Research in Microbiology 165 (2014) 468–471

www.elsevier.com/locate/resmic

Brief note

Use of pyrosequencing to explore the benthic bacterial community structure in a river impacted by wastewater treatment plant discharges

Elisabet Marti, José Luis Balcázar*

Catalan Institute for Water Research (ICRA), Scientific and Technological Park of the University of Girona, Emili Grahit 101, 17003 Girona, Spain

Available online 13 April 2014

Abstract

In this study, we determined the diversity and composition of benthic bacterial communities collected in river sediments upstream and downstream from a wastewater treatment plant (WWTP). Pyrosequencing of bacterial 16S rRNA genes revealed notable differences between the communities from upstream and downstream sites. In particular, a higher relative abundance of *Acidobacteria*, *Chloroflexi*, *Deltaproteobacteria* and *Firmicutes* and a lower proportion of *Gammaproteobacteria* and *Verrucomicrobia* sequences were detected at the downstream site compared to the upstream site. These findings represent a first approximation of the impact of WWTP discharges on environmental microbial communities.

Keywords: Wastewater treatment plant; Microbial diversity; Sediment; Aquatic ecosystem

1. Introduction

Several studies have suggested that wastewater treatment plant (WWTP) discharges may affect the structure and function of microbial communities in the receiving river, both bacterioplankton from the water column and natural biofilms, since these discharges supply large amounts of organic matter, nutrients, bacteria and many chemical compounds to the receiving water bodies [1,2]. Bacteria residing on the benthos, which includes organisms living in or on sediments at the bottom of an aquatic system [3], play a substantial role in the degradation of organic pollutants, biogeochemical cycles and microbial food webs [4]. However, few studies have explored the potential effects of WWTP discharges on benthic bacterial communities.

Because conventional detection and identification of microorganisms have depended on cultivation-based techniques, our understanding of benthic bacterial communities is restricted to those that can be cultured. Nevertheless, the development of molecular techniques is providing new ways to study microbial communities in their natural environments. Current high-throughput sequencing technologies, including the Roche 454 FLX pyrosequencer, the Illumina genome analyzer and ABI Solid, generate large amounts of sequence data very rapidly and at substantially lower costs than traditional Sanger sequencing methods [5]. Moreover, these technologies provide a higher resolution and more in-depth analysis of the composition and diversity of microbial communities than traditional molecular techniques, such as sequence analysis of 16S rRNA gene clone libraries or fingerprinting methods like restriction fragment length polymorphism (RFLP), denaturing gradient gel electrophoresis (DGGE) and an automated ribosomal intergenic spacer analysis (ARISA), which may exclude a significant proportion of rare taxa [6]. Among the next-generation sequencing technologies, the 454-pyrosequencing of 16S rRNA genes is being used to explore microbial diversity from complex samples [7]. We therefore used this technology to explore the taxonomic diversity and composition of benthic bacterial communities in a Mediterranean river impacted by WWTP discharges.

* Corresponding author. Tel.: +34 972183380; fax: +34 972183248.
E-mail address: jlbalcazar@icra.cat (J.L. Balcázar).

http://dx.doi.org/10.1016/j.resmic.2014.04.002
0923-2508/

2. Materials and methods

The study was performed in September 2010 and samples were obtained from the Ripoll WWTP discharge point and from the Ter River, 100 m upstream and downstream of the WWTP discharge point. The Ter River is located in the northeast of Spain and supplies water to most cities in Catalonia. The studied WWTP operates with conventional activated sludge and receives an average daily flow about 8000 m^3 made up mostly of urban wastewater. Sediment samples (about 50 g) from each site were collected from the top layer (0–5 cm) of the river bed and were processed for bacterial community analysis. Briefly, samples were weighed, homogenized in phosphate-buffered saline solution (PBS; 10 mM sodium phosphate, 150 mM sodium chloride, pH 7.2) and resuspended in lysis buffer (20 mM Tris–HCl, pH 8.0; 2 mM sodium EDTA; 1.2% Triton X-100; and 20 mg/ml lysozyme), and genomic DNA was then extracted using a standard phenol-chloroform method. The hypervariable region V1–V3 of the bacterial 16S rRNA gene was amplified using the forward primer 27F (3′-GAG TTT GAT CNT GGC TCAG-5′) and the reverse primer 519R (3′-GTN TTA CNG CGG CKG CTG-5′). The amplicons were sequenced using Roche 454 GS-FLX titanium technology as described previously [8]. Sequences were quality-trimmed using the MOTHUR software package [9], restricted to the first 250 bp and aligned using the SILVA reference database [10]. Sequences were grouped into operational taxonomic units (OTUs) with cutoffs of 97 and 95% sequence similarity. A threshold value of 97% 16S rRNA gene sequence similarity was used to define a core set of representative sequences, which were used for phylogenetic analyses. Diversity and richness estimators were also calculated. The parsimony [11] and weighted UniFrac [12] tests were applied to determine whether two or more communities have the same structure. A Bonferroni correction was used for multiple comparisons, and the significance level was set at 0.0167 ($p \leq 0.05/3$). The Ribosomal Database Project (RDP) pipeline and classifier function were used to assign identities at a confidence threshold of 80% [13]. A phylogenetic tree was constructed using the neighbor-joining method implemented in MEGA, version 5.0 [14], and heatmaps were generated using iTOL software [15]. The sequences from this study have been deposited in the NCBI Short Read Archive under accession number SRP037755.

3. Results and discussion

A total of 29,213 sequence reads for the three samples were obtained after quality filtering and trimming. The number of sequence reads obtained per sample varied from 4468 to 7945; thus, all sequence libraries were randomly subsampled to contain the same number of sequences for α- and β-diversity comparisons. The number of OTUs was estimated at two different cutoffs, 95 and 97% 16S rRNA gene sequence similarity. A total of 1817, 1529 and 1994 OTUs (defined at 97% sequence similarity) were found in sediment samples from the WWTP discharge point, upstream and downstream sites, respectively. A more conservative approach (defined at 95% gene similarity) revealed values ranging from 1268 to 1671 OTUs (Table 1). The species diversity and richness estimators (Shannon and Chao1) showed that downstream sediment samples had higher bacterial diversity than upstream samples (Table 1), suggesting that WWTP discharges may have a significant effect on bacterial community diversity in aquatic ecosystems. This result is in agreement with those of a previous study performed in the same geographic area [16]. Direct comparisons between these studies are, however, difficult because they may be affected by several environmental factors. In fact, the sediment samples used in the previous study were obtained at the end of the spring season (water flow is maximum), whereas the samples from the present study were collected at the end of the summer season (water flow is minimum).

The effect of WWTP discharges was further confirmed using the parsimony and weighted UniFrac tests, as implemented by MOTHUR, which showed significant differences ($p < 0.001$) in community structure between samples (Table 2). Benthic bacterial communities were dominated by *Actinobacteria* and *Proteobacteria* (mainly represented by members belonging to the *Betaproteobacteria* class); however, notable differences were observed at the genus level (defined at 97% similarity) between samples collected at the upstream and downstream sites, as revealed by the phylogenetic tree in conjunction with heatmaps (Fig. 1). A higher relative abundance of *Acidobacteria*, *Chloroflexi*, *Deltaproteobacteria* and *Firmicutes* and a lower proportion of *Gammaproteobacteria* and *Verrucomicrobia* sequences were detected at the downstream site.

The phylum *Acidobacteria* is one of the most abundant phylogenetic groups in the environment and its members have been detected in different locations, including soil, sediment, freshwater, marine and polluted environments [17]. Although

Table 1
Measures of α-diversity for the sediment samples.

Samples[a]	Total no. of OTUs at		Shannon diversity index		Chao1 richness estimator	
	97% sequence similarity	95% sequence similarity	97% sequence similarity	95% sequence similarity	97% sequence similarity	95% sequence similarity
US	1529	1268	6.31	5.96	4523	2934
WWTP	1817	1506	7.20	6.79	3726	3134
DS	1994	1671	7.17	6.85	6721	4526

[a] Sediment samples were collected from the WWTP discharge point (WWTP) and from the river upstream (US) and downstream (DS) of the WWTP.