BACTERIAL COMMUNITY STRUCTURE AND THEIR CHANGES IN CONTAMINATED ECOSYSTEMS

Extended abstract

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Background

Within the different ecosystem compartments, bacterial diversity reflects the natural and stressed environmental conditions to which they are subject. The presence of contaminants can lead to the disappearance of key species and/or to the selection of microbial groups able to partially or completely remove such molecules.

Environmental contamination may result in changes in the microbial ecology, possibly changing the types of bacteria that carry out important ecosystem processes such as nutrient transformation and biomass decomposition. Microbial biodiversity in fact has a functional importance in the maintenance of soil and water biological processes, because most of the transformation involved in biogeochemical cycles is mediated exclusively by microorganisms. It has been reported that shifts in bacterial community structure, associated with a reduction in microbial biodiversity, lead to losses of functional stability (Girvan et al., 2005). Owing to their small size, large numbers and ubiquitous distribution in the environment, microorganisms are valuable indicators of the occurrence of disturbances due to exogenous physical-chemical stressors. The assessment of variations in microbial community structure is of basic importance in making it possible to evaluate the impact of an environmental stressor. The presence of toxic chemicals in microbial ecosystems induces the synthesis of detoxifying or degradative enzymes and certain stress proteins. Effects due to chemical toxicity tend to narrow the spectrum of microbial diversity because organisms that are not capable of resisting the toxic effects either die or enter a static metabolic phase, leaving those that have evolved resistance mechanisms and are thus capable of utilizing the excess chemicals as nutrients, to proliferate and become dominant members of the impacted ecosystems (Ogunseitan, 2000).

In recent years, it has been recognized that biodegradation and/or mineralization of some contaminant molecules is only possible through the presence of microorganisms able to transform them (Topp et al., 2013). Consequently some relationships between microbial communities and pollutants have been established. Among pollutants, pharmaceuticals including antibiotics, for both human and veterinary use, are frequently found as microcontaminants both in water and in soil ecosystems. Pharmaceuticals are molecules designed to produce a therapeutic effect on the body, usually active at very low concentrations, can pass through biological membranes and persist in the body long enough to avoid being inactivated before having an effect. These compounds are excreted through feces and urine as a mixture of metabolites and substances which are often unchanged. The primary sources of pharmaceutical contamination are represented by domestic, urban, hospital, and industrial wastewater, as well as by effluents from sewage treatment plants (STPs), aquaculture, and intensive livestock farming. Moreover, re-use of solid and liquid livestock manure and sewage sludge in agriculture, in order to recycle nitrogen compounds as fertilizers, can contribute to the dispersion of pharmaceuticals into soil and, under certain conditions, into water bodies. At present, the presence of antibiotics, steroids, blood lipid regulators, estrogens, painkillers, anti-inflammatories, antiseptics, antihypertensive drugs, anti-epileptics, antineoplastic agents and other substances in surface water bodies and in soils receiving livestock manure or sewage sludge, even at very low concentration (ng-µg/L or Kg), is well-documented (Boxall, 2004). Percolation of pharmaceuticals into groundwater and their presence in sea coastal water have been detected as well. The environmental occurrence of these intrinsically and biologically active molecules may cause direct (toxicological effects on non-target organisms) and indirect effects such as...
antibiotic resistance (Bottoni et al., 2010). People can be exposed to pharmaceuticals through polluted water or by consumption of contaminated food.

Non-PCR-based methods commonly used in environmental studies are epifluorescence microscopy techniques, such as Fluorescence In Situ Hybridization (FISH) (Barra Caracciolo et al., 2013; Barra Caracciolo et al., 2010). They make it possible to characterize in situ bacterial populations in their natural ecosystems. In particular FISH investigates the overall taxonomic composition of bacterial communities by using rRNA-targeted fluorescent probes. This technique is also used for testing the efficient remediation of xenobiotic pollutants by microbial communities (Whiteley and Bailey, 2000). In fact the ability to monitor diversity structuring, stability and long-term resilience during process management are key requirements for monitoring and predicting bioremediation efficiency.

**Objectives**

In this work, through the sensitive Fluorescence In Situ Hybridization molecular method, we analyze the bacterial community of two different contaminated ecosystems, water and soil, and changes in their structure related to the presence of two different group of pharmaceuticals (anti-inflammatory drug and veterinarian antibiotics) in order to better investigate the effect of the selected compounds on the natural microbial community and to assess the microbial populations involved in their degradation. A description of Fluorescence In Situ Hybridization (FISH) technique, used in the two study cases, is reported; it was able to assess the phylogenetic characterization of active microbial communities in their natural environments (water and soil). The sensitivity of the FISH method in determining changes in microbial community structure related to the presence of contaminants is presented.

**Outline of the work**

This work reports two study cases:

- First, we considered a freshwater bacterial community in the presence of a non-steroidal anti-inflammatory drug, naproxen, in the Tiber River, Rome, Italy. Experiments with Naproxen, considered a high priority pharmaceutical, were conducted on water samples collected in the North of Rome. An experimental set consisting of 32 destructive closed microcosms each (100 mL capacity) was set up. Aliquots from the drug working standard solution were spiked to treated microcosms to a final concentration of 100 µg/L of naproxen. Spiking and all operations were conducted under sterile conditions and, once the solvent (acetonitrile) had completely evaporated at room temperature in order to eliminate any additional carbon source, 50 mL of the river water (natural or previously sterilized) was added to each microcosm. All microcosms were incubated at 20°C on an orbital shaker (125 rpm) in the dark. FISH analyses were conducted on the natural initial condition and at the end of the experiment, when chemical analyses confirmed the biodegradation of the naproxen.

- The second case deals with experimental plots chronically contaminated by some veterinarian antibiotics (mixture of Sulfamethazine, Tylosin and Chlorotetracycline), located in the Agriculture and Agri-Food Canada experimental farm in London, Canada. These antibiotics are commonly used as livestock production for growth promotion, prophylaxis and treatment of illness. This experiment was conducted on twelve 2 m² plots that received annually for 11 years a mixture of the three selected antibiotics to achieve concentrations for each drug of 0.01, 0.1 and 1 mg/kg soil to a depth of 15 cm. Drugs were applied each June by mixing in aqueous mixtures of the antibiotics to 1 kg of soil from each plot. Three plots were left untreated as controls. Before the experiment the soil had not been treated with materials that could have carried drugs. Plots were cropped continuously to soybeans during each growing season, and received no further management. The control plots were otherwise managed exactly as described for the treated. Starting in 2005, the annual applications were increased 10-fold to achieve concentrations of 0.1, 1 and 10 mg/kg. Each microplot was sampled for FISH analysis 5 months after the last antibiotic application in order to evaluate the long term effect of the antibiotics on the bacterial community.

**Methods**

The evaluation of bacterial biodiversity is mainly limited by their small size, by the absence of distinguishing phenotypic characters, and by the fact that most of these organisms cannot be cultivated. The number of techniques for studying microbial communities has increased exponentially over the last 20 years and the advent of culture-independent methods, such as molecular biological techniques, has changed the view of microbial diversity. Fluorescence in situ hybridization (FISH) method makes it possible to identify microorganisms in situ using fluorescently labeled ribosomal RNA targeted oligonucleotide probes. FISH combines the precision of molecular genetics with the visual information from microscopy, allowing visualization and identification of individual cells in their natural microhabitat, so that nucleic acid sequences can be examined inside cells without altering their morphology or the integrity of their various compartments.

The FISH technique for detecting RNAs has been introduced into living cells using probes that fluoresce only when hybridized. In situ identification of individual microbial cells with fluorescently labeled rRNA-targeted oligonucleotide probes (phylogenetic stains), is based on the high cellular content of ribosomes, which can be found in living organisms and, consequently, like many 16S or 23S rRNA molecules. rRNAs are the main target molecules because they are relatively stable and include both variable and highly conserved sequence domains. The selection of particular regions of the rRNA molecule then enables phylogenetic specificity to be varied from the universal to the subspecies level. Under appropriate reaction conditions, complementary sequences in the probe and target cell
anneal, and the site of probe hybridization is detected by fluorescence microscopy. The most common target sequences are in bacterial 16S rRNA, but other ribosome subunit sequences have also been used. In the FISH approach it is assumed that actively growing microbes have many ribosomes and should theoretically yield brighter fluorescence signals owing to higher rRNA-targeted probe hybridizations. Probes hybridize to whole bacterial cells, resulting in the selective staining of target cells. Fluorescent probes suitable for use in FISH have been developed at a variety of taxonomic levels (e.g., universal, or domain-, family-, or species-specific) and they can be designed based on phylogenetic trees. The FISH protocol includes four steps: fixation and permeabilization of the sample, hybridization, washing to remove un-bound probe and detection of labeled cells by microscopy. Prior to hybridization, bacteria must be fixed and permeabilized in order to allow penetration of the fluorescent probes into the cell and protect the RNA from degradation by endogenous RNAs. Hybridization must be carried out under stringent conditions for proper annealing of the probe to the target sequence. For this crucial step of the FISH procedure, a pre-heated hybridization buffer is applied to the sample containing fluorescently labeled probes complementary to the target RNA. The hybridization takes place in a dark humid chamber, usually at temperatures between 37 and 50°C. Stringency can be adjusted by varying either the formamide concentration or the hybridization temperature. The oligonucleotide probes typically consist of 18 to 30 nucleotide bases conjugated to a fluorescent marker on the 5' end which allows detection of probes bound to cellular rRNA by fluorescence microscopy. Multiple group-specific rRNA probes targeting microbial taxa can be used in a FISH experiment for simultaneous phylogenetic classification as well as quantification of physiologically active microbial populations in an environmental sample. The phylogenetic composition of the bacterial communities of the two ecosystems we considered was analyzed. At the domain level, EUB338 probes (EUBI, II and III) targeting Bacteria were used. Inside this domain various Cy3-labelled commercially synthesized oligonucleotide probes were applied (Table 1). Each surface water or soil subsample was filtered through a 0.2 µm polycarbonate membrane using a gentle vacuum (soil subsamples were pre-treated in order to eliminate particulate loads that interfere with probe fluorescence, Barra Caracciolo et al., 2005). FISH of the harvested cells, counterstained with DAPI, was performed according to published protocols (Barra Caracciolo et al., 2005). The average number of cells binding each of the probes was calculated as a proportion of the total DAPI positive cells from 10 to 20 randomly selected fields on each filter section (corresponding to 500-1000 stained cells). The slides were examined and counted on a Leica DM 4000B epifluorescence microscope at x1000 magnification.

### Table 1. Probes and their characteristics applied in hybridization

<table>
<thead>
<tr>
<th>Probes</th>
<th>Corresponding Taxa</th>
<th>Sequence (5'3')</th>
<th>rRNA position</th>
<th>% Formamide</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALF1b</td>
<td>Alpha-Proteobacteria</td>
<td>CGTTGCTGTCCTGAGCCAG</td>
<td>16S(19-35)</td>
<td>20</td>
</tr>
<tr>
<td>BET42a</td>
<td>Beta-Proteobacteria</td>
<td>GCTTCCCCACTTCGTTT</td>
<td>23S(1027-1043)</td>
<td>35</td>
</tr>
<tr>
<td>GAM42a</td>
<td>Gamma-Proteobacteria</td>
<td>GCTTCCCCAATGTTT</td>
<td>23S(1027-1043)</td>
<td>35</td>
</tr>
<tr>
<td>DELTA43a</td>
<td>Delta-Proteobacteria</td>
<td>AGTACCGGTAGTCTCTCT</td>
<td>16S(495-512)</td>
<td>35</td>
</tr>
<tr>
<td>DELTA43b</td>
<td>Delta-Proteobacteria</td>
<td>AGTACCGGTAGTCTCTCT</td>
<td>16S(495-512)</td>
<td>35</td>
</tr>
<tr>
<td>EPS51d</td>
<td>Epsilon-Proteobacteria</td>
<td>AGTACCGGTAGTCTCTCT</td>
<td>16S(495-512)</td>
<td>35</td>
</tr>
<tr>
<td>PLA46</td>
<td>Planctomycetes</td>
<td>GACTTGCATGGCTTA</td>
<td>16S(46-63)</td>
<td>30</td>
</tr>
<tr>
<td>PLA86</td>
<td>Planctomycetes</td>
<td>GCTTGCATGCCTCAA</td>
<td>16S(46-63)</td>
<td>30</td>
</tr>
<tr>
<td>CF319a</td>
<td>Bacteroidetes</td>
<td>TTGTCGCGTCCTCGTAC</td>
<td>16S(319-336)</td>
<td>35</td>
</tr>
<tr>
<td>LGCG35a</td>
<td>Firmicutes</td>
<td>TGGGAAGATTCCCTACGTC</td>
<td>16S(354-371)</td>
<td>35</td>
</tr>
<tr>
<td>LGCG35b</td>
<td>Firmicutes</td>
<td>CCGAAGATTCCCTACGTC</td>
<td>16S(354-371)</td>
<td>35</td>
</tr>
<tr>
<td>LGCG35c</td>
<td>Firmicutes</td>
<td>CCGAAGATTCCCTACGTC</td>
<td>16S(354-371)</td>
<td>35</td>
</tr>
<tr>
<td>HGL69a</td>
<td>Actinobacteria</td>
<td>TATATTACCCCGCGGT</td>
<td>23S(1901-1918)</td>
<td>23</td>
</tr>
</tbody>
</table>

### Results and discussion

In natural river water samples the Bacteria dominant group was the Beta-Proteobacteria (data not shown). The comparison of the autochthonous bacterial community analyzed 20 days after the naproxen-treatment in the microcosms shows changes in the abundance and shifts in the dominance of several groups. In fact their values were more than 10 times lower than those in the control soil. This effect at low concentrations may be due to a suppressive combination in which multiple antibiotics were used, associated with an acquired resistance to the treatment, except bacterial groups were unaffected 5 months after the treatment, as it is possible to see in the graph. As it is possible to see in the graph, most of bacterial groups were unaffected 5 months after the treatment, except Planctomycetes (Pla) and Bacteroidetes (CF). Planctomycetes displayed a significant decrease in number (t test, p <0.01) in soils treated with low and medium concentrations (0.1 and 1 mg/kg) of antibiotics; in fact their values were more than 10 times lower than those in the control soil. This effect at low concentrations may be due to a suppressive combination in which multiple antibiotics were used, associated with an acquired resistance.
to one antibiotic used and enhanced susceptibility to the others (suppressive combination). The decrease in their number may also have been due to a competitive advantage of populations belonging to other bacterial groups that did not undergo inhibitory effects from the presence of the antibiotics, such as the Alpha-, Beta- and Gamma-Proteobacteria groups. However, the Planctomycetes in soil treated at a high concentration (10 mg/kg) had abundance values comparable to those of the control soil, showing no long-term effects at that concentration. Planctomycetes is a microbial population with intrinsic resistance to many antibiotics due to the absence of the peptidoglycan layer in their cell wall and they are naturally resistant, for example, to penicillins, which interfere with the synthesis of peptidoglycan.

**Bacteroidetes** phylum (CF) increases in number at the highest concentration (10 mg/kg) and this could be due to a resistance mechanism. The overall results show not only that antibiotic occurrence can affect bacterial population dynamics, but they also can induce resistance mechanisms via bacterial conjugation among different soil populations. The impacts of antibiotics on microbial soil populations are therefore surprisingly complex and further studies are necessary to better investigate induction of antibiotic resistance. The latter cause particular concern because can be acquired by pathogenic microorganisms, reducing the effectiveness of antibiotics.

**Concluding remarks**

In this work we report, as example, the effects of selected contaminants on bacterial communities of two different ecosystems, soil or surface water. Contaminants affect natural bacterial communities causing directly or indirectly changing in community structure like dominance and/or disappearance of some bacterial groups with potential loss of important ecosystem functions. The overall results suggest the usefulness of bacterial structure and functioning studies as microbiological indicators for assessing freshwater and soil quality states.

**Keywords:** antibiotics, contaminants, FISH, microbial community structure, pharmaceutical

**Acknowledgements**

The authors thank particularly Ed Topp - Agriculture and Agri-Food Canada - for his help and for supplying soil samples from the experimental farm in London, Canada.

**References**


