequence of its adaptation to the specific ecological conditions present in sewage infrastructure (Fisher et al., 2014). In contrast, the second most abundant component in the STP, the phylum *Firmicutes,* is characteristic of the human gastrointestinal tract and can make up to 80% of gut microbiota (Rajilić-Stojanović and deVos, 2014). Among *Firmicutes*, the family *Lachnospiraceae* (class *Clostridia*), was the most common. *Lachnospiraceae* are considered a part of the core fecal community in untreated sewage waters (Shanks et al., 2013). Their reduction after the primary treatment could be ascribed to the fact that they are anaerobes. However, their ability to form spores facilitates their survival in a variety of aerobic and anaerobic environments (Galperin, 2013). The third major component of the STP microbial community consisted of

358 *Gammaproteobacteria*. This class includes many opportunists and pathogens, such as
359 *Acinetobacter*, *Aeromonas*, *Escherichia/Shigella*, *Enterobacter*, *Pseudomonas* and *Vibrio*
360 which are considered as consistent members of sewage influents (VandeWalle et al., 2012).
361 The primary treatment seemed to have the lowest impact on the members of this class.
362 *Bacteroidetes* and *Betaproteobacteria* were two minor components of the STP bacterial
363 community. Their presence corresponded to the typical structure of fecal communities found
364 in the human intestine. The distribution of PPB in the STP showed that the system was not
365 effective in their reduction. Moreover, an increase in number of detected genera and their
366 relative abundance after the treatment indicated the probable survival of PPB introduced
367 during previous inputs into the system.

368 The trace of the allochthonous bacteria (mainly *Firmicutes* and *Arcobacter*) in the marine
369 environment corresponded to the trends and distributions of FIB and fecal sterols. Even when
370 the volumes of the released sewage waters and the resident population varied during the
371 investigated period, the variation in the concentrations of FIB and fecal sterols in the sea
372 between the months was not significant. In the polluted waters of Cuvi bay the maximum
373 COP concentration was 15.1 μg L^{-1} while the average levels were 0.33±1.34 μg L^{-1},
374 corresponding to slightly contaminated coastal areas (Isobe et al., 2002). In Cuvi bay the
375 concentration of all fecal pollution indicators significantly decreased with distance from the
376 sewage outfall. In August and October the concentration of FIB and fecal sterols was the
377 highest below the thermocline, while in February and March it was lower and more evenly
378 distributed in the water column. May represented the transition between the aforementioned
379 conditions. The overall effect of the waste water input on the HB abundance was very limited
380 being positive only in the closest vicinity of the outfall in August and October, most probably
381 due to the local increase of organic substrate, ammonia and phosphates released through the
382 outfall. One of the reasons responsible for such a limited effect might be ascribed to the rapid
383 dilution and spreading of the waste waters due to the relatively intense marine currents (~0.15
384 ms^{-1}). The rates of bacterial production even in the waters with the highest concentration of
385 the fecal pollution indicators were within the natural ranges for the northern Adriatic Sea
386 (Ivančić et al., 2010).

387 COP showed a remarkable correlation with other indicators of fecal pollution, FC and FS.
388 Although those indicators were strongly associated, some differences emerged between warm
389 and cold months as already observed in previous studies (Leeming and Nichols, 1996; Isobe
390 et al., 2002). During August relatively low concentrations of COP (i.e. <0.5 μg L^{-1}) were

coupled with high FIB counts (>10000 CFU/100 mL), while in February higher COP (i.e. >0.5 μg L^{-1}) corresponded to low numbers of FIB (<50 CFU/100 mL). The reasons for these relations during winter could be ascribed to the greater dissipation of the sewage in the whole water column which exposed FIB to a higher degree of predation by microzooplankton. The opposite situation during August could be caused by the maintenance of favorable conditions for the survival of FIB inside the polluted and nutrient-rich plume spreading only below the thermocline.

The community structure of the less contaminated layers differed substantially from the polluted ones. The detection of PPB in some unpolluted waters could be explained by the fact that these genera include many autochthonous marine bacteria such as *Pseudomonas* and *Vibrio* which are commonly found in coastal waters. Among the autochthonous bacteria in unpolluted layers *Alphaproteobacteria* were ubiquitous and very abundant. Within this class a temporal pattern was recognized. The AEGEAN-169 marine group showed a very high relative abundance only in August while the SAR11 clade was abundant during the rest of the year. Previously, the AEGEAN-169 marine group has been found to be closely linked to the SAR11 clade during dinoflagellate blooms (Yang et al., 2015). In addition, the SAR11 clade is a typical representative of oligotrophic Mediterranean (Alonso-Saez et al., 2007) and Adriatic waters (Korlević et al., 2015) having a major role in the oxidation of low-molecular-weight dissolved organic matter. *Gammaproteobacteria* were the second largest group which showed a different structure in polluted and unpolluted waters. In the latter the SAR86 clade was relatively more abundant, especially in colder months when there was an increase of Chl *a*, a proxy for phytoplankton biomass. Generally, *Gammaproteobacteria* are involved with phytoplankton blooms and degradation of algal biomass, where *Reinekea* spp. and clade SAR92 are commonly present (Teeling et al., 2016). However, it seems that in oligotrophic waters the SAR86 clade takes over their ecological role (Korlević et al., 2015). The SAR86 clade displays metabolic streamlining containing an expanding capacity for the degradation of lipids and polysaccharides (Dupont et al., 2012). Another prominent gammaproteobacterial clade detected was the OM60 (NOR5) clade that appeared in the whole water column in March and increased in abundance in May (comparable to the SAR86 clade). The OM60 (NOR5) clade has been described as a marine cosmopolitan member with clear preference for coastal marine waters (Yan et al., 2009).

Within the unpolluted layers actinobacterial genus "*Candidatus* Actinomarina" appeared first in October in association with genera typical for oligotrophic environments (e.g. SAR11 and

SAR86). During February, the increased relative importance of "*Candidatus* Actinomarina" characterized the entire water column. This genus is described as the smallest among free-living prokaryotes, having an enhanced performance in oligotrophic environments or nutrient-depleted conditions (Ghai et al., 2014). Moreover, these bacteria appear to be associated with zones of maximal photosynthetic production (Mizuno et al., 2015). In February, the specific rate of the bacterial biomass production was the highest and was not balanced with rates of DNA synthesis resulting in very high Leu/TdR ratios. In less favorable environmental conditions, such as low temperature and lack of substrate supply, bacteria might have reduced protein and especially DNA synthesis rates thus increasing the Leu/TdR ratio (Chin-Leo and Kirchman, 1990; Shiah and Ducklow, 1997). Bacterial cells with high Leu/TdR ratios are presumably processing carbon without cell division thus showing lower bacterial growth efficiency (Gasol et al., 1998). In our case the outfall and phytoplankton supplied the system with some substrate but the low winter temperature might have slowed the rate of DNA synthesis. In addition, besides the resources and the environmental conditions the intrinsic responses related to the bacterial community structure might have affected the pattern of bacterial metabolism and consequently the ratio of Leu/TdR (del Giorgio et al., 2011). A large spatial variability was generally observed for all bacterial production descriptors in accordance with other studies, which is probably due to the characteristics of water masses that coexist vertically in a certain area (Longnecker et al., 2006; del Giorgio et al., 2011).

In May, the presence of *Bacteroidetes*, commonly associated with phytoplankton blooms, might have indicated the availability of a substrate typically present in these conditions. In general, this phylum (order *Flavobacteriales*) appears recurrently, in successions, in response to the availability of phytoplankton-derived polysaccharides, i.e. transparent exopolymer particles. *Bacteroidetes* are considered fast-growing r-strategist with specialization on the initial attack of highly complex organic matter (Teeling et al., 2016). However, instead of having typical flavobacterial groups *Ulvibacter* and *Formosa*, our samples were dominated by NS4 and NS5 marine groups which seem to be better adapted to phytoplankton blooms in more oligotrophic conditions (Korlević et al., 2015). The increasing importance of the betaproteobacterial genus *Limnobacter* in March and May could be partly ascribed to the higher humic matter content (Hutalle-Schmelzer et al., 2010) potentially introduced with sewage or storm waters, but mainly to an increase in the autotrophic biomass. It has been recently shown that *Limnobacter* has been found associated with different diatoms (e.g. *Pseudo-nitzschia multiseries*) in the Atlantic and the North Pacific Ocean and specific

bacterial clades belonging to *Alpha*-, *Gammaproteobacteria* and *Bacteroidetes* (Amin et al., 2012), also relevant in our samples. The obtained community structure in May suggested that there was an increase in phytoplankton biomass in agreement with an increase in Chl *a* concentration in the area. This input of substrate combined with higher temperatures could have enabled an increase in the rate of bacterial rates of DNA synthesis leading to a relatively low Leu/TdR ratio. A similar imbalance in bacterial production rates was observed around deep-chlorophyll maximum during an autotrophic biomass increase in the oligotrophic South Adriatic (Najdek et al., 2014).

5. Conclusions

The primary treatment did not affect substantially the bacterial community structure and did not reduce the concentration of PPB, COP and FIB. All fecal indicators were mutually significantly correlated. However, there was a temporal fluctuation in their correlation due to the variable survival of FIB. The distribution of the sewage plume was governed by the vertical stratification and the currents. The rapid dispersion of the sewage plume greatly mitigated its effect on the marine environment. In the recipient waters characterized by high concentrations of FIB and COP the presence of bacteria typical for sewage systems (*Arcobacter* and *Firmicutes, Bacteroides*) was evident within the community. Bacterial abundance and production in the sea receiving waste waters depended predominantly on environmental conditions. Throughout the year the autochthonous bacterial communities were dominated by taxa typical for coastal waters which included alphaproteobacterial clades SAR11 and AEGEAN-169, gammaproteobacterial clade SAR86 and NS4 and NS5 marine groups of the *Bacteroidetes*. The detection of "*Candidatus* Actinomarina" and *Limnobacter* appeared to be associated with increases of phytoplankton biomass but also with a possible leaching of coastal soils and an increased input of storm waters within the sewage system. The latter relations should however be further investigated.

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664

665 Figure captions

666 Fig. 1. Sampling stations in the Cuvi bay.

667 Fig. 2. Distribution of salinity (S), temperature (T), dissolved inorganic nitrogen (DIN), fecal
668 coliforms (FC), fecal streptococci (FS) and coprostanol (COP) in the water column along the
669 NW-SE and NE-SW profiles during August 2010, October 2010, February 2011, March 2011
670 and May 2011.

671 Fig. 3. Distribution of heterotrophic bacteria (HB) and cell specific incorporation rates of
672 leucine (Leu) and thymidine (TdR) in the water column along the NW-SE and NE-SW
673 profiles during August 2010, October 2010, February 2011, March 2011 and May 2011.

674 Fig. 4. Principal component analysis for the water temperature (T), salinity (S), concentration
675 of orthophosphates (PO_4), dissolved inorganic nitrogen (DIN), ammonium (NH_4), fecal
676 coliforms (FC) and streptococci (FS), coprostanol (COP), bacterial abundance (HB) and cell
677 specific incorporation rates of leucine (Leu) and thymidine (TdR) in the water column during
678 August 2010 (red triangle), October 2010 (yellow triangle), February 2011 (blue triangle),
679 March 2011 (black triangle) and May 2011 (green triangle).

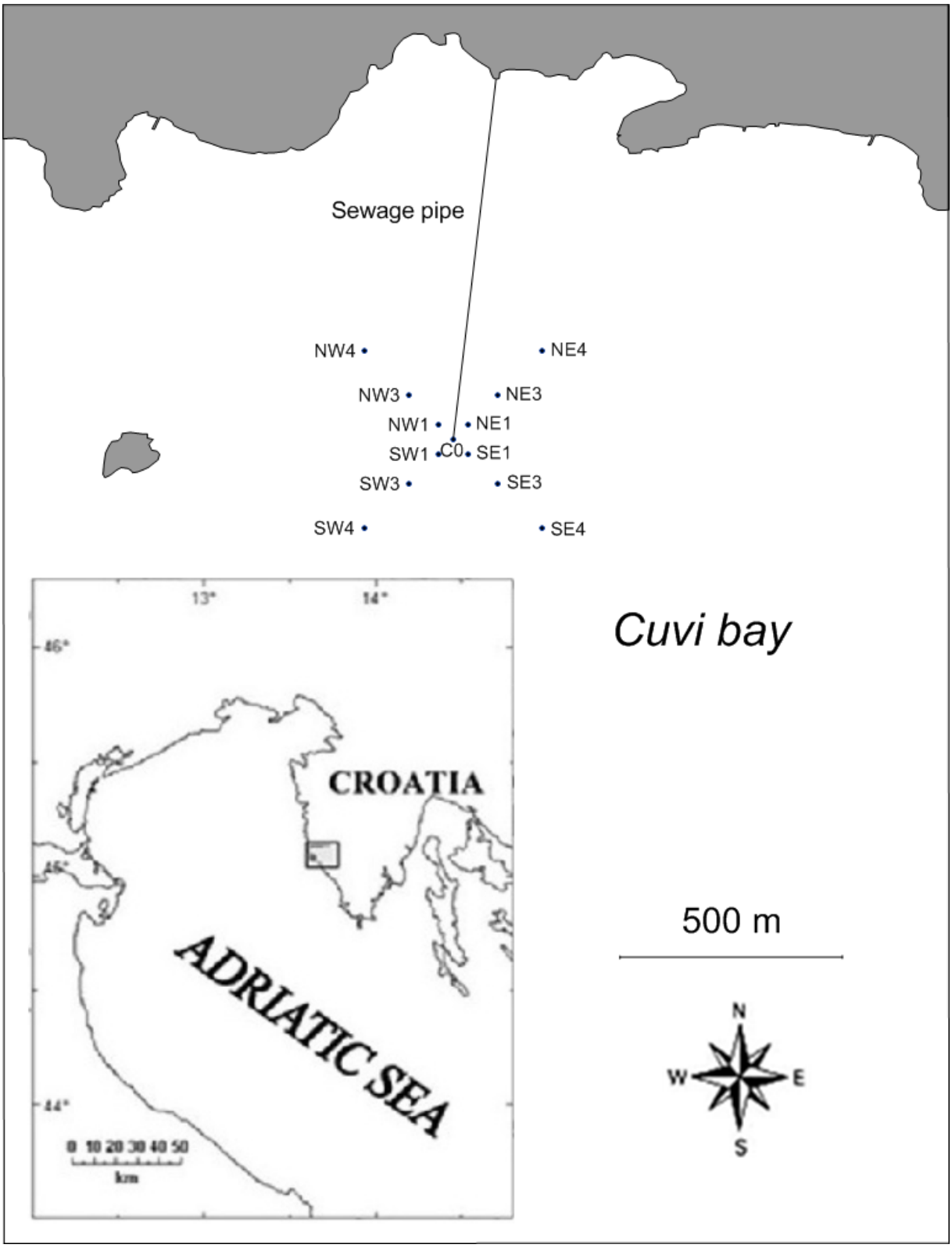
680 Fig. 5. Taxonomic classifications and relative contribution of the most common bacterial 16S
681 rRNA sequences before and after the primary treatment at the sewage treatment plant (STP).

682 Fig. 6. Taxonomic classifications and relative contribution of the most common bacterial 16S
683 rRNA sequences and bacterial abundances (HB) at the stations C0, SE1 and SE3 in August
684 2010, October 2010, February 2011, March 2011 and May 2011.

685 Fig. 7. Relative abundances (percentage of a specific pathogenic genus in total identified
686 pathogenic bacteria) of potentially pathogenic bacterial genera in the study area.

687 Fig. S1. Rarefaction curves for the Cuvi bacterial communities sampled in STP, August,
688 October, February, March and May at stations C0, SE1 and SE3 at different depths.

689


Sewage pipe
NW4
NE4
NW3
NE3
NW1
NE1
C0
SW1
SE1
SW3
SE3
SW4
SE4
13°
14°
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44°
CROATIA
ADRIATIC SEA
0 10 20 30 40 50
km
Cuvi bay
500 m
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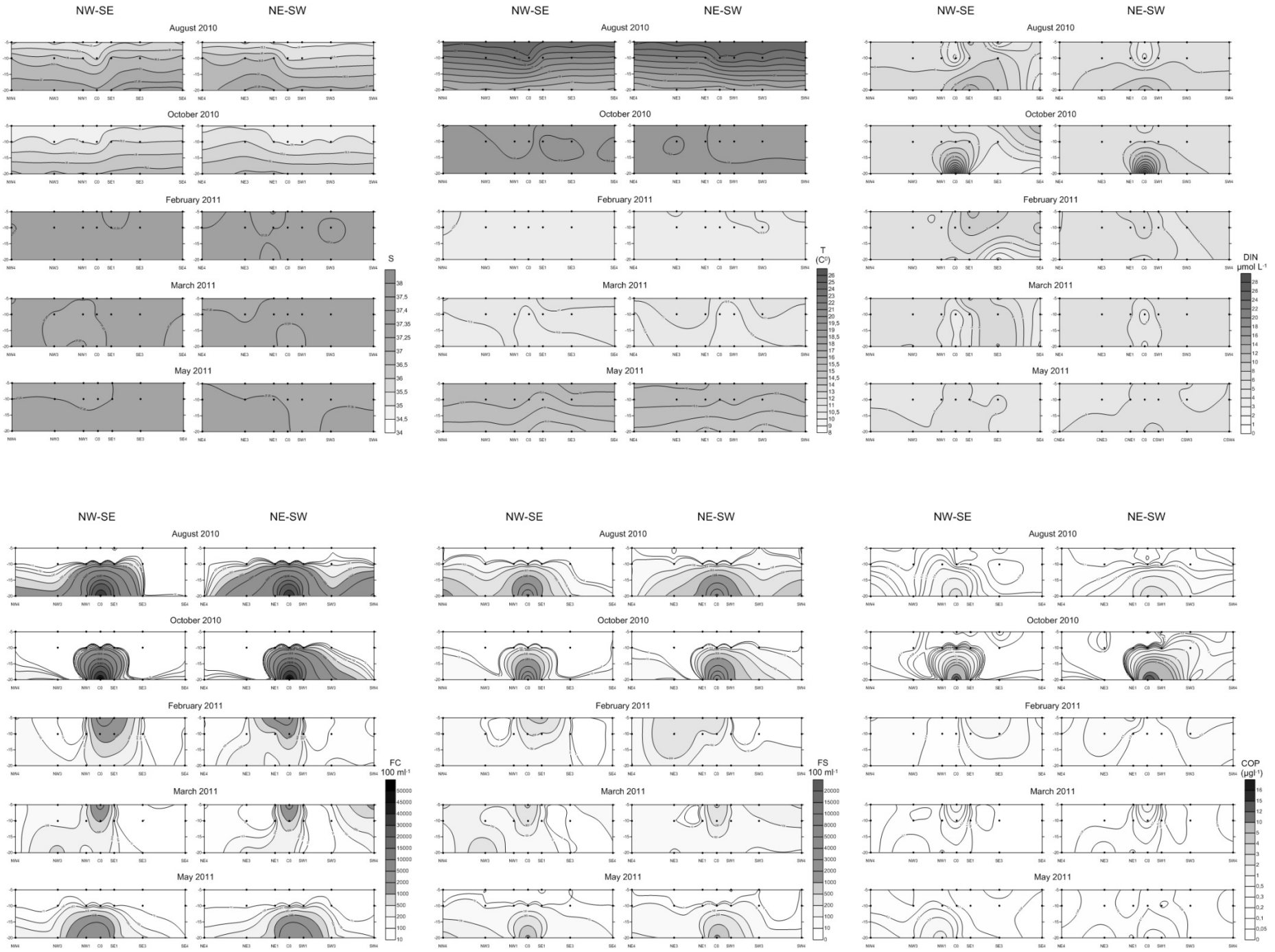
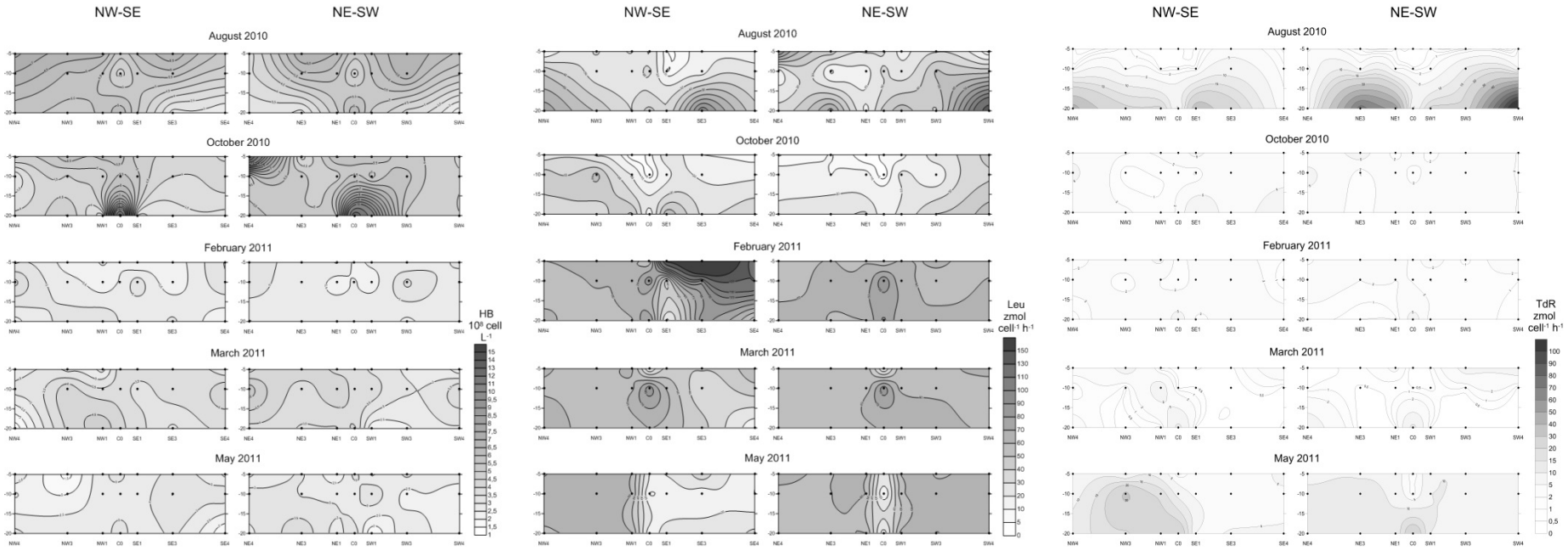
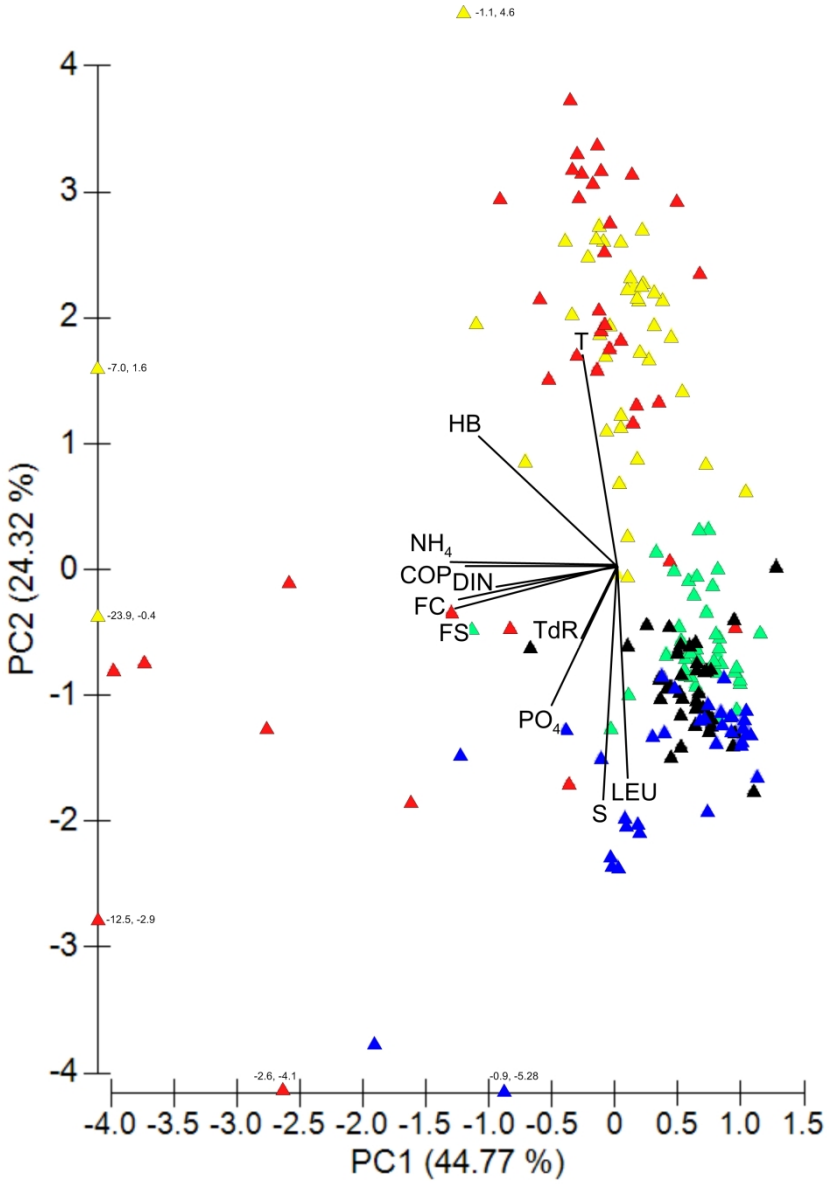
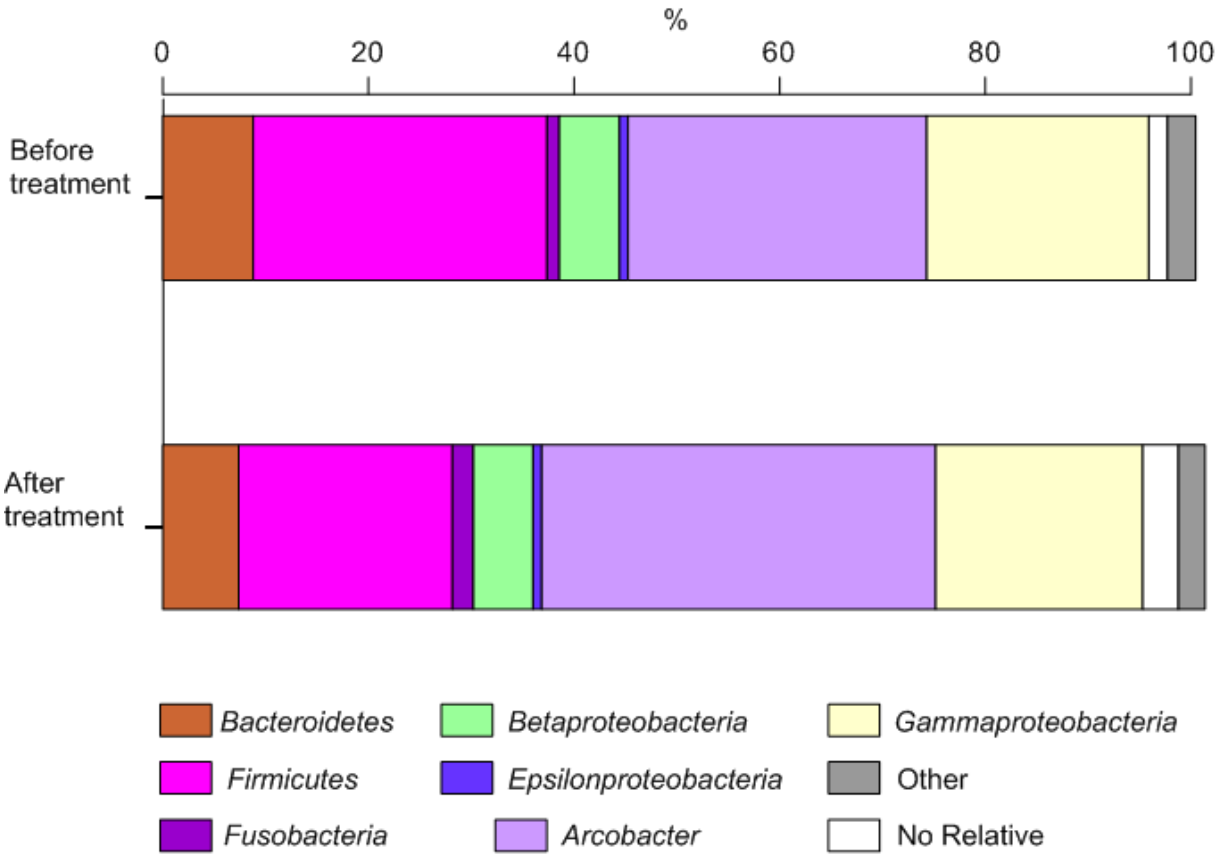
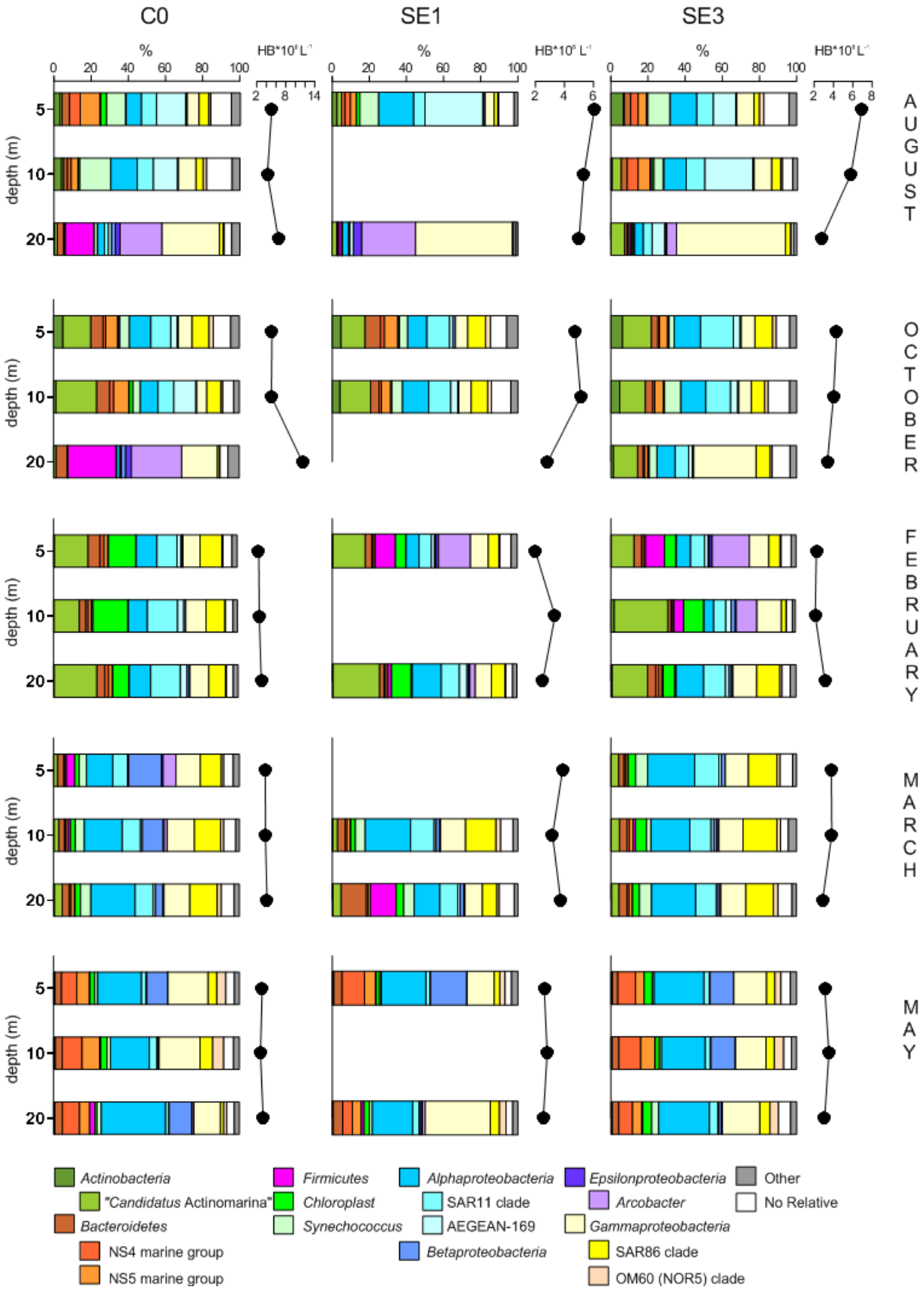

NW-SE
NE-SW
August 2010
October 2010
February 2011
March 2011
May 2011
S
T (C°)
DIN µmol L-1
FC 100 ml-1
FS 100 ml-1
COP (µgl-1)


NW-SE
NE-SW
August 2010
October 2010
February 2011
March 2011
May 2011
HB
10⁸ cell L⁻¹
Leu
zmol cell⁻¹ h⁻¹
TdR
zmol cell⁻¹ h⁻¹


-1.1, 4.6
-7.0, 1.6
-23.9, -0.4
-12.5, -2.9
-2.6, -4.1
-0.9, -5.28
T
HB
NH4
COP
DIN
FC
FS
TdR
PO4
LEU
S
PC2 (24.32 %)
PC1 (44.77 %)
4
3
2
1
0
-1
-2
-3
-4
-4.0 -3.5 -3.0 -2.5 -2.0 -1.5 -1.0 -0.5 0 0.5 1.0 1.5


%
0
20
40
60
80
100
Before treatment
After treatment
Bacteroidetes
Betaproteobacteria
Gammaproteobacteria
Firmicutes
Epsilonproteobacteria
Other
Fusobacteria
Arcobacter
No Relative


C0
SE1
SE3
%
HB*10⁶ L⁻¹
depth (m)
5
10
20
AUGUST
OCTOBER
FEBRUARY
MARCH
MAY
Actinobacteria
"Candidatus Actinomarina"
Bacteroidetes
NS4 marine group
NS5 marine group
Firmicutes
Chloroplast
Synechococcus
Alphaproteobacteria
SAR11 clade
AEGEAN-169
Betaproteobacteria
Epsilonproteobacteria
Arcobacter
Gammaproteobacteria
SAR86 clade
OM60 (NOR5) clade
Other
No Relative

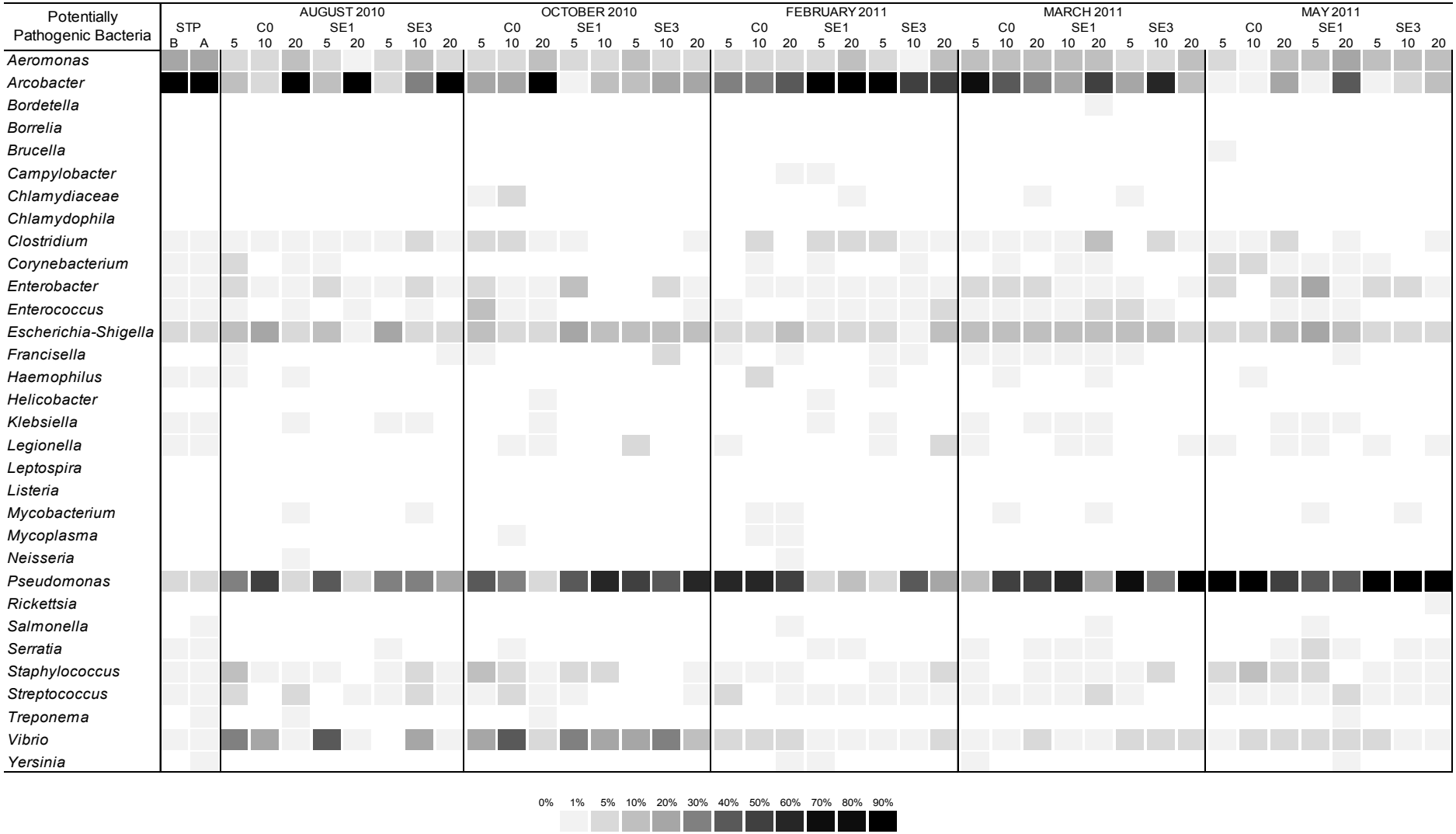
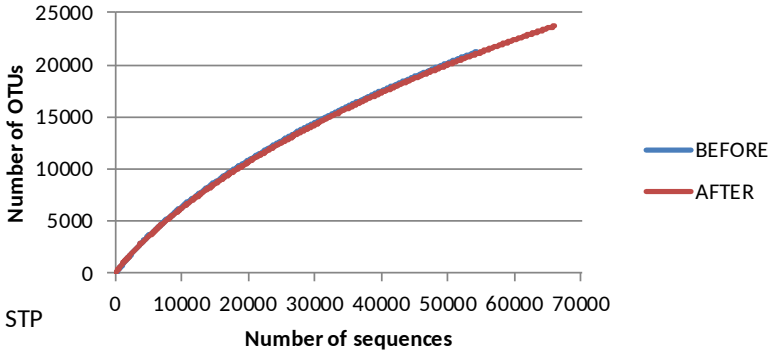
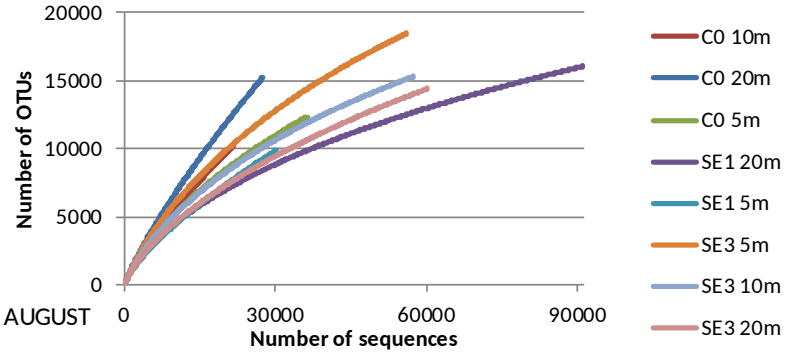

Potentially Pathogenic Bacteria
AUGUST 2010
OCTOBER 2010
FEBRUARY 2011
MARCH 2011
MAY 2011
STP
C0
SE1
SE3
B A 5 10 20 5 20 5 10 20 5 10 20 5 10 20 5 10 20 5 10 20 5 20 5 10 20 5 10 20 10 20 5 10 20 5 20 5 10 20
Aeromonas
Arcobacter
Bordetella
Borrelia
Brucella
Campylobacter
Chlamydiaceae
Chlamydophila
Clostridium
Corynebacterium
Enterobacter
Enterococcus
Escherichia-Shigella
Francisella
Haemophilus
Helicobacter
Klebsiella
Legionella
Leptospira
Listeria
Mycobacterium
Mycoplasma
Neisseria
Pseudomonas
Rickettsia
Salmonella
Serratia
Staphylococcus
Streptococcus
Treponema
Vibrio
Yersinia
0% 1% 5% 10% 20% 30% 40% 50% 60% 70% 80% 90%

Table 1. Summary of one way ANOVA (N – number of samples, df – degrees of freedom, F-ratios and p-values) for the monthly, depth and distance changes of the concentrations of fecal coliforms (FC), fecal streptococci (FS), coprostanol (COP), heterotrophic bacteria (HB), cell specific incorporation rates of leucine (Leu) and thymidine (TdR) and their ratios (Leu/TdR). Sample means are ordered by magnitude according to Tukey post hoc test.

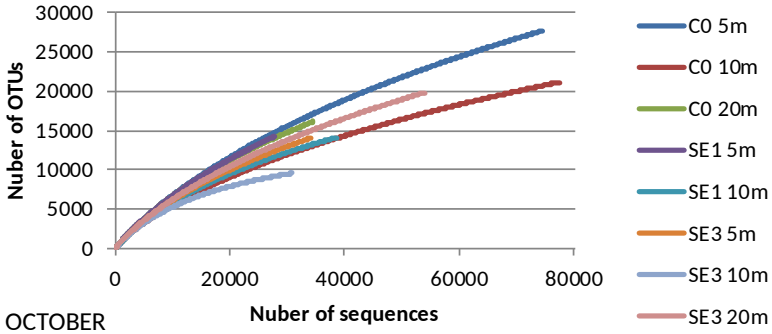
Factor	Parameter	N	df	F-ratio	p-value	Tukey rank
month	FC	195	4	1.544	>0.05	−
	FS	195	4	1.709	>0.05	−
	COP	195	4	1.673	>0.05	−
	HB	195	4	39.404	<0.05	F~May~Mar<A~O
	Leu	195	4	15.343	<0.05	May~O~A~Mar<F
	TdR	195	4	2.958	<0.05	Mar~F~O~A<May
	Leu/TdR	195	4	7.710	<0.05	May~O <Mar~F~A
depth	FC	195	2	6.145	<0.05	5 m~10 m <20 m
	FS	195	2	8.495	<0.05	5 m~10 m <20 m
	COP	195	2	3.992	<0.05	5 m~10 m <20 m
	HB	195	2	0.980	>0.05	−
	Leu	195	2	1.176	>0.05	−
	TdR	195	2	7.370	<0.05	5m~10m<20m
	Leu/TdR	195	2	2.157	>0.05	−
distance	FC	195	3	9.658	<0.05	300 m~150 m~50 m <C0
	FS	195	3	10.080	<0.05	300 m~150 m~50 m <C0
	COP	195	3	4.070	<0.05	300 m~150 m~50 m <C0
	HB	195	3	0.961	>0.05	−
	Leu	195	3	0.844	>0.05	−
	TdR	195	3	0.224	>0.05	−
	Leu/TdR	195	3	3.316	>0.05	−



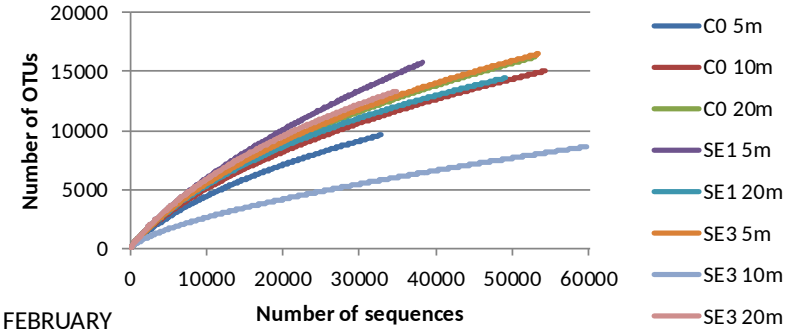
STP



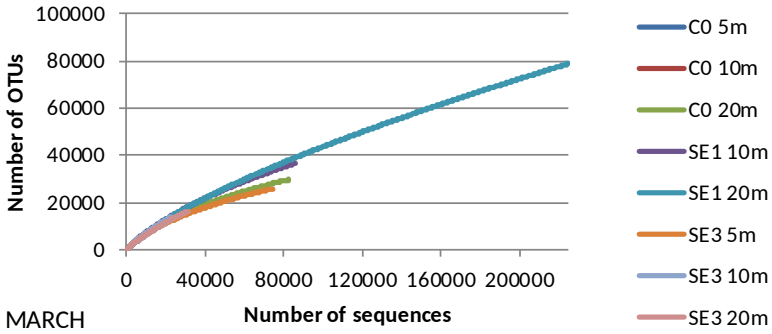
AUGUST



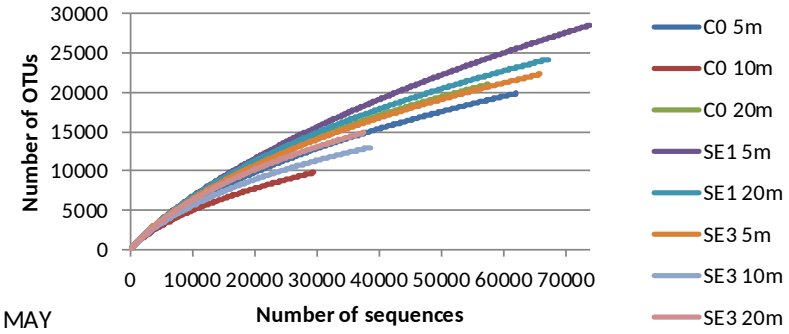
OCTOBER



FEBRUARY



MARCH



MAY

Table S1. Sequence statistics obtained by SILVAngs pipeline

Month	Station	Depth (m)	Min. Length (bp)	Avg. Length (bp)	Max. Length (bp)	Number of sequences	Number of Clustered Sequences	Number of Replicates	Number of Classified Sequences	Number of "No Relative" Sequences	Number of Rejected Sequences	Number of OTUs	Number of Singletons
	STP	Before	35	392	502	63367	23076	9917	52804	1439	9124	21247	13097
		After	35	421	501	71343	28277	14043	64373	1691	5279	23735	14129
August 2010	C0	5	35	330	502	52805	17164	7472	35634	1820	15351	12372	7439
		10	35	307	501	33225	10113	1483	21087	910	11228	10268	7684
		20	35	319	501	39935	10392	1918	26426	1222	12287	15293	12018
	SE1	5	36	404	501	34429	16261	4471	30409	611	3409	9966	6918
		20	35	439	502	94609	45532	29661	90412	931	3266	16059	7871
	SE3	5	36	431	502	59101	24822	12936	54102	2280	2719	18493	10005
		10	35	414	501	63298	26703	15810	56576	1470	5252	15300	7705
		20	36	431	501	64517	31483	14615	59670	1057	3790	14414	8123
October 2010	C0	5	35	432	501	78631	30938	16358	71830	3460	3341	27741	15708
		10	35	421	502	85569	36268	21451	77107	2379	6083	21180	11553
		20	36	411	501	37676	11401	6995	32490	2088	3098	16172	10011
	SE1	5	35	427	502	29877	10801	2833	26360	1696	1821	14319	9284
		10	36	429	501	40715	15924	8543	37254	1465	1996	14144	7596
	SE3	5	35	432	501	35771	13416	6708	33015	1271	1485	14045	8244
		10	35	433	502	32358	12608	8836	29942	1162	1254	9583	3488
		20	36	435	502	56690	23038	11171	52246	1923	2521	19855	11409
February 2011	C0	5	35	314	502	58701	19035	9079	37998	1030	19673	9660	5399
		10	35	406	502	75286	32654	17262	65828	1565	7893	15070	8064
		20	35	406	502	64616	26547	13975	56488	1520	6608	16226	8784
	SE1	5	36	412	502	44492	18660	5519	39412	1321	3759	15811	10023
		20	35	413	502	59794	24611	14707	53802	1256	4736	14477	7066
	SE3	5	35	410	501	62844	24765	14899	55635	1471	5738	16521	8657
		10	35	322	502	95543	34371	23034	66136	1030	28377	8657	5255
		20	35	425	501	39847	15757	7609	36384	1147	2316	13383	7336
March 2011	C0	5	35	293	502	31222	7132	805	18763	625	11834	11193	8812
		10	35	404	501	37474	13854	1448	32641	621	4212	17513	13587
		20	35	422	501	91595	34937	19979	83178	2299	6118	29762	15856
	SE1	10	35	441	501	89774	40045	10303	85516	2309	1949	36571	22688
		20	35	413	502	254535	128167	25881	231274	4581	18680	78865	56315
	SE3	5	35	424	501	82699	33230	17559	75968	1695	5036	25943	14113
		10	37	446	502	24968	8767	690	23300	1081	587	14122	10855
		20	35	394	501	37390	14032	1429	31777	735	4878	16460	12594
May 2011	C0	5	35	438	502	66439	29985	13179	61885	1669	2885	19889	10984
		10	35	431	501	32426	14235	6152	29671	951	1804	9846	5587
		20	35	437	501	60634	23485	13555	56808	1561	2265	21070	11362
	SE1	5	35	433	502	79625	36553	9534	72657	2585	4383	28534	17479
		20	35	441	502	71462	29865	14454	67324	1922	2216	24248	13156
	SE3	5	35	429	501	73274	29946	15606	66530	2298	4446	22327	12025
		10	36	445	502	40512	17929	8296	38495	1100	917	13063	6808
		20	35	436	501	41058	15505	8429	38195	1251	1612	14896	8208

Table S2. Number of OTUs, richness estimates (Chao1 and Abundance-based Coverage Estimator [ACE] and Shannon's diversity index following the normalisation step.

Month	Station	Depth (m)	Number of OTUs	Chao1	ACE	Shannon's Diversity Index
	STP	before	10485	29881	35249	8,75
		after	10226	29129	34367	8,62
August 2010	C0	5	8077	19536	22135	8,24
		10	9149	38312	41967	8,31
		20	11216	53584	56932	8,79
	SE1	5	7079	24745	25985	7,70
		20	6724	14751	15727	8,00
	SE3	5	9458	23778	27004	8,61
		10	7933	17982	20749	8,24
		20	7033	17599	19470	7,99
October 2010	C0	5	11225	33079	39660	8,90
		10	8786	22657	26502	8,39
		20	10846	26839	30203	8,81
	SE1	5	10838	27112	31695	8,84
		10	9215	19676	22559	8,59
	SE3	5	9640	21537	23877	8,66
		10	7660	11619	12760	8,41
		20	10123	26637	31000	8,68
February 2011	C0	5	6900	15602	16734	7,98
		10	7914	18976	21475	8,23
		20	8597	20401	23908	8,37
	SE1	5	9575	26808	31482	8,55
		20	8313	17762	20814	8,28
	SE3	5	8768	20892	24334	8,32
		10	4001	11408	11359	6,55
		20	9137	19211	21648	8,53
March 2011	C0	5	11193	59848	65846	8,74
		10	11293	53444	58180	8,80
		20	11381	32916	38608	8,97
	SE1	10	12390	45935	52303	9,10
		20	12138	49658	54574	9,02
	SE3	5	10727	28827	33781	8,87
		10	12092	46276	55159	9,01
		20	10981	50770	53808	8,77
May 2011	C0	5	9485	23760	27152	8,66
		10	7570	16548	17539	8,31
		20	10414	26021	30122	8,83
	SE1	5	11088	36054	41769	8,87
		20	10842	28605	33730	8,90
	SE3	5	10314	25660	29839	8,83
		10	8594	17554	19876	8,51
		20	9835	21461	24001	8,78

Table S3. Summary of one way ANOVA (F-ratios and p-values) for the monthly and spatial changes of total number of OTUs, richness estimates (Chao1 and ACE) and Shannon's Diversity Index. Sample means are ordered by magnitude according to Tukey post hoc test.

Factor	Richness estimator / Diversity index	F-ratio	p-value	Tukey rank
Month	OTUs	9,369	< 0,001	F ~ A ~ May ~ O < Mar
	Chao1	11,538	< 0,001	F ~ O ~ May ~ A < Mar
	ACE	11,783	< 0,001	F ~ A ~ O ~ May < Mar
	Shannon's Diversity Index	7,221	< 0,001	F ~ A ~ O ~ May < Mar
Station	OTUs	0,307	> 0,05	-
	Chao1	0,685	> 0,05	-
	ACE	0,659	> 0,05	-
	Shannon's Diversity Index	0,176	> 0,05	-