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Method paper

Taxonomic and functional diversity of a coastal planktonic bacterial community in a river-influenced marine area

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ABSTRACT

The Gulf of Naples is a dynamical area with intense exchanges between offshore oligotrophic and coastal eutrophic waters with frequent freshwater inputs. The Sarno River, one of the most polluted rivers in Europe, strongly contributes to the pollution of the area, discharging high amounts of heavy metals and organic wastes from heavily cultivated and industrial areas. This paper reports on the diversity and community structure of the marine residential Bacteria and Archaea of the Gulf of Naples in an area close to the river Sarno plume and investigates their small-scale taxonomic diversity and expression patterns as a proxy of potential metabolic activity using metagenomics and metatranscriptomics. Bacteria and Archaea were mainly represented by marine clades, with only minor contributors from freshwater ones. The community was dominated by *Alpha-* and *Gammaproteobacteria*, of which *Rhodospirillales*, *Pelagibacteriales*, and *Oceanospirilalles* were most represented. However, *Alteromonadales* and *Rhodobacterales* were the most active, despite their relative lower abundance, suggesting that they are important for overall ecosystem functioning and nutrient cycling. Nitrification and a reversed form of dissimilatory sulfate reduction were the major metabolic processes found in the metatrascriptomes and were mainly associated to *Nitrosopumilales* and *Pelagibacter*, respectively. No clear indication of transcripts related to stress induced by heavy metals or organic pollutants was found. In general, despite the high loads of pollutants discharged continuously by the Sarno River, the microbial community did not show marks of stress-induced changes neither structural nor functional, thus suggesting that this river has little or no effect on the planktonic bacterial community of the Gulf of Naples.

1. Introduction

River plumes are highly dynamic areas where environmental factors change rapidly in time and space due to dilution of freshwater into the sea. The salinity gradients are usually very steep, so as those of pH, temperature, nutrients, and organic matter, significantly challenging the physiology of the bacterial and archaeal communities (Lozupone and Knight, 2007; Lindh et al., 2013; Fodelianakis et al., 2014). In general, transitions are observed, where freshwater communities, dominated by *Alphaproteobacteria*, *Betaproteobacteria*, *Bacteroidetes*, *Verrucomicrobia*, and *Actinobacteria* (Zwart et al., 2002; Crump and Hobbie, 2005; Winter et al., 2007; Read et al., 2015) are replaced by marine communities dominated by *Alphaproteobacteria* (mainly *Pelagibacterales* and *Rhodobacteraceae*), *Gammaproteobacteria* (mainly SAR86 clade), *Synechococcus*, *Prochlorococcus*, and marine members of *Bacteroidetes* (Bouvier and del Giorgio, 2002; Crump et al., 2004, 2007).

Despite several studies describing microbial taxonomical diversity in river influenced marine systems, functional diversity in terms of metabolic functions and transcription of functional genes has not been investigated intensely. Investigations in the Amazon River (Brazil) plume found higher abundances of C- and N-related transcripts in the free living community as compared to the particle-attached microbes, where transcripts related to vitamin biosynthesis and S-cycle dominated. Both communities were dependent on nutrients and light availability (Satinsky et al., 2014a,b). Along a transect from the Columbia River (USA) to the ocean, transcripts related to photosynthesis and denitrification increased towards marine waters, even though the metabolic potential in terms of total transcripts was highly similar along the transect and the gene expression was independent from salinity changes (Fortunato and Crump, 2015). These studies point to a large variability in bacterial and archaeal taxonomic and functional diversity occurring in river-influenced marine areas.

The Gulf of Naples is a wide and rather deep coastal embayment. Its circulation patterns are ruled by the offshore Tyrrhenian Sea, which,

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mainly in winter, occupy a vast area of the bay, conferring an oligotrophic character to the water masses (Casotti et al., 2000). In its eastern part freshwater is often detected, after heavy rains or due to urban discharges along the coast. The main contributor of the freshwater presence in the marine realm is the Sarno River, whose inputs and their dispersal strongly depend upon the season and the general circulation of the area and are clearly marked during surveys. The Sarno River is not only the major source of freshwater, but also transports high amounts of different pollutants. Among these are fertilizers, pesticides and other chemicals used for agriculture, heavy metals (e.g. arsenic, mercury, cadmium, and copper) from tanneries, and inadequately treated waste waters from civil conglomerations and pharmaceutical plants (Arienzo et al., 2001; De Pippo et al., 2006; Montuori and Triassi, 2012; Montuori et al., 2013). Hence, when the Sarno River encounters the sea, the microbial communities present in the area experience not only gradients of environmental factors, such as salinity, but are also exposed to high concentrations of toxins and pollutants which potentially influence their taxonomic and functional diversity. This, in turn, may have detrimental effects on the whole ecosystem functioning and also on human health (Nogales et al., 2011).

The present study represents a snapshot of the marine bacterial community at a marine site where salinity indicates a little residual dilution of freshwater from the Sarno River. It is stressed here that our aim was to investigate the marine coastal area which is affected by the river and not the river community that encounters the sea. This is the first thorough investigation of the residential bacterial and archaeal communities in the Gulf of Naples using cultivation-independent methods. The hypothesis followed is that the influence of the pollutants carried by the river into the sea is reflected either in the structure or the activity of the marine planktonic bacterial community. This is based on the observation that many bacteria show tolerance or adaptation to pollutants such as Pb, As and Hg (e.g. Pepi et al., 2016).

Based on this hypothesis, we have investigated the residential microbial community using high throughput sequencing (Illumina) and combined with flow cytometry and a wide array of environmental data, so to assess how the river waters affect the residential marine microbes and their metabolic functioning.

2. Materials and methods

2.1. Sampling

Surface samples were taken by a rosette sampler equipped with Niskin bottles on board the RV Vettoria on November 28th 2013 at four stations (S1: 40.7245°N 14.3775°E; S2: 40.7250°N 14.3998°E; S3: 40.7255°N 14.4477°E; S4: 40.7268°N 14.4613°E) (Fig. 1).

Samples for dissolved inorganic nutrient (DIN) analyses (20 ml) were frozen on board, while those for chlorophyll (1 l) were immediately filtered onto GF/F filters (Whatman, GE Heathcare LifeSciences, Milan, Italy) and frozen in liquid nitrogen. Duplicate samples for flow cytometry (1 ml) were fixed with a mix of paraformaldehyde (0.1% final concentration) and glutaraldehyde (0.05% final concentration), flash frozen in liquid nitrogen and stored at −80 °C as in Casotti et al. (2003). For DNA and RNA sequencing, duplicate samples of 2 l of seawater were filtered sequentially through a 20 μm net and a GF/A filter (Whatman, GE Heathcare LifeSciences, Milan, Italy, nominal pore size 1.6 μm), and collected onto GSWP nitrocellulose membrane filters (Millipore, Milan, Italy; pore size 0.22 μm), except at S1, where SVGV Sterivex filters (Millipore, Milan, Italy) were used as last step, instead. All filters were immediately frozen in liquid nitrogen and stored at −80 °C.

2.2. DIN and chlorophyll a analyses

DIN concentrations were measured by colorimetry as in Hansen and Grasshoff (1983), using a FlowSys Autoanalyzer (Systea SpA, Italy). Chlorophyll samples were analyzed by spectrofluorometry as in Holm-Hansen et al. (1965).

2.3. Flow cytometry

Cell concentrations of *Synechococcus*, *Prochlorochoccus* and total heterotrophic bacteria were estimated using a FACScalibur flow cytometer equipped with a 488 nm Ar laser and standard filter set (BD Biosciences, Franklin Lake, USA). Heterotrophic bacteria were stained with SYBR Green I (Invitrogen, ThermoFisher, Monza, Italy), final concentration 10^{-3} of stock solution, for 15 min in the dark before flow cytometry, while *Synechococcus* and *Prochlorochoccus* cell counts were determined from unstained samples, based on natural fluorescence from phycoerythrin (orange) and chlorophyll (red), respectively as in Casotti et al. (2003). Data acquisition was performed by CellQuest software (BD Biosciences, Franklin Lake, USA) and analysis using FCS Express 4 Plus Flow Research Edition software (DeNovo Software, Glendale, USA).

2.4. Metagenomics

DNA of a single sample from each station was extracted using the Qiagen DNAeasy Blood and Tissue kit (Qiagen, Milan, Italy). Due to the lack of replicates, statistical analyses of the results were not performed and therefore the results should be considered only qualitative.

Sequencing for metagenomic analyses was realized by Genomix4life S.r.l. (Baronissi, Italy) using Illumina MiSeq platform retrieving 300 bp long paired-end reads. The retrieved metagenomes were deposited in

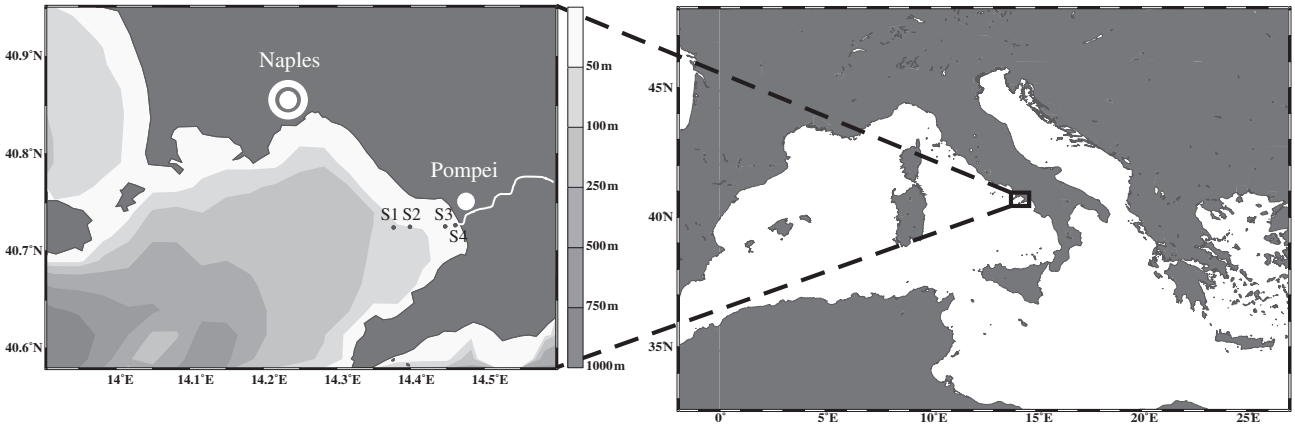

Fig. 1. Map of the sampled area (https://odv.awi.de/). The insert indicates a detail of the Gulf of Naples with the four sampled stations (S1 to S4) from the more offshore (S1) to the most river-influenced (S4) in terms of salinity.