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 km² 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 m³) and October (127,925 m³) when the population was 44000 and 20000, respectively (touristic office of Rovinj, pers. comm.), the lowest in February (73,533 m³) and March (81,105 m³) when the population was 14000 (only local residents), while May (17000 residents) was close to the monthly average (104,878 m³).

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, NH₄, DIN, PO₄, 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).
High concentrations of DIN (up to 28.96 μmol L\(^{-1}\)) with the highest contribution of NH\(_4\) (up to 96%) were recorded along the 20 m layer in August in a radius of 300 m and in October only in the proximity of the outfall (C0). In February and March DIN decreased with respect to the warmer months, and was highest at 5 m and 10 m. In May, DIN concentrations were similar to the winter months but showed a distribution similar to the summer months with relative increase at 20 m (Fig. 2). During the summer the concentrations of phosphates (PO\(_4\)) were increased only in the proximity of the outfall (up to 0.36 μmol L\(^{-1}\)), while all the other values (during all samplings) were comparable to the ones typical for the northern Adriatic coastal waters (0.01 to 0.08 μmol L\(^{-1}\), data not shown). The average monthly Chl \(a\) in the area of Cuvi were within the long-term measurements for the northeastern Adriatic waters. The lowest values of Chl \(a\) were detected in August (0.28 μg L\(^{-1}\)) and October (0.31 μg L\(^{-1}\)) followed by an increase in February (0.48 μg L\(^{-1}\)) and the maximum values in May (0.55 μg L\(^{-1}\)).

3.2. Fecal pollution indicators; FIB and COP

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

At the STP the concentrations of fecal pollution indicators were very high and displayed no relevant differences before (FC: 8.3·10^8 CFU/100 ml, FS: 6.1·10^7 CFU/100 ml, COP: 1256 μg L\(^{-1}\)) and after (FC: 7.9·10^8 CFU/100ml, FS: 5.4·10^7 CFU/100 ml, COP: 1162 μg L\(^{-1}\)) the 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
Arcobacter were concentrated only around the outfall, while in February allochthonous bacteria were present in the whole water column especially near the surface (5 and 10 m) at SE1 and SE3. Interestingly, at C0 their contribution was negligible. In March, Arcobacter and Firmicutes were detected at C0 (5 m and 10 m) while a major share of Firmicutes was present at SE1 (20 m). In May, a smaller portion of allochthonous bacteria was present at C0 and SE1 only at 20 m (Fig. 6).

In August and October, Gammaproteobacteria dominated the community at 20 m increasing with distance, but were much less present in the upper layer where the gammaproteobacterial clade SAR86 became quite recognizable. During February, March and May the share of Gammaproteobacteria remained in general constant (13.9%-42.2%) in the entire water column. The presence of the SAR86 clade was remarkably high during the colder months (up to 18.4%), while it decreased in May when a higher contribution of the gammaproteobacterial clade OM60 (NOR5) was observed. Alphaproteobacteria were also abundant in marine samples throughout the year (up to 56%). In warmer months the upper layers were much enriched with this class; however in the polluted layers a consistent decrease was observed.

From February to May Alphaproteobacteria were uniformly distributed in all layers. However, different relative proportions of alphaproteobacterial members, the SAR11 clade and AEGEAN-169 marine group, appeared in various sampling months. The AEGEAN-169 marine group was important in August; the SAR11 clade in October, February and March, while in May both of them became marginal (Fig. 6). Betaproteobacteria were a minor component of the community until March and May when they became relevant, with the prevalence of the genus Limnobacter. They showed a different spatial distribution between the two months being present in polluted superficial waters in March, while in May they were characteristic for the other layers.

Regarding Bacteroidetes, the genus Bacteroides was found in marine samples at the most polluted sites. The share of other members of Bacteroidetes (NS4 and NS5 marine groups) decreased with depth during warm months, while during the rest of the year they remained fairly homogenous. In August and May, these marine groups were quite relevant in the community. Actinobacteria, consisting principally of the genus “Candidatus Actinomarina”, were mainly present in October and February with a similar abundance at all depths, except at C0 (20 m) (Fig. 6). Among the Cyanobacteria, the genus Synechococcus was recorded in August in the upper layers and only marginally in October, March and May. Prochlorococcus 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 173,580 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
Gammaproteobacteria. This class includes many opportunists and pathogens, such as Acinetobacter, Aeromonas, Escherichia/Shigella, Enterobacter, Pseudomonas and Vibrio which are considered as consistent members of sewage influents (VandeWalle et al., 2012).

The primary treatment seemed to have the lowest impact on the members of this class. Bacteroidetes and Betaproteobacteria were two minor components of the STP bacterial community. Their presence corresponded to the typical structure of fecal communities found in the human intestine. The distribution of PPB in the STP showed that the system was not effective in their reduction. Moreover, an increase in number of detected genera and their relative abundance after the treatment indicated the probable survival of PPB introduced during previous inputs into the system.

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

COP showed a remarkable correlation with other indicators of fecal pollution, FC and FS. Although those indicators were strongly associated, some differences emerged between warm and cold months as already observed in previous studies (Leeming and Nichols, 1996; Isobe 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 \(\alpha\), 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...
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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Figure captions

Fig. 1. Sampling stations in the Cuvi bay.

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

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

Fig. 4. Principal component analysis for the water temperature (T), salinity (S), concentration of orthophosphates (PO$_4$), dissolved inorganic nitrogen (DIN), ammonium (NH$_4$), fecal coliforms (FC) and streptococci (FS), coprostanol (COP), bacterial abundance (HB) and cell specific incorporation rates of leucine (Leu) and thymidine (TdR) in the water column during August 2010 (red triangle), October 2010 (yellow triangle), February 2011 (blue triangle), March 2011 (black triangle) and May 2011 (green triangle).

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

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

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

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

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

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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.

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