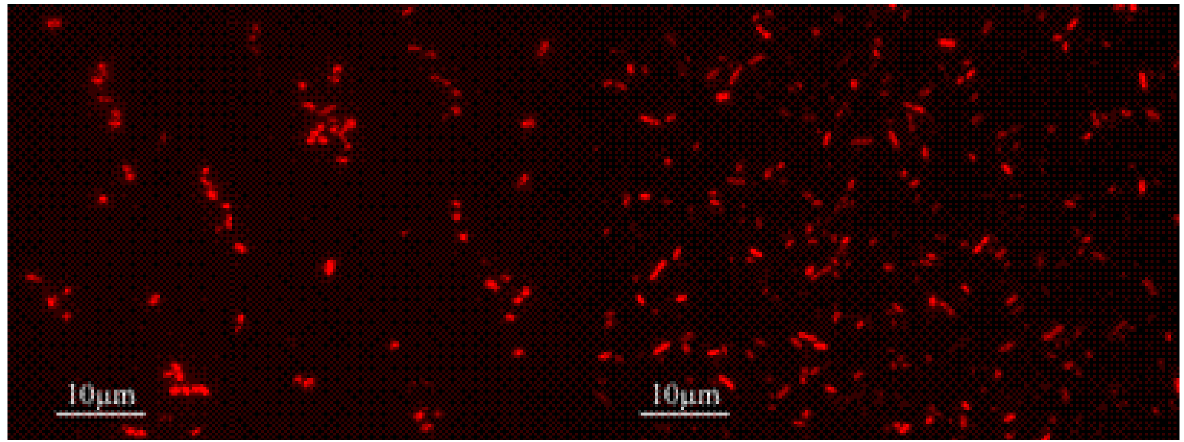
EFFECTS OF PESTICIDES AND PHARMACEUTICALS ON SOIL AND WATER BACTERIAL COMMUNITIES



Paola Grenni

PhD thesis in Environmental Science
University of Milano-Bicocca

UNIVERSITA' DEGLI STUDI DI MILANO-BICOCCA

Facoltà Di Scienze Matematiche, Fisiche, Naturali
Dipartimento di Scienze dell'Ambiente e del Territorio

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EFFECTS OF PESTICIDES AND PHARMACEUTICALS ON SOIL AND WATER BACTERIAL COMMUNITIES

Coordinatore Dottorato
Prof. Marco Vighi

Tutor
Dott. Antonio Finizio

Dottoranda
Paola Grenni

Revisore esterno
Dott. Albero Puddu

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Cover illustration - Images of Fluorescence *in situ* Hybridization assays, under the Confocal Laser Microscope, of pure cultures of *Advenella incenata* and *Jantinum lividum* isolated from groundwater (see experimental research *Role of a groundwater bacterial community in terbuthylazine herbicide degradation: laboratory microcosms experiment with groundwater samples collected from a s-triazine chronically contaminated aquifer*).

Effects of pesticides and pharmaceuticals on soil and water bacterial communities

Paola Grenni

Università Milano-Bicocca, Facoltà Di Scienze Matematiche, Fisiche, Naturali, Dipartimento di Scienze dell'Ambiente e del Territorio

Subject headings: biodegradation; microbial community of soil, surface and groundwater; herbicides (terbuthylazine, linuron, simazine); pharmaceuticals (antiviral drug Tamiflu, macrolide antibiotics erythromycin and josamycin).

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ABSTRACT

Studying soil and water ecosystems using a microbial ecology approach, which analyses the diversity and functioning of microbial communities, can help in evaluating the impact of environmental stressors, such as xenobiotics. In fact, soil and water microorganisms play an important role in maintaining ecosystem environmental quality. For instance, the ability of soil and water to recover from chemical contamination is primarily dependent on the presence of a microbial community with the ability to remove it. Furthermore the microbial community characteristics of an ecosystem can indicate changes in resource availability and the presence of pollution. In this way, the microbial community represents an important key to understanding the impacts of environmental and anthropogenic factors on ecosystems.

This thesis focuses on two groups of xenobiotics frequently detected in soil and water: pesticides and pharmaceuticals. The effects of *s*-triazine and phenylurea herbicides (terbuthylazine, simazine and linuron) and pharmaceuticals (such as the antiviral drug Tamiflu and two macrolide antibiotics, erythromycin and josamycin) on microbial communities in soil, surface and groundwater were studied. The researches was carried out utilizing innovative molecular techniques in order to identify the natural microbial populations involved in the chemical degradation and evaluate the direct and indirect effects of the herbicides and pharmaceuticals on the microbial community structure and functioning.

For this purpose several investigations involving the selected chemicals in soil and water ecosystems were carried out:

- Research activity regarding *s*-triazine groundwater contamination caused by diffuse sources. Although the groundwater was considered to have been without life for a long period and unable to recover from herbicide contamination, the experiments performed showed the presence of an autochthonous bacterial community able to degrade the herbicide terbuthylazine. Moreover some bacterial strains such as *Janthinobacterium lividum, Advenella incenata* and *Rhodococcus wratislaviensis*, with the capability of growing on various *s*-triazines were isolated. Among the three isolates, *Rhodococcus wratislaviensis* was also capable of mineralising the triazine-ring. Finally, an oligonucleotide probe was designed to detect this strain in soil and water samples by applying the *in situ* fluorescence hybridization technique. This probe can be very useful for monitoring the presence of active *R. wratislaviensis* populations with the potential to degrade *s*-triazines in contaminated aquifers and agricultural soils. The research activity was accomplished thanks to active cooperation between IRSA-CNR in Rome and Madrid Complutense University.
- Research activity regarding soil herbicide contamination caused by point sources. The experiments aimed at evaluating the effects of the co-presence of herbicides (linuron and terbuthylazine) and wood amendments on soil bacterial communities. These amendments have recently been proposed for their adsorption capacity, which prevents the mobility of

pesticides caused by point sources of contamination. The herbicide degradation and the microbial activity in the presence/absence of pine and oak amendments were assessed and compared. The amendments did not negatively affect bacterial community functioning in terms of dehydrogenase activity. The use of wood amendments can thus be effective in limiting the mobility of herbicides in soils. However the capacity of pine-amended soils to adsorb linuron and terbuthylazine was so high that it hampered herbicide degradation and this should be taken into account since it can modify the persistence of these herbicides. This research was performed by IRSA-CNR together with Spanish CSIC.

- Research on the assessment of the effects of pharmaceutical waste disposal on bacterial communities in soil and groundwater. The research regarded an open quarry contaminated by pharmaceutical residuals (erythromycin and josamycin) owing to its previous improper use for disposal of pharmaceutical waste by a factory producing macrolide antibiotics. The microbiological community characteristics (bacterial abundance, diversity, viability and activity), proposed as microbial indicators, together with chemical investigations of soil and groundwater samples, made it possible to evaluate the quality state of the site.
- Research on the effects of the antiviral drug Tamiflu (recommended for the treatment of cases of avian and swine influenza) on the bacterial community of a surface ecosystem. The role of the bacterial community in the antiviral degradation was demonstrated in microcosm experiments and some bacterial groups, analyzed by fluorescence *in situ* hybridization, were found to increase in number when there was a halving of the pharmaceutical. This research was performed by IRSA-CNR together with Bologna University.
- Research on variations in bacterial community structure in soil, surface water and groundwater in the presence of herbicides (terbuthylazine, simazine and linuron) or pharmaceuticals (Tamiflu). The research shows that fluorescence *in situ* hybridization was a useful tool for following the dynamics of individual microbial populations in the ecosystems considered and highlighted the presence of particular groups presumably involved in chemical degradation.

INTRODUCTION

1. Microbial ecology approach for evaluating the effects of contaminants on soil and water ecosystems

Microbial ecology studies the relationship between microorganisms and their environment. A major aim of it is to study the abundance, localization and activities of microorganisms *in situ* in order to understand their ecophysiological roles in natural ecosystems. Understanding microbial ecology is extremely important, because the relationships between microorganisms and their environments have a crucial role in environmental restoration, food production and bioengineering of useful products such as antibiotics, food supplements and chemicals. Using microbes to study ecology helps to understand complex ecosystems because bacteria are small but with very large populations and can be studied thanks to their speed of reproduction, which is rapid when compared to larger organisms. Biological and biochemically mediated processes in soil and water are significant for ecosystem functions (Zabaloy et al., 2008). In fact, microorganisms have a key role in biogeochemical cycles and ecosystem energy flow, plant productivity and environmental health in pristine ecosystems (Desai et al., 2009). Since microbes play a critical role in carbon and nutrient transformations, any change in their population and activity may affect the cycling of nutrients as well as their availability, thus indirectly affecting soil and water functions (Wang et al., 2008). For example, soil microflora, mainly bacteria, fungi, algae and protozoa makes a valuable contribution to making soil fertile through their primary catabolic role in the degradation of plants and animal residues and in the recycling of the organic and inorganic nutrients content of soil. In fact, arable soils have been reported to harbour several hundred species per gram of soil and pasture and forest soils may harbour more complex communities consisting of several thousand species (Torsvik et al., 2002). Microbial ecology recognizes that microorganisms have a large homeostatic capability *vis-à-vis* xenobiotic substances. In fact, natural microbial communities harbour an amazing physiological versatility and catabolic potential for the breakdown of an enormous number of organic molecules, thanks to their great adaptability to different conditions. Microbial versatility also extends to the majority of synthetic compounds introduced into natural metabolic cycles (Alexander, 1971; Knackmuss, 1996). Microbes are thus among the most important biological agents in removing and degrading waste materials, enabling their recycling in the environment. They are able to colonize contaminated sites and metabolize some recalcitrant xenobiotics, for example pesticides (Galvao et al., 2005). For these reasons they are essential in global processes contributing to the state of human health and environmental quality in pristine ecosystems (Atlas & Bartha, 1997; Desai et al., 2009). However the presence of a xenobiotic, for instance after pesticide application, may favour certain species, resulting in these communities becoming less complex (Torsvik et al., 2002). Agricultural treatments have been reported to influence soil microbial community structures

(Rousseaux et al., 2003; Marschner et al., 2004; Hartmann et al., 2006; Widmer et al., 2006) and to decrease soil bacterial diversity (Lupwayi et al., 1998; Torsvik et al., 2002). Moreover pesticides that disrupt the activities of soil microorganisms can be expected to affect the nutritional quality of soils and, therefore, have serious ecological consequences (Handa et al., 1999).

The assessment of variations in microbial community structure is of fundamental importance for the evaluation of the impact of an environmental stressor.

The occurrence of contaminants in natural microbial communities can significantly affect the abundance and activity of microbial populations and harm some crucial ecological functioning.

In particular, it is possible to observe:

1. changes in community structure in terms of dominance and/or disappearance of some bacterial groups with consequent loss of important ecosystem functions;
2. occurrence of some bacterial populations adapted to xenobiotics and able to use it as a source of carbon and/or nitrogen.

Laboratory studies suggest that there is a relationship between disturbance, such as the presence of a xenobiotic, bacterial diversity and spatial biological diversity (Buckling et al., 2000; Johnsen et al., 2001; Kozdroj & Van Elsas, 2001; Horner-Devine et al., 2004), (Figure 1).

1.1 Degradation of xenobiotics by microorganisms

Studying soil or water systems using an ecological approach is a necessary pre-requisite for improving the understanding of their structure (biodiversity) and functioning (Bardgett, 2002; Windinga et al., 2005). In particular studying microbial communities in presence/absence of a xenobiotic may improve the knowledge of the ecosystems. In fact, the fate of environmental pollutants is determined by abiotic processes (e.g. photooxidation) and, overall, by metabolic activities driven by microorganisms.

In recent years it has been generally recognized that xenobiotic biodegradation and/or mineralisation is only possible with the presence of microorganisms (mainly bacteria and microfungi), which transform the molecules through metabolic and co-metabolic processes (Topp et al., 1997; Sinha et al., 2009). Consequently, some relationships between microbial communities and pollutants have been established. For example, in chemically polluted soils, microbial diversity is reduced and community structure is altered in comparison to control soils (Barra Caracciolo et al., 2005a).

Since bacterial metabolism is very versatile, strains with the specific capability to remove xenobiotic compounds could be used for the bioremediation of polluted sites and waters (Desai et al., 2010). There are several mechanisms, or combinations of them, by which microbial communities can adapt to the presence of xenobiotics in their environment. Firstly, there can be an increase in population size of those organisms that tolerate or even degrade the compounds by induction of appropriate genes. Secondly, their cells can adapt through

mutations of various kinds, such as single nucleotide changes or DNA rearrangements that result in resistance to or degradation of the compounds.
Microorganism pesticide degradation characters are located on plasmids and transposons. Understanding of the characters provides clues to the evolution of degradative pathways and makes the task of gene manipulation easier through the creation of genetically engineered microbes capable of degrading the pollutants.

Thirdly, they may acquire genetic information from either related or phylogenetically distinct populations in the community by horizontal gene transfer (HGT) – also called ‘lateral gene transfer’, or xenobiotic catabolic mobile genetic elements (MGEs), like plasmids (Liu & Sulfita 1993; Springael & Top, 2004). It has been widely accepted that pollutant-degrading enzymes have evolved from isozymes (enzymes that differ in amino acid sequence but catalyse the same chemical reaction) in response to industrial production and environmental release of xenobiotics (Wackett, 2004; Singer, 2006). Distinct catabolic genes are either present on mobile genetic elements, such as transposons (a small segment of DNA that can move from one region of DNA to another) and plasmids (small molecules of circular, extrachromosomal DNA found in some bacteria), or the chromosome itself, which facilitates horizontal gene transfer and enhances the rapid microbial transformation of toxic xenobiotic compounds (Sinha et al., 2009). Eventually the individual cells best adapted to resisting or degrading the xenobiotic get selected and their populations increase in number compared to others in the microbial community (Top & Springaely, 2003; Janssen et al., 2005).

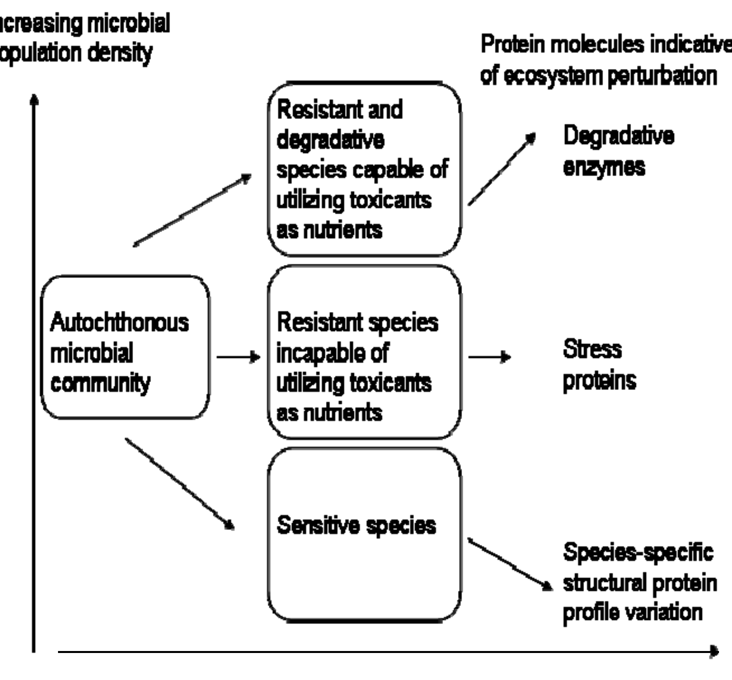

Figure 1. Schematic representation of microbial community in response to environmental perturbations. Toxic chemicals, for example, can cause changes in microbial population densities and diversity (Ogunseitan, 2000).

Protein molecules mediate these effects by virtue of the ability of each species to synthesize degradative enzymes or otherwise engage in repair mechanisms through the activities of stress proteins and modified structural components (Ogunseitan, 2000). Monitoring these proteins provides information on toxic chemical fates (biodegradative enzymes) and effects (toxicity-induced changes in protein profiles). Complex microbial communities may therefore serve as ideal and ecologically relevant toxicity indicators (Brandt et al., 2004).

A number of microbiologically-driven processes have been proposed for evaluating the effects of xenobiotics on ecosystems (Wagner-Döbler et al., 1992; Nazaret et al., 1994; Moyer et al., 1994; Griebler & Slezak, 2001). Proteins, genes, metabolites, or lipids that, when expressed, present a pattern of molecular change in an organism in response to a specific environmental stressor, can be defined as environmental biomarkers.

1.2 Groundwater ecosystems and their contamination

Until the 1970s, scientific concepts and methods limited the knowledge of groundwater microbiology. It was common to assume that the groundwater environment was devoid of life because methods for sampling them for microbes were very limited. Moreover, it was generally assumed that water passing through the soil was purified by active microbial processes and by filtration; there was therefore little concern about groundwater contamination. As it became more and more evident during the 1980s and awareness grew of the importance of aquifer microbiota for ecosystem services and functioning, including the provision of drinking water, the motivation for understanding groundwater environments increased. In addition, new methods in microbiology, based on advances in molecular biology, provided microbiologists with new tools to explore these ecosystems. In fact, the development of sampling techniques suitable for microbiological investigations and the application of both cultivation-based and molecular methods have yielded a much greater insight into microbial communities in contaminated aquifers (Griebler & Lueders, 2009). However knowledge of microbial biodiversity in natural ecosystems is still poor at present.

Groundwater ecosystems are generally devoid of photosynthesis and lack inputs of fresh, easily available organic carbon. These factors are among the most important differences from surface and groundwater ecosystems (Gibert, 1994). Microbial communities within aquifers are thus expected to consist largely of heterotrophs well adapted to the nutrient-poor and oligotrophic groundwater environment (de Lipthay et al., 2003). Lithoautotrophs, which fix carbon dioxide and meet their energy requirements by oxidising inorganic electron donors, are another important component of groundwater microbial communities (Stevens & McKinley, 1995).

In groundwater microorganisms have the same roles in nutrient cycles and they mitigate numerous chemical processes in subsurface ecosystems, including contaminant degradation and immobilization, redox cycling and nutrient transport (Griebler & Lueders, 2009). For examples, natural attenuation of contaminated groundwater has been achieved *in situ* (Tuxen

et al., 2002; Williams et al., 2003) or in a laboratory setting (using indigenous bacteria from contaminated sites) with a limited number of pesticides (Mirigain et al., 1995; Johnson et al., 2000; Pucarevic et al., 2002; Harrison et al., 2003). To date, however, there have been few studies on natural attenuation in *s*-triazine contaminated groundwater.

Several novel phylogenetic lineages have been found in groundwater habitats, but to date no clearly 'endemic' subsurface microbial phyla have been identified. The future will show if the rather low diversity generally found in pristine oligotrophic aquifers is a fact or just a result of the low abundances and insufficient resolution of today's methods. Factors identified for controlling microbial diversity in aquifers include spatial heterogeneity, temporal variability and disturbances such as pollution by chemical anthropogenic contaminants.

Although first insights into the importance of individual biogeochemical processes may be obtained from surveys of microbial diversity within functional groups, direct links to groundwater ecosystem functioning have rarely been established so far. A large number of contaminants have been found in groundwater, including pesticides and pharmaceuticals. Pesticides are intrinsically toxic because of their biocide properties.

They are among the most commonly found and known groundwater contaminants and are therefore regulated (Dir. 91/414/EEC, Dir. 2000/60/EC, Dir. 2006/118/EC). Furthermore the frequent occurrence of their metabolites, which are often toxic like their parent compounds, causes concern. Pharmaceuticals are considered "emerging contaminants". They have only recently been detected in water and groundwater and are not yet regulated (Richardson & Ternes, 2005). Finally, there is a general concern about the co-presence of different chemical compounds (e.g. pharmaceuticals, pesticides etc.) which have different modes of action and can constitute multiple stressors for ecosystems.

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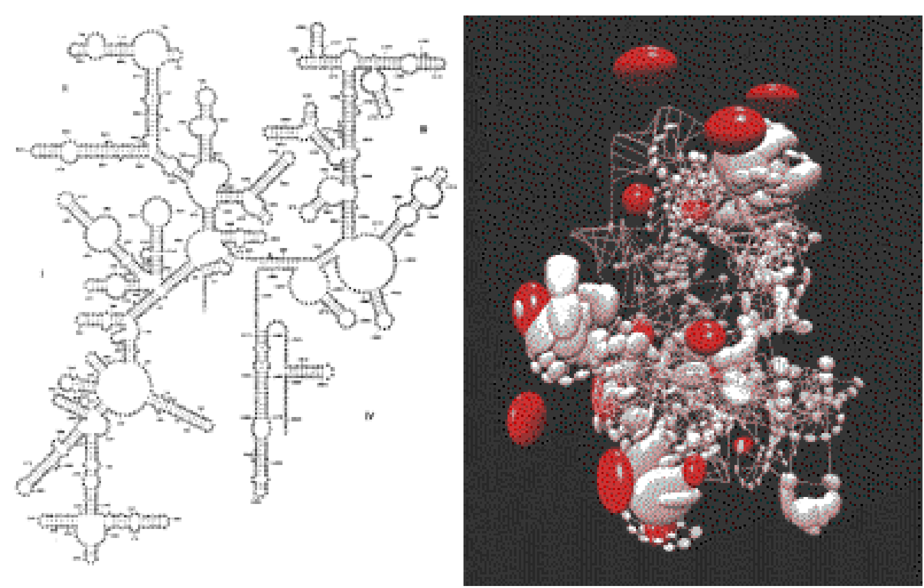
1.3 Microbial ecology methods

The assessment of variations in microbial community structure and functioning is of basic importance for the evaluation of the impact of an environmental stressor. Despite the importance of soil and water microbial communities in biogeochemical cycles and in xenobiotic biodegradation, there has been little research into such communities because this would necessarily involve methods capable of identifying and characterizing the community structure and functioning. Traditional methods for microbial identification are based on the cultivability of the microorganisms on selective media, which is laborious, time-consuming and prone to statistical and methodological errors (Moter & Göbel, 2000). According to classical microbiology, in order to study a microorganism it is necessary to isolate it from the original matrix, and this isolation can be done only on the appropriate medium, from the plates showing separately grown colonies, i.e. from the plates corresponding to the so-called countable dilutions. Moreover, it has been demonstrated how any environmental modification

during cultivation could affect the structure of microbial communities, thus preventing a complete view of the ecosystem considered. Therefore, with isolation and cultivation in an agar medium it is possible to characterize only those microorganisms capable of growing, multiplying and forming colonies in the selected medium and growth conditions, with the loss of information about the microbial component, which presents a vitality condition despite not being able to duplicate it in a culture medium. Moreover it has been estimated that only < 1% of bacterial species are currently known and cultivable in soil, water and groundwater (Amann et al., 1995). In addition, traditional methods are based on differences in morphology, growth, enzymatic activity and metabolism, but these phenotypic characters are not always enough to define genera and overall species (Torsvik et al., 2002).

On the contrary, with molecular methods, it is possible to overcome this kind of identification limitation and classify all organisms. With advances in molecular biology and the sequencing era, a modern classification tool has appeared based on the small subunit of ribosomal ribonucleic acid (16S rRNA for *Bacteria* and *Archaea*, and 18S rRNA for *Eucarya,* Figure 2).

Because of their universal distribution, high conservation and absence of interspecies transfer, rRNAs are considered the most useful biopolymers for comparative analyses. Sequencing the small-subunit 16/18S rRNA and comparative analysis of the sequences have been used most commonly to construct the natural universal phylogenetic tree which divides organisms into three main branches: *Bacteria*, *Archaea* and *Eucarya* (Woese et al., 1990; Woese, 2000). Consequently, full and partial 16S rRNA gene sequencing methods have emerged as useful tools for identifying microorganisms.



Left: two-dimensional map of the secondary structure of 16S RNA.
Right: low-resolution model of the large ribosomal subunit in bacteria.

Figure 2.

Whole groups of uncultivated and uncharacterized microorganisms, known only from their nucleic acid sequences, have now been classified using comparative analyses of the nucleotide sequences of genes encoding ribosomal 16s rRNA (Woese et al., 1990; Woese, 2000). Figure 3 shows the relationships between major branches of life, the *Archaea*, *Bacteria* (Prokaryotes) and *Eucarya*, as well as the major branches within the Prokaryotes based on these gene sequences (Woese, 2000).

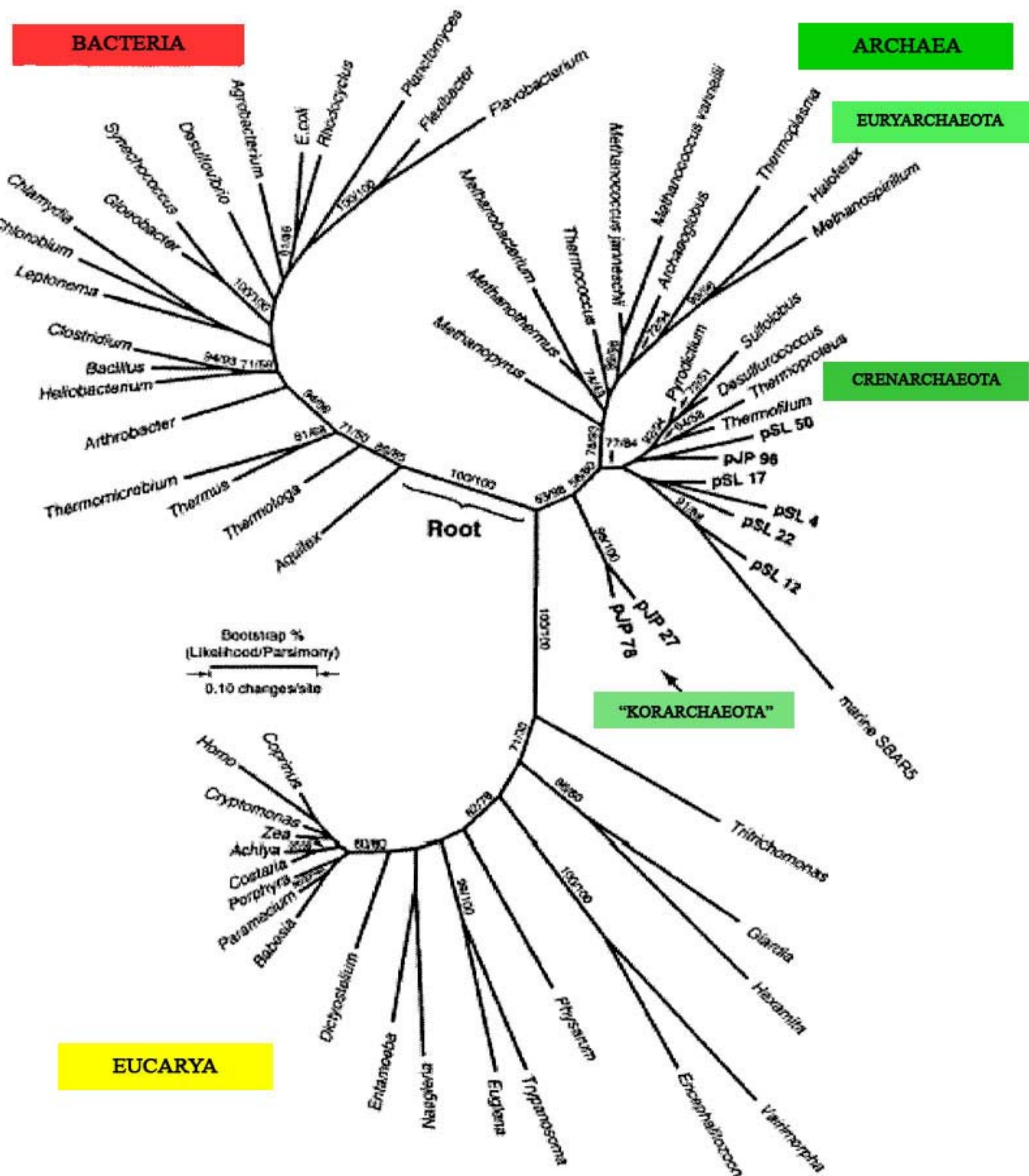


Figure 3. Universal phylogenetic tree based on small-subunit 16S rRNA sequences. There are three Domains, *Bacteria, Archaea* and *Eucarya*. The evolution distance between two organisms is a proportion of the measurable distance between the branches (Barns et al., 1996, modified).

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 (Rossello-Mora & Amann, 2001). Consequently, while microbiologists were previously limited by their inability to characterize uncultured organisms, the advent of so-called 'cultivation independent' methods has provided researchers with the ability to determine the composition of microbial communities and identify numerically important, but not yet cultured, organisms (Forney et al., 2004). The study of natural microbial communities could lead to the identification of new bacterial species with specific characteristics *vis-à-vis* contaminants. However, to identify a new species both phenotypic and genotypic methods are necessary and polyphasic taxonomy is the most appropriate approach for bacterial classification, because it takes into account all available phenotypic and genotypic data and combines them (Uilenberg & Goff, 2006). Moreover, the presence of certain bacterial indicators or bacterial genes involved in the degradation of a pollutant can indicate sources of contamination in an environment and complex microbial communities, determined by molecular techniques, may serve as ideal and ecologically important toxicity indicators (Brandt et al., 2004). There are a wide array of molecules, including nucleic acids, lipids and proteins, which are useful for diagnosing microbial responses to pollution and for monitoring environmental management strategies. The presence of toxic chemicals in microbial ecosystems, for example, induces the synthesis of detoxifying or degradative enzymes and certain stress proteins. Among these techniques it is possible to distinguish between those which are primarily based on the use of Polymerase Chain Reaction (PCR) and those that are non-PCR-based (Figure 4).

PCR uses specific primers to amplify a DNA target sequence. The bacterial 16S rDNA gene is today the most commonly used for assessing overall diversity in microbial communities and for studying the phylogeny of microorganisms. Sequence variations in PCR fragments are detected either by a cloning/sequencing analysis, which provides a complete characterization of the fragments, or by an electrophoretic analysis, which provides a visual separation of the mixture of fragments. Fragment separation is based on sequence polymorphism, in Denaturing Gradient Gel Electrophoresis (DGGE) or length polymorphism, Terminal-Restriction Fragment Length Polymorphism (T-RFLP) and Automated Ribosomal Intergenic Spacer Analysis (ARISA). DGGE is frequently used in environmental studies (Ibekwe et al., 2001; Guo et al., 2009).

Quantitative Polymerase Chain Reaction (qPCR and qRT-PCR) has become a commonly used technique for the detection and quantification of microorganisms in the environment because of its high sensitivity at low concentrations (Dionisi et al., 2003; Devers et al., 2004; Zhang & Fang, 2006; Kim et al., 2007).

It can be used to detect changes in gene expression a pattern induced by adverse conditions and also does not require prior knowledge of expected contaminants, using non-specific stress responses as general indicators of deleterious conditions (Van Dyk et al., 1995).

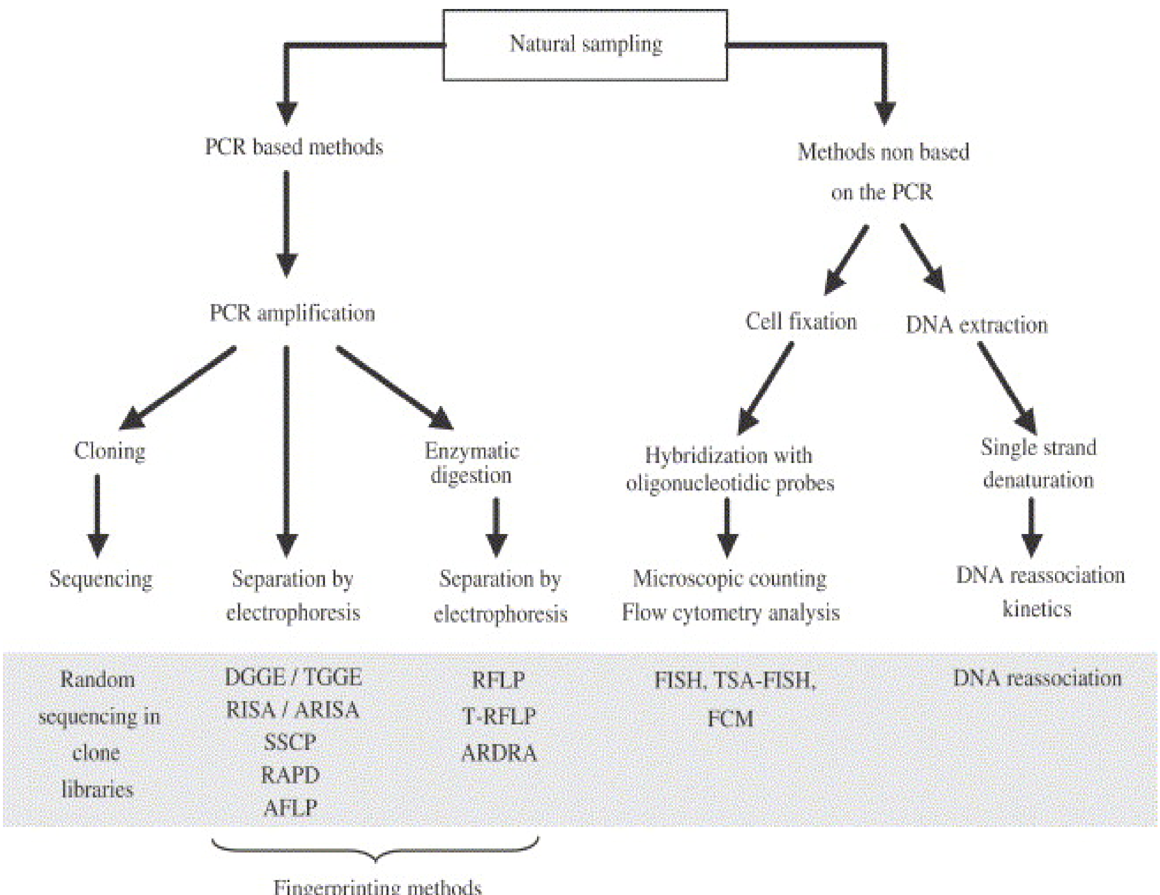


Figure 4. Diagram of the different molecular approaches for assessing the genetic diversity of microbial communities. FISH (Fluorescence *In Situ* Hybridisation) was used in this study (from Dorigo et al., 2005).

Thanks to the new methodological approaches, the exploration of microbial biodiversity has taken a quantum leap forward and permits the observation of microbial populations in complex communities. Methods that allow *in situ* observation of microorganisms and microbial activities have been especially important in the development of our understanding of the roles of microorganisms in nature. The use of molecular tools, such as gene probes and polymerase chain reaction (PCR), are essential for further scientific advances in microbial ecology.

Non-PCR-based methods commonly used in environmental studies are epifluorescence microscopy techniques, such as direct count of bacterial abundance (DAPI count), vitality (Live/Dead cell viability assay) and Fluorescence *In Situ* Hybridization (FISH). They make it possible to characterize *in situ* bacterial populations in their natural ecosystems.

Bacterial abundance is a structural parameter in assessing bacterial communities (Eaton et al., 2005; Hammes et al., 2008; Kepner et al., 1994; Pascaud et al., 2009). An abundant bacterial community is an essential pre-requisite for its response to environmental stress. The more bacteria there are in an ecosystem, the greater the likelihood that some bacterial

populations can resist and/or adapt to it. The direct count method using the fluorochrome staining technique is widely employed for enumerating bacteria in natural environments. This technique is the most reliable and inexpensive procedure for estimating total bacterial abundance. The most commonly used dye, 4',6- diamidino-2-phenylindole (DAPI) (Kepner & Pratt, 1994; Seo et al., 2010; Barra Caracciolo et al., 2005a, 2005b, 2005c) makes it possible to distinguish bacteria (which appear in a luminescent blue colour) from non-living bacterium-sized particles (which appear yellow) under epifluorescence microscopy.

Cell viability is generally measured on the basis of cell membrane integrity, which is a well-accepted criterion for characterising viable (active or inactive) cells and distinguishing them from damaged and membrane-compromised ones by using different stains (e.g. SYTO9 or SYBR Green II and propidium iodide; Handbook of Fluorescent Probes and Research Chemicals, 6th ed., Molecular Probes, Inc.). These stains produce green fluorescent staining of bacteria with intact cell membranes (SYTO9 or SYBR Green II) and red fluorescent staining of bacteria with damaged membranes (propidium iodide), which can be visualized under an epifluorescence microscope. The ratio of green to red fluorescence intensities provides a quantitative index of bacterial viability (Amalfitano et al., 2008; Alonso et al., 2002). This information is of major importance in research into the function of microbial communities in natural environments, because it assigns the bulk activities measured by various methods to the active cells that are effectively responsible for them. The application of cell fluorescence-based methods makes facilitates fast and accurate viability analysis and monitoring of microbial populations in ecological environments (Hammes & Egli, 2010).

Fluorescence *in situ* hybridisation (FISH) investigates the overall taxonomic composition of bacterial communities by using rRNA-targeted fluorescent probes.

Each of the above-mentioned techniques can provide different information for the analysis of environmentally significant genes in microbial communities exposed to toxic chemicals and direct detection of genes involved in maintaining key biochemical functions at the microbial level (Ogunseitan, 2000).

1.4 Fluorescent *in situ* hybridisation

The presence of xenobiotics can affect microbial community structure and change microbial diversity. In some cases, a xenobiotic presence narrows the spectrum of microbial diversity, because organisms that are not capable of resisting the toxic effects either die or enter a static metabolic phase. On the contrary, microorganisms which have evolved resistance mechanisms, can utilize the excess chemicals as nutrients, to proliferate and become dominant members of the impacted ecosystem (Ogunseitan, 2000).

Of the various microbiological methods mentioned above this section includes a detailed description only of Fluorescent *In Situ* Hybridisation (FISH) because it is that is able to identify active microbial communities in their natural environment. The sensitivity and reliability of this method are shown in several research activities in the experimental part of this thesis (see Experimental Research section).

The technique, first applied by De Long et al. (1989), is based on the hybridization of synthetic dye-labelled oligonucleotide probes to specific regions within the bacterial ribosome and does not require cultivation (Figure 5). FISH originated in medicine and developmental biology for the localization of particular DNA sequences in mammalian chromosomes. Owing to its sensitivity, it has subsequently been applied primarily in environmental bacteriology (Amann et al., 1995) and to a lesser extent in ecology (Lim et al., 1996). In fact, this technique is considered a powerful tool for phylogenetic, ecological, diagnostic and environmental studies in microbiology. FISH is a rapid and highly specific method for the whole-cell identification of bacteria (Amann et al., 1995) and investigation of the overall taxonomic composition of bacterial communities.

The FISH technique is also used for testing the efficient remediation of xenobiotic pollutants by microbial communities (Whiteley & Bailey, 2000). In fact the ability to monitor diversity structuring, stability and long-term resilience during process management is key requirements for monitoring and predicting bioremediation efficiency.

Figure 5. FISH schematic protocol.

FISH has so far been applied to the study of microbial symbiosis and microbial diversity in environmental samples and wastewater treatment (Pernthaler et al., 2001). It is also routinely used in medicine as a diagnostic tool for the identification of bacteria in complex communities colonising the oral cavity and the respiratory and gastro-intestinal tracts, as well as for the detection of pathogens within human and animal tissues (Moter & Göbel, 2000). FISH combines the precision of molecular genetics with the visual information from microscopy, allowing visualization and identification of individual cells within their natural microhabitat or diseased tissue, so that nucleic acid sequences can be examined inside cells without altering their morphology or the integrity of their various compartments (Moter & Göbel, 2000).

FISH with rRNA target probes has been developed for the *in situ* identification of single microbial cells and is the most commonly applied among the non-PCR based molecular techniques (Amann et al., 1990; Pernthaler et al., 2001; Moter & Göbel, 2000; Martín et al., 2008). It allowed significant advances in resolution, speed and safety, and later paved the way for the development of simultaneous detection of multiple targets, quantitative analysis and live-cell imaging (Levsky & Singer 2003). This technique allows a deeper study of live gene expression in a minimally disturbed context, but their interpretation needs to take into consideration the possible artefacts that may result as physiological ramifications of hybridization (Levsky & Singer 2003). 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 labelled rRNA-targeted oligonucleotide probes, the so-called phylogenetic stains, is based on the high cellular content of ribosomes, which can be found in all living organisms and, consequently, like many 16S and 23S rRNA molecules (Amann & Kuhl, 1998). rRNAs are the main target molecules because they are relatively stable and include both variable and highly conserved sequence domains (Pernthaler et al., 2001). The selection of particular regions of the rRNA molecule then enables phylogenetic specificity to be varied from the universal to the subspecies level (DeLong et al., 1989; Amann et al., 1990), even if because of the relatively slow mutation rate of rRNA, this molecule generally possesses no target sites that differentiate between strains of prokaryotic species (Wagner et al., 2003). Under appropriate reaction conditions, complementary sequences in the probe and target cell anneal, and the site of probe hybridization is detected by fluorescence microscopy (DeLong et al., 1989; Amann et al., 1990).

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 hybridisations. Probes hybridize to whole bacterial cells, resulting in the selective staining of target cells (Amann et al., 1995; Moter & Göbel, 2000).

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 (Figure 3). A typical FISH protocol (Figure 5) includes four steps: fixation and permeabilisation of the sample, hybridization, washing to remove unbound probe and detection of labelled cells by microscopy or flow cytometry (Pernthaler et al., 2001).

Prior to hybridization, bacteria must be fixed and permeabilised in order to allow penetration of the fluorescent probes into the cell and protect the RNA from degradation by endogenous RNAs (Moter & Göbel, 2000). 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 labelled 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. These conditions vary from probe to probe and optimal conditions maximize probe binding while minimizing nonspecific binding (Bouvier & del Giorgio, 2003). Detailed protocols for FISH are available in published literature (Hugenholtz et al., 2001; Daims et al., 2005; Bottari et al., 2006).

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 (Figure 6), confocal laser scanning microscopy or flow cytometry. The probes generally applied in experimental research for detecting the main known bacterial groups are shown in Table 1.

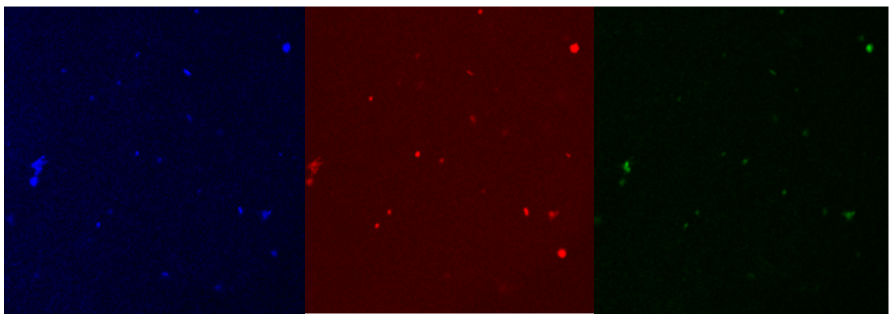


Figure 6. Images in a confocal microscope of bacteria detectable by DAPI-staining (blue colour), by EUB338 for *Bacteria* domain (red, Cy3-labeled) and by a specific probe, α-*Proteobacteria* (green, Flu-labelled) in environmental samples.

At the domain level, EUB338 probes (I, II and III) target *Bacteria*, while *Archaea* are stained by the ARCH915 probe and *Eukaryota* by the EUK516 probe. More specific probe sets for lower taxonomic levels are described in published literature, or can be constructed using consensus rDNA data published on large public access databases (e.g., the Ribosomal Database Project II, Cole et al., 2005). Alternatively, a useful public access database for

selection of FISH probes at various taxonomic levels that have been catalogued from published literature is probeBase (http://www.microbial-ecology.net/probebase/default.asp; Loy et al., 2007). As new 16S rDNA sequences and oligonucleotide probes are continuously being identified and added to public access databases, the consensus sequences need to be periodically checked. Multiple group-specific rRNA probes targeting prokaryotic and eukaryotic 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.

By conjugating probes with different fluorochromes (Table 2) to select microbial targets, several target organisms can be visualized at the same time. Probe fluorochromes should be chosen to yield the largest degree of contrast under autofluorescence to components of soil or water. Epifluorescence microscopes utilised in FISH analysis, use epi-illuminators with small 50 or 100 watt super pressure mercury lamps.

Using a matched set consisting of an exciter filter, dichroic beam splitter and barrier filter, the epi-illuminator allows only light of the correct wavelength to strike the sample preparation and only light of the longer emitted wavelength to reach the eyepiece or the camera. The microscopes are often accompanied by a digital camera, which offers the possibility of using digital images for enumeration.

Although FISH is employed in various matrices, its application to soil samples is hampered by the presence of inorganic particles and clays owing to their high interference with visualization using epifluorescence microscopy. The main drawback is that it is difficult and often impossible to distinguish cells among particles and this strongly hinders the identification and enumeration of bacteria in such samples. Thus, the availability of an optimized procedure for detaching and separating cells from inorganic particles would make it possible to apply this powerful technique for monitoring bacterial populations in soil or similar matrices like aquifers and sediment (Wallner et al., 1993). For this reason we have published (Barra Caracciolo et al., 2005b) a cell extraction procedure based on the use of density gradient cell separation, which makes it possible to apply FISH to the analysis of native soil and sediment bacteria without any pre-enrichment on cultivation media. This extraction procedure is successful and permits a perfect visualization and quantification of bacteria. This approach is therefore promising for *in situ* detection of indigenous bacterial communities in complex samples.

Table 1. Oligonucleotide probes used for bacterial community characterization.

Probe name and target group	Ribosomal target and position	Stringency (% formamide)
ARCH915 *Archaea*	16S rRNA 915 – 934	20 %
EUB338 *Bacteria*	16S rRNA 338 – 355	0-50 %
EUB338II *Bacteria*	16S rRNA 338 – 355	0-50 %
EUB338III *Bacteria*	16S rRNA 338 – 355	0-50 %
ALF1b *α-Proteobacteria*	16S rRNA 19 – 35	20 %
BET42a *β-Proteobacteria*	23S rRNA 1027-1043	35 %
GAM42a *γ-Proteobacteria*	23S rRNA 1027-1043	35 %
HGC69A Gram+ High DNA G+C content	23S rRNA 1901-1918	25 %
Pla46 *Planctomycetes*	16S rRNA 46 – 63	30 %
Pla886 *Planctomycetes*	16S rRNA 886 – 904	30 %
CF319a Cytophaga– Flaviobacterium cluster phylum CFB	16S rRNA 319 – 336	35 %
LGC354a *Firmicutes* (Gram+ bacteria with low G+C content)	16S rRNA 354 – 371	35 %
SRB385 Sulfate-Reducing Bacteria of the *Deltaproteobacteria* group	16S rRNA 385 – 402	30 %
EPS710 Sulfur-Reducing heterotrophic *Epsilon*	16S rRNA 710 – 728	30 %

Table 2. Most commonly employed fluorescent dyes to label oligonucleotides for FISH analysis

Fluorochrome	Colour	Max. excitation λ (nm)	Max. emission λ (nm)
Alexa488	Green	493	517
AMCA	Blue	399	446
CY3	Red	552	565
CY5	Red	649	670
CY7	Violet	743	767
DAPI	Blue	350	456
Fluorescein	Green	494	523
Rodamine	Red	555	580
TAMRA	Red	543	575
Texas red	Red	590	615
TRITC	Red-orange	550	580

Abbreviations: *AMCA* methyl cumarinic acetic acid, *CY* carbocyanine, *DAPI* 4′6-diamidino-2-phenylindole dihydrochloride, *TAMRA* tertramethyl rhodamine, *TRITC* tetramethylrodamine-isotiocianate.

2. Xenobiotics as environmental contaminants

Environmental pollution caused by the release and/or use of a wide range of compounds as a consequence of human development has now assumed serious proportions. Global anthropogenic pollution has led to the accumulation of a wide variety of organic xenobiotic compounds causing detrimental effects to human health and pristine ecosystems. In particular xenobiotics are compounds that do not exist as natural products or may contain structural elements that cannot be synthesized biochemically (Rieger et al., 2002; Leisinger, 1983). As a result, they are a potential risk for the environment and human health.

Among xenobiotics, pesticides and pharmaceuticals are the most frequently found as pollutants in soil and water ecosystems, because they are used widely and in large quantities.

2.1 Pesticides

Pesticides include herbicides, fungicides, insecticides and rodenticides, which are specifically aimed at weeds, fungi, insect and rodents, respectively.

Pesticide usage has gradually become an integral part of modern agriculture systems, improving the quantity and quality of world food production, as illustrated by the fact that more than half a million tons of pesticide products are used each year in the United States (US EPA, 2010) and about 320,000 tons of active substances are sold each year in the European Union (EC, 2010). Pesticides are also used in large quantities in Italy; it has been estimated that about 150,000 tons of agrochemicals are used every year on about 13,000 hectares (ISTAT, 2010).

There are several active compounds and related pesticide products currently authorised in Italy (Ministry of Health, List of active ingredients; ICPS, PESTIDOC database; FOOTPRINT Pesticide Properties Database) and listed in Annex 1, Dir. 91/414 CEE.

2.1.1 Contamination pathways

As generally recognised, as a consequence of the widespread use of pesticides, one important unwanted effect of their use is their frequent and widespread transfer from the site of application to surface waters (river, lakes and wetlands) and groundwater, determining water resource deterioration, including of that used for drinking purposes, as reported in several data monitoring reports (ISPRA, 2010a).

Once contaminated these sources are not usable for drinking purposes if above legal limits (>0.1 $\mu g\ L^{-1}$). Pesticides in drinking water (Cabras & Angioni, 2000) may have adverse effects on human health: carcinogenesis (Blair et al., 1985), neurotoxicity (Tanner & Laangston et al., 1990) and effects on cell development (Gray et al., 1994) are the possible chronic effects deriving from these compounds. The scientific community has shown great concern about these risks, which are confirmed by results from major monitoring studies

performed over 20 years ago (Hörmann et al., 1979) and by more recent investigations (Otto et al., 2007; Guzzella et al., 2006a).

Pesticide contamination is caused by diffuse sources and point sources. Diffuse sources are mainly related to drift losses and run-off during application and drainage discharge from treated fields. Point sources are mainly related to the handling of pesticides during transport, storage, filling, cleaning, and management of liquid residues and disposal of empty packages (Figure 7).

Research results show that point source pollution can be the main entry route for pesticides into surface water and contributes to more than 50% of total pesticide contamination (Bach et al., 2005; Müller et al., 2002).

Figure 7. Illustration of the phases of pesticide management which can cause point source water contamination. Source: TOPPS (www.topps-life.org). PPP: pesticide products

Table 3 summarises the different routes which have been identified and classifies them as deriving from diffuse or point sources.

In the case of agricultural land, once they are applied, pesticides and their metabolites can enter the soil, air and water. In fact they are involved in several simultaneous processes, including volatilization, emission, wash off, sorption/desorption, runoff, leaching, plant uptake and finally degradation (Figure 8).

Wash off amends the pesticide load in the soil and thus alters the amount of pesticide available for movement by runoff and sediment.

Pesticide volatilization (diffusion of pesticides through the soil and atmosphere) and pesticide emission from the plant canopy or soil surface to the atmosphere usually depends on vapour pressure and the chemical vaporization heat of the chemicals, the partition coefficient between the atmosphere and any other phase and the air flow mass which transports the chemicals when airborne. In addition to these factors, pesticide volatilization and emission are affected by the form of pesticide application (spray application is more susceptible to volatilization than soil incorporation.

Pesticides can be adsorbed/desorbed by soil particles. Adsorption refers to the adhesion or attraction of ions or molecules to the soil particles' surface and it depends on soil characteristics (organic matter content, pH, soil particle size distribution, temperature and moisture content) and pesticide characteristics (molecular structure, electrical charge, solubility). The degree of adsorption-desorption between soil and pesticide influences the bioactivity, leachability and degradability of these chemicals in a given environment and affects their distribution through the soil profile.

Table 3: Sources of water contamination by pesticides

Diffuse	Point
spray drift	tank filling
volatilisation and precipitation	spillages
surface runoff/overland flow	faulty equipment
leaching	washing and waste disposal
Through flow/inter flow	sumps, soakaways and drainage
Drain flow	direct contamination including overspray
base flow seepage	consented discharges

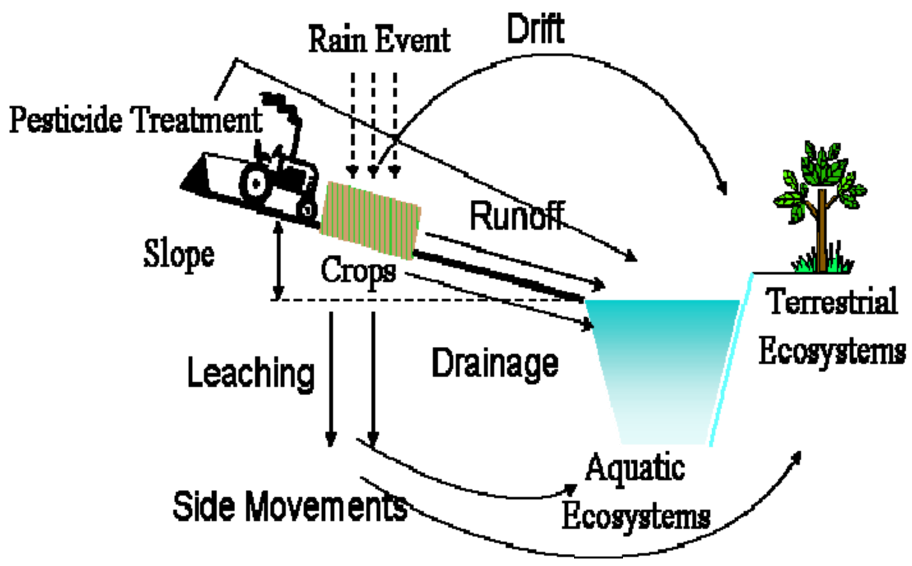


Figure 8. Environmental fate of pesticides.

Pesticides carried by surface runoff from agricultural areas constitute a significant portion of the pesticide pollutants entering surface water bodies. The transport of pesticides in surface runoff depends on the form in which the compounds exist in water and the hydrodynamics of the system. A pesticide molecule can exist either in the dissolved phase or it can be associated with a particle or colloid. In the dissolved phase, transport of the pesticide will be governed essentially by water flow.

Leaching of pesticides through soil is an environmental concern because of the possibility of their reaching deeper soil and/or the water table and contaminating groundwater. However, whether a pesticide reaches groundwater depends not only upon its movement through the soil, but also upon its disappearance from the soil. If, for instance, the rate of degradation is sufficiently rapid compared to the rate of leaching, the chemical disappears before it can reach the groundwater and, therefore, does not pose an environmental problem. The determination of soil leaching rates is important because the rate at which a pesticide is leached indicates how long it is retained in the top soil, where it is most subject to degradation or dissipation. However, leaching of a pesticide through the soil profile is strongly influenced by preferential flow. Preferential flow is the process in which water and solutes rapidly move through soil macropores, bypassing much of the soil matrix. Several factors such as size, geometry and distribution of macropores affect preferential flow (Köhne et al., 2009a and 2009b).

The uptake of pesticides by plants is affected by many factors. The most important are the plant species, growth stage and intended use. Soil characteristics such as pH, temperature, clay fraction, moisture content and particularly organic matter content also influence the uptake of pesticides by plants. In addition, the type of pesticide, its formulation, the application method and the way in which it works affect plant uptake.

Pesticide degradation is the major process by which most pesticides are lost after their application. Pesticide degradation refers to the breakdown of pesticides in the environment. A pesticide is susceptible to photochemical, chemical and microbial decomposition (biodegradation). Photochemical degradation (photolysis) is the degradation process whereby radiant energy in the form of photons breaks the chemical bonds of a molecule. Chemical degradation includes hydrolysis, oxidation-reduction and ionization. It usually occurs through the presence of excess acidity or alkalinity and is therefore related to pH. As soil pH becomes extremely acidic or alkaline, microbial activity usually decreases, but such conditions may result in increased chemical degradation.

Biodegradation is the result of microbial metabolism of pesticides and is often the main route of pesticide degradation in soils. It occurs when fungi, bacteria and other microorganisms in the soil use pesticides as a food or other energy source, or consume the pesticides along with other sources of food or energy. Soil organic matter content, moisture, temperature, aeration and pH all affect microbial degradation. Microbial activity is generally high in warm, moist soils with a neutral pH.

Depending on the specific chemical, the biological degradation can be very fast owing to the presence of microbial enzymes; for other compounds the process may be very slow. Various

kinetic formulations have been proposed, including first- and second-order forms. The rate coefficients are known to be a function of temperature, pH and available nutrients. The second-order kinetic formulations describe the degradation rate as a function of the concentration of the compound and the size of the bacteria population, which is changing as the compound is degraded. A variety of organic compounds may be subject to biodegradation.

How long the pesticide remains in the soil depends on how strongly it is bound by soil components and how readily it is degraded. It also depends on the environmental conditions at the time of application, e.g., soil water content. Soil temperature, which affects the rates of both microbial growth and death, influences the rate of pesticide degradation. The pesticide degradation rate actually decreases as temperature increases, if the temperature lies between the optimal and maximum growth temperatures for microorganisms (Li et al., 2001; Barra Caracciolo et al., 2001).

2.1.2 Herbicides

Of the pesticides used in Italy herbicides represent just 10% of the total (ISPRA, 2010a, b). However, together with their main metabolites, they are among the most frequently detected contaminants, both in surface and ground water (Guzzella et al., 2003).

Examples of water and groundwater contamination by herbicides are shown in Table 4 A and B, in which terbuthylazine and simazine (*s*-triazine herbicides) and linuron (a phenylurea herbicide), together with their metabolites desethyl-terbuthylazine and 3,4-dichloroaniline, are among the herbicides most detected in surface and groundwater (Table 4A and B).

In particular, terbuthylazine and desethyl-terbuthylazine are the most frequently detected herbicides.

Some types of herbicides or their metabolites are resistant to degradation and can persist and accumulate in aquatic ecosystems and modify their balance by eliminating or reducing the populations of various organisms, including endangered species. In addition, they can destroy the food sources of higher organisms, or reduce the amount of vegetation available for habitats or stabilization of soft sediments. Herbicides can be persistent in soil and water ecosystems owing to their intrinsic characteristics and/or can degrade to more toxic metabolites (Guzzella et al., 2006a).

Table 4A. Terbuthylazine, simazine and linuron herbicide and their metabolites concentration in surface and groundwater detected in 2007. Data from ISPRA (2010a).

	Surface water			Groundwater		
Herbicide or metabolite	Sampling points and % detection	Max Conc. Detected (μg L^{-1})	Samples with residues: concentrations (μg L^{-1}) and its percentile	Sampling points and % detection	Max Conc. Detected (μg L^{-1})	Samples with residues: concentrations (μg L^{-1}) and its percentile
Terbuthylazine	751 40.3%	14.210	0.040 - 50th 0.100 - 75th 0.210 - 90th 0.400 - 95th	1678 9.8%	45.500	0.030 - 50th 0.070 - 75th 0.160 - 90th 0.372 - 95th
Terbuthylazine desethyl	696 29.2%	0.900	0.030 - 50th 0.040 - 75th 0.080 - 90th 0.140 - 95th	1544 12.8%	3.83	0.060 - 50th 0.105 - 75th 0.184 - 90th 0.247 - 95th
Simazine	807 7.9%	0.370	0.030 - 50th 0.040 - 75th 0.070 - 90th 0.123 - 95th	1631 4.4%	0.740	0.020 - 50th 0.040 - 75th 0.061 - 90th 0.086 - 95th
Linuron	596 0.2%	0.040	0.010 - 50th 0.025 - 75th 0.034 - 90th 0.037 - 95th	1063 0	-	-
3,4-dichloroaniline	73 6.8%	0.210	0.075 - 50th 0.120 - 75th 0.170 - 90th 0.190 - 95th	-	-	-

Table 4B. Concentration of terbuthylazine, simazine and linuron herbicides and their metabolites detected in surface and groundwater in 2008. Data from ISPRA (2010a).

	Surface water			Groundwater		
Herbicide or metabolite	Sampling points and % detection	Max Conc. Detected ($\mu g\ L^{-1}$)	Samples with residues: concentration and its percentile	Sampling points and % detection	Max Conc. Detected ($\mu g\ L^{-1}$)	Samples with residues: concentration and its percentile
Terbuthylazine	875 23.3%	0.690	0.020 - 50th 0.050 - 75th 0.115 - 90th 0.370 - 95th	1734 9.6%	0.296	0.020 - 50th 0.040 - 75th 0.070 - 90th 0.164 - 95th
Terbuthylazine-desethyl	849 15.0%	0.320	0.020 - 50th 0.030 - 75th 0.080 - 90th 0.160 - 95th	1544 12.8%	0.261	0.030 - 50th 0.060 - 75th 0.110 - 90th 0.181 - 95th
Simazine	877 0.8%	0.720	0.020 - 50th 0.030 - 75th 0.065 - 90th 0.178 - 95th	-	-	-
Linuron	545 0.1%	0.757	0.108 - 50th 0.355 - 75th 0.625 - 90th 0.724 - 95th	1191 1%	0.770	0.770 - 50th 0.770 - 75th 0.770 - 90th 0.770 - 95th
3,4-dichloroaniline	72 1.0%	0.153	0.038 - 50th 0.085 - 75th 0.133 - 90th 0.146 - 95th	-	-	-

2.1.3 Groundwater contamination

Soil and water contamination by pesticides is due to their widespread use (diffuse contamination) and/or improper use (point sources of contamination) (Carter, 2000; Finizio et al., 2011). Groundwater is of special interest because it is used in irrigation and is often the main drinking water source in many countries. Groundwater contamination by pesticides, and in particular herbicides, is generally thought to be caused by diffuse sources. Contamination of groundwater is directly linked to the transport of the pollutant within the soil column supporting the adjective and diffusional flow system, the geochemistry of the groundwater, and the overall groundwater flow. It is in particular the result of the intrinsic herbicide characteristics (e.g. water solubility, K_{oc}, biodegradability etc.) and specific vadose zone ones (e.g. presence of preferential flow paths, climatology conditions, etc.), which determine whether herbicides can be leached towards it (Jaynes et al., 2001; Guzzella et al., 2003; Guzzella et al., 2006b; Barra Caracciolo et al., 2005c). The chemical properties of the soil

particles, their distribution and size, and the amount of organic matter will influence the capacity of the soil to retain more hydrophobic compounds.

Other sources of pesticide contamination are discharges arising from pesticide storage, handling and waste disposal. Indeed, it has been recently pointed out that point sources (high concentration in a small area), such as spills from the devices used to apply herbicides and uncontrolled disposal in soil of waste and equipment-washing water, can be a more significant source of soil and water pollution than agricultural use (Fait et al., 2007; Reichenberger et al., 2007). It has been shown that at least in some regions point-source inputs are the main causes of the detected pesticide loads in rivers (Müller et al., 2002; Neumann et al., 2002).

The retention of a pesticide by soil can prevent its short-term access to ground or surface waters and its effects on non-target organisms, but the persistence of un-degraded pesticide or harmful metabolites constitutes an ever-present – and cumulative – risk to the environment and, potentially, to human health. It is therefore necessary to understand both the processes involved in the retention and release of pesticides by soil – and the factors influencing these processes – and the processes by which degradation occurs.

Since organic matter controls the adsorption of pesticide to soil, organic amendments in soils have recently been proposed for reducing soil and groundwater contamination (Rodríguez-Cruz et al., 2007a; Dolaptsoglou et al., 2009). In fact the organic amendments increase the organic matter content of the soil, enhancing herbicide sorption and thereby decreasing the availability for both transport and degradation processes. Organic amendments are proposed especially for reducing the amounts of pesticide available for leaching to groundwater from point sources. This is why their influence on pesticide sorption and movement through the soil profile is studied with the intention of reducing the risk of water pollution associated with rapid run-off or leaching of pesticides in soil (Albarrán et al., 2002; Morillo et al., 2002). Other research into modifying soil physic-chemically is relevant in particular when the sorption capacity of soils with low organic matter contents needs to be increased to combat mobility from pollution point sources and prevent the pollution of waters (Sánchez-Martin, 2006). Organic matter-rich agricultural by-products are being produced in high quantities and can be applied to soil as a disposal strategy.

2.1.4 *s*-Triazines and Phenylureas

Three different herbicides were considered in our research: terbuthylazine, simazine and linuron (Table 5). They were selected because they are common soil and groundwater contaminants (Di Corcia et al., 1999; Barra Caracciolo et al., 2005b and 2005d, Guzzella et al., 2006a and 2006b).

The **phenylurea herbicides**, such as **linuron**, are an important group of pesticides used predominantly in either pre- or post-emergence treatment of cotton, fruit, cereal or other agricultural crops. The degradation data reported are quite variable, with DT_{50} values in the range of 38–135 days in laboratory studies and 13–82 days in field ones (Caux et al., 1998;

Rodríguez-Cruz et al., 2001; Rasmussen et al., 2005; FOOTPRINT Pesticide Properties Database).

Table 5. Herbicides studied in the experimental research. GUS index indicates the leaching potential of a pesticide. Data from FOOTPRINT Pesticide Properties Database and Pubchem Compounds Database (Geer et al., 2010).

Substance Name	**Terbuthylazine**	**Simazine**	**Linuron**
IUPAC Name	2-N-tert-butyl-6-chloro-4-N-ethyl-1,3,5-triazine-2,4-diamine	6-chloro-N2,N4-diethyl-1,3,5-triazine-2,4-diamine	3-(3,4-dichlorophenyl)-1-methoxy-1-methylurea
Chem. Formula	$C_9H_{16}ClN_5$	$C_7H_{12}ClN_5$	$C_9H_{10}Cl_2N_2O_2$
CAS	5915-41-3	122-34-9	330-55-2
Chem. Structure			
type	*s*-triazine	*s*-triazine	phenylurea
GUS index	3.07	3.35	2.03

With these values, combined with the K_{oc} values, it is possible to calculate the GUS index (Groundwater Ubiquity Score), according to the following equation (Gustafson, 1989):

$$GUS = (\log DT_{50}) \times (4 - \log K_{oc})$$

Using this equation, it is possible to classify pesticides into leachers (GUS > 2.8), non leachers (GUS < 1.8) and transient, which have intermediate properties (1.8 < GUS < 2.8). A GUS index over 2.8 indicates that the compound will not be degraded or retained in the organic matter in the soil. However, the K_{oc} and the half-life of a compound are not the sole parameters which can be used to explain the transport of pesticides within the soil matrix and eventually to groundwater. In fact, leaching is favoured when the vapour pressure of the pesticide is low or its solubility in water is high.

Linuron's GUS index (Table 5) indicates that it can be considered a potential leacher, as well as its metabolite 3,4-dichloroaniline, which has a GUS index of 3.77 (FOOTPRINT Pesticide Properties Database).

Given its slow natural attenuation rate in various soils, in particular as regards mineralisation of the phenyl structure (Caux et al., 1998), and the potential carcinogenic risk of this herbicide and its potential intermediates such as the chloroanilines (Scassellati-Sforzolini et al., 1997; Tixier et al., 2001 and 2002), there is a serious need to develop remediation processes to eliminate or minimize contamination of surface and groundwater.

Moreover the linuron metabolite 3,4-dichloroaniline, with toxicological properties similar to or greater than its parent compound (Barra Caracciolo et al., 2005d, Guzzella et. al 2006b; Lintelmann et al., 2003; Tixier et al., 2001 and 2002), poses a risk to both ecosystems and human health.
Although photo-chemical and physical processes may be involved in the removal of this compound, biodegradation is reported to be the most significant mechanism for its dissipation from soil (Caux et al., 1998). Several studies suggest the involvement in degradation of a bacterial consortium rather than a single strain (El-Fantroussi et al., 2000; Sørensen et al., 2003) and Dejonghe et al. (2003) isolated a single strain capable of degrading linuron, stimulated by a synergistic interaction with other strains.

***s*-Triazines** are among the most commonly used herbicides in the world for pre-emergence and post-emergence weed control, encompassing both a number of agricultural crops, forests and annual grasses in non-crop fields (Laird & Koskinen, 2008). The *s*-triazine chemical family includes a large number of herbicides and is characterized by a symmetrical hexameric ring consisting of alternating carbon and nitrogen atoms, such as terbuthylazine, simazine and atrazine. In recent years, concern has been growing about the persistence, mobility and toxicity of triazines and their metabolites, owing to the residual concentrations of these compounds detected in aquifers (Tappe et al., 2002). In many European countries, a significant proportion of monitored groundwater is contaminated by triazines, primarily atrazine, terbuthylazine and their desethyl-degradation metabolites; the concentration of these contaminants is often >0.1 µg L^{-1}, the maximum admissible concentration under the EC legislation (Directive 98/83EC).

Atrazine (6-chloro-N2-ethyl-N4-isopropyl-1,3,5-triazine-2,4-diamine), the most commonly used triazine, is used extensively in many parts of the world to control a variety of weeds, primarily during production of corn. It was sold worldwide starting in the 1960s, but in the early 1990s European regulators banned its use because of its persistence in the environment. Subsequently, terbuthylazine was introduced as an atrazine substitute.
Atrazine biodegradation has been found to occur via several pathways. Workers have isolated a variety of fungi and bacteria which dealkylate and dechlorinate atrazine but do not mineralize the *s*-triazine ring. More recently, however, there have been several reports of rapid atrazine mineralization in agricultural soils, and a variety of atrazine-mineralizing bacteria, including members of the genera *Pseudomonas*, *Rhizobium*, *Acinetobacter*, and *Agrobacterium*, have been isolated from soils that have come in contact with this chemical (Topp et al., 2000).

Terbuthylazine (Table 5) has replaced atrazine in Italy and in many other countries where the latter's use has been banned. In 2008 more than 470,000 kg of terbuthylazine (figure referring to the active ingredient) were sold (APPA Fitofarmaci), primarily in the maize producing regions. Terbuthylazine has not included in the Annex 1, the 'positive' list of active substances that are authorised for use in the Plant Protection Products Directive (91/414/EEC, 'The Authorisations Directive'), but has been re-submitted. Currently it is the only *s*-triazine

sold in Italy, with its use restricted to maize and sorghum, mixed with other substances. Since 2008 there has been restriction of its use (in particular in vulnerable areas).
Among *s*-triazines, terbuthylazine is the most persistent in surface environments (Guzzella et al., 2006a; Carafa et al., 2007). Metabolite formation is mainly due to biochemical processes such as dealkylation, dechlorination and hydroxylation, deamination and ring cleavage of the parent compounds.

Herbicide degradation does not always produce a lower phytotoxicity; for example, desethyl-atrazine [6-chloro-N-isopropyl-(1,3,5)-triazine-2,4-diamine] (DES) has the same toxicity as atrazine. According to studies by Dousset et al. (1997), the prominent dealkylation product of terbuthylazine is desethyl-terbuthylazine. Dealkylated products can be considered hazardous contaminants for groundwater pollution as they are generally more persistent and water soluble than the parent compound (Guzzella et al., 2003).

Simazine belongs to the *s*-triazine herbicides and is a widely used selective systemic herbicide. It is used in various formulations as a pre- and post-emergence herbicide in agriculture including orchards, container plant production, Christmas trees and recreational areas. It has been found as one of the main pollutants in shallow and deep groundwater in United States, Europe and Australia (Spalding et al., 2003; Flores et al., 2009). Such widespread use makes considerable non-target organism exposure likely, in both terrestrial and adjacent aquatic areas (Strandberg & Scott-Fordsmand, 2002). Many studies have dealt with the effect of target organisms, especially laboratory studies, but much less is known of the potential effect on non-target organisms in the field.
Simazine is included in the EU Priority Pollutants List (Annex X, Dir. 2000/60/EC, consolidated version), owing to its persistence, toxicity, moderate leaching capacity and potential to adsorb onto soils and sediments. It has two main degradation pathways yielding either 2-hydroxysimazine (OH-Sim) or desethyl-simazine (DES). 2-Hydroxy-simazine can be formed via both biotic and abiotic processes but desethyl-simazine only via biotransformation. Desethyl-simazine is known to have similar phytotoxic properties to its parent compound (Strandberg & Scott-Fordsmand, 2002).

In recent years concerns about the persistence, mobility and toxicity of triazines and their metabolites have been growing, owing to the detection of residual concentrations of these herbicides in groundwater. In fact terbuthylazine, DES and simazine have a GUS index of 3.07, 3.8 and 3.35, respectively (FOOTPRINT Pesticide Properties Database) and they are considered leacher compounds (Guzzella et al., 2003).

The widespread presence of terbuthylazine and its metabolite DES in water bodies may be traced back not only to the high amounts of the parent compound used in agriculture, but also to agricultural practices, the chemical's intrinsic and chemiodynamic properties (mainly water solubility, soil and water half-lives, soil adsorption and K_{oc}) and the hydrogeological characteristics of the receiving environment. In fact groundwater monitoring data show that terbuthylazine and DES often occur at levels above the 0.1 µg L^{-1} limit established in the Directive for Drinking Water (Directive 98/83/EC; Guzzella et al., 2003; Guzzella et al.,

2006a; Tappe et al., 2002; Otto et al., 2007). In Italy most drinking water sources are groundwater and, once contaminated above the legal limit, they can never be used for drinkable purposes. Since DES has a very similar chemical structure to desethyl-atrazine, which is toxic like atrazine (Tu, 1992; Winkelmann & Klaine, 1991), it may be considered a hazardous contaminant for groundwater and may pose environmental and health problems for man and aquatic organisms.

s-Triazines are commonly found to be biodegraded by microorganisms, mainly bacteria and fungi. Bacteria generally use the herbicide as a carbon and nitrogen source (Dinamarca et al., 2007; Barra Caracciolo et al., 2005d). To establish the natural capability to recover from pollution and the bioremediation processes that remove these compounds from soil and water, especially from groundwater considered to have been for a long time without life, the detection of active indigenous microbial communities able to degrade *s*-triazines is required. Typical procedures for soil or water analysis combine chemical and molecular approaches. Molecular biological approaches assess the presence and diversity of microorganisms and the catabolic genes for *s*-triazine degradation. In fact it is known that plasmidic genes such as *Atz*A, B, C and *Trz*N, encoding triazine degradation enzymes, are responsible for *s*-triazine degradation and mineralization (Figure 9).

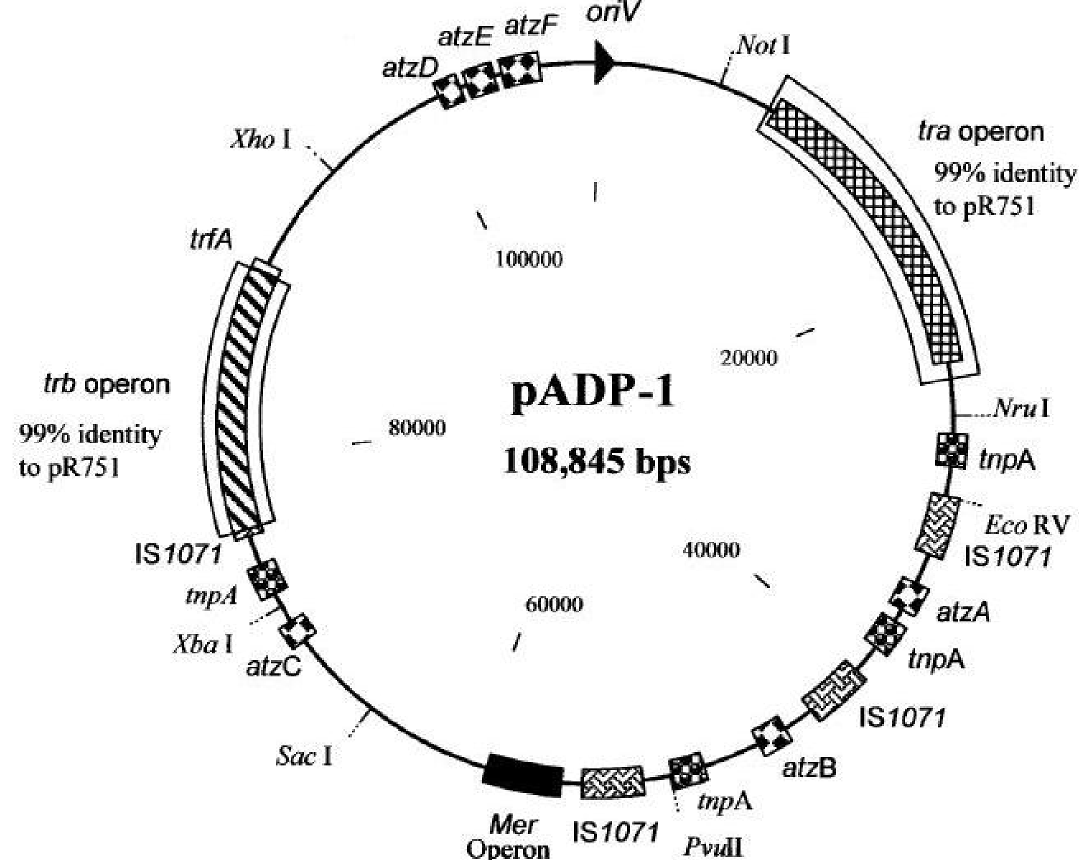


Figure 9. Circular physical map of the catabolic plasmid pADP-1 from the *Pseudomonas* sp. strain ADP. The map positions of selected restriction sites, genes and operons on pADP-1 are indicated. Genes involved in atrazine catabolism are indicated. Copies of similar putative transposons have the same shading (Martinez et al., 2001).

The *Pseudomonas* sp. strain ADP plasmid, encoding *s*-triazine genes, has been cloned and characterized (de Souza et al., 1995).
Most commonly, the bacterial metabolism of *s*-triazines has been reported to occur via two different upper pathways. The first pathway involves the enzymes encoded by the *atz*A-*atz*B-*atz*C genes (Figure 10).

The second pathway utilizes the initiating enzymes of the hydrolytic reactions encoded by the *trz* gene family, such as *trz*N, and the *atz*B-*atz*C genes (Shapir et al., 2007). These genes are widespread, highly conserved in bacteria, and are often associated with transposable elements on plasmids (Devers et al., 2007; Shapir et al., 2007). Both of these pathways lead to the formation of cyanuric acid and alkylamines as common intermediates, which are ultimately degraded by the enzymes encoded by the *atz*DEF genes and by amine oxidases, respectively (Shapir et al., 2007).
Although there have been several studies showing biotic and abiotic triazine degradation in soil (Di Corcia et al., 1999; Barra Caracciolo et al., 2005a, b), degradation of these compounds in groundwater remains, to our knowledge, to be investigated. The fact that bacterial strains with the potential capability to degrade these compounds can be found in groundwater has important implications for planning remediation strategies and, in particular, for assessing the natural attenuation time and the implementation of bacterial strains for bioaugmentation purposes.

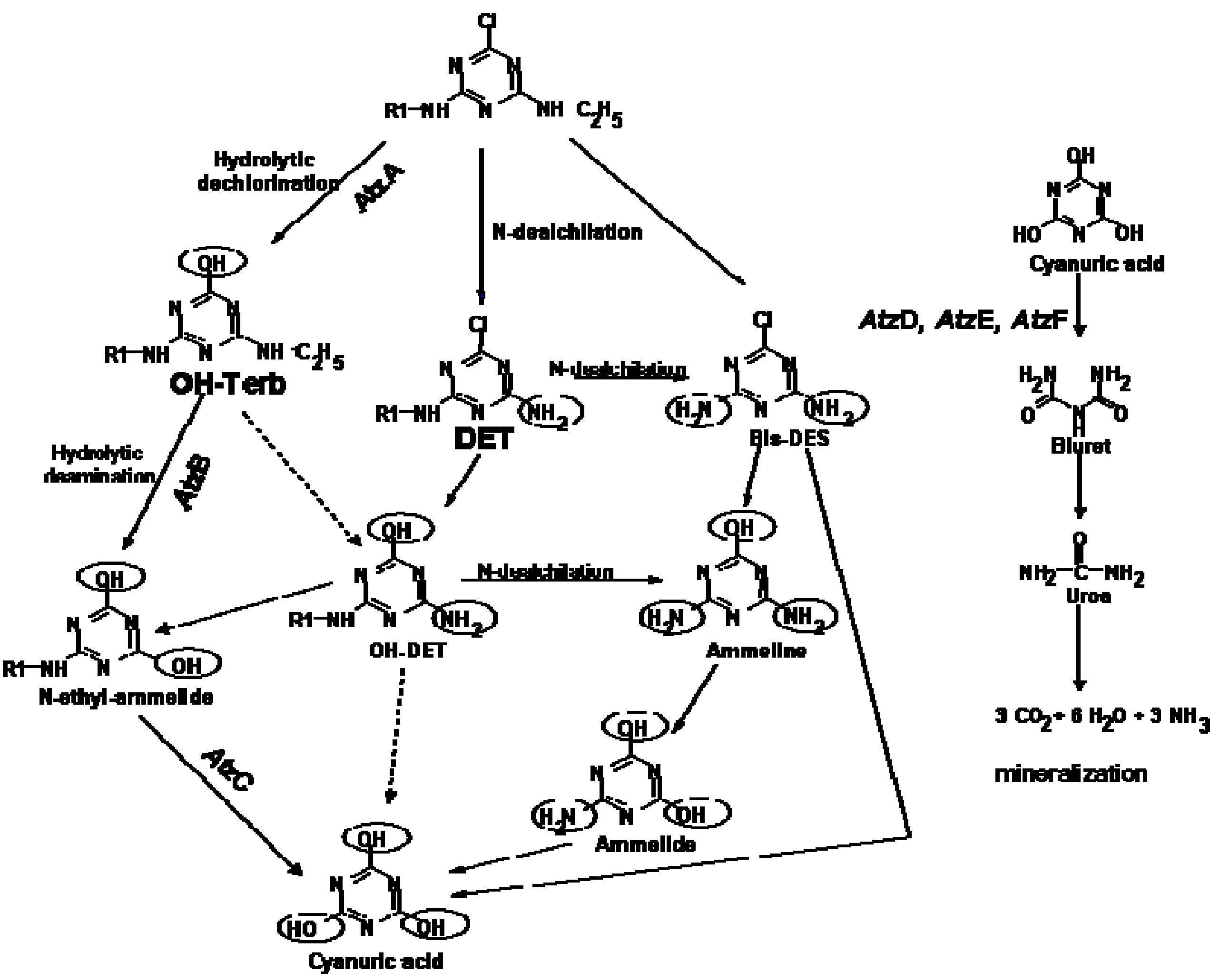


Figure 10. Terbuthylazine degradation pathway and bacterial genes involved.
R1 = $C(CH_3)_3$. OH-Terb: hydroxyl-terbuthylazine; DET: desethyl-terbuthylazine; OH-DET: hydroxy-desethyl-terbuthylazine; BIS-DES: desethyl-debuthyl-terbuthylazine.

2.1.5 Legislation

In the European Union the quality of water for human consumption is controlled by various regulations, which establish very low concentrations for toxic pesticides and their degradation products (e.g. Water Framework Directive 2000/60/EC): 0.1 µg L^{-1} is the maximum permissible concentration for a single pesticide and/or its metabolite and 0.5 µg L^{-1} for the total load of all pesticides (Prammer, 1998; WHO, 1993).
As regards water resources, a major policy issue currently is the implementation of the WFD (Water Framework Directive) in European countries. It stresses that an assessment is required to implement efficient measurement programs to preserve or restore the good ecological status of water bodies (to be achieved in 2015). Compliance with this WFD implies a reduction in the impact of agricultural pressure on the environment and the implementation of

measures designed to reduce pollution of surface and groundwater by agriculture. River basins have been designated as the appropriate level for integrated water management. With this approach both point and non-point sources (NPS) of pollution are subject to control. Pollution from NPS typically includes nutrients and pesticides applied to the arable land surface. The European Parliament recently approved new EU pesticides legislation (Dir. 2009/128/EC, to be implemented in 2011), which establishes a framework for Community action to achieve the sustainable use of pesticides. The Directive demands that each Member State adopts a National Action Plan to set quantitative objectives, targets, measures and timetables to reduce risks and impacts of pesticide use on human health and the environment, including timetables and targets for a reduction in use. Furthermore, each Member State must encourage the development of Integrated Pest Management (IPM) and of alternative approaches or techniques in order to reduce dependency on the use of pesticides. Member States have until December 2012 to communicate these National Action Plans to both the European Commission and to other Member States.

2.2 Pharmaceuticals

Pesticides are only one component in a group of chemicals which are being continually introduced into the environment. In fact, during the past decade, the increasing introduction on the market of new chemicals and the development of more accurate analytical methods have added various new and 'emerging' environmental contaminants to the already large array of pollutants.

Emerging contaminants are defined as *any synthetic or naturally occurring chemical that is not commonly monitored in the environment, although it has the potential to enter soil and aquatic ecosystems, causing known or suspected adverse ecological and/or human health effects* (USGS, 2009). Among emerging contaminants, pharmaceuticals are a cause of concern as they have been detected in various ecosystems.

The term "pharmaceuticals" refers to a complex class of compounds used worldwide in human and veterinary medicine. Pharmaceutically active compounds are used for prevention, diagnosis or treatment of diseases and for restoring, correcting or modifying organic functions (Daughton & Ternes 1999, Kümmerer, 2009a; Bottoni et al., 2010) and include more than 4000 molecules with different physic-chemical and biological properties (antibiotics, anticancer, anti-inflammatory, lipid regulators, antiviral etc.) and distinct modes of biochemical action (Beausse, 2004). They are grouped according to the organ or system on which they act and/or their therapeutic and chemical characteristics in the Anatomical Therapeutic Chemical (ATC) Classification System, which is controlled by the WHO Collaborating Centre for Drug Statistics Methodology (WHOCC).

The quantity of pharmaceuticals and personal care products (PPCPs) entering the environment each year in the world is reported to be similar to the amounts of pesticides (Daughton & Ternes, 1999) and levels of pharmaceuticals sold amount to thousands of tons per year, which is similar to the amount of fertilizers and other chemicals used in agriculture (OECD 2001).

Pharmaceuticals have only recently started to be found in the environment (Zuccato et al., 2006; Jones et al., 2001 USGS, 2002; Jørgensen et al., 2000). Although they have been detected in the environment in low concentrations, in the range of ng-µg L^{-1}, most of these 'micropollutants' raise considerable toxicological concerns because they are intrinsically and biologically active molecules (Allen et al., 2010; Martínez, 2008). The environmental concentrations of pharmaceuticals have been measured and generally fall within a range 10^3-10^7 times lower than the known LC_{50} or EC_{50} values for various organisms (Peake & Braund, 2009). Hence it is unlikely that lethal or acute toxicity effects will occur. However, these concentrations can have sub-lethal or chronic toxic effects. Moreover, this ecotoxicology issue is particularly challenging as regards two factors: pharmaceuticals are present in the environment in mixtures; these mixtures are constituted of diverse chemicals with different modes of action and unknown sub-therapeutic side effects, especially on non-target organisms

(Schwarzenbach et al., 2006; Brack et al., 2007) and this is a prime concern for the health of aquatic organisms and humans (Pomati et al., 2006; 2008).

Pharmaceuticals are considered “pseudo-persistent” compounds owing to their constant introduction into the environment (Daughton, 2003a,b). This phenomenon transforms what are mainly degradable substances into persistent ones, owing to their on-going replacement; consequently non-target organisms present in environmental ecosystems can be chronically exposed to them (Hernando et al., 2006; Bendz et al., 2005).

Although pharmaceutical and therapeutic products are widely found in the natural environment, there is limited understanding of their potential unintended environmental impact and the ecological effects on receiving environments remain largely unknown.

2.2.1 Environmental fate and ecotoxicological assessment

Pharmaceuticals can enter the environment through a variety of sources. Figure 11 shows the possible sources and pathways for the occurrence of pharmaceutically active products in the environment.

After administration of drugs only a limited quantity is assimilated and metabolized by the organisms and the rest is excreted in urine and faeces (Carlsson et al., 2006) as parent compounds and/or metabolites and enters the sewage treatment system. Patients are therefore considered the main pollution source and waste water treatment plants represent important points for pharmaceutical environmental pollution control. However current treatment systems are unable to remove drugs effectively (Halling-Sørensen et al., 1998; Zuccato et al., 2005; Singer et al., 2008), so that they pass into either surface waters or groundwater.

It has been estimated that about 70-80% of pharmaceutical pollution is due to the inefficiencies of waste treatment plants and the remaining 20-30% derives from other sources, mainly industrial pollution, improper or illegal waste disposal, runoff from farm animal operations (e.g. drainage from land that has been irrigated with wastewater or fertilized with biosolids), hospital discharges and aquaculture industry. Among the different ways that drugs enter the environment, one that has to be considered is pharmaceutical waste disposal if this is done improperly (Figure 12). In fact manufacturers have to be considered a point source of environmental intake. Once a pharmaceutical compound is deposited as a solid in a landfill, it is subject to all the aerobic and anaerobic degradation processes that occur with any other type of organic solid waste. These lead to the formation of an organic-rich liquid leachate that can be contained or released in a controlled manner into nearby soil, where it can make a contribution to the groundwater, or into a natural river course, where it is rapidly diluted (Peake & Braund, 2009). Although waste disposal from pharmaceutical production is normally considered only a point source of contamination, with discharges affecting a limited area, the concentration of drugs in soil and groundwater down gradient from a landfill formerly used for the waste disposal can be significant (Hirsh et al., 1999).

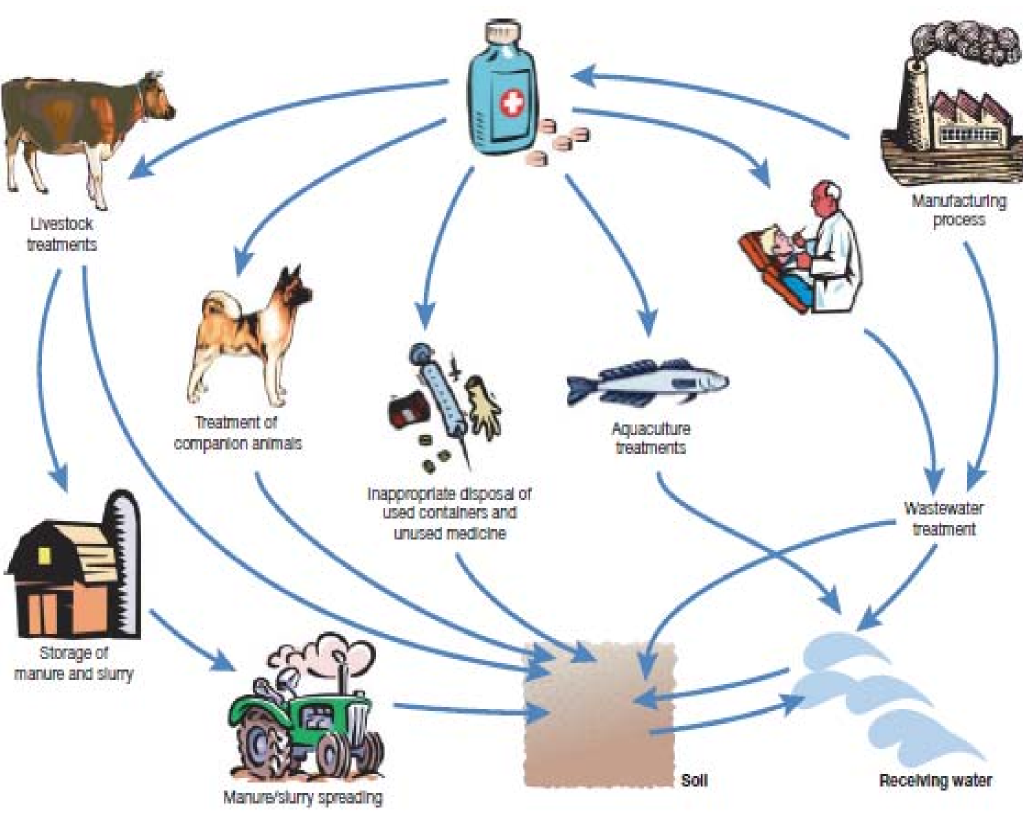


Figure 11. Routes by which pharmaceuticals enter the environment (Boxall, 2004).

Chemicals can enter surrounding aquifers as a part of the leachates if waste from pharmaceutical industries is disposed of at landfills with no leachate collection systems (Holm et al., 1995). Very little is known about the exposure routes of the medical substances to the environment and they may also have unintended effects on animals and microorganisms in the environment. Although the side effects on human and animal health are usually investigated in thorough safety and toxicology studies, the potential environmental impacts of drugs is less well understood and has only recently become a topic of research interest.

Pharmaceuticals are often designed to target specific metabolic and molecular pathways in humans and animals. Considering their widespread use and their potential risk for the environment, the European Medicines Agency (EMEA) issued an environmental risk assessment guideline (Guideline on the Environmental Risk Assessment of Medicinal Products for Human Use, CPMP/SWP/4447/00, European Medicines Agency, 2006). The new legislation is apply to all new marketing authorisations with the exclusion of vitamins, electrolytes, amino acids, peptides, carbohydrates and lipids; vaccines and herbal medicinal products are also excluded.

In line with traditional environmental risk assessment procedures (Figure 13), the characterization of the environmental risk of pharmaceuticals is also based on the PEC/PNEC

ratio (PEC: Predicted Environmental Concentration; PNEC: Predicted No Effect Concentration).

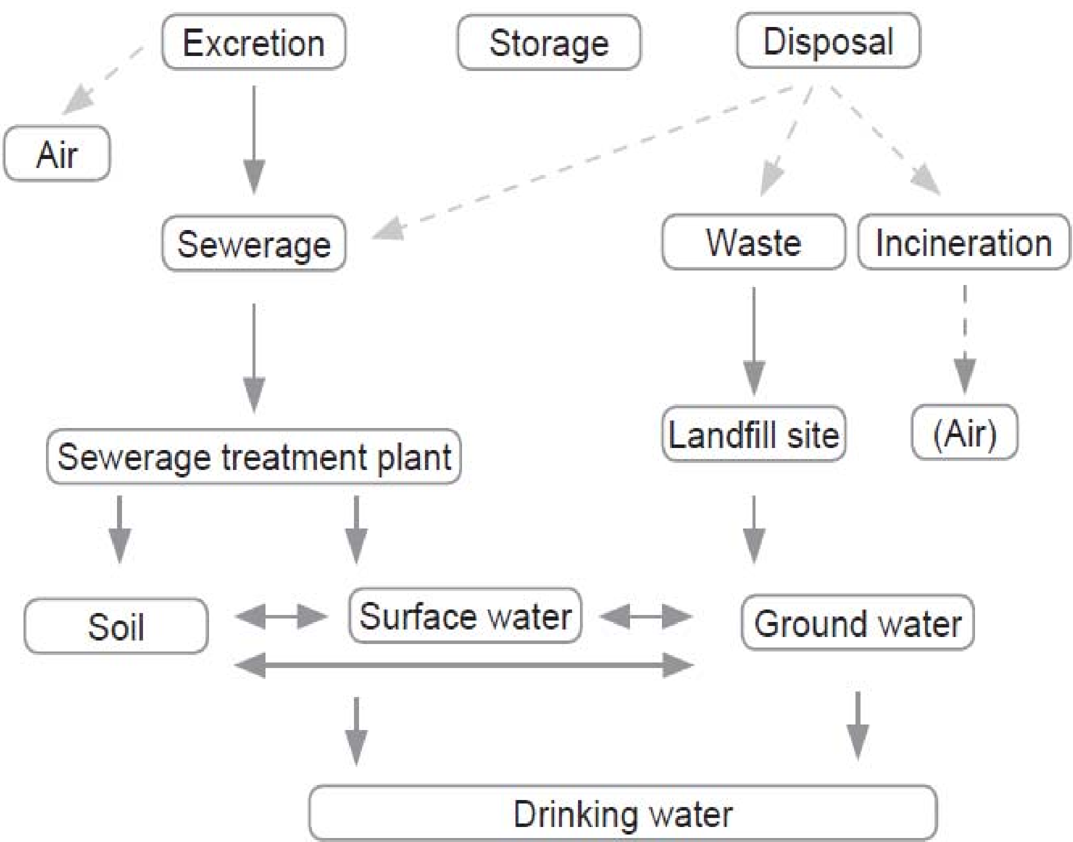


Figure 12. Potential pathways for entry of human pharmaceuticals into the environment (European Medicines Agency Document EMEA/CHMP/SWP/444700, 2006).

It is thus necessary to get information both on exposure and effects on living organisms.

In phase I the predicted environmental concentration (PEC) of a pharmaceutical in surface water is estimated considering parameters such as a default value for the percentage of market penetration, maximum daily dose consumed per inhabitant, amount of wastewater per inhabitant per day and a dilution factor. If the $PEC_{surface\ water}$ is below 0.01 µg L^{-1} and no other environmental concerns are apparent, it is assumed that the pharmaceutical is unlikely to represent a risk for the environment. However, a screening for persistent, bioaccumulative and toxic (PBT) substances is performed in Phase I if the logarithmized n-octanol water coefficient is higher than 4.5. In case the $PEC_{surface\ water}$ value is equal or above 0.01 µg L^{-1}, a phase II environmental fate and effect analysis is performed according to the EMEA guideline. During this Tier A of Phase II, the fate in the sewage treatment plant and the environment is assessed in ready biodegradability tests, by analysing the sorption behaviour

of the substance to sewage sludge and soil, the distribution between water and octanol, and by a transformation test in water–sediment systems. Depending on the outcome of the fate tests, further testing in Tier B might be necessary, for example on fate and effects in soil or on bioaccumulation (Christen et al., 2010). The key aspects to be evaluated are exposure, physical-chemical properties - such as water solubility (S), the n-octanol/water partition coefficient (K_{ow}), vapour pressure (P) and DT_{50} (degradation half-life) in soil and water. It is also important to know the quantity released into the environment. These are all used as input parameters for predictive PEC calculation models. The effects are generally evaluated by ecotoxicological tests on non-target water and soil organisms. Ecotoxicological assessments of pharmaceuticals are based on acute and chronic toxicity experiments performed using standard tests on organisms belonging to different trophic levels such as algae, zooplankton, other invertebrates and fish (e.g. Algal growth inhibition test, OECD Guidelines 201; Fish Early Life Stage test OECD Guidelines 210). The overall results of these tests make it possible to calculate PNEC (predicted no-effect concentration).

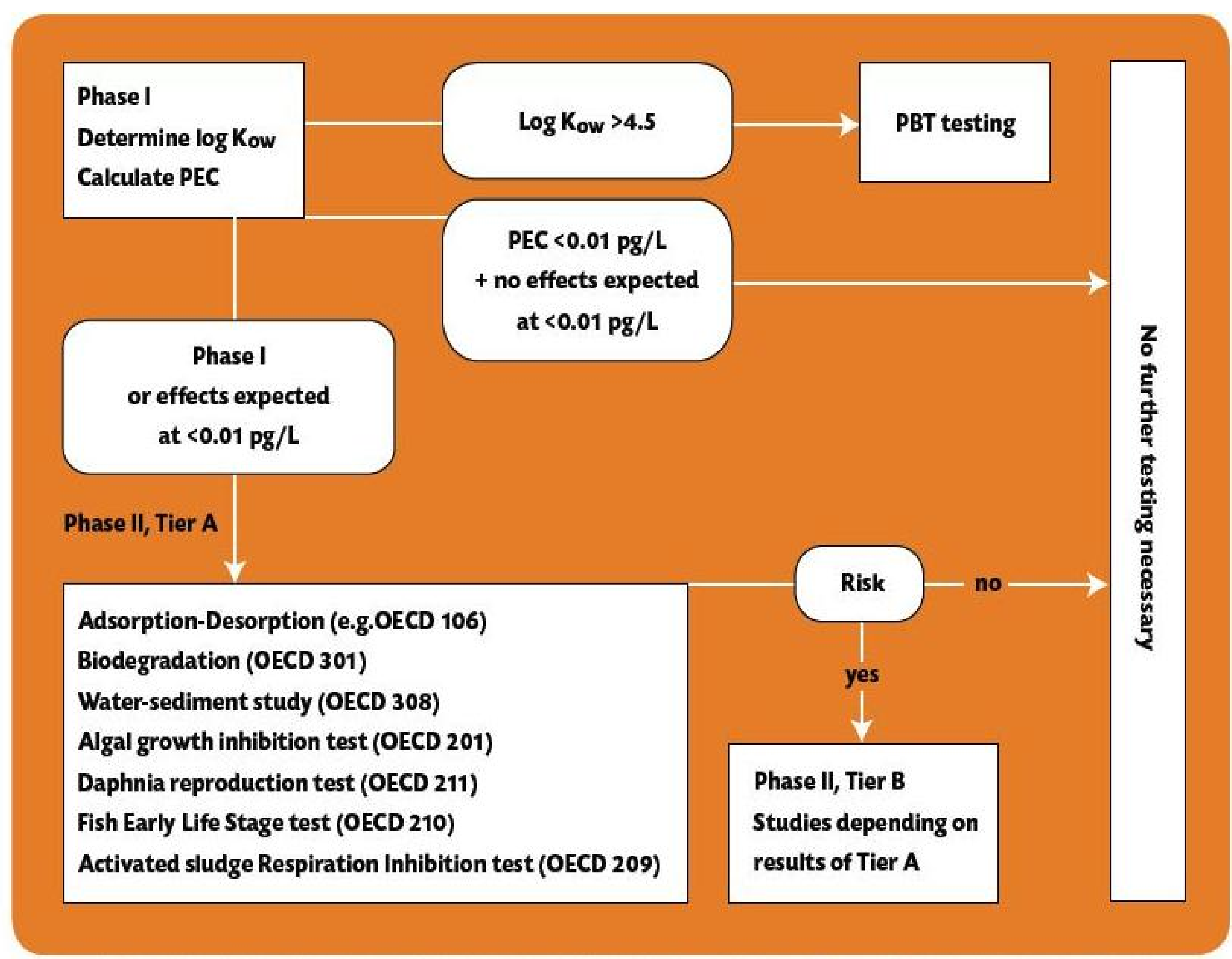


Figure 13. The step-wise structure of the EMEA guideline on environmental risk assessment of medicinal products for human use.

However, for pharmaceuticals there is a scarcity of information both on exposure and effect parameters and this frequently makes a realistic risk analysis difficult. Furthermore, very little is known about possible counterparts of human target receptors/biomolecules of pharmaceuticals in vertebrates and even more so in invertebrates. In addition, for some pharmaceuticals the specific mode of action is not well characterized and often not only one but different modes of action are present.

As for the environmental fate of antibiotics and their effects on bacteria and other organisms in the environment, it is not clear whether the standardized tests used for risk assessment of chemicals are appropriate for antibiotics and other pharmaceuticals. Studies using test systems indicate that various antibiotics remain active against different groups of bacteria present in waste water (Kümmerer, 2001; Kümmerer & Henninger, 2003). Effects on algae and daphnids have been reported at surprisingly low concentrations (5-100 µg L^{-1}; Holten-Lützhøft et al., 1999; Wollenberger et al., 2000; Macri et al., 1988).

2.2.2 Antibiotics and Antivirals

Among pharmaceuticals detected in the environment, antibiotics and antivirals are the most worrying substances.

Antibiotics have been widely used in human and veterinary medicine for therapeutic (disease control), prophylactic (disease prevention) and sub-therapeutic (growth promotion) purposes, contributing significantly to the improvement of our quality of life. In fact they are used to prevent or treat microbial infections as well as to promote the growth of animals in livestock production. Nevertheless, the knowledge of what happens with antibiotics after their use, once they are released into the environment, has been very limited until now. For this reason the European Union (EU) recommends the prudent use of antimicrobial agents in human medicine (The Council of the European Union, 2002).

Many thousands of tons of antibiotics are produced every year. According to data supplied by the European Federation of Animal Health (FEDESA, 2001), about 13,000 tons of antibiotics were used in the EU and Switzerland in 1999, of which 65% was used in human medicine, 29% was used in the veterinary field and 6% as growth promoters. Various antibiotics used at sub-therapeutic dose as growth promoters have now been banned within the EU and only four compounds remain in this group of feed additives. This explains the decline in the use of antibiotics in animal husbandry compared with human medicine.

In the USA about 16,000 tons were produced in 2000 (Union of Concerned Scientists, 2001) of which 70% was used in livestock farming, i.e. eight times the amount used in human medicine (Mellon et al., 2001). Wise (2002) estimated total antibiotic market consumption world-wide to lie between 100,000 and 200,000 tons.

The use of these antibiotics in humans and the livestock industry plus aquaculture has resulted in their release into the environment through different pathways as either parent compounds or their primary metabolites in the forms of conjugated, oxidized or hydrolysed products. Fifty to ninety per cent of these pharmaceuticals or their primary metabolites are excreted by humans and animals after their administration (Kümmerer, 2004b).

Wastewater treatment plants (WWTPs) and sewage treatment plants (STPs) are considered the major source by which human-used antibiotics enter the environment owing to their only partial removal efficiency (Beausse, 2004; Kim & Carlson, 2005; Hao et al., 2007).

Antibiotics used for veterinary purposes enter the environment mostly through manure, waste lagoon water and sewage sludge applications in fields as plant fertilizers (Schlusener et al., 2003; Kim & Carlson, 2005; Díaz-Cruz & Barcelo, 2006). Runoff from animal agricultural operations or overflow and leakage from storage lagoons or tanks are likely to contribute to the release of these compounds into the environment as well (Hao et al., 2006; Kim & Carlson, 2007). Moreover another source of pollution is the improper disposal of pharmaceutical waste arising from industrial activity. These are point sources that can contribute to high pharmaceutical concentrations in the environment.

In soil naturally occurring antibiotics from, amongst others, bacteria and fungi control the dynamics of bacterial populations. In contrast to these, most of the compounds used nowadays are semi-synthetic or synthetic. They are often much more stable and are not biodegradable by bacteria and may therefore persist in the environment. Furthermore, they often have a different, e.g. broader, activity spectrum.

The most common response of cells to antibiotics is to cease growing (bacteriostasis), or to grow but with an inhibition of the target in the organism, leading indirectly to cell death. In the treatment of an infection, bacteriostasis is often effective because the killing and elimination of the pathogen are mediated through host immune defences. Such augmentation is typically absent in the environment. In this respect, there is much less knowledge about environments such as waste water, sludge, surface water and soil than about the medical use and effectiveness of antibacterials. Furthermore, in waste water, surface water, sediments, sludge and soil a cocktail of different active compounds may be present as a result of the medical and veterinary application of antibiotics and disinfectants.

Antibiotics and antivirals produced for humans and animals are typically found in the environment in sub-therapeutic concentrations. Antibiotics have been detected in the $\mu g\ L^{-1}$ range in municipal sewage, the effluent of sewage treatment plants (STPs), surface water and groundwater (Zuccato et al., 2000; Golet et al., 2001; Sacher et al., 2001).

These concentrations can promote bacterial resistance (Sacher et al., 2001; Kümmerer, 2003) and consequently cause particular concern. In fact recently increasing numbers of bacterial and viral diseases are becoming resistant to traditional treatments, such as antibiotic and antiviral drugs (Kümmerer, 2004a,b; Kümmerer, 2009; Allen et al., 2010; Lipsitch et al., 2007; Singer et al., 2007). Moreover antimicrobial resistance increases mortality and the cost

of treating infectious diseases (Kümmerer, 2004a,b; Kümmerer, 2009; Allen et al., 2010; Lipsitch et al., 2007; Singer et al., 2007).

There are a number of recent publications about the impact of very low antibiotic concentrations on the expression of bacterial virulence factors (Boxall, 2004; Allen et al., 2010), in which resistant bacteria may be selected by antibiotic substances in hospital effluent, municipal sewage, aeration tanks, the anaerobic digestion process of STPs or in soil. Furthermore, resistant bacteria are excreted and discharged into sewage or soil and other environmental compartments. The risk of contaminating soil or surface water run-off after application of manure or sewage sludge for land amendment is high, increasing the selective pressure for resistant bacteria (Kümmerer, 2009b).

The selection and development of antibiotic-resistant bacteria is one of the greatest concerns with regard to the use of antimicrobials (The Council of the European Union, 2002; Wise et al., 1998; Morris & Masterton, 2002). In fact antibiotics are typically found in the environment at sub-therapeutic concentrations, which can in fact promote bacterial resistance (Kümmerer, 2003; Zuccato et al., 2000; Sacher et al., 2001). An example of antibiotic resistance mechanisms in a Gram-negative bacterium is shown in Figure 14.

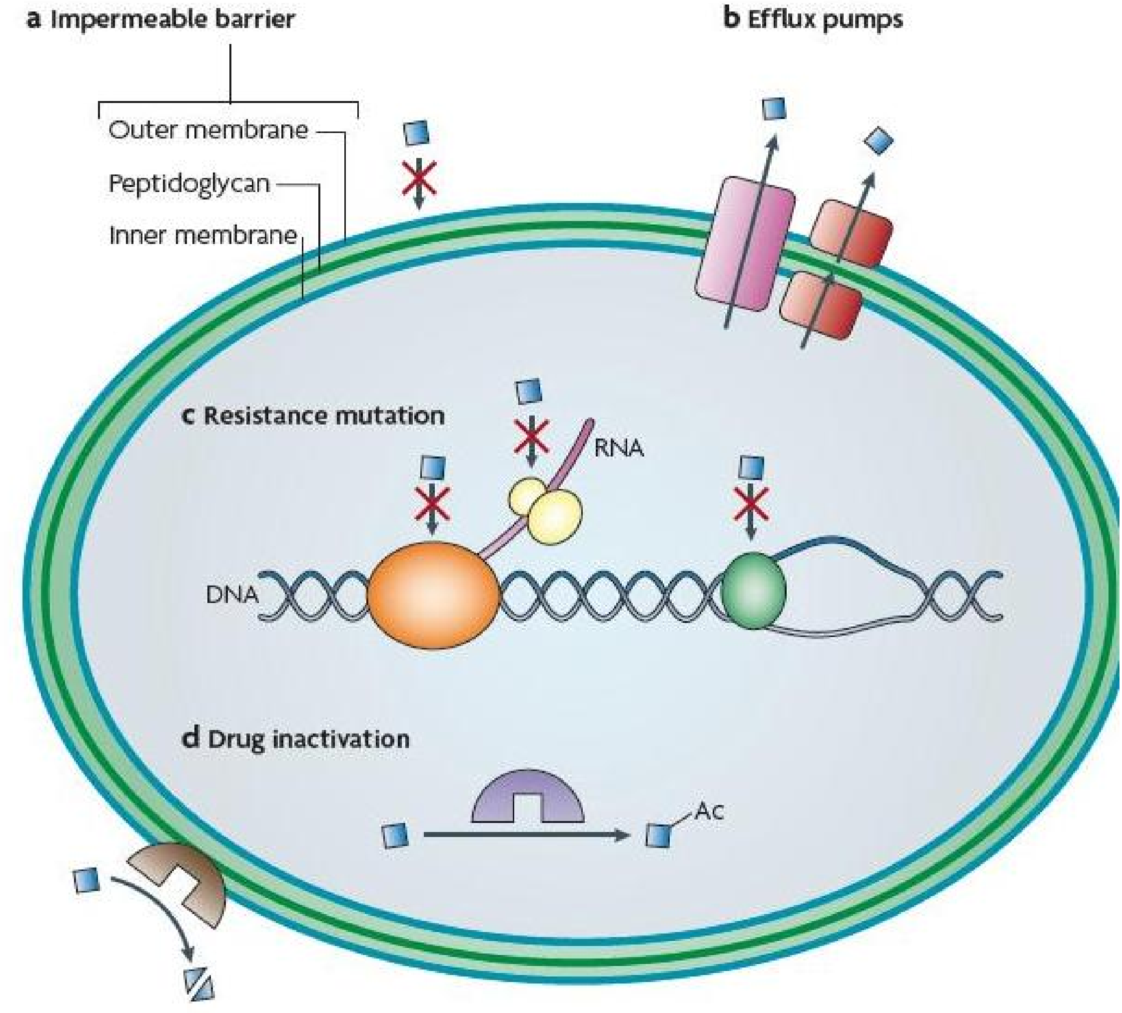


Figure 14. Antibiotic resistance mechanisms in a Gram-negative bacterium (from Allen et al., 2010). Ac, acetyl group.

In this Figure is shown:

a: Impermeable barriers. Some bacteria are intrinsically resistant to certain antibiotics (blue squares) simply because they have an impermeable membrane or lack the antibiotic target.

b: Multidrug resistance efflux pumps. These pumps secrete antibiotics from the cell. Some transporters, such as those of the resistance–nodulation–cell division family (pink), can pump antibiotics directly outside the cell, whereas others, such as those of the major facilitator superfamily (red), secrete them into the periplasm.

c: Resistance mutations. These mutations modify the target protein, for example by disabling the antibiotic-binding site but leaving the cellular functionality of the protein intact. Specific examples include mutations in the gyrase (green), which cause resistance to floroquinolones, in RNA polymerase subunit B (orange), which cause resistance to rifampicin, and in the 30S ribosomal subunit protein S12 (encoded by rpsL) (yellow), which cause resistance to streptomycin.

d: Inactivation of the antibiotic. Inactivation can occur by covalent modification of the antibiotic, such as that catalysed by acetyltransferases (purple) acting on aminoglycoside antibiotics, or by degradation of the antibiotic, such as that catalysed by β-lactamases (brown) acting on β-lactam antibiotics.

Recent works have suggested that some antibiotics may serve for signalling purposes at the low concentrations probably found in natural ecosystems, whereas some antibiotic resistance genes were originally selected in their hosts for metabolic purposes or for signal trafficking. However, the high concentrations of antibiotics released in specific habitats (for instance, clinical settings) as a consequence of human activity can shift these functional roles (Martinez, 2009). Several plasmids encode toxin–antitoxin systems (Hayes, 2003). If these plasmids contain antibiotic resistance genes (Moritz & Hergenrother 2007; Perichon et al., 2008; Sletvold et al., 2008), it may be highly likely that resistance will persist (Figure 15).

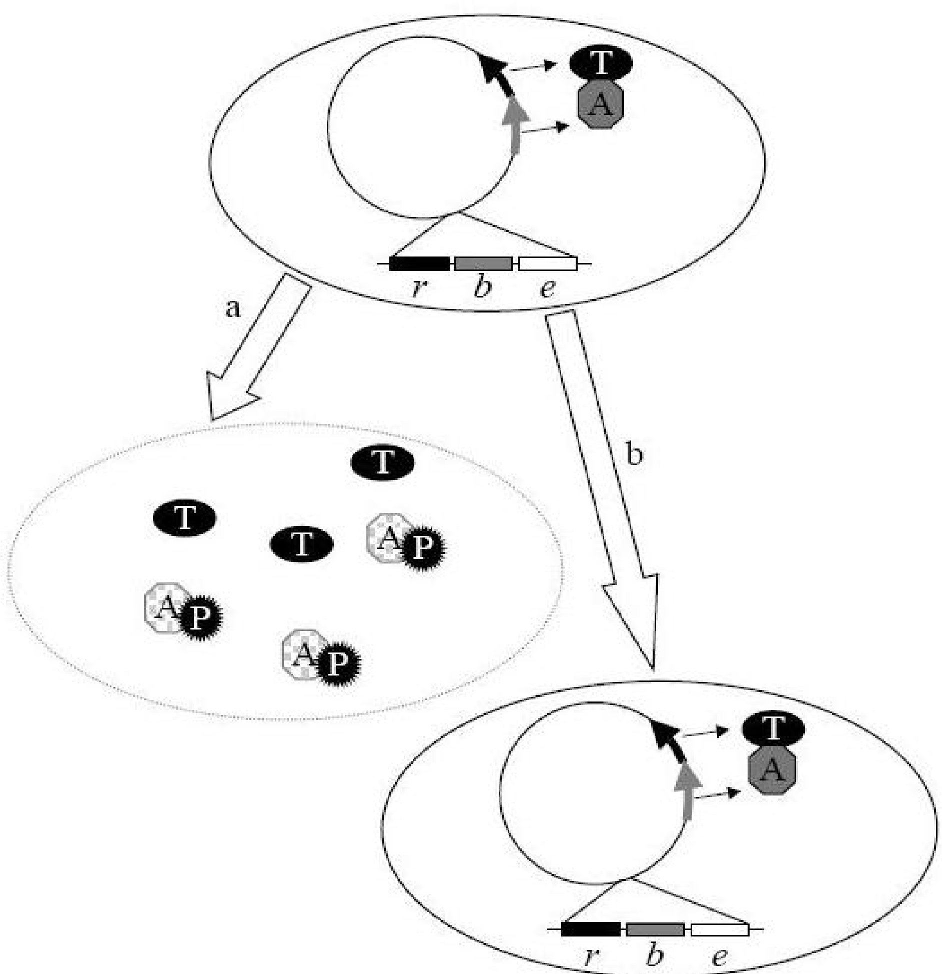


Figure 15. Maintenance of antibiotic resistance platforms in the absence of antibiotic selective pressure. Antibiotic resistance genes are acquired by genetic platforms that can disseminate among bacterial populations (Martínez, 2009).

The Figure 15 represents a bacterium carrying one plasmid. The plasmid can contain different genes, which encode determinants with adaptive values for the recipient bacterium. This includes antibiotic resistance genes (**r**), biocide resistance or heavy metal resistance determinants (**b**) or ecologically rewarding elements (**e**), such as siderophores, microcins or toxins among others.

Even in the absence of antibiotics, the presence of these determinants associated in the same plasmid can favour its selection and thus co-selection of antibiotic resistance. On the other hand, several plasmids express toxin (**T**)/antitoxin (**A**) systems that impede their loss. The antitoxin binds the toxin and thus impedes bacterial death. However, if the plasmid is lost (**a**), the production of the two proteins is blocked and the antitoxin is rapidly degraded by a protease (**P**), liberating the toxin and allowing bacterial killing. If the plasmid is maintained (**b**), there is no interruption to antitoxin production and the activity of the toxin is inhibited. If this type of plasmid contains resistance genes, their loss, even in the absence of antibiotic

selective pressure, is unlikely. Bacterial resistance can be transferred to other bacteria living in other environments such as ground water or drinking water (Figure 16).

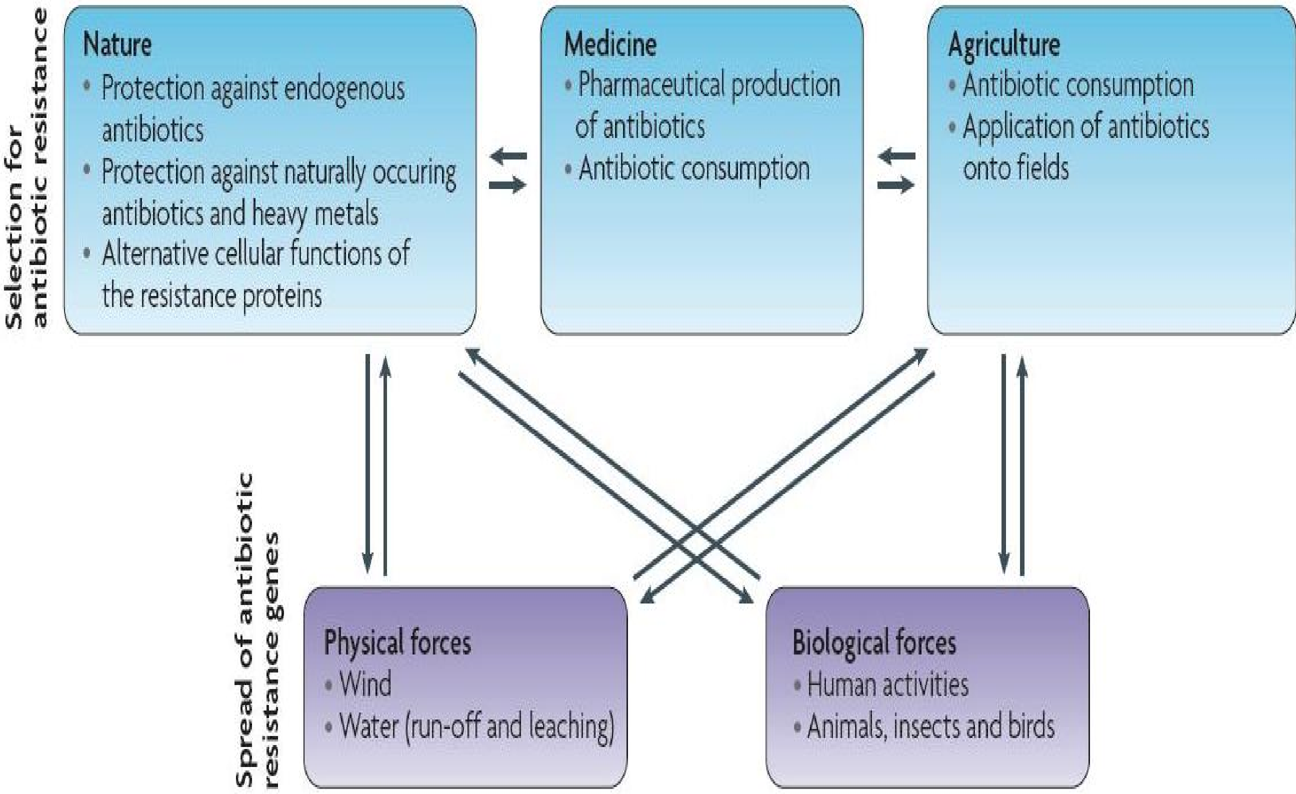


Figure 16. Sources and movement of antibiotic resistance genes in the environment. Resistance genes exist naturally in the environment owing to a range of selective pressures in nature. Humans have applied additional selective pressure for antibiotic resistance genes because of the large quantities of antibiotics that we produce, consume and apply in medicine and agriculture. Physical and biological forces also cause widespread dissemination of resistance genes throughout many environments (Allen et al., 2010).

All this raises questions for human health and the stability of the ecosystem.

Resistant and even multi-resistant pathogenic bacteria have been detected in wastewater and STPs, as well as in other environmental compartments (Guardabassi et al., 1998. Witte, 1998; Sacher et al., 2001). Furthermore, in arid regions, wastewater containing resistant bacteria and antibiotics is used for irrigation and sewage sludge serves as a fertilizer.

This allows resistant bacteria to enter the food chain directly. Aquatic and soil ecosystems seem to act as reservoirs of antibiotic-resistant bacteria (Biyela et al., 2004; D’Costa et al., 2006), although the implications for human health and the stability of the ecosystem are still unknown.

Once antibiotics enter ecosystems, they can be treated as an ecological factor, driving the evolution of the community structure (Aminov & Mackie 2007).

Accordingly, a change in community structure influences the ecological functions of soil and water ecosystems, such as biomass production and nutrient transformation (Kotzerke et al., 2008).

In order to update our knowledge of the effects of antibiotics, it is important to know how they alter the microbial community structure, inhibit or promote ecological functions and affect the magnification of resistance, in particular in the context of the natural environment. Antibiotic concentrations, exposure time, substrate and multiple antibiotics are mainly factors influencing microbial communities in the natural environment (Ding & He, 2010). Effects of antibiotics on ecological functions have also been discovered, including nitrogen transformation, methanogenesis and sulphate reduction.

Microbial community structure and functioning can change upon exposure to antibiotics (Ding & He, 2010). The inherent reason is that antibiotics in general, even those designed to be broad-spectrum drugs, have their selective effects on various groups of microbes. Here, the group of microbes could be as broad as fungi or bacteria (Mohamed et al., 2005) or as narrow as a single genus (Yang et al., 2009) or species (Moenne-Loccoz et al., 2001), Table 6. As a result, the selective antibiotic effects alter the relative abundance of microbial species and subsequently interfere with the interactions among different species. Notably, the effects on the microbial population are dependent on the original soil properties (Čermák et al., 2008), microbial groups (Hammesfahr et al., 2008) and the dose of antibiotics added (Zielezny et al., 2006). Additionally, the ratios of bacteria/fungi and Gram-positive/Gram-negative bacteria (G+/G−) are the two most often used indicators to indicate changes in microbial community structure.

The effects on microbial communities include phylogenetic structure alteration, resistance expansion and ecological function disturbance in the micro-ecosystem (Ding & He, 2010). Our knowledge of the indirect effects of antibiotic disturbance on the micro-ecosystem are limited, but it is expected that such disturbance might have significant and long-term effects on the rate and stability of ecosystem functioning (Perry et al., 1989).

Table 6. Some antibiotics and their mode of action (Ding & He, 2010).

Antibiotic class	Example	Gram +	Gram -	Bactericidal action	Bacteriostatic action	Mode of action
Aminoglycosides	Kanamycin		X	X		A
	Streptomycin					
β-lactams	Amoxicillin	X	(X)	X		B
	Ampicillin					
	Benzylpenicillin					
	Penicillin					
Glycopeptides	Vancomycin	X		X		B
Macrolides	Erythromycin	X			X	A
	Tylosin					
Polypeptides	Bacitracin	X	(X)	X		B
	Colistin		X	X		C
	Polymyxin B		X	X		C
Quinolones	Ciprofloxacin	(X)	X	X		D
	Flumequine					
	Ofloxacin					
	Oxolinic acid					
Sulfonamides	Sulfachloropyridazine	X	X		X	E
	Sulfadiazine					
	Sulfamethoxazole					
	Sulfapyridine					
Tetracyclines	Chlortetracycline	X	X		X	A
	Doxycycline					
	Oxytetracycline					
	Tetracycline					

[a]Antibiotic mode of action: A inhibition of protein synthesis, B inhibition of cell wall synthesis, C impairment of cell membrane function, D inhibition of DNA synthesis and replication, E inhibition of folinic acid and consequently DNA synthesis.

2.2.3 Surface and groundwater contamination by pharmaceuticals

The quantities of pharmaceuticals reaching surface water depend on several factors, some theoretically predictable, like metabolism and degradation, some unpredictable, such as improper disposal (Zuccato et al., 2005). Monitoring environmental contamination by pharmaceuticals is advisable for several reasons, including reliable assessment of risks for the environment and, through the food chain, for man.

Studies undertaken in USA, Europe and Canada have detected a wide range of drugs in groundwater, surface water and even drinking water systems (Ternes, 2001; Zuccato E. et al.,

2000). Pharmaceuticals have been detected in rivers (Kolpin et al., 2002; Zuccato et al., 2005; Zuccato et al., 2010), seas (Weigel et al., 2002), lakes (Tixier et al., 2003), groundwater (Sacher et al., 2001; Jones et al., 2002), sediments (Zuccato et al., 2000; Loos et al., 2010) and soils (Díaz-Cruz et al., 2003) and this widespread contamination can pose potential risks to humans and the environment (Kümmerer, 2001).

The following tables report examples of antibiotic concentrations in waters, PECs PNECs and bioconcentration factors and the chemical-physical and mobility parameters of various pharmaceuticals (Table 7, 8 and 9).

Table 7. Examples of antibiotic concentrations (Louvet et al., 2010).

	Antibiotic	**Type**	**Concentrations (μg L^{-1})**			**Ref.**
			Median	Min	Max	
Raw urban wastewater influents	Erythromycin-H_2O	Macrolide	0.34	0.07	1.2	A
	Erythromycin-H_2O	Macrolide	0.63	0.47	0.81	B
	Erythromycin-H_2O	Macrolide		0.25	1.98	C
	Roxithromycin	Macrolide		0.07	0.16	C
	Clarithromycin	Macrolide	0.11	0.11	0.72	D
	Azithromycin	Macrolide	0.17	0.09	0.38	E
Hospital effluents	Erythromycin-H_2O	Macrolide	0.94		6.11	F
	Sulfamethoxazole	Sulfonamide	0.03		1340	F
	Ciprofloxacin	Fluoroquinolone		28,000	31,000	G
	Norfloxacin	Fluoroquinolone		320	390	G
	Enoxacin	Fluoroquinolone		150	300	G
Urban WWTP effluents	Erythromycin-H_2O	Macrolide		0.11	0.2	A
	Erythromycin-H_2O	Macrolide	0.27	0.09	0.3	H
River water	Erythromycin-H_2O	Macrolide	0.034		0.044	I
	Erythromycin-H_2O	Macrolide			75.5	L
	Erythromycin-H_2O	Macrolide	0.46		0.636	M
	Erythromycin-H_2O	Macrolide	0.03		0.423	M
	Azithromycin	Macrolide	0.017	0.009	0.068	I
	Sulfamethoxazole	Sulfonamide	0.037	0.022	0.169	I

WWTP: wastewater treatment plant

Ref.: A: Karthikeyan KG, Meyer MT, 2006. *Sci. Total Environ.* 361:196-207; **B**: Gulkowska A, Leung HW, So MK, Taniyasu S, Yamashita N, Yeung LWY, Richardson BJ, Lei AP, Giesy JP, Lam PKS, 2008. *Water Res.* 42:395-403; **C**: Xu W, Zhang G, Li X, Zouc S, Lia P, Hua Z, Lia J. 2007. *Water Res.* 41:4526–4534; **D:** Spongberg AL, Witter JD, 2008. *Sci. Total Environ.* 397:148-157; **E:** Göbel A., Thomsen A, Mcardell CS, Joss A, Giger W, 2005. *Environ. Sci. Technol.* 39:3981-3989; **F:** Yu-Chen Lin A, Tsai Y-T, 2009. *Sci Total Environ.* 407:3793-3802; **G:** Larsson DGJ, de Pedro C, Paxeus N, 2007. J. *Hazard. Mater.* 148:751–755; **H:** Batt AL, Bruce IB, Aga DS, 2006. *Environ. Pollut.* 142:295-302; **I:** Gros M, Petrović M, Barceló, D, 2007. *Environ. Chem.* 26:1553-1562; **L:** Lin AYC, Tsai YT, 2009. *Sci. Total*

Environ. 407:3793-3802; **M:** Xu WH, Zhang G, Zou SC, Li XD, Liu YC, 2007. *Environ. Pollut.* 145:672-679.

Table 8. PECs PNECs and bioconcentration factors for same pharmaceuticals (Jones et al., 2002, modified).

Compound name	Amount used (kg)	PEC (mg L^{-1})	PNEC (mg L^{-1})	PEC:PNEC	BCF
Paracetamol	390,954.26	11.96	136	0.09	3.162
Metformin hydrochloride	205,795.00	6.30	511.57	0.01	3.162
Ibuprofen	162,209.06	4.96	9.06	0.55	3.162
Amoxycillin	71,466.83	2.19	0.0037	588.02	3.162
Carbamazepine	40,348.75	1.23	6.359	0.19	15.36
Ferrous sulphated	37,538.52	1.15	7.1	0.16	3.162
Naproxen	35,065.98	1.07	128	0.01	3.162
Oxytetracycline	27,195.11	0.83	0.23	3.60	3.162
Erythromycin	26,483.78	0.81	74	0.01	45.31
Quinine sulphate	16,731.26	0.51	20000	2.55×10^{-05}	5.623
Mefenamic acid	14,522.77	0.44	0.428	1.03	5.623

BCF: Bioconcentration factor

Table 9. Chemical-physical and mobility parameters (Bottoni et al., 2010, modified).

Compound	**Water sol.**	**pKa**	**K_{oc}**	**log K_{ow}**	**Kd ($L\ kg^{-1}$)**	**DT_{50} (days)**	
						water	**soil**
clofibric acid	insoluble			2.57		> 63	
mefenamic acid	0.00041 $g\ L^{-1}$	4.2	461.0	5.12	18917		
amoxycillin	4.0 $mg\ mL^{-1}$		865.0	0.87	1.06		
aspirin	4600 $mg\ L^{-1}$	3.5	10.0	1.19	2.22		
atenolol	slightly sol.	9.2	148.0	0.16	0.21		
carbamazepine	insoluble			2.45		63; 100	
benzimidazole	slightly sol.	5.48	110	1.32-6.2			15
carbamazine	insoluble	13.9	3870	2.25	25.5		
diclofenac	~ 9 $mg\ mL^{-1}$	4.2	883.0	0.70-4.51	0.72	5.0	
diltiazem hydrochloride	soluble	7.7	9500	2.70	72		
erythromycin	2 $mg\ mL^{-1}$	8.9	10.0	3.06	165		
fenoxymethylpenicilline	5.88 $mg\ L^{-1}$		177	2.09	17.6		
ibuprofen	25 $mg\ L^{-1}$	4.91	394.0	3.97-4.9	454	20; 32	
mebeverine hydrochloride			6.66×10^5	3.82	948		
metformin hydrochloride	soluble	12.4	110	-1.43	0.0003		
naproxen	insoluble	4.15	349.0	3.18-3.24	217	14	
oxytetracycline	slightly sol.	3.3	97.2	-0.90	0.02		
paracetamol (acetaminofen)	slightly sol.	9.4	61.7	0.46	0.41		
propanolol	soluble	9.45		-0.45 pH 2		16.8	
quinine sulfate	~1 $g\ L^{-1}$	5.07	1.85×10^8	5.40	36045		
ranitidine hydrochloride	soluble	3.5					
sodium valproate	slightly sol.		24.1	-0.85	0.02		
sulfate iron (III)	156.5 $g\ L^{-1}$		14.6	-0.37	0.06		
sulfasalazine	insoluble		1840	3.81	926		

2.2.4 Macrolide Antibiotics and Antiviral drug Tamiflu

Macrolides are among the thousands of pharmaceuticals that have been used in humans, agriculture and aquaculture and they have a significant impact upon ecosystems and human health (Table 10).

Table 10. Macrolide antibiotics considered in the experimental research. Data from Pubchem Compounds Database (Geer et al., 2010).

Name	Erythromycin	Josamycin
CAS	114-07-8	16846-24-5
Molecular Formula	$C_{37}H_{67}NO_{13}$	$C_{42}H_{69}NO_{15}$
IUPAC name	(3R,4S,5S,6R,7R,9R,11R,12R,13S,14R)-6-[(2S,3R,4S, 6R)-4-(dimethylamino)-3-hydroxy-6-methyloxan-2- l]oxy-14-ethyl-7,12, 13-trihydroxy-4-[(2R,4R,5S,6S)-5-hydroxy-4-methoxy-4,6-dimethyloxan-2-yl]oxy-3,5,7,9,11,13-hexamethyl-oxacyclotetradecane-2, 10-dione	[(2S,3R,4R,6S)-6-(2R,3S,4R,5R,6S)-6-[(4R,6S,7R,9R,10R,11E,13E, 16R)-4-acetyloxy-10-hydroxy-5-methoxy-9, 16-dimethyl-2-oxo-7-(2-oxoethyl)-1-oxacyclohexadeca-11, 13-dien-6-yl]oxy]-4-(dimethylamino)-5-hydroxy-2-methyloxan-3-yl]oxy-4-hydroxy-2,4-dimethyloxan-3-yl] 3-methylbutanoate
production	Produced by *Streptomyces erythreus*.	produced by *Streptomyces narbonensis*

Macrolides are a group of antibiotics that have been widely used for human medical and veterinary purposes. They are a group of basic and lipophilic antibiotics that are highly potent against a wide variety of Gram-positive and Gram-negative organisms. They exert their antibiotic effect by binding irreversibly to the 50S subunit of bacterial ribosomes. Ribosomes are the protein factories of cells and by binding to them macrolides inhibit translocation of tRNA during translation (the production of proteins under the direction of DNA).

Macrolides (which include **erythromycin and josamycin**, Table 10) are considered high priority pharmaceuticals based on their consumption, physical/chemical properties, toxicity, occurrence, persistence and resistance to treatment (de Voogt et al., 2009). They have been detected in Italian rivers (Po and Lambro), at 0.9 - 15.90 ng L^{-1} in 1997 and 2001 for josamycin and erythromycin respectively (Castiglioni et al., 2004). Moreover erythromycin is detected in Italian STP effluent (52 ng L^{-1}; Zuccato et al., 2010) and is reported to be stable in fresh water for considerable periods ($DT_{50} \geq 1$ year; Zuccato et al., 2005).

Erythromycin is one of the most important macrolide antibiotics. It is a bacteriostatic antibiotic macrolide widely used in human medicine (principally in ambulatory care), as well as by farmers to control bacterial diseases and promote animal growth. It is produced by fermentation of a strain of *Streptomyces erythraeus* (Wang, 2009) and is most effective in vitro against mycoplasma, Gram-positive cocci such as *Staphylococcus aureus* (penicillin G-sensitive or resistant) and some strains of *Haemophilus influenzae*, *Pasteurella multocida*, *Brucella*, *Rickettsia* and *Treponema*. In veterinary medicine, erythromycin is used for the treatment of clinical and subclinical mastitis in lactating cow infectious diseases due to erythromycin-sensitive bacteria in cattle, sheep, swine and poultry and chronic respiratory diseases due to mycoplasma in poultry (EMEA, 2008). In the aquaculture industry, erythromycin is employed to treat infection from Gram-positive cocci such as *Lactococcus garvieae* in trout (Lucchetti et al., 2005), to medicate various bacterial infections in multiple species of food-fish and non-food aquarium fish and to control the bacterial kidney disease caused by the Gram-positive bacillus *Renibacterium salmoninarum* in salmonids (Bullock & Leek, 1986; Billedeauet al., 2003).

The presence of macrolide residues in the food supply and environment as a result of human medical and veterinary practices has raised concerns among scientists and others. With regard to environmental contamination, erythromycin A was more frequently detected in 139 United States stream sites than 21 other veterinary and human antibiotics (Kolpin et al., 2002).

The treatment of influenza pandemic is possible with **antiviral drugs.** An influenza pandemic, which could occur through mutation of the present deadly avian strain H5N1, has galvanized global efforts to understand the potential benefits and limitations of mitigation strategies. Over recent years influenza A viral infections have posed serious risks to public health. Since 2003, 286 lethal human cases of 'avian' influenza H5N1 have been confirmed by the WHO and worldwide several countries have reported laboratory-confirmed cases of 'swine' influenza H1N1, including several deaths. Although vaccination is the primary strategy for prevention, antiviral drugs are recommended for the treatment and prevention of influenza A.

There are two groups of antiviral drugs: M2 inhibitors (amantadine and rimantadine) and neuraminidase inhibitors (oseltamivir and zanamivir).

Oseltamivir phosphate **(Tamiflu)** is the drug of choice for treatment and prophylaxis of influenza viruses (influenza A H5N1, 'avian' influenza and H1N1, 'swine' influenza) by the World Health Organization (WHO, 2006). It is a specific inhibitor of influenza A and B virus neuraminidase.

Hundreds of millions of courses of Tamiflu have been stockpiled worldwide since 2003 and in the last year sales of it have grown very significantly.

Despite the effectiveness of these drugs in reducing influenza-related illness and mortality, the emergence of drug resistance poses a critical limitation for their application. The incidence of viral resistance to M2 inhibitors has been associated with an increasing rate of

seasonal influenza, possibly through the widespread or indiscriminate use of the drugs (Bright et al., 2005). Neuraminidase inhibitors are less prone to selecting for resistant mutations (Moscona, 2005; Regoes & Bonhoeffer, 2006) and therefore offer a better option for pandemic preparedness. However, the recent emergence of oseltamivir resistance has raised concerns about how we might deal with influenza pandemic (Kiso et al., 2004; de Jong et al., 2005; Moscona, 2005; Regoes & Bonhoeffer, 2006). There is therefore particular concern if they are present in groundwater both because it could be used as drinking water (Hirsch et al., 1999) and because of its naturally slow remediation capacity.

Previous modelling studies have considered pharmaceutical and non-pharmaceutical interventions (Ferguson et al., 2003, 2005, 2006; Longini et al., 2004, 2005; Gani et al., 2005; Germann et al., 2006) and rationalized the use of antiviral drugs as the first-line defence against a new pandemic strain. The effects of these drugs are twofold: (i) they reduce the infectivity and duration of infectiousness by inhibiting virus replication and (ii) reduce susceptibility. These in turn decelerate the spread of infection in the population and provide time for the development of new vaccine candidates.

Oseltamivir phosphate (OP) is the pro-drug of the active metabolite oseltamivir carboxylate (OC), (Kim et al., 1997). Pharmacological studies have demonstrated that after oral administration of OP and absorption in the gastrointestinal tract, it is converted by the hepatic esterase to OC. It has been shown to be clinically active for the treatment and chemoprophylaxis of influenza in adults and children (Ward et al., 2005). It reduces viral infection by binding to the highly conserved active site of the neuraminidase of the virus, inhibiting the release of progeny virions from the surface of infected cells (Bardsley-Elliot & Noble 1999). Some characteristics of OP and OC are given in Table 11. More than 80% of each oral dose of OP is eliminated by renal excretion as OC (Ward et al., 2005).

Given its resistance to degradation and its hydrophilicity, the active metabolite OC is expected to enter the aquatic ecosystem from sewage treatment plants. In fact, recent publications have shown that the active metabolite OC is not degraded in sewage treatment plants and is also persistent in aquatic environments (Singer et al., 2008; Fick et al., 2007; Saccà et al., 2009; Accinelli et al., 2010). Consequently, in the case of urban areas with a large number of patients receiving Tamiflu, there is a potential risk of OC contaminating the aquatic ecosystem (Fick et al., 2007; Singer et al., 2007, 2008). This implies that OC will be present in aquatic environments in areas where oseltamivir is prescribed to patients for therapeutic use, owing to its limited mineralisation based on a standard Organization for Economic Co-operation and Development (OECD) biodegradability test (EMEA, 2005). Hence the OC has the potential to be maintained in rivers receiving treated wastewater. In countries where oseltamivir has already been used for some time, e.g. Japan, where it is used to treat seasonal flu, it is detectable in waterways and in sewage treatment plants at high levels (from 58 to 190.2 ng L^{-1} OC during the peak of the flu season; Ghosh et al., 2010; Söderström et al., 2009).

Table 11. Chemical structure and main chemical-physical characteristics of Tamiflu (OP) and of its active form OC. (Singer et al., 2007; American Hospital Formulary Service, 2006).

Pharmaceutical:	Pro-drug Tamiflu (OP)	Active form OC
	OP	OC
IUPAC name	Ethyl(3R,4R, 5S)-4-acetamido-5-amino-3-pentan-3-yloxycyclohexene-1-carboxylate; phosphoric acid	-
CAS	196618-13-0	-
Molecular weight	312.41	284.35
Solubility (water), 25°C	> 200 mg L^{-1}	> 500 mg L^{-1}
Melting point	~193°C (OP-PO_3)	
Vapour pressure	~≤1.4 x 10^{-3}	~≤7.3 x 10^{-10} Pa
partition coefficient (log P)		1.1

Predicted environmental concentrations (PEC) of OC, calculated in catchments with particularly low flow and high populations, could be over 20 μg L^{-1}, which is significantly higher than that observed for most other pharmaceutical contaminants (Singer et al., 2008). Chronic ecotoxicity tests, conducted in the light of the EMEA guidelines (2006) on environmental risk assessment for human pharmaceuticals, revealed that the ecotoxicity level of concern is quite low. The preliminary no observed effects concentrations (NOECs) resulted in a PNEC of 100 μg L^{-1}, applying an assessment factor of 10 (Singer et al., 2007).

The natural reservoir of the influenza virus, dabbling ducks, is thus exposed to oseltamivir, which could promote the evolution of viral resistance, as reported in recent studies (Singer et al., 2007; Fick et al., 2007); questions about the widespread use of Tamiflu in seasonal epidemics and the potential ecotoxicological risk associated with its use in the event of a pandemic have thus been raised (Straub, 2002).

Considerable concerns are the potential inhibition of non-target neuraminidases in different organisms than influenza viruses and the fact that the presence of OC in rivers can be a risk for the generation of OC resistance in influenza viruses. A recent analysis of isolated A viruses revealed a high increase in Tamiflu-resistant strains in different countries worldwide (Hurt et al., 2009).

Previous studies have indicated that the fate of OC in surface water is mainly governed by microbial and photochemical processes (Accinelli et al., 2007; Bartels & von Tümpling, 2008) and it has a negligible degradation in river water in the absence of an active bacterial community (Accinelli et al., 2007). Owing to the generally low metabolic potential of surface waters, OC is not rapidly removed from them (Accinelli et al., 2007; Saccà et al., 2009). Consequently, widespread use of Tamiflu may be considered a potential contaminant of aquatic ecosystems, leading to the development of OC-resistant strains of the viruses (Fick et al., 2007; Singer et al., 2007), and a mass administration of Tamiflu could pose a risk for drinking water safety and ecological health. Moreover the effects of Tamiflu on microbial communities have not well studied, in particular that on the bacterial community structure of a surface water ecosystem.

2.2.5 Legislation

Over recent decades legislation on the impact of chemical pollution has focused almost exclusively on conventional 'priority pollutants', especially those acutely toxic/carcinogenic pesticides and industrial intermediates displaying persistence in the environment (US EPA, 2009). Governments now regulate the use and disposal of toxic chemicals more rigorously than in the past and several forms of legislation have alternated in the past decades to control water pollution in the European Union. The first steps in European water legislation focused mainly on quality standards for certain types of waters (bathing waters, aquaculture and drinking waters), leading to the passing of the Drinking Water and the Bathing Water Directives. Within the European Union the quality of water for human consumption is determined by the Drinking Water Directive (Dir. 98/93/EC). Of the 48 parameters within the directive, none is related to pharmaceuticals.

In 2000, the Water Framework Directive, WFD (2000/60/EC), expanded EU water policy to all waters and addressed all sources of impacts. It defines the ecological quality according to hydro-morphological, physic-chemical and biological (biodiversity on the three levels: genetic, population, community) parameters and priority pollutant concentrations in water, sediments and organisms. The WFD Directive, its daughter Directive for the protection of groundwater (2006/118/EC, GWD), and its other daughter Directive on Priority Substances 2008/105/EC, which states the EU List of Priority Substances (also known as Annex X to WFD) for surface waters and related Environmental Quality Standards (EQSs), are the European legislation currently in force as regards the protection of aquatic environments and related organisms. The Directive 2008/105/EC identifies 33 substances or groups of substances, which have been shown to be of major concern for European waters and for which control measures should be adopted over the next 20 years. A further 14 substances were identified as being subject to review for identification as possible priority hazardous substances. The list includes selected chemicals, plant protection products, biocides, metals and other groups like Polyaromatic Hydrocarbons (PAH), which are mainly incineration by-products, and Polybrominated Biphenylethers (PBDE), used as flame retardants. Additionally

member countries have undertaken their own national reviews to identify future emerging contaminants. The revised List will come into force in the early months of 2011.

The much wider range of emerging pollutants that are now widely used is not included in the list however the priority substance list will be updated every 4 years and has identified future emerging priority candidates. In fact, due to the known pressures of pharmaceuticals on water ecosystems and their potential impacts to aquatic organisms, some relevant pharmaceuticals could be included among new priority candidates in the current or future revision of the EU List of Priority Substances covered by the Water Framework Directive 2000/60/EC (Bottoni et al., 2010). A simplified and pragmatic methodology was developed under the WFD Common Implementation Strategy taking into consideration both monitoring data and modelling data. Among the possible priority pharmaceuticals, it should be noted that some cytotoxic/genotoxic/reprotoxic substances (tamoxifen, cyclophosphamide etc.) synthetic estrogens and hormones, have already been identified as candidates for Point 4, Annex VIII WFD, (Indicative List of Main Pollutants: *"Substances and preparations, or the breakdown products of such, which have been proved to possess carcinogenic or mutagenic properties or properties which may affect steroidogenic, thyroid, reproduction or other endocrine-related functions in or via the aquatic environment)*. On the other hand, the inclusion of key pharmaceuticals in the EU List of Priority Substances should lead to extensive monitoring of ambient water, sediment and biota in EU countries, supported in this by the latest Daughter Directive 2009/90/EC, which defines the minimum performance criteria for chemical analysis methods applied in monitoring activities of significance for the WFD and GWD.

The U.S. Environmental Protection Agency currently has an active program called the Contaminant Candidate List (CCL) to identify contaminants in public drinking water that warrant detailed study. The most recent Contaminant Candidate List, CCL3, finalized on Sept. 22, 2009, includes, for the first time, 10 pharmaceutical compounds. The list includes one antibiotic (erythromycin) and nine hormones: 17 alpha-estradiol, 17 beta-estradiol, equilenin, equilin, estriol, estrone, ethinyl estradiol, mestranol and norethindrone.

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EXPERIMENTAL RESEARCH

Pesticides and pharmaceuticals are widely used in large quantities (tons/year) for different purposes (the former to control weeds in agricultural areas, the latter to control, prevent and treat human and animal diseases), and they can be considered ubiquitous pollutants (Schwarzbauer & Ricking, 2010; Götz et al., 2010; Loosa et al., 2010; Weigel et al., 2002). In particular, agricultural activity (use of pesticides) and drug administration or improper disposal of drug waste are the main ways in which pesticides and pharmaceuticals enter the environment using a variety of mechanisms.

The environmental chemistry, fate, toxicology and impact of pesticides, and in particular herbicides, used for selective control of annual weeds in fruit and field crops and non-crop areas, have been extensively studied in surface ecosystems, while the environmental fate and ecological effects of pharmaceuticals remain largely unknown both in surface and subsurface ecosystems, including groundwater. However, the risks posed by pesticides for the environment are also still largely unknown. For instance, in recent years there has been growing concern about the persistence, mobility and toxicity of triazines and their metabolites, owing to the detection of residual concentrations of these herbicides in groundwater. Since the number of aquifers that cannot be used for drinking purposes is increasing owing to contamination, there is a need to study the natural capacity of groundwater to recover from pesticide contamination. Studies exploring the ability of the indigenous microbial communities to degrade herbicides and the impact of these chemicals on microbial community structure may contribute to our understanding of how and when microorganisms indigenous to aquifers acclimatized to xenobiotic compounds. However, the presence in groundwater of bacterial strains with a degrading capability has not been thoroughly studied until now. Natural attenuation of contaminated groundwater has been achieved *in situ* (Williams et al., 2003; Tuxen et al., 2002) or in the laboratory setting (using indigenous bacteria from contaminated sites) only with a limited number of pesticides (Harrison et al., 2003; Johnson et al., 2000; Mirigain et al., 1995; Pucarevic et al., 2002) and never with *s*-triazines. Although there are many studies showing biotic and abiotic triazine degradation in soil and surface water (Di Corcia et al., 1999; Barra Caracciolo et al., 2005a, b), to our knowledge their degradation in groundwater remains to be explored. In order to fill this knowledge-gap, groundwater degradation experiments on *s*-triazines were performed and some bacterial populations able to transform them were isolated.

As already reported (see the Introduction and Figure 7), point sources of pesticide contamination, resulting from agricultural activities have recently been identified as causes of

soil and water pollution potentially more significant than diffuse contamination (Fait et al., 2007). The use of organic materials has been proposed for preventing the mobility of pesticides coming from point sources of contamination (Rodríguez-Cruz et al., 2007a). In particular, adsorbent wood residues, such as oak and pine, have recently been investigated as biomaterials for the immobilization of several pesticides in soil, including linuron (Rodríguez-Cruz et al., 2007b). However, any organic matter and nutrients added to soil can strongly affect the structure and activity of bacterial and fungal populations as a result of their increased metabolism of these readily available nutrients (Briceño et al., 2007; Sánchez-Camazano et al., 2000) and can consequently affect pesticide biodegradation. Some organic amendments may stimulate biodegradation, but others can reduce it (Moorman et al., 2001; Briceño et al., 2007). However, the effects of wood amendments on the soil microbial community and on the potential of this community for linuron and terbuthylazine degradation were not investigated in the past. For this reason degradation experiments on linuron and terbuthylazine in the presence of oak and pine amendments were performed.

Although pharmaceuticals and other health care products are extensively tested in mammals, their potential unintended environmental and ecological effects on receiving environments remain largely unknown. In recent years several authors have reported the occurrence of pharmaceuticals in environmental receiving water samples. Pharmaceutical substances were found in sewage treatment plants and in surface and groundwater samples (Lawrence et al., 2005). The industrial and domestic disposal routes were identified as the main contamination pathways for drugs into aquatic environments (Richardson & Bowron 1985). Industrial contamination may occur as a result of accidental discharge during production but is usually limited because of the high value of the chemicals. Domestic waste sources therefore represent the main and most consistent contamination pathway. The use of various drugs for treatment of human and animal medical conditions results in parent compounds and metabolites being excreted and entering aquatic receiving environments via sewage treatment plants. Although pharmaceutical and therapeutic products are widely found in the natural environment, there is limited understanding of their ecological effects and in particular their effects on microbial communities.

Several research experiments were performed in laboratory microcosms and/or in the field in order to better investigate the environmental fate of the selected compounds (such as terbuthylazine, simazine, linuron, oseltamivir carboxylate, josamycin and erythromycin) and the microbial populations involved in their degradation. Moreover, by using enrichment cultures and molecular techniques some bacterial species able to grow using the xenobiotic as the only source of carbon were isolated and identified.

The experimental research was, therefore, aimed at improving our knowledge of the response of soil and water microbial communities to xenobiotics (such as some classes of herbicides and pharmaceuticals) and was as follows:

1st PART: Research on the effects of triazine and phenylurea herbicides on groundwater and soil bacterial communities

A. Pesticide diffuse contamination: herbicide biodegradation experiments in groundwater

- ✓ Role of a groundwater bacterial community in terbuthylazine herbicide degradation and identification and isolation of the bacterial strains involved in *s*-triazine degradation.
- ✓ Isolation of *Rhodococcus wratislaviensis* able to use *s*-triazines as the carbon source and design of a new oligonucleotide probe for its detection by fluorescence *in situ* hybridization in contaminated groundwater and soil samples.

B. Point sources of pesticides: herbicide biodegradation experiments in agricultural soils in the presence of wood residues

- ✓ Changes in microbial activity in a soil amended with oak and pine residues and treated with linuron herbicide.
- ✓ Changes in microbial activity in a soil amended with oak and pine residues and treated with terbuthylazine herbicide.

2nd PART: Research activity regarding the effects of antibiotic and antiviral drugs on soil and water bacterial communities

- ✓ Characterization of the microbial community in an abandoned open quarry used for disposal of pharmaceutical waste.
- ✓ Effect of the antiviral drug oseltamivir (Tamiflu) on the bacterial community structure in a surface water ecosystem analysed using fluorescence *in situ* hybridization.

1st PART: Research on the effects of triazine and phenylurea herbicides on groundwater and soil bacterial communities

A. Pesticide diffuse contamination: herbicide biodegradation experiments in groundwater

Groundwater contamination by pesticides, and in particular herbicides such as terbuthylazine and its metabolite desethyl-terbuthylazine, is generally recognized as being caused by diffuse sources. In many European countries, in fact, a significant proportion of monitored groundwater is contaminated by triazines, at concentrations often above the maximum admissible concentration under the EC legislation (EC 98/83EEC), 0.1 mg L^{-1}. Triazine contamination of groundwater is a very serious environmental problem and risk to human health, especially when the water is used for drinking purposes. Although there have been several studies showing biotic and abiotic triazine degradation in soil, the natural capacity of groundwater to recover from pesticide contamination remains to be investigated. For this purpose we evaluated the capacity of the autochthonous bacterial community of a shallow aquifer, chronically contaminated with residual concentrations of *s*-triazines, to degrade terbuthylazine. In the course of the study, several bacterial strains able to use *s*-triazines as a sole carbon source were isolated and classified. However, only the strain *Rhodococcus wratislaviensis* FPA1 showed an ability to mineralise *s*-triazine. To the best of our knowledge, this is the first report showing the isolation, from groundwater, of a bacterial strain able to degrade *s*-triazines. Finally, a specific probe was designed for detecting *R. wratislaviensis* in natural soil and groundwater samples.
The results are published in the following papers:

- Barra Caracciolo A., Grenni P., Saccà M.L., Amalfitano S., Ciccoli R., Martin M., Gibello A., 2010. The role of a groundwater bacterial community in the degradation of the herbicide terbuthylazine. FEMS Microbiology Ecology, 71 (1): 127-136.
- Gibello A., Vela A.I., Martín M., Barra Caracciolo A., Grenni P., Fernández-Garayzábal J.F., 2009. Reclassification of the members of the genus *Tetrathiobacter* Ghosh et al. 2005 to the genus *Advenella* Coenye et al. 2005. International Journal of Systematic and Evolutionary Microbiology, 59: 1914-1918.
- Grenni P., Gibello A., Barra Caracciolo A., Fajardo C., Nande M., Vargas R., Saccà M.L., Martinez-Iñigo M.J., Ciccoli R., Martín M., 2009. A new fluorescent oligonucleotide probe for *in situ* detection of *s*-triazine-degrading *Rhodococcus wratislaviensis* in contaminated groundwater and soil samples. Water Research, 43: 2999-3008.

- Grenni P., Barra Caracciolo A., 2010. Un batterio in grado di degradare gli erbicidi. Cnr.it, Highlights 2008-2009. Edizioni Darwin-Consiglio Nazionale delle Ricerche, p. 26. ISSN: 1365-8816. http://www.cnr.it/documenti/Highlights2008_2009.pdf

Role of a groundwater bacterial community in the terbuthylazine herbicide degradation: laboratory microcosm experiments with groundwater samples collected from an *s*-triazine chronically contaminated aquifer

Abstract

A bacterial community in an aquifer contaminated by *s*-triazines was studied. Groundwater microcosms were treated with terbuthylazine at a concentration of 100 mg L^{-1} and degradation of the herbicide was assessed. The bacterial community structure (abundance and phylogenetic composition) and function (carbon production and cell viability) were analysed. The bacterial community was able to degrade the terbuthylazine; in particular, *Betaproteobacteria* were involved in the herbicide biotransformation. Identification of some bacterial isolates by PCR amplification of the 16S rRNA gene revealed the presence of two *Betaproteobacteria* species able to degrade the herbicide: *Advenella incenata* and *Janthinobacterium lividum*. PCR detection of the genes encoding *s*-triazine-degrading enzymes indicated the presence of the *atz*A and *atz*B genes in *A. incenata* and the *atz*B and *atz*C genes in *J. lividum*. The nucleotide sequences of the PCR fragments of the *atz* genes from these strains were 100% identical to the homologous genes of the *Pseudomonas* sp. strain ADP. In conclusion, the results show the potential for the use of a natural attenuation strategy in the treatment of aquifers polluted with the terbuthylazine. The two bacteria isolated could facilitate the implementation of effective bioremediation protocols, especially in the case of the significant amounts of herbicide that can be found in groundwater as a result of accidental spills.

Introduction

Protection of ground and surface water quality is critical to human health and environmental quality, as well as economic viability. In fact groundwater is an important drinking water resource. The presence of contaminants in groundwater is a common phenomenon and derives from many anthropogenic activities. Among these activities most likely to pollute water resources are the use of fertilizers, pesticides, application of livestock, poultry manure, and urban sludge. Therefore, agriculture results to be a significant contributor to diffuse and point sources of groundwater contamination. Studies exploring the potential of the indigenous microbial communities to degrade herbicides and the impact of these chemicals on microbial community structure may contribute to our understanding of how and when microorganisms indigenous to aquifers acclimate to xenobiotic compounds.

s-Triazines are among the most commonly used herbicides in the world. In recent years, concern has been growing about the persistence, mobility and toxicity of triazines and their metabolites, owing to the residual concentrations of these compounds detected in aquifers

(Tappe et al., 2002). In many European countries, a significant proportion of monitored groundwater is contaminated by triazines, primarily atrazine, terbuthylazine (TBA) and their desethyl-degradation metabolites; the concentration of these contaminants is often >0.1 mg L^{-1}, the maximum admissible concentration under the EC legislation (EC 98/83EEC) for potable water (Tappe et al., 2002; Guzzella et al., 2003, 2006; Hildebrandt et al., 2008). There may also be no sealed wells located in agricultural areas that become significantly (in the agricultural dose range) contaminated by accidental spills from the devices used to apply the herbicide (Fait et al., 2007).

Among *s*-triazines, terbuthylazine is the most persistent in surface environments (Guzzella et al., 2006; Carafa et al., 2007); in groundwater, its half-life ranges from 263 to 366 days (Navarro et al., 2004a). Toxic pollutant contamination of groundwater is a very serious environmental problem and is a risk to human health, especially because many communities depend on groundwater as the sole or as a major source of drinking water. In fact, >65% of the drinking water produced in Europe is sourced from groundwater. As the number of aquifers that cannot provide potable water is increasing, it is becoming necessary to study methods that will allow the removal of herbicides from groundwater, including physical, chemical and biological approaches.

The majority of studies addressing herbicide contamination of groundwater have focused on the rate of herbicide degradation. Only in the past few years has microbial acclimation to herbicides in subsurface aquifer environments been investigated (De Lipthay et al., 2003). Natural attenuation has been found in contaminated groundwater *in situ* (Tuxen et al., 2002; Williams et al., 2003) and in laboratory experiments using indigenous bacteria from contaminated sites (Mirigain et al., 1995; Franzmann et al., 2000; Johnson et al., 2000; Pucarevic et al., 2002; Harrison et al., 2003). To date, however, there have been few studies on natural attenuation in *s*-triazine-contaminated groundwater.

The natural attenuation of other herbicides has only been found when the concentrations exceed 40 mg L^{-1} and when it is associated with acclimated bacterial communities displaying positive degradation of the compound (Johnson et al., 2000; Broholm et al., 2001; Tuxen et al., 2002). In fact, the recalcitrant behaviour of the herbicide in groundwater could be a result of concentrations that are too low to induce bacterial degradation (Tappe et al., 2002). Biodegradation and mineralisation of *s*-triazines have been shown to be carried out by bacterial consortia and by strains isolated from contaminated sites (Aislabie et al., 2005; Grenni et al., 2009b).

Most commonly, the bacterial metabolism of *s*-triazines has been reported to occur via two different upper pathways. The first pathway involves the enzymes encoded by the *atz*A-*atz*B-*atz*C genes (Figure 9 and 10, pag. 30-32). The second utilizes the initiating enzymes of the hydrolytic reactions encoded by the *trz* gene family, such as *trz*N, and the *atz*B-*atz*C genes (Shapir et al., 2007). These genes are widespread, highly conserved in bacteria and are often associated with transposable elements on plasmids (Devers et al., 2007; Shapir et al., 2007). Both of these pathways lead to the formation of cyanuric acid and alkylamines as common

intermediates, which are ultimately degraded by the enzymes encoded by the *atz*DEF genes and by amine oxidases, respectively (Shapir et al., 2007). Although there have been several studies showing biotic and abiotic triazine degradation in soil (Di Corcia et al., 1999; Barra Caracciolo et al., 2005a, b), to our knowledge, degradation of these compounds in groundwater remains to be investigated. The fact that bacterial strains with the potential capability to degrade these compounds can be found in groundwater has important implications for planning remediation strategies and, in particular, for assessing the natural attenuation time and the implementation of bacterial strains for bio-augmentation purposes.

In this study, we assessed the capacity of the autochthonous bacterial community of a shallow aquifer that is chronically contaminated with residual concentrations of *s*-triazines to degrade terbuthylazine in microbiologically active and sterile groundwater microcosms. Specifically, we studied the bacterial community of the groundwater microcosms, analysing their structure (bacterial abundance and phylogenetic composition) and function [cell viability and bacterial carbon production (BCP)] throughout the experimental period. We also investigated the putative herbicide degradation pathway in the bacteria isolated from this aquifer.

Materials and methods

Groundwater collection and characteristics of the aquifer

The criteria used for selection of the site were as follows: an intensive agricultural area in which the use of the herbicide terbuthylazine was a common practice; intrinsic aquifer vulnerability (Daly et al., 2000). We selected an alluvial aquifer located near Assisi (PG), Central Italy, on the Petrignano Plane, at 216 m a.s.l. (Figure 1).

Figure 1. Groundwater sampling from a well.

The water table was surficial at 12 m depth. According to the Umbria Regional Environmental Agency's monitoring surveys (2000–2008), the herbicide terbuthylazine and its metabolite desethyl-terbuthylazine are always found in the groundwater at this site in

concentrations >0.1 µg L^{-1}. It is also common to find significant nitrate contamination at this site (>100 µg L^{-1}).

Some parameters (pH, O_2, redox potential, depth and conductivity) were analysed on site, and others were examined in the laboratory. Groundwater samples were collected by a sterile bailer from a well and placed in sterile polyethylene bottles to avoid any contamination. Subsamples were fixed or treated immediately for different purposes. The collected groundwater was stored (24 h maximum) at 4°C before use.

Following a 0.45-µm filtration, dissolved organic carbon was measured using a Shimadzu ASI-5000A Total Organic Carbon Analyser aqueous carbon analyser with a detection limit of 0.050 mg L^{-1}. Moreover, the total cell abundance (No. of bacteria mL^{-1} water) and cell viability (% live cells/live+dead) were assessed on the same day as the sampling in three replicates, as described below.

Microcosm setup for degradation studies

The experimental set-up (Figure 2) consisted of 80 closed vessels (100 mL capacity). Twenty microbiologically active microcosms (TBA) were set up with 50 mL of groundwater and the herbicide terbuthylazine (Dr Ehrenstorfer, Augsburg, Germany) at a concentration of 100 µg L^{-1}.

Figure 2. Groundwater microcosms.

In order to compare the degradation without bacteria, 20 additional microcosms (sterile) were set up with previously sterilized groundwater (120 °C, 20 min) and treated with terbuthylazine (100 µg L^{-1}). Moreover, 20 additional microcosms without terbuthylazine (control) were used as microbiological controls, to assess the effect of the herbicide on the bacterial community. The pH and oxygen content were constantly monitored throughout the

experimental period in another 20 herbicide-treated microcosms used only for this purpose. All microcosms were maintained in the dark, gently shaken and incubated at 15°C (the same temperature as that recorded in the aquifer). Two sacrificial microcosms for each experimental condition (TBA, sterile, control) were collected and sub-sampled for chemical and/or microbiological analyses at selected times (0, 7, 14, 28, 40, 60, 80, 124 and 175 days). For each analysis, we collected two subsamples from each microcosm (four replicates in total).

Chemical analysis

Terbuthylazine and desethyl-terbuthylazine concentrations were measured immediately after treatment and at various times until day 175. Cyanazine was added to the subsamples (four replicates for each condition) as an internal standard and each sample was extracted twice with methylene chloride. The extract was passed through a layer of anhydrous sodium sulphate to remove any residue of water and dried under a stream of nitrogen at room temperature. The extract was then reconstituted with 150-200 µL of methylene chloride and injected into a GC. The analyses were performed using a Thermo Finnegan Trace 2000 GC/MS (Waltham, MA) equipped with a model AS 2000 auto-sampler, operating with an electronic impact at 70 eV. The mean recovery was >80%.

Total cell number, viability, BCP and bacterial community composition

The total cell abundance (No. of bacteria mL^{-1}) was determined in four replicates of ethanol-fixed subsamples (four replicates of 5 mL each) by direct count, using 4'-6-diamidino-2-phenylindole (DAPI) as a DNA fluorescence agent (Barra Caracciolo et al., 2005a, b). Cell viability (% live cells/live+dead) was assessed in fresh replicate subsamples (5 mL each) using a two-dye fluorescent bacterial viability kit (Kit Live/Dead Bacterial Viability Kit, BacLight^{TM}) that distinguishes between viable (green) and dead (red) cells under a fluorescence microscope (Alonso et al., 2002).We calculated the live cell abundance (No. of live bacteria mL^{-1}) from the total cell abundance, obtained by DAPI counts, multiplied by viability (expressed as % live cells/live+dead).

BCP was estimated by [^{3}H]leucine (NEN Life Science Products, Boston) incorporation measurements, using the micro-centrifugation method (Smith & Azam, 1992). Briefly, subsamples of groundwater (1.7 mL each) were amended with 20 nM radiotracer (saturation value) and incubated for 1 h at 20°C. Zero-time controls were run by killing samples with 100% trichloroacetic acid (TCA, 5% final concentration) 15 min before leucine addition. The extraction of labelled macromolecules was carried out by washing with 5% TCA and 80% ethanol. Each washing step was performed by centrifugation at 15800 *g* for 10 min at room temperature. The supernatant was discarded and 1 mL of liquid scintillation cocktail was added to all samples. Radioactivity was detected using a TRICARB 4430 (Packard

Bioscience) scintillation counter. The rates of leucine incorporation were converted into units of C per millilitre (ng C mL^{-1} h^{-1}) by applying the conversion factor of 3.1 kg C produced per mole of incorporated leucine (Kirchman, 2001).

The phylogenetic composition of the bacterioplancton was analysed in four replicates of fixed subsamples (5 mL each) by FISH, using Cy3-labelled commercially synthesized oligonucleotide probes (Biomers.net, Ulm, Germany). The probes used were ARCH915 (*Archaea* domain), EUB338I-III (*Bacteria* domain), ALF1b (*Alphaproteobacteria*), BET42a (*Betaproteobacteria*), GAM42a (*Gammaproteobacteria*), HGC69A (Gram-positive with a high DNA G+C content), Pla46 (*Planctomycetes*), CF319a (*Cytophaga- Flaviobacterium* cluster phylum CFB), LGC354a (*Firmicutes*, Gram-positive bacteria with a low G+C content), EPS710 (*Epsilonproteobacteria*) and SRB385 (sulphate-reducing *Deltaproteobacteria*). Further details of these probes are available at http://www.microbial-ecology.net/probebase (Loy et al., 2003, 2007).

Each groundwater subsample was filtered through a 0.2-μm polycarbonate membrane using a gentle vacuum (<0.2 bar), followed by 70%, 90% and 95% (v/v) ethanol series for 10 min each at room temperature, and then air dried. FISH of the harvested cells, counterstained with DAPI, was performed according to published protocols (Pernthaler et al., 2001; Barra Caracciolo et al., 2005c).

The averages of the number of cells binding each of the probes were 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 mounted with a drop of Vecta-Shield and the preparations were examined and counted on a Leica DM 4000B epifluorescence microscope at ×1000 magnification.

Isolation and characterization of bacterial strains from terbuthylazine-treated groundwater microcosms

In order to test for the presence of cultivable bacterial strains able to grow on the terbuthylazine herbicide as the sole carbon source, aliquots (10 μL) of TBA microcosm groundwater (collected at 40 and 60 days) were used as inoculum. The aliquots were plated on minimal medium MB (K_2HPO_4, 1.6 g L^{-1}; KH2 PO_4, 0.4 g L^{-1}; $CaSO_4 \times 2H_2O$, 0.1 g L^{-1}; $MgSO_4 \times 7H_2O$, 1.0 g L^{-1}; $FeSO_4 \times 7H_2O$, 0.02 g L^{-1}; $(NH_4)_2SO_4$, 2 g L^{-1}; agar, 15 g L^{-1}), supplemented with 100 μg L^{-1} terbuthylazine as the carbon source and were incubated at two different temperatures (15 and 20 °C).

Once some bacterial colonies appeared, they were sub-cultured on plates of the same media to obtain pure cultures. The isolates were subjected to morphological and Gram-staining characterization and subsequently to phylogenetic analysis by FISH.

Bacterial isolates were cultivated aerobically (in duplicate) at 28°C in 100-mL flasks containing 30 mL of Luria-Bertani (LB) medium, supplemented with 10 mg L^{-1} of terbuthylazine and 0.03% casaminoacid.

Chemical analysis of terbuthylazine was performed, as reported above, in order to assess the biodegradation capability of the two isolated strains. Moreover, cells growing in the exponential phase were harvested by centrifugation and resuspended in phosphate-buffered saline. A volume of 30 µL of this cell suspension was adjusted to a concentration of 10^5–10^7 cells cm^{-2} and filtered onto 0.2-µm pore size polycarbonate filters (47 mm diameter, Isopore GTTP, Millipore, Germany). Samples were fixed using 70%, 90% and 95% (v/v) ethanol series for 10 min each at room temperature. Filters were stored at -20°C and FISH was performed using the specific probes described above.

Species-level identification of the bacterial isolates was carried out by biochemical characterization (determined using the API 20NE and, when necessary, the API 50CH systems of BioMérieux SA), and by sequencing of the 16S rRNA genes.

Identification of the environmental isolates was performed by comparative 16S rRNA gene sequence analysis. DNA from each isolate was extracted using the method described in Casas et al. (1995). The amplification of a 1500-bp fragment corresponding to the 16S rRNA gene was performed in a Mastercycler Personal (Eppendorf), using the universal primers and conditions described by Willems & Collins (1996). Amplicons were purified using the Qiaquick PCR Purification kit (Quiagen GmbH, Hilden, Germany), and both strands of the 16S rRNA gene were sequenced using the DyeDeoxy (dRhodamine) Terminator Cycle Sequencing kit in an automatic ABI Prism 373 DNA sequencer (Applied Biosystems) from SECUGEN facilities (Centro de Investigaciones Biologicas, CSIC, Spain). DNA sequences corresponding to the 16S rRNA gene were compared with those available in the GenBank/EMBL databases using the BLAST software (http://www.ncbi.nlm.nih.gov/BLAST).

Analysis of the genes encoding s-triazine degrading enzymes in bacterial isolates

The pathway of terbuthylazine degradation in bacterial isolates was studied by detection of the *atz* genes, which encode the *s*-triazine-degrading enzymes, using two different methods: FISH and PCR.

AtzB-FISH analyses

Before filtering, isolated bacteria were incubated with lysozyme (12 mg L^{-1}) for 20 min at 37°C to permeabilize the cells. Filters with cells fixed as described previously were analysed by FISH using the specific probe 5’FAM-AtzB1 (5’-GGA GAG CAC CGA TAC TTT TCT T-3’), under the conditions described previously (Martin et al., 2008). Percentages of *atz*B-harbouring cells were calculated based on the total number of cells stained with DAPI.

PCR amplification of atz genes

PCR was performed using the primers designed (de Souza et al., 1998; Mulbry et al., 2002; Devers et al., 2004) to amplify the conserved DNA regions of the *s*-triazine catabolic genes *atz*A (atrazine chlorohydrolase), *atz*B (hydroxyatrazine ethylaminohydrolase), *atz*C (N-

isopropylammelide isopropylamidohydrolase), *atz*D (cyanuric acid amidohydrolase) and *trz*N (triazine hydrolase). Reactions were conducted in a final volume of 100 μL, containing a DNA template (30–50 ng of bacterial DNA), 0.2 mM of dNTPs, 0.4 μM of both primers and 0.05 U μL^{-1} of Taq DNA polymerase (Biotools B & M laboratories SA). Following an initial denaturation step of 95°C for 1 min, the amplifications were carried out in a Mastercycler gradient (Eppendorf) as follows: 40 cycles of 1 min at 94°C, 1 min at 55°C and 1 min at 72°C, plus an additional 10-min cycle at 72°C. PCR products were separated by electrophoresis on 1% agarose gels. Negative (no template DNA) and positive (50 ng of purified DNA from *Pseudomonas* sp. ADP) PCR controls were performed in the case of all *atz* genes. Amplification products were purified from the agarose gels or PCR reactions using the Geneclean Turbo Kit (MP Biomedicals LLC).

Statistical analysis

Results were expressed as mean ±SE and were statistically analysed using Student's *t*-test (at $p<0.05$).

Results

The main characteristics of the sampled aquifer and of the groundwater samples are reported in Table 1.

Table 1. Main characteristics of the aquifer and of the groundwater samples

Lithology	Alluvial sands, gravels
Geochemical facies	Alkaline-bicarbonate
Temperature (°C)	15
Eh (mV)	210
Conductibility (μS cm^{-1})	930
O_2 (mg L^{-1})	9.01
pH	6.87
DOC (mg L^{-1})	0.56
Terbuthylazine (μg L^{-1})	> 0.1
Desethyl-terbuthylazine (μg L^{-1})	> 0.1
Nitrate contamination (mg L^{-1})	> 100
Total cell abundance (No. bacteria mL^{-1})	2.0×10^4
Cell viability (%)	72

The detection of *s*-triazines, together with the high nitrate concentration, demonstrates that this groundwater represents a contaminated ecosystem. Moreover, the bacterial abundance and the high percentage of cell viability, similar to or greater than that found in some surface

soils studied (Martin et al., 2008; Grenni et al., 2009a), indicated the presence of a natural microbial community quite active in this aquifer.

Terbuthylazine degradation in groundwater microcosms

Figure 3 shows the terbuthylazine concentration as a percentage of original concentration vs. time (days) under the two different experimental conditions (TBA and sterile).

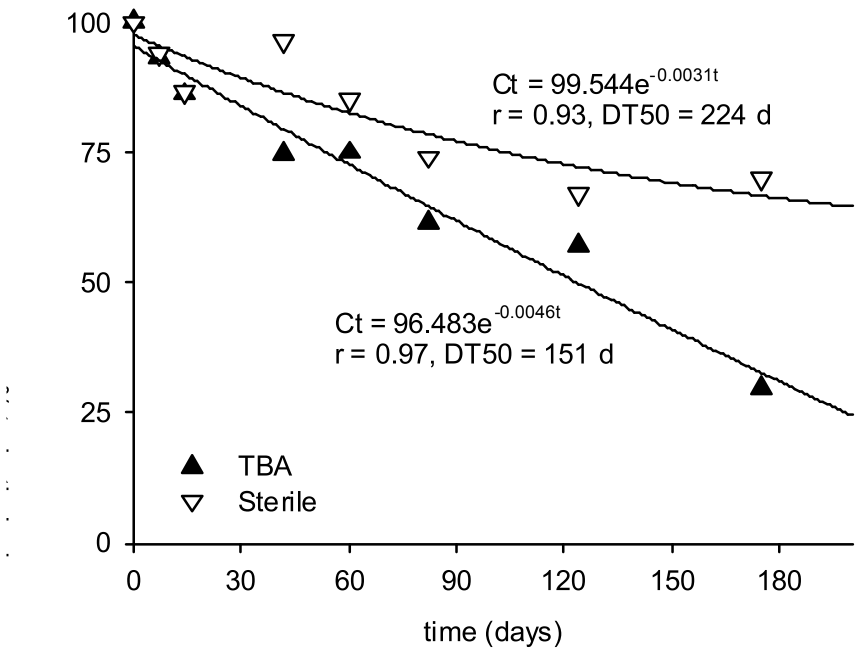

Figure 3. Decrease (%) of terbuthylazine concentrations in microbiologically active (TBA) and sterile (sterile) groundwater microcosms. The DT_{50} of the herbicide was calculated by the exponential equation obtained from the regression between concentrations (Ct) vs. time (days) in each experimental condition.

The disappearance time of 50% (DT_{50}) values (expressed in days) calculated from the regression curve between the detected concentrations (Ct) and the sampling times (t) show that there was a halving of the initial concentration of terbuthylazine in 151 ±0.9 days (r = 0.97) in the microbiologically active microcosms (TBA). Under sterile conditions, about 70% of the initial herbicide concentration remained at the end of the experiment and the theoretical DT_{50} value was 224 ±3 days (r = 0.93). These results indicate that the autochthonous bacterial community played an active role in degradation of the herbicide. The decrease in herbicide in the sterile condition was presumably due to terbuthylazine dehalogenation to its hydroxylated form, which, as is well known, occurs through hydrolysis (Mandelbaum et al., 1993; Di Corcia et al., 1999).

The metabolite desethyl-terbuthylazine was detected at increasing concentrations, starting from day 80 and reaching about 10 μg L^{-1} at day 175. This compound was never found in the

sterile microcosms. Oxygen and pH did not change significantly during the entire experimental period (*t*-tests not significant) under any of the conditions, with average values of 7.8 ±0.1 mg L^{-1} and 7.0 ±0.2, respectively. Consequently, we can exclude the effect of these parameters on the herbicide degradation pattern.

Live cell abundance

The live cell abundance in the treated (TBA) and the untreated (control) microcosms was similar, increasing to 10^5 live bacteria mL^{-1} until day 60. However, in the TBA microcosms, a significant increase was observed at both day 80 and day 124 (*t*-test, $p < 0.05$), with values of 2.8–3×10^5 and 2.5–2.9×10^5 live bacteria mL^{-1}, respectively. At the end of the experiment (175 days), the live cell abundance was again comparable between the two conditions, reaching a value of approximately 4.0×10^4 live bacteria mL^{-1}.

BCP

The BCP, expressed as ng C $mL^{-1}h^{-1}$, is reported in Figure 4 (on the left y-axis). The BCP values were higher in the control than in the TBA microcosms at days 14 and 40. A reversal of this trend was observed at days 80 and 124, when BCP was higher in the TBA microcosm (*t*-test, $p<0.05$). Using the live cell abundance and BCP values, the bacterial growth rate (μ) and doubling time or turnover time [$T_2 = (\ln 2)/\mu$] can be calculated using the exponential growth model (Koch, 1994; Amalfitano et al., 2008):

$$\mu = \ln((\text{live cell abundance}+\text{BCP})/\text{live cell abundance})/\text{time}$$

The doubling time values obtained by this analysis (Figure 4, on the right *y*-axis) show that the cell turnover time was higher in the control than in the TBA microcosms at both day 80 and day 124, indicating a faster growth rate in the presence of the herbicide.

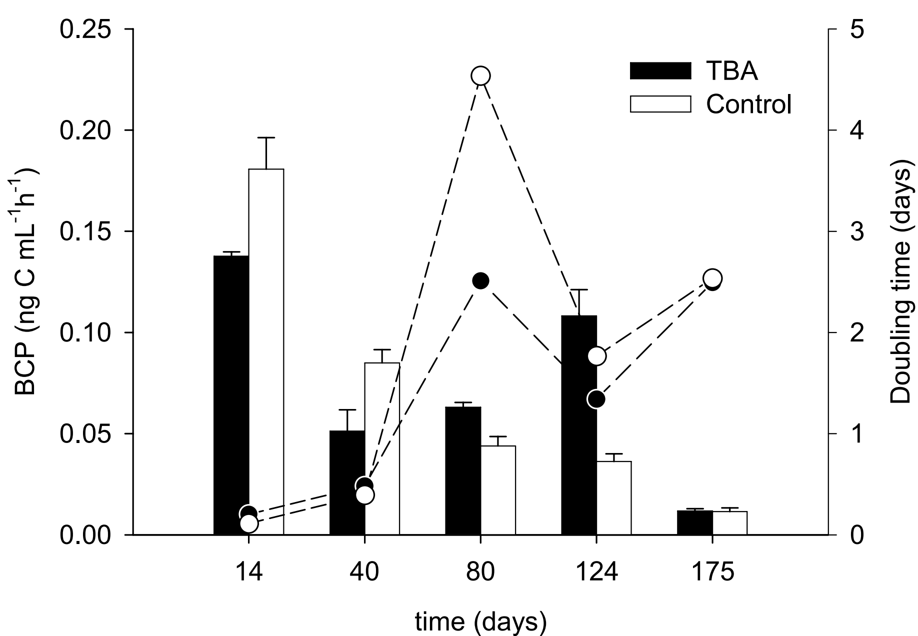

Figure 4. BCP in columns and active cell-doubling time in dots [T_2 = (ln2)/growth rate; see text] at different incubation times. The error bars indicate SEs of four independent values.

Analysis of bacterial community composition by FISH

The use of 16S rRNA gene-targeted oligonucleotide probes made it possible to determine the structure of the autochthonous bacterial community at the phylogenetic level. At day 0, about 80% of the cells detected by DAPI belonged to the *Bacteria* domain (Figure 6a), and only 1% belonged to the *Archaea* domain.

In all microcosms, the percentage of *Archaea* did not display a significant variation in time or between the different conditions, remaining at approximately 1% in all the samplings, with a transient increase to 3% at day 124 under both conditions. In the TBA microcosms, the % of cells positive to the general EUB probes diminished drastically from day 0 (about 80%) to days 80 (35%), 124 (20%), and 175 (35%). A decrease over time was also observed in the control microcosms, although the values were always higher than in the TBA samples and never > 50% of DAPI-positive cells (data not shown).

The lower percentages of *Bacteria* detected in the TBA samples indicated that most *Bacteria* were negatively affected by the herbicide, except the *Betaproteobacteria* subclass. In fact, the *Betaproteobacteria* were quite dominant, representing 23%, 95% and 45% of the *Bacteria* domain at days 80, 124 and 175, respectively (Figure 5b).

Bacterial strain isolation from terbuthylazine-treated microcosms

We obtained four different bacterial isolates from the terbuthylazine-treated microcosms. All four isolates were Gram-negative and their phylogenetic characterization by FISH, using all the available probes (listed previously), showed that they were *Bacteria* belonging to the *Betaproteobacteria* subclass (98–100% of DAPI-stained cells).

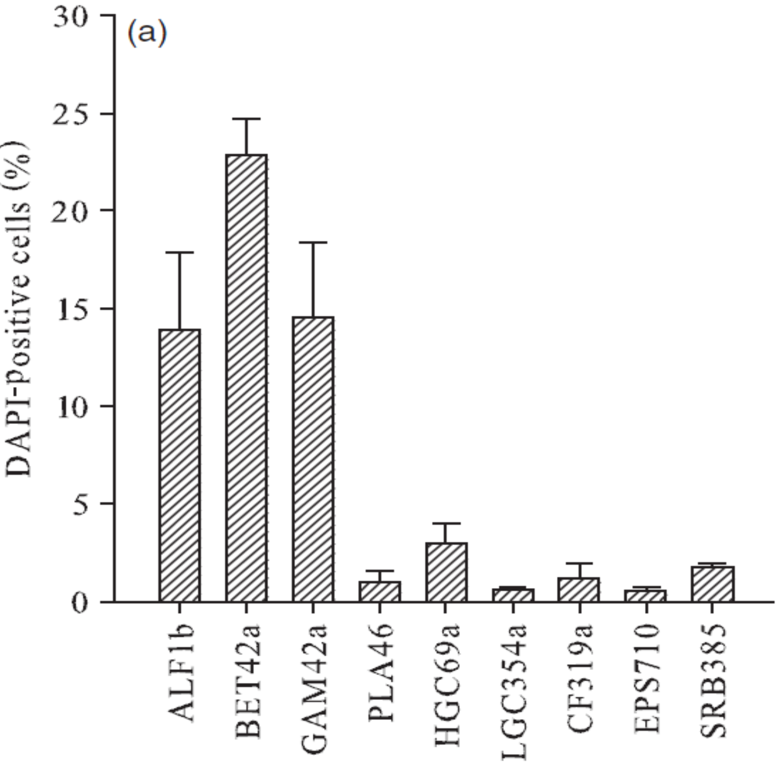

Figure 5. (a) Bacterial community structure detected by FISH in natural groundwater. Probes used for *Bacteria* subgroups detection: ALF1b, *Alphaproteobacteria*; BET42a, *Betaproteobacteria*; GAM42a, *Gammaproteobacteria*; PLA46, *Planctomycetes*; HGC69A, Gram-positive with a high DNA G+C content; LGC354a *Firmicutes*, Gram-positive bacteria with a low G+C content; CF319a, *Cytophaga-Flaviobacterium* cluster phylum CFB; EPS710, *Epsilonbacteria* and SRB385, sulphate-reducing bacteria. The SE for each value is shown (±).

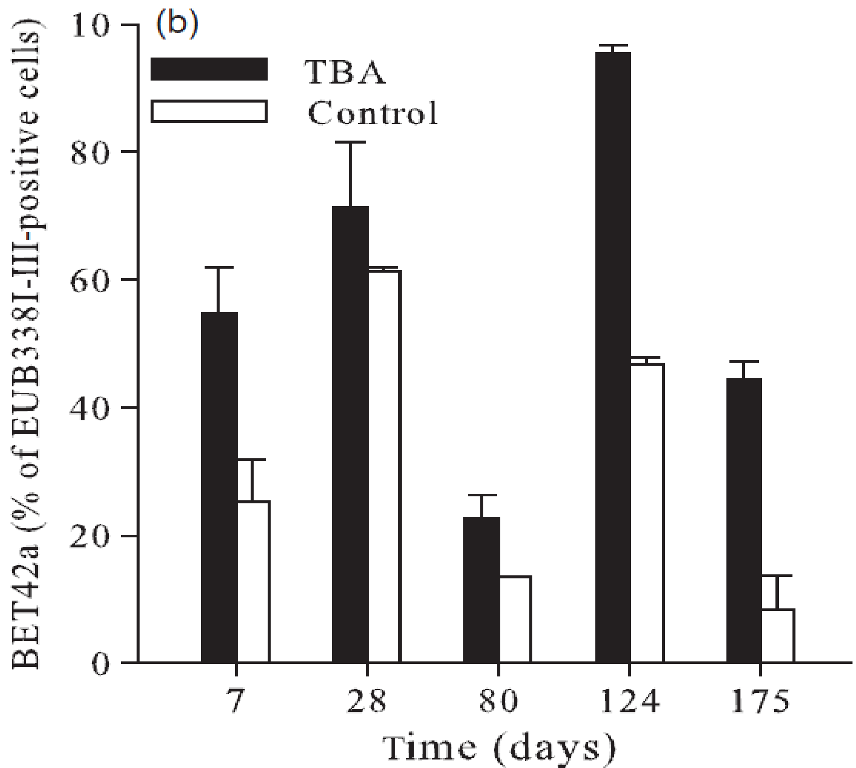

Figure 5. (b) Dynamics over time of the *Betaproteobacteria* subclass (% of the *Bacteria* domain) detected with the probe BET42a during microcosm incubation. The values are means of four analyses. The SE for each value is shown (±).

Further identification of these groundwater isolates was carried out by PCR amplification and sequencing of their 16S rRNA genes. Based on 16S rRNA gene alignment, strain 2-GA-2008 showed a close phylogenetic relationship with *Janthinobacterium lividum*, displaying 99.6% similarity in a 1380-bp overlap with the type strain of this bacterium (accession number Y08846).

With the other species of the genus, *Janthinobacterium agaricidamnosum* (accession number Y08845), the percentage of 16S rRNA gene sequence similarity was 99.0%.

The biochemical characteristics of the strain 2-GA-2008 determined using the API 20NE system were in accordance with those described for *J. lividum* DSM 1522 T (Lincoln et al., 1999). Therefore, the phylogenetic and biochemical results indicate that the isolate 2-GA-2008 belongs to this species. The other three environmental isolates, 4GA-2008, 6GA-2008 and 7GA-2008, exhibited the highest level of 16S rRNA gene sequence similarity (99.4%) to the strain *Advenella incenata* (accession number AM944734), and, using a polyphasic approach that included phenotypic, genetic and phylogenetic studies, were identified as *A. incenata* (Gibello et al., 2009).

Biodegradation of terbuthylazine by the environmental isolates

Both *J. lividum* and *A. incenata* were able to grow in liquid culture (MB) supplemented with terbuthylazine as the sole carbon source, displaying the capacity to degrade the herbicide with a DT_{50} of 121 ±10 days (r = 0.94) and 88 ±6 days (r = 0.98), respectively. Using the AtzB1 probe, FISH analysis allowed us to detect the presence of *atz*B in the gene pool of both *J. lividum* and *A. incenata*. In both cases, the percentage of AtzB1-positive bacteria was >70% (Figure 6).

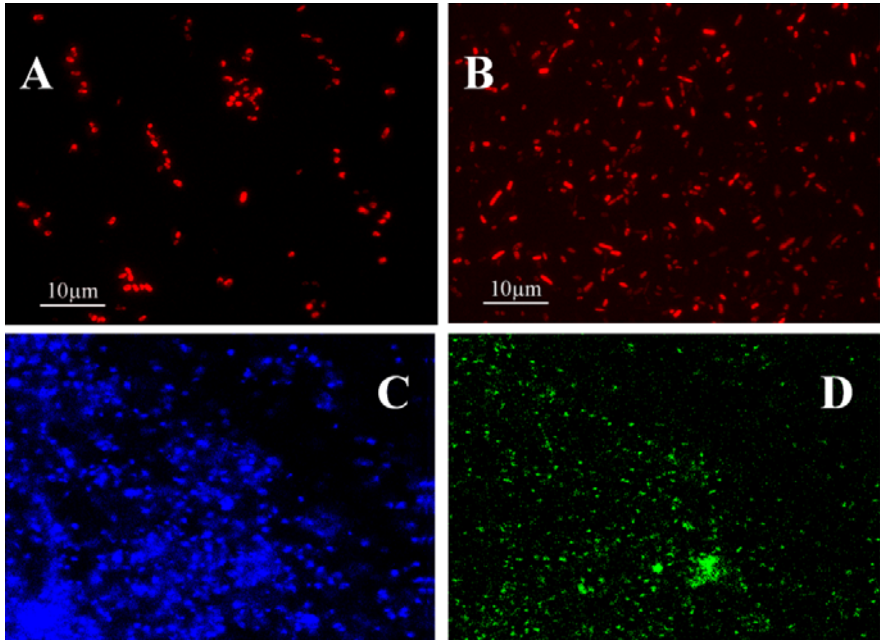

Figure 6. Images of FISH assays under the Confocal Laser Microscope, of pure cultures of bacterial isolates, using the BET42a (*β-Proteobacteria*) Cy3-labeled probe (red): *A. incenata* (A) and *J. lividum* (B). Detection by FISH of target *atz*B gene in *A. incenata* pure cultures: DAPI-stained cells (blue), (C) and the AtzB-probe positive cells (green), (D).

Figure 7 shows the PCR fragments of the DNA amplicons generated with the primer sets used for the detection of each catabolic *s*-triazine gene (200 nt for *atz*A, 204 nt for *atz*B and 228 nt for *atz*C).

The fact that hybridization signals were obtained in the pure cultures of these bacteria may suggest that they possess the genetic potential for *s*-triazine degradation. Additional data on the presence of terbuthylazine-degrading genes in these bacteria were obtained by PCR analysis.

The results show that *A. incenata* contained the *atz*A and *atz*B genes (Figure 7a), while only the *atz*B and atzC genes were found in *J. lividum* (Figure 7b). However, neither *atz*D nor *trz*N could be detected using this standard PCR method. Nucleotide sequences of the PCR

fragments of the atz genes and from the low molecular- weight DNA band from *A. incenata atz*B (Figure 7a) were 100% identical to the homologous genes of the *Pseudomona*s sp. strain ADP.

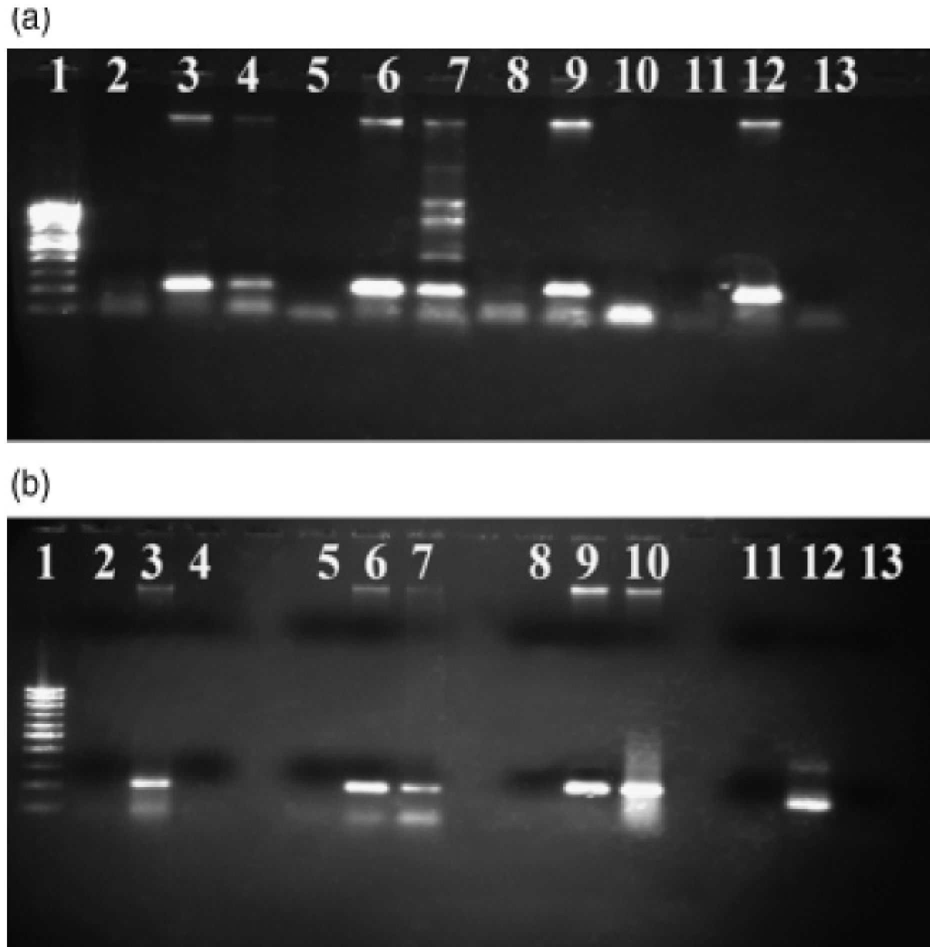

Figure 7. Amplification products obtained in the PCR assays for screening the *atz* genes in *Advenella incenata* (a) *and Janthinobacterium lividum* (b).
Lane 1, 100-bp DNA ladder (Biotools B & M laboratories SA); lane 2, negative control of *atz*A PCR; lane 3, positive control of *atz*A PCR (DNA from *Pseudomonas* sp. strain ADP); lane 4, *atz*A-PCR amplification of the DNA from a bacterial isolate; lane 5, negative control of *atz*B PCR; lane 6, positive control of *atz*B PCR (DNA from *Pseudomonas* sp. strain ADP); lane 7, *atz*B PCR amplification of the DNA from a bacterial isolate; lane 8, negative control of *atz*C PCR; lane 9, positive control of *atz*C PCR (DNA from Pseudomonas sp. strain ADP); lane 10, *atz*C-PCR amplification of the DNA from a bacterial isolate; lane 11, negative control of *atz*D PCR; lane12, positive control of *atz*D PCR (DNA from *Pseudomonas* sp. strain ADP); lane 13, *atz*D-PCR amplification of the DNA from a bacterial isolate.

Discussion

Terbuthylazine degradation has been reported to be quite variable (Barra Caracciolo et al., 2005a), and depends on bacterial activity and on abiotic factors (such as organic matter

content, pH and, above all, temperature) that directly or indirectly influence the degradation rate. In previous studies on terbuthylazine degradation in soil at two different depths (Di Corcia et al., 1999; Barra Caracciolo et al., 2001), it was found that at 15°C the DT_{50} was 180 days at the surface and 200 days in the subsoil.

Our result of 151 days, found in TBA microcosms, agrees with the conclusions of these findings that terbuthylazine is highly persistent at relatively low temperatures. Temperature may be one environmental factor that affects the persistence of terbuthylazine in groundwater, where its half-life was found by Navarro et al. (2004a, b) to range from 263 to 366 days.

Groundwater is very different from surface ecosystems (absence of light, low carbon and oxygen availability and relatively low temperatures) for a community mainly composed of microorganisms adapted to these physical and chemical characteristics (de Lipthay et al., 2003).

Thus, the prolonged persistence of terbuthylazine in groundwater may be an indication of lower microbial activity or the absence of herbicide-degrading organisms. In this study, the autochthonous bacterial community found in the groundwater was able to degrade the herbicide terbuthylazine at the relatively high concentration used. This capability was presumably acquired through chronic exposure to contamination with the herbicide due to intensive agriculture and the high permeability of the aquifer studied (Daly et al., 2000).

The microbial community present in the groundwater degraded the herbicide at a significant rate (Figure 3). The fact that the bacterial community played a role in terbuthylazine degradation was confirmed by the changes in the bacterial community function (viability, carbon production) and structure (abundance and phylogenetic structure determined by FISH) observed in the TBA microcosms.

The significant increase in live cell abundance observed in the TBA microcosms at days 80 and 124, in correspondence with the simultaneous increase in BCP and decrease of the corresponding cell-doubling time (Figure 4), can be ascribable to the activity of specific degrading populations of the bacterial community. The lower values of $T_{1/2}$ in the TBA compared with control microcosms, both at 80 and 124 days, showed that the bacterial populations favoured by the presence of the herbicide were more active. In fact, 124 days represented the closest sampling time to the half-life of the initial herbicide concentration.

The FISH phylogenetic results may further support the fact that the herbicide exerted selective pressure on the bacterial community, promoting the dominance of the *Betaproteobacteria* group. These data suggest that the *Betaproteobacteria* were better able to survive under this condition or that they could be involved in the degradation of the herbicide. This last hypothesis was confirmed by the isolation of two bacterial species (*J. lividum* and *A. incenata*), belonging to this bacterial group, that are able to degrade terbuthylazine (DT_{50} of 121 ±10 and 88 ±6 days). Both these isolates contain some *atz*-degradation genes. Detection of the *atz*B gene was first screened by FISH (Figure 6C and D) and, in addition, *s*-triazine genes encoding the degrading enzymes were further analysed by PCR in both bacterial isolates. The results showed that A. incenata contained the *atz*A and *atz*B genes, while the

*atz*B and *atz*C genes were found in *J. lividum* (Figure 7). Curiously, although *atz*A was not detected in *J. lividum*, this bacterium was able to degrade terbuthylazine (DT_{50} of 121 ±10 days), presumably through the catabolic activity of the enzyme encoded by the *atz*B gene, which might catalyse both deamination and dechlorination reactions at different rates, as was suggested previously (Seffernick et al., 2007). None of these strains appeared to contain the *atz*D gene, suggesting that these bacteria were able to degrade terbuthylazine, transforming it to desethyl-terbuthylazine and cyanuric acid, but they were unable to complete the herbicide mineralisation. Thus, in contrast to *Pseudomonas* ADP and other *s*-triazine degraders (Sadowsky et al., 1998; Wackett et al., 2002), the growth of these bacteria on terbuthylazine as the sole carbon source is only supported by the production of the putative alkylamidohydrolase products (alkylamines). On the other hand, the dominant presence of *Betaproteobacteria* in the TBA microcosm at day 175 did not ensure that the residual terbuthylazine (about 40 mg L^{-1}, corresponding to 30% of the initial concentration) was further removed. In fact, at the end of the experiment, a residual concentration of 30 mg L^{-1} terbuthylazine was still detected.

Bacterial degradation of *s*-triazines in groundwater has not been observed by other authors (Johnson et al., 2000; Pearson et al., 2006); however, we recently isolated a bacterial strain capable of using *s*-triazines as its sole carbon source in a pure liquid culture (Grenni et al., 2009b). To the best of our knowledge, the results of this study are the first to show that terbuthylazine (at 100 mg L^{-1}) can be effectively biodegraded by the autochthonous bacterial community in groundwater microcosms. Interestingly, the variable distribution of the *atz* genes in these bacteria suggests the hypothesis that their terbuthylazine degradation pathways may result from different genetic elements in the microbial communities, rather than from the acquisition through horizontal transfer of a genetic ‘cassette’ encoding the entire set of *atz* genes. A similar situation has been described in the atrazine-degrading bacteria of genus *Pseudaminobacter* (Topp et al., 2000). However, the sequences of the *atz* genes were identical to those reported in every atrazine-degrading species examined to date (de Souza et al., 1998; Aislabie et al., 2005; Shapir et al., 2007), indicating that these genes are highly conserved and widely distributed between the different bacteria genera.

In conclusion, our study shows the potential for the use of a natural attenuation strategy in the treatment of aquifers polluted with the terbuthylazine herbicide. Moreover, the two bacteria isolated (*A. incenata* and *J. lividum*) could facilitate the implementation of effective bioremediation protocols in the case of the significant amounts that can be found in groundwater as a result of accidental herbicide spills. However, at the end of the experiment, a residual concentration of 30 µg L^{-1} was still detected and whether the biodegradation may occur at even lower concentrations still has to be investigated. The complete removal of residual concentrations is in fact a crucial factor in the success of recovery strategies. Our results encourage further studies on the role of the bacterial populations in groundwater contaminated at lower concentrations.

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A new fluorescent oligonucleotide probe for *in situ* detection of *s*-triazine-degrading *Rhodococcus wratislaviensis* in contaminated groundwater and soil samples

Abstract

A bacterial strain (FPA1) capable of using terbuthylazine, simazine, atrazine, 2-hydroxysimazine, deethylatrazine, isopropylamine or ethylamine as its sole carbon source was isolated from a shallow aquifer chronically contaminated with *s*-triazine herbicides. Based on its 16S rDNA sequence analysis, the strain FPA1 was identified as *Rhodococcus wratislaviensis*. The disappearance time of 50% of the initial terbuthylazine concentration in the presence of this strain (DT_{50}) was 62 days. This strain was also able to mineralise the [U ring ^{14}C] triazine-ring, albeit at a slow rate. A 16S rRNA target oligonucleotide probe (RhLu) was designed, and the FISH protocol was optimised, in order to detect *R. wratislaviensis* in *s*-triazine-contaminated sites. The RhLu probe gave a positive signal (expressed as % of total DAPI-positive cells) in both the groundwater (2.19 ±0.41 %) and soil (2.10 ±0.96 %) samples analysed. Using the RhLu probe, *R. wratislaviensis* can be readily detected, and its population dynamics can be easily monitored, in soil and in water ecosystems contaminated with *s*-triazine. To the best of our knowledge, this is the first report showing the isolation, from groundwater, of a bacterial strain able to degrade *s*-triazines.

Introduction

s-Triazines are among the most commonly used herbicides in the world. In recent years, concerns about the persistence, mobility and toxicity of triazines and their metabolites have been growing, owing to the detection of residual concentrations of these herbicides in groundwater. In Italy and Spain, a considerable number of monitored aquifers are contaminated by simazine, terbuthylazine and their deethylate metabolites, with concentrations greater than 0.1 $\mu g\ L^{-1}$, which is the maximum admissible concentration under the EC drinking-water legislation (EC 98/83EEC). Since the number of aquifers that cannot be used for drinking purposes is increasing due to contamination, there is a need to study the natural capacity of groundwater to recover from pesticide contamination. However, until now, the presence in groundwater of bacterial strains with potential degrading ability had not been thoroughly studied.

Natural attenuation of contaminated groundwater has been achieved *in situ* (Williams et al., 2003; Tuxen et al., 2002) or in the laboratory setting (using indigenous bacteria from contaminated sites) only for a limited number of pesticides (Harrison et al., 2003; Johnson et al., 2000; Mirigain et al., 1995; Pucarevic et al., 2002) and never against *s*-triazines. Furthermore, in these studies, biological degradation occurred only when aquifer pesticide concentrations exceeded 40 $\mu g\ L^{-1}$. Although there are many studies showing biotic and

abiotic triazine degradation in soil and surface water (Di Corcia et al., 1999; Barra Caracciolo et al., 2005a,b), to our knowledge, their degradation in groundwater remains to be explored.

Groundwater bacterial populations are adapted to physical and chemical environmental conditions that are very different from those of surface ecosystems (de Lipthay et al., 2003). Examples of these are: the absence of light, low carbon and oxygen availability, and relatively low temperatures. The use of fluorescence *in situ* hybridization (FISH) allows for the direct and selective detection of both cultivable and uncultivable bacteria in environmental samples, even in particular ecosystems like groundwater (Detmers et al., 2004). Furthermore, fluorescently-labelled 16S rRNA-targeted oligonucleotide probes can be used to detect microorganisms at different levels of taxonomic specificity (Amann et al., 1995).

We describe the isolation of a terbuthylazine-degrading bacterium, *Rhodococcus wratislaviensis* strain FPA1, from a contaminated aquifer. To the best of our knowledge, this is the first time that these bacteria have been found in groundwater. We also designed a 16S rRNA-targeted oligonucleotide probe, and applied it to environmental samples using an optimised FISH protocol to monitor the attenuation capability of this bacterial strain in a soil-groundwater system.

Materials and methods

Field site, groundwater and soil sampling

The field site near Assisi (PG, Central Italy) is an intensive agriculture area with a shallow alluvial aquifer (water table at 12 m depth, geochemical facies alkaline-bicarbonate) of intrinsic vulnerability (Daly et al., 2002). Field parameters were: 15°C, pH 6.87, dissolved oxygen 9.01 mg L^{-1}, Eh 210 mV and conductibility 930 µS cm^{-1} (determined at the sampling point by using portable meters from WTW Instruments).

According to the Umbria Regional Environmental Agency's monitoring surveys (2000–2008), terbuthylazine, a frequently used pesticide, and its metabolite, desethyl-terbuthylazine, are commonly found in this groundwater (>0.1 µg L^{-1}parametric value). It is also common to find significant nitrate contamination at this site (>100 mg L^{-1}). Groundwater samples were collected with a sterile sampler (bailer) from a well and then placed directly into sterile polyethylene bottles. Samples were kept at 4°C until laboratory processing, which was performed within 5 h of sampling. The dissolved organic carbon (DOC), which was measured using the Total Organic Carbon Analyser (Shimadzu model TOC500A- ASI-5000A) in accordance with the manufacturer's instructions, was 0.56 mg L^{-1}.

Agricultural soil samples were collected from the surface horizon (0–20 cm depth) of the same area. The soil was silty-clay, with 0.46% organic carbon content, 0.13% nitrogen content and a pH of 7.

Bacterial abundance, cell viability and microbial characterization in groundwater samples

The bacterial abundance (No. bacteria mL^{-1}) was determined in four fixed sub-samples (5 mL each) by direct count using DAPI as the DNA stain agent (Barra Caracciolo et al., 2005a, b, c). The cell viability, expressed as the percentage of live cells compared to total (sum of live and dead) cells, was determined in four fresh sub-samples (5 mL) using a two-dye fluorescent bacterial viability kit (Kit Live/Dead Bacterial Viability Kit, BacLight; Alonso et al., 2002). Finally, the phylogenetic composition of the indigenous bacterioplancton was analysed in sub-samples (5 mL) by applying the FISH technique using Cy3-labelled commercially-synthesised oligonucleotide probes against different taxa: ARCH915 (*Archaea* domain), EUB338I-III (*Bacteria* domain), ALF1B (*α-Proteobacteria*), BET42a (*β-Proteobacteria*), GAM42a (*γ-Proteobacteria*), HGC69A (*Actinobacteria*, high G+C content Gram-positive bacteria), Pla46 and Pla886 (*Planctomycetes*), CF319a (*Cytophaga–Flaviobacterium* cluster phylum CFB), LGC354a (*Firmicutes* with low G+C content), EPS710 (*Epsilonbacteria*) and SRB385 (some sulfatereducing bacteria of *Deltaproteobacteria*, other *Deltaproteobacteria* and Gram-positive bacteria), (Biomers.net, Ulm, Germany). Further details on these probes are available at probeBase (http://www.microbial-ecology.de/probebase/; Loy et al., 2003, 2007).

For the FISH analysis, each groundwater subsample was filtered through a 0.2 μm polycarbonate membrane, fixed by using a 70, 90 and 95% (v/v) ethanol series (10 min each, at room temperature), and then air-dried. FISH of the fixed cells was performed following published protocols (Pernthaler et al., 2001; Barra Caracciolo et al., 2005b; Amalfitano et al., 2008). The average number of probe-labelled cells was calculated as the percentage of the total number of DAPI-positive cells. Cells were counted from 10 to 20 randomly selected fields on each filter section corresponding to 500-1000 stained cells.

Bacterial strain isolation and characterization

In order to isolate terbuthylazine-degrading bacteria, groundwater aliquots (1 mL) were serially diluted in PBS (9 mL) and plated on minimal medium MB plates (Gerhardt et al., 1981). Cultures were supplemented with 100 μg L^{-1} terbuthylazine as the sole carbon source and incubated both at 15°C (groundwater temperature) and 28°C. The resulting isolate was grown on Luria Bertani (LB) agar and then tested for purity and for microbiological characterization (Figure 1).

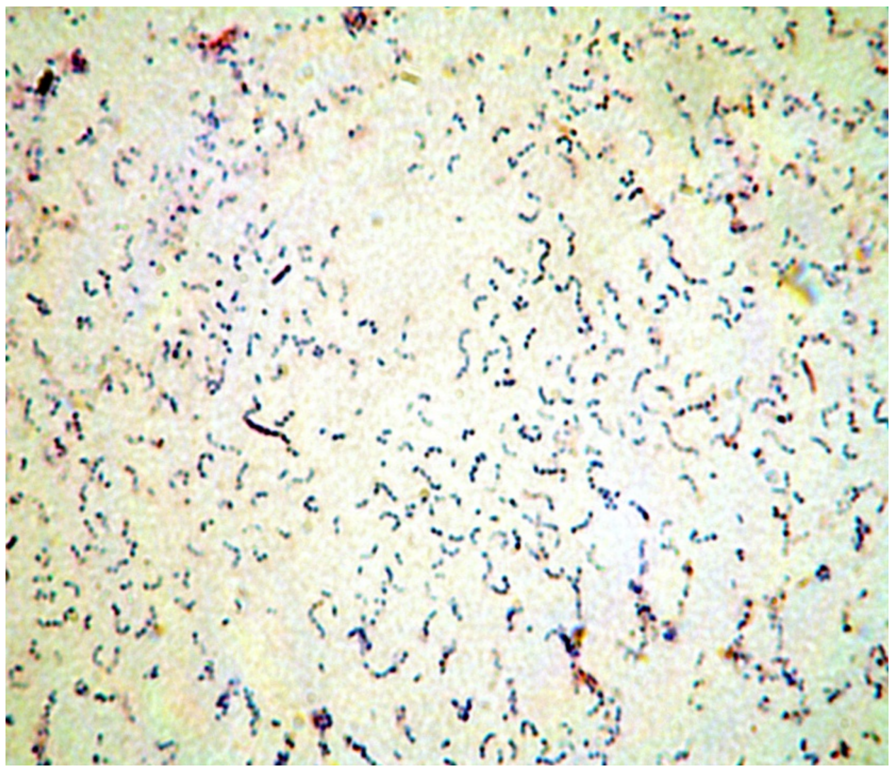

Figure 1. Gram-staining of the bacterial strain isolated.

Bacterial identification

Biochemical characteristics of the isolate were determined using the API Coryne strips (BioMerieux S.A.) according to the manufacturer's instructions. Finally, the identification of isolated FPA1 to species level was carried out by PCR amplification and further sequencing of the 16S rRNA gene.

The amplification of a PCR fragment of 1500 bp of the 16S rRNA gene was performed using the universal primers previously described (Willems and Collins, 1996; Aranaz et al., 2008). The PCR products were cleaned according to the recommended instructions of the QIAquick PCR purification kit (Qiagen), and then sequenced using an ABI Prism 377 DNA sequencer (Applied Biosystems) from SECUGEN facilities (Centro de Investigaciones Biologicas, CSIC, Spain). Sequence data were analysed by Blast search analysis (http://www.ncbi.nlm.nih.gov). The partial 16S rRNA gene sequence corresponding to the isolate FPA1 has been deposited in the GenBank Database under the accession number FM999002.

A rooted phylogenetic tree was performed under maximum likelihood with the HKY85 model using PHYML_v2.4.3 (Guindon and Gascuel, 2003). DNA sequences corresponding to the 16S rRNA were retrieved from GenBank and aligned with Clustal W (Thompson et al., 1994).

Isolate growth on terbuthylazine, simazine and other related compounds

The FPA1 strain was cultivated aerobically at 28°C in 100 mL flasks containing 30 mL minimal medium (MB) supplemented with 0.03% casaminoacid and one of the following carbon sources: terbuthylazine (100 μg L^{-1}), atrazine (5 mg L^{-1}), deisopropyl-atrazine (5 mg L^{-1}), simazine (5 mg L^{-1}), 2-hydroxysimazine (5 mg L), isopropylamine (5 mg L^{-1}) or ethylamine (5 mg L^{-1}). The growth was monitored during the incubation with a spectrophotometer at 600 nm (OD 600). The terbuthylazine disappearance time (DT_{50}) was calculated from the chemical analysis data. All the experiments were run in triplicate.

The mineralisation of [U-ring ^{14}C] simazine by the isolate was also measured. The rate of $^{14}CO_2$ released from the ^{14}C-labelled ring simazine by the bacterial isolate was determined in the MB medium supplemented with 5 mg L^{-1} unlabelled simazine and 2 μCi of [U-ring^{14}C]simazine, as described elsewhere (Martín et al., 2008a). Cultures (three replicates) were incubated at 28°C in 250 ml biometer flasks sealed with Teflon stoppers. A control was prepared with an un-inoculated medium. $^{14}CO_2$ from mineralisation was trapped in a vial with 1 mL of 1N NaOH solution. Radioactivity was measured at 24 h intervals by scintillation counting with a scintillation spectrometer (Hewlett–Packard, model 2500TR).

Chemical analysis

Terbuthylazine and its metabolite desethyl-terbuthylazine were analysed by gas-chromatography (GC), in accordance with Navarro et al. (2000). Cyanazine was added to 3 mL water samples as an internal standard and it was then sequentially extracted three times with 3 mL of methylene chloride. The extracts were dried in sodium sulphate and the volume reduced to 150 μL under a stream of nitrogen before analysis by GC/MS using a Thermo Fisher (Waltham, MA) trace system with a capillary J&W DB5-MS column (60 m length; 0.25 mm id; 0.25 μm film thickness). The injection port was set to 250°C and the transfer line was kept at 280°C. The temperature program for the GC oven was: initial temperature 50°C maintained for 1 min, followed by a temperature gradient at 30°C min^{-1} up to 150°C and then at 5°C min^{-1} up to 180°C. Finally, the target temperature of 260°C was reached at 30°C min^{-1}. The final temperature was maintained for 10 min. Under these conditions, the retention times for the target compounds were: 9.6 min for the internal standard cyanazine; 14.3 min for desethyl-terbuthylazine, and 15.0 min for terbuthylazine. The mean recoveries were greater than 80% for all the compounds.

Oligonucleotide probe design, test, and stringency optimization for its use in FISH analysis of the isolated strain FPA1

Based on the structure and the alignment of 16S rDNA gene sequences from different bacterial species belonging to the genus *Rhodococcus* (EMBL database), we used the ARB software (http://www.arb-home.de; Kumar et al., 2005) to design a 16S RNA-targeted probe. The probe, henceforth called ‘RhLu’ (5’-TGCATGGCTGAGGGTGG-3’), was designed after positions 157–173 of DNA codifying the 16S-RNA from *Rhodococcus wratislaviensis*

(accession number Z37138). This probe was covalently labelled at the 5’ end with the isothiocyanate derivative (Cy3) for FISH analysis.

The hybridization efficiency of the RhLu-labelled oligonucleotide probe was estimated by monitoring the fluorescence intensity of pure cultures of the target *Rhodococcus wratislaviensis* FPA1 strain and those of other phylogenetically related and not related bacteria. We tested: *Burkholderia cepacia* CECT 322, *Pseudomonas fluorescens* CG5 (Garbi et al., 2006), *Rhodococcus equi* CECT555, *Rhodococcus erytropolis* CECT 3013, *Rhodococcus ruber* and *Rhodococcus rhodochrous* (soil isolates supplied by Universidad Complutense), *Staphylococcus aureus* CECT 4013, *Corynebacterium xerosis* CECT 538 and *Mycobacterium peregrinum* isolate (Aranaz et al., 2008). Whole cell hybridization was performed at different temperatures (47–55°C) and with increasing concentrations of formamide (from 0 to 50% v/v, in 10% increase steps) to determine the optimal conditions for the RhLu probe to distinguish target and non-target organisms.

Simultaneously, probe EUB338 (GCT GCC TCC CGT AGG AGT), complementary to a region of the 16S rRNA specific for the *Bacteria* domain, was used as a positive control to test the efficiency of the hybridization (Amann et al., 1995). Cells growing in the exponential phase were harvested by centrifugation and resuspended in PBS. A volume of 30 µL of this cell suspension, adjusted to get 10^5–10^7 cells cm^{-2}, was filtered on 0.2 µm pore size polycarbonate filters (47 mm diameter, Isopore GTTP, Millipore, Germany) with a gentle vacuum and then fixed using a 70, 90 and 95% (v/v) ethanol series (10 min each, at room temperature), and finally air-dried. All the filters were stored at -20°C until further processing.

FISH analysis of environmental samples: soil and groundwater

In order to test the designed probe on the natural bacterial community of the area studied, fresh groundwater and soil samples were analysed. Groundwater samples (four replicates of 5 mL each) were filtered and fixed in ethanol as described above. Fresh soil samples (four replicates of 1 g each) were fixed and the cells were extracted by high speed centrifugation with the non-ionic density gradient medium Nycodenz, as previously described (Barra Caracciolo et al., 2005b; Martin et al., 2008b). Cells were filtered on a 0.2 µm polycarbonate membrane.

In situ hybridization on membrane filters

The *Rhodococcus* sp. pure culture, groundwater and soil samples were permeabilized with different lysozyme concentrations. Each filter was cut into sections and then treated with a 10 mg mL^{-1} lysozyme solution (Sigma–Aldrich, St Louis) at 37°C in a pH 8 buffer, containing 100 mM Tris–HCl and 50 mM EDTA. The incubation time was varied in order to optimise cell permeabilisation. This is a crucial step for the effectiveness of FISH in the case of Gram-positive bacteria such as *R. wratisalviensis*, in which the cell wall limits the access of the probe. Cells were washed twice in MilliQ water for 10 min. Each section from the same filter was used with both the designed Cy3-labelled RhLu probe and the general FAM-labelled

EUB338 bacterial probe. Filter sections were heated with 2× SSC formamide solution at 70°C for 2 min, then covered with 50 µl of hybridisation buffer and the probes (final concentration of each 8 ng μL^{-1}), and finally incubated overnight.

The FISH protocol, including the washing step, was the same as described in detail in a recent work by Martín et al. (2008b). The results are expressed as the percentage of DAPI-positive cells that hybridised with the fluorescent probe. Negative controls were used to assess potential non-specific binding to non-target reference bacteria. Cell autofluorescence was also determined using the negative controls (Martin et al., 2008b).

Laser confocal microscopy and analysis

Fluorescent probes were detected and imaged using a MRC-1024 confocal microscope (Bio–Rad, Hempel Hempstead, UK). The Cy3-labelled probes were excited using a 543 nm emitting Ar laser and the fluorescence recovered through a 550/570 band-pass (BP) filter. The FAM-labelled probes were excited using a He–Ne laser emitting at 488 nm and the fluorescence was recovered using a 492/518 BP filter. When the FAM- and Cy3-labelled probes were used in the same sample, sequential image acquisition was used. Lasersharp and Laserpix softwares (Bio–Rad) were used to analyse the images. Cells binding the probes (EUB338 and RhLu) were reported as percentage of the total DAPI-positive cells and quantified as previously described.

Results

Groundwater analysis

Although the detection of *s*-triazine and the high nitrate concentration showed this groundwater to be a contaminated ecosystem, the bacterial number (2.0×10^4 bacteria mL^{-1}), the cell viability (72%), and the positive results for all of the applied FISH probes (Table 1) suggested the presence of an active natural bacterial community. As regards the phylogenetic composition, the results of the fluorescence *in situ* hybridization showed a prominent presence of the *Bacteria* domain (78%), and a detectable presence of the *Archaea* domain (>1%). Moreover, within the *Bacteria* domain, a positive signal was found for the nine taxa-specific probes tested (Table 1).

Table 1. Phylogenetic composition of the *Bacteria* domain detected by FISH in the natural groundwater samples. Values are means of four analyses and are expressed as percentages of total DAPI counts for each sample.

Taxa	% detected vs DAPI ±SE
α-Proteobacteria	13.9 ± 4.0
β-Proteobacteria	22.89 ± 1.8
γ-Proteobacteria	14.58 ± 3.8
Actinobacteria, high G + C Gram-positive bacteria	3.00 ± 1.00
Planctomycetes	1.00 ± 0.6
Cytophaga-Flaviobacterium	1.19 ± 0.79
Firmicutes with low G+C content	0.60 ± 0.1
Epsilonbacteria	0.57 ± 0.2
*Sulfate Reducing Bacteria**	1.79 ± 0.2

SE: standard error.

Bacterial strain isolation and characterization

Through the usage of selective culture medium the bacterial strain FPA1 was isolated from the aquifer, and was found to be capable of growing on terbuthylazine as its sole source of carbon. The isolate was aerobic, Gram-positive and capable of morphological differentiation in response to changes in its environment (e.g., cocci in the MB or bacillus filaments in the rich-medium, LB). Phenotypical characterization of the isolate supported its assignment to the genus *Rhodococcus*. Genotypical characterization completed the taxonomic identification. Comparative analysis of the 16S rDNA sequences from the isolate (accession number FM999002) revealed that it belongs to the *Rhodococcus* genus, displaying 100% sequence similarity to the type strain of *Rhodococcus wratislaviensis* (GenBank accession number Z37138). Figure 2 shows how the isolate FPA1 is related to other strains of the same species and to other *rhodococci* species.

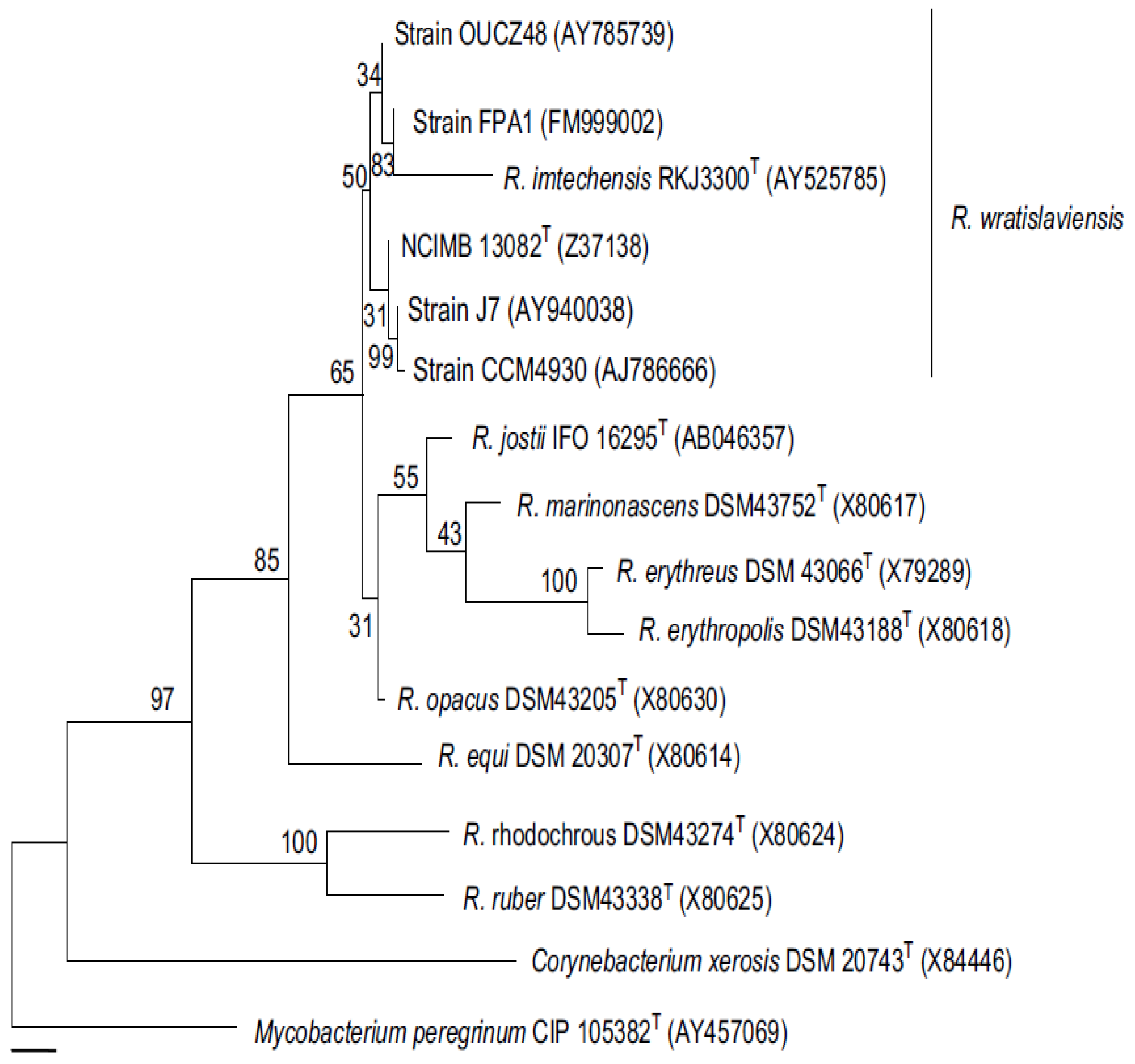

Figure 2. Phylogenetic tree showing the evolutionary relationships between *R. wratislaviensis* strains with other *Rhodococcus* species used in this work. Numbers above branches indicate a bootstrap values (1000 replicates). GenBank accession numbers are given in parentheses. *Corynebacterium xerosis* and *Mycobacterium peregrinum* were used as out-group. Bar, five substitutions per 1000 nucleotide positions.

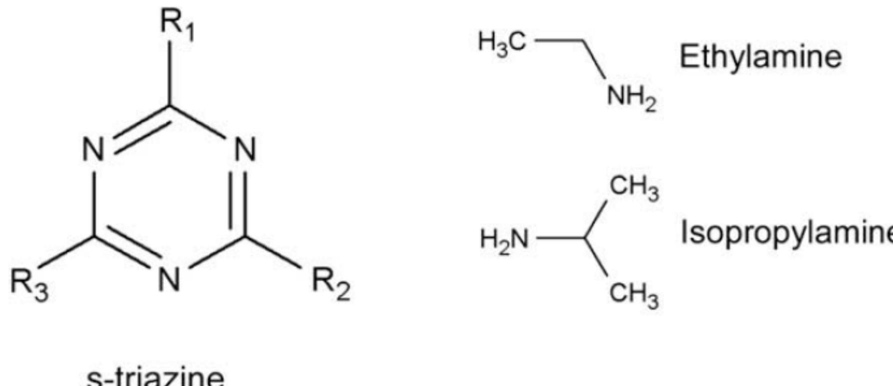

Figure 3. *s*-Triazine structure (on the left) and metabolised lateral chains (on the right). (Also see Table 2).

Table 2 – Metabolization of *s*-triazines by the *R. wratisalviensis* FPA1 strain, when provided as the sole carbon source in liquid medium (MB).

Chemical name	Common name	Substituents			Growth (O.D.) 7d
		R1	R2	R3	
2-Chloro-4,6-bis(ethylamino)-*s*-triazine	Simazine	Cl	Ethylamine	Ethylamine	1.062 ±0.003
2-Hidroxy-4,6-bis(ethylamino)-*s*-triazine	2-OH-simazine	OH	Ethylamine	Ethylamine	0.68 ±0.002
2-Chloro-4-ethylamino-6-isopropylamino-*s*-triazine	Atrazine	Cl	Ethylamine	Isopropyl-amine	0.76 ±0.002
2-Chloro-4-ethylamino-6-amino-*s*-triazine	Deisopropyl-atrazine	Cl	Ethylamine	Amine	0.71 ±0.02

Terbuthylazine degradation and s-triazine metabolism

The growth of *R. wratislaviensis* FPA1 in the presence of 100 µg L^{-1} terbuthylazine as the sole carbon source on the minimal liquid medium MB was monitored by measuring the OD 600 (Figure 4A). The chemical analysis showed the degradation capability to be 62 ±1 days, as determined by the DT_{50} of the initial concentration of 100 µg L^{-1} (Figure 4B).

The range of *s*-triazine growth substrates metabolised by *R. wratislaviensis* was determined in liquid media containing these compounds as the sole carbon source. Atrazine, simazine, 2-hydroxy-simazine, deethyl-atrazine, ethylamine and isopropylamine were shown to support bacterial growth. A list of the carbon sources and their corresponding OD values at day 7 are shown in Table 2 and Figure 3.

The ability of *R. wratislaviensis* to grow on N-alkylamines as the sole carbon source (ethylamine and isopropylamine, Figure 3) was consistent with the capacity of this bacterial strain to use the herbicides atrazine and simazine as a source of carbon.
In addition to these experiments, [U-ring^{14}C]simazine was also provided as a substrate to measure the ability of *R. wratislaviensis* to cleave the triazine-ring.

Simazine was mineralised by the isolated strain and converted to carbon dioxide at a rate of 0.24 µg L^{-1} day^{-1}.

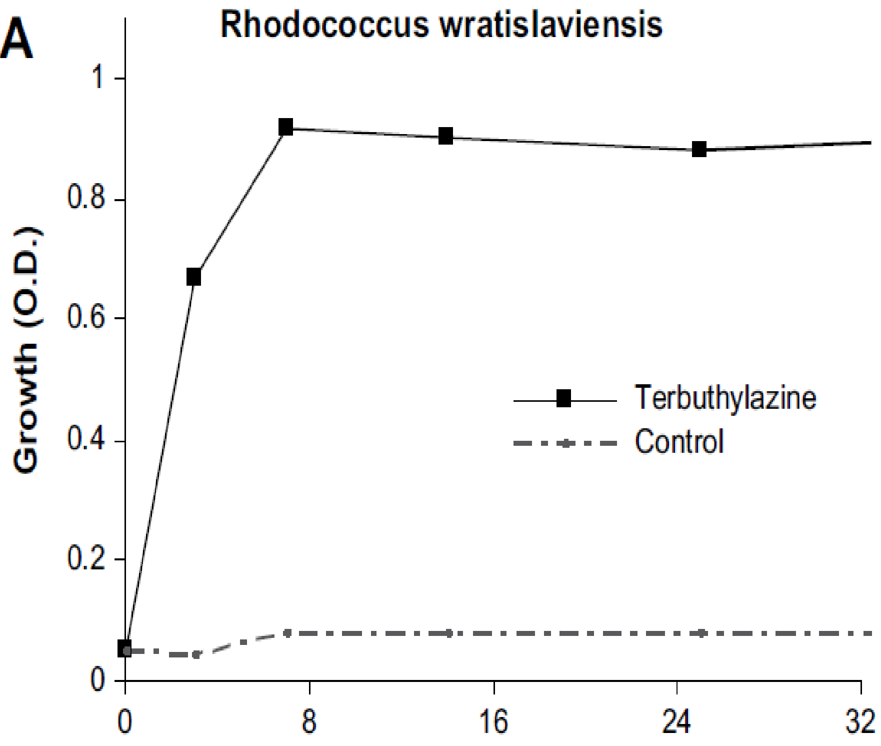

Figure 4. (A) Growth curve of *R. wratislaviensis* strain FPA1 cultured at 28°C in minimal medium MB with 100 µg L^{-1} terbuthylazine as the sole carbon source. Control: only MB with 100 µg L^{-1} terbuthylazine, without the bacterial strain.

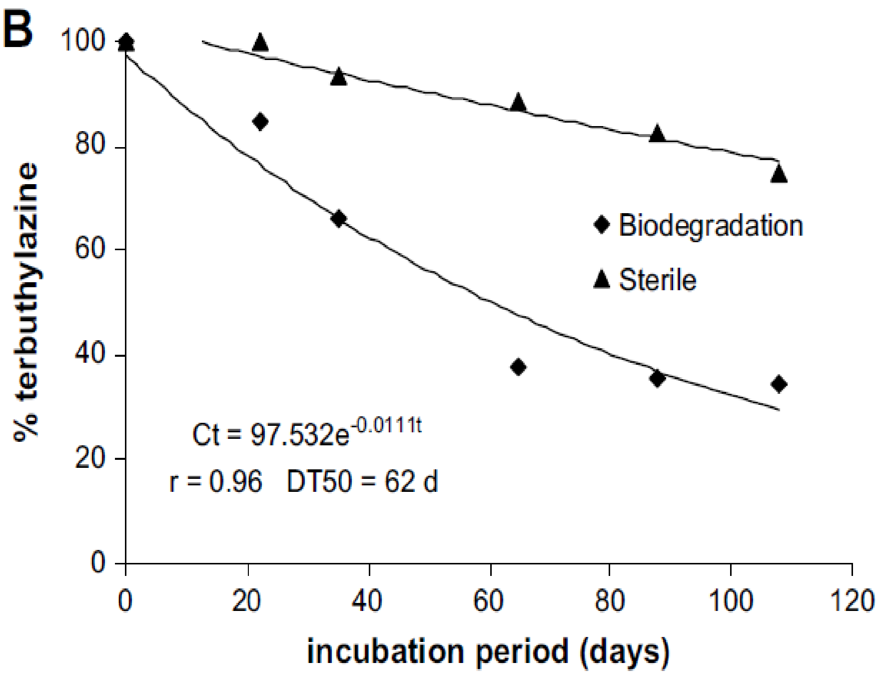

Figure 4. (B) Terbuthylazine degradation (%) in liquid cultures of *R. wratislaviensis* strain FPA1 (Biodegradation) or in sterile MB (Sterile).

Optimization of hybridization conditions and probe specificity

Optimal FISH conditions for the designed RhLu probe, together with its specificity, were analysed both on the isolated *R. wratislaviensis* and on selected control strains. A weak positive response to hybridisation with the RhLu probe was observed in un-permeabilized *R. wratislaviensis* cells, whereas no positive signal was detected in any of the selected control strains. The effects of different temperature conditions, formamide concentrations, and lysozyme treatments were further evaluated in order to enhance the FISH hybridization sensitivity (i.e., the percentage of DAPI stained cells that hybridised with the probe). The FISH hybridisation sensitivity, using either the EUB338 or the RhLu probe, increased from 28 ±8 % in the un-permeabilized samples to 95 ±5 % after 90 min of lysozyme treatment (10 mg mL^{-1}). Comparing DAPI and FISH with EUB338 probe counts, we could not detect significant cell number differences after the permeabilisation treatment. The highest FISH hybridisation efficiency was observed when *R. wratislaviensis* cells were harvested in the exponential growth phase, and hybridisation was performed with 15% formamide at 53°C.

The usefulness of the oligonucleotide probe RhLu identifying *R. wratislaviensis* cells was tested by performing FISH assays with pure cultures of bacterial isolate and with reference strains under the conditions described above. The results obtained with the RhLu probe showed that *R. wratislaviensis* cells were successfully labelled with this probe (Figures 5A, B, Table 3), whereas no hybridisation was observed in control strain cultures.

Table 3 – Average percentage of DAPI-stained cells binding EUB and RhLu probes in pure cultures and environmental samples

Strain/Sample	RhLu (%)	EUB 338 (%)
R. wratislaviensis	97 ±0.9	96 ±1.2
R. equii CECT55	N.D.	92 ±0.5
R. erytropolis CE	N.D.	94 ±0.3
R. rubber	N.D.	94 ±0.5
R. rhodochrous	N.D.	93 ±0.6
P. fluorescens C(	N.D.	96 ±1.5
P. cepacia CECT	N.D.	96 ±0.7
S. aureus CECT	N.D.	95 ±1.2
C. xerosis CECT	N.D.	94 ±1.2
M. peregrinum	N.D.	95 ±1.2
Soil	2.10 ±0.96	96 ±1.8
Groundwater	2.19 ±0.41	94 ±0.2

The standard error for each value is shown (±). N.D.: Not detected

FISH assays using the RhLu probe on groundwater and soil samples

We next used the FISH assay with the RhLu probe to investigate the occurrence of *R. wratislaviensis* in natural samples. We used this assay on groundwater and soil samples contaminated by the herbicide terbuthylazine. The optimised treatment for cell permeabilisation (10 mg lysozyme mL^{-1}, 90 min) was applied to both groundwater and soil samples.

All analysed samples were highly positive to the universal probe for Bacteria, which indicated high ribosome content in the sample and presumably, an overall high level of metabolic activity by the communities being studied (Christensen et al., 1999). Moreover, we also detected signals in both the groundwater and the soil samples analysed when we used the RhLu probe (2.19 ±0.41 and 2.10 ±0.96 % respectively; Figures 5C–F, Table 3).

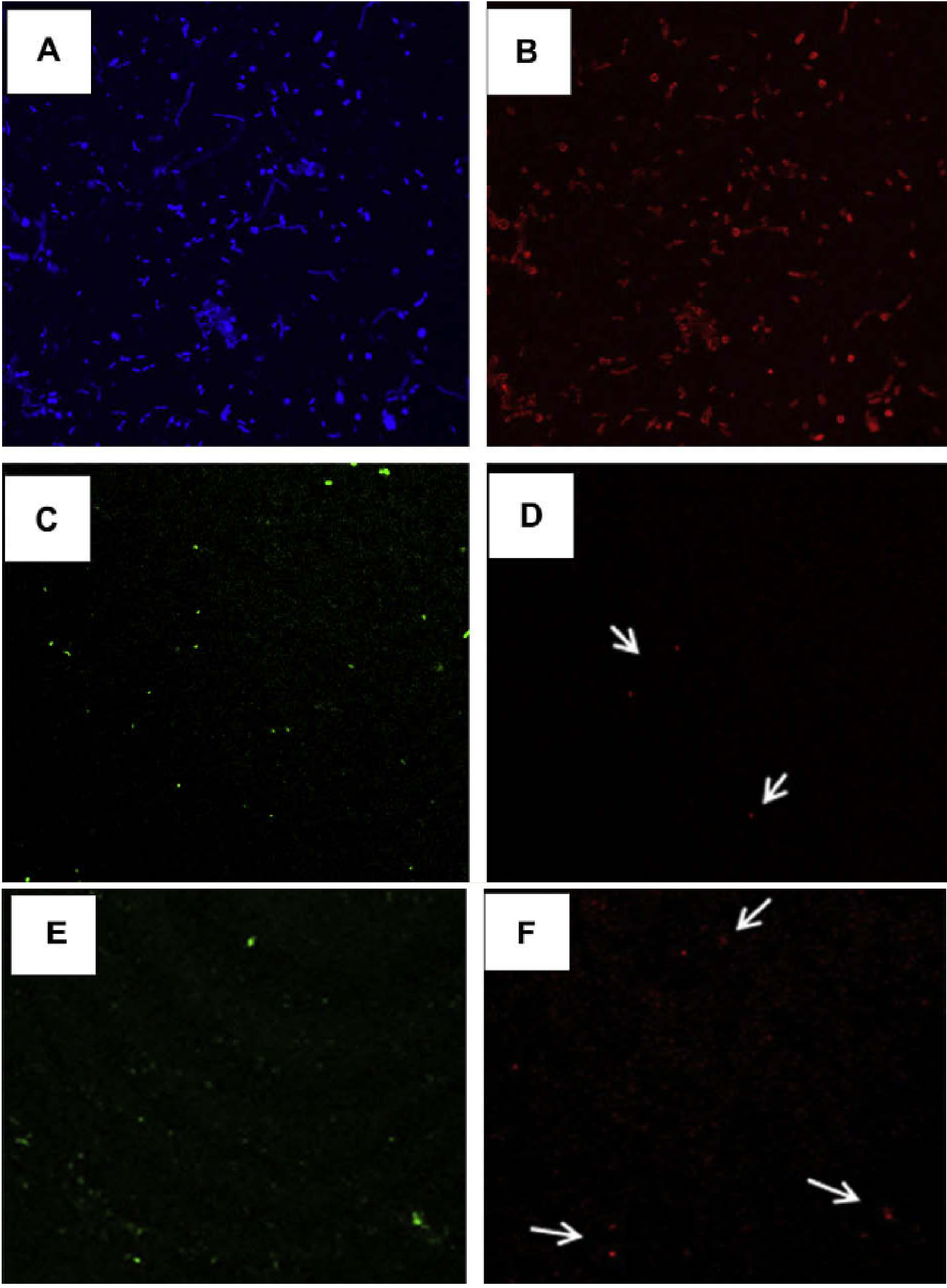

Figure 5. Images of FISH assays under the laser confocal microscope using the designed Cy3-labelled RhLu probe. (A, B) *R. wratislaviensis* FPA1 pure culture: (A) DAPI-stained cells (blue); (B) the RhLu-probe positive cells (red).(C, D) Detection of target bacteria in soil samples.(C) FAM-labelled EUB338 probe (green); (D) RhLu-positive bacteria. (E, F). Images of FISH assays under the laser confocal microscope using the designed Cy3-labelled RhLu probe. Detection of bacteria in groundwater samples: (E) FAM-labelled EUB338-positive (green); (F) RhLu-positive cells (red).

Discussion

It is known that FISH may fail to detect bacteria in oligotrophic habitats or metabolically inactive populations (as those generally found in groundwater ecosystems) because of the low cellular ribosomal content of the samples (Daims and Wagner, 2007). However, our results regarding the cell viability and FISH identification (Table 1) showed the presence of a naturally active microbial community, which was a necessary pre-requisite for carrying out a successful isolation experiment.

In this study, the *s*-triazine-degrading bacterium isolated from the shallow aquifer was identified as *Rhodococcus wratislaviensis* on the basis of its 16S ribosomal DNA sequencing. The rhodococci themselves are a very diverse group of bacteria, possessing the ability to degrade a large number of organic compounds, particularly many that are recalcitrant and toxic (Larkin et al., 2005; Martinkova et al., 2009), including *s*-triazines (Behki et al., 1993). Furthermore, these bacteria are found in very diverse habitats. As regards their environmental significance, metabolic versatility, and potential for biotechnological applications, rhodococci are in some aspects similar to the pseudomonads and related bacteria, but their catabolism is less known. The long-term survival of rhodococci in the environment seems to rely on a remarkable metabolic versatility (Bell et al., 1998). Our strain FPA1 was able to grow on terbuthylazine over a wide range of temperatures (4-30°C) (data not shown) and this characteristic allowed its cultivation both at 15 and 28°C. This result is in accordance with the ability of rhodococci to degrade organic compounds under potentially adverse conditions such as low temperatures, a characteristic which is very important for an effective bioremediation process.

Terbuthylazine can be considered a persistent compound in water; e.g. Navarro et al. (2004a, b) report DT_{50} values of 76 days in sea water, 196 days in river water and 366 days in groundwater. Among *s*-triazines, terbuthylazine is the most persistent and the most frequently detected in surface and groundwater (Guzzella et al., 2006; Carafa et al., 2007). The DT_{50} value (62 days) found in our degradation experiment (Figure 4B) is lower than all the values reported by Navarro et al. (2004a, b). Moreover, *R. wratislaviensis* strain FPA1 displayed the capacity to grow efficiently using not only terbuthylazine but also atrazine, simazine, deisopropyl-atrazine and 2-hydroxysimazine (Figure 4A, Table 2). This is due to its ability to use alkylamine chain substituents. In fact, this strain grew on ethylamine or isopropylamine considerably faster than on atrazine and simazine (data not shown). These results indicate that the ethylamine (or isopropylamine) residues from the herbicide are removed hydrolytically, yielding the corresponding hydroxyl-triazine products. In fact, we detected desethyl-terbuthylazine as a metabolite when isolated cells grew on terbuthylazine. Moreover, the fact that 2-hydroxysimazine was detected in the liquid culture when simazine was used as the sole carbon source, demonstrated the existence of chlorohydrolase-type enzymes in the catabolic machinery *of R. wratislaviensis.* Chloro-*s*-triazine metabolites maintain the toxicity of their parent compounds and *R. wratisalviensis*, unlike other native soil bacteria with only deaminoalkylase enzymes, is capable of mineralising the triazine ring. Thus, *R.*

wratisalviensis could be used as a biological agent for the complete detoxification of herbicide-contaminated water and soil. However, further studies are necessary in order to assess its efficiency for bioremediation purposes. Other authors have reported some *Rhodococcus* sp. that were able to degrade triazines in soil or surface water (Behki et al., 1993; Jones et al., 1998; Kodama et al., 2001; Fujii et al., 2007). However, to the best of our knowledge, this is the first time that a strain capable of mineralising the triazine-ring has been isolated from groundwater.

The fact that bacterial strains with potential degrading capability can be found in groundwater has important implications for the planning of remediation strategies; in particular, for assessing the natural attenuation time once the contamination source has been eliminated, and for planning the implementation of bacterial strains for bioaugmentation purposes. In light of the importance that *R. wratislaviensis* could have in the process of natural attenuation of groundwater contaminated with *s*-triazine compounds, we extended the application of the FISH method to the detection of this strain in the natural habitat (Table 1).

In natural samples, FISH has been successfully used to identify bacteria at different phylogenetic levels using rRNA-targeted probes (Rossetti et al., 2003; Barra Caracciolo et al., 2005b; Daims and Wagner, 2007; Amalfitano et al., 2008). The access of rRNA-targeted oligonucleotide probes to their target site can sometimes be hindered by the three-dimensional structure of the ribosome (Kumar et al., 2005; Yilmaz et al., 2006). The high sensitivity showed by the RhLu probe can be partially attributed to the capacity of the probe to access the rRNA structure, since it was designed with the ARB software, taking into consideration its accessibility to the target (Kumar et al., 2005; Yilmaz et al., 2006). Here we applied a simultaneous FISH using two rRNA targeted probes (EUB338 and RhLu) to detect specific bacteria in pure cultures and natural samples (Figure 5, Table 3). The optimised FISH protocol (lysozyme cell wall permeabilisation for 90 min, 15% formamide, and hybridisation temperature of 53°C) and, in the case of soil samples, the extraction of cells by centrifugation with the non-ionic density gradient medium Nycodenz, made it possible to successfully use FISH to detect the Gram-positive *R. wratislaviensis* in all the samples analysed (pure culture, soil and groundwater).

The fact that RhLu hybridisation signals were obtained in pure cultures of *R. wratislaviensis* strain FPA1 pointed to the selectivity of the probe. At the time of writing, among the more than 40 species recognised as belonging to the genus *Rhodococcus* (Wang et al., 2008), the RhLu probe could also target (by in silico analysis) the homologous genes from *Rhodococcus opacus* (accession number X80630), *Rhodococcus imtechensis* (AY525785) and *Rhodococcus jotsii* (CP000431). This is due to the fact that *R. wratislaviensis* has a high 16S RNA sequence homology to *R. opacus* (99.58% identity, with only six substitutions across 1462 bp), to *R. imtechensis* (99.03% identity with 14 nt differences in 1456 overlapping nts), and to *R. jotsii* (98.56% identity, with only 17 nt differences across the 16S rRNA sequence).

The rooted phylogenetic tree based on 16S rRNA gene sequences (see Figure 2), showed the relationship between *R. wratislaviensis* strains and related type strains of *Rhodococcus*

species. From an evolutionary perspective *R. imtechensis* may have a common lineage to *R. wratislaviensis*. Nevertheless, *R. wratislaviensis* was the only rhodococci species that we isolated in the groundwater samples.

The RhLu probe was then applied to soil and groundwater samples labelling 2% of all DAPI-positive cells (Table 3 and Figure 5). This is consistent with the presence of terbuthylazine presence in both the soil and groundwater. The detection and the quantification of phylogenetic groups by FISH in environmental samples depend not only on cell viability but also on the cellular rRNA content, which is itself linked to cellular metabolic activity (Detmers et al., 2004). Therefore, the RhLu probe could be very useful for monitoring the presence of active *R. wratislaviensis* populations with the potential to degrade *s*-triazines in contaminated aquifers and agricultural soils. With further data on the evolution of contaminant levels and the abundance of specific degraders in environment samples, it will be possible to assess the viability of the *R. wratislaviensis* strain FPA1 as a potential biomarker of contamination. Knowledge of the presence of autochthonous bacterial populations with natural remediation capacity can be useful for the development of management strategies for natural attenuation of contaminated soils and aquifers. In addition, it would also encourage further studies on the effect of the bacterial populations in herbicide-contaminated groundwater.

Conclusions

- The *Rhodococcus wratislaviensis* FPA1 strain was isolated from a contaminated aquifer. It was capable of growing on *s*-triazines and to mineralise the [U-ring ^{14}C] triazine-ring.
- The designed 16S rRNA target oligonucleotide probe (RhLu) was successfully applied to detect *R. wratislaviensis* FPA1 in natural soil and groundwater samples.
- FISH method has proved to be a reliable tool to properly manage complex microbial systems, such as groundwater.
- *R. wratislaviensis* FPA1 could be considered an adequate sentinel organism for bio-monitoring the natural attenuation capacity of terbuthylazine-contaminated groundwater and also a biological agent for the complete detoxification of herbicide-contaminated water and soil.

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B. Point Sources of pesticides: Herbicide biodegradation experiments in soil in the presence of wood residues

Organic matter-rich agricultural by-products such as wood amendments are being produced in high quantities. They can be applied to soil as a disposal strategy and have recently been proposed for limiting the mobility of pesticides coming from point sources of contamination. Any organic matter and nutrients added to soil can strongly affect the structure and activity of bacterial and fungal populations as a result of their increased metabolism of these readily available nutrients, and can consequently affect pesticide biodegradation. In this context, two different ***herbicide degradation experiments in agricultural soil amended with low-cost material*** (pine and oak residues) and ***treated with linuron or terbuthylazine herbicides*** were performed in which changes in the microbial activity were assessed.

The aim of these studies was to assess how an agricultural soil bacterial community was influenced by the presence of wood amendments (pine and oak residues) both in its general functions, such as dehydrogenase activity and viability (broad scale processes), and in its specific linuron or terbuthylazine degradation capability (narrow niche soil process). For this purpose, the number of live bacteria and soil dehydrogenase activity were assessed in soil treated with linuron or terbuthylazine and either amended with pine or oak residues or unamended. The ability of microbiologically active soils to degrade the herbicide linuron was evaluated by comparing the compound half-lives ($t_{1/2}$), in various scenarios, to those in sterile soil.

Two experimental sets, using two different herbicides (linuron or terbuthylazine) and agricultural soils (one sandy-loam and the other silty-clay), were performed.

This research was performed thanks to a cooperation agreement between IRSA-CNR of Rome and IRNSA-CSIC of Salamanca.

The results are reported in the following papers:

- Grenni P., Barra Caracciolo A., Rodríguez-Cruz M.S., Sánchez-Martín M.J., 2009. Changes in the microbial activity in a soil amended with oak and pine residues and treated with linuron herbicide. Applied Soil Ecology, 41: 2-7

- Grenni P., Rodríguez-Cruz M.S., Herrero-Hernández E., Marín-Benito J.M., Sánchez-Martín M.J., Barra Caracciolo A. Changes in the microbial activity in an agricultural soil amended with pine and oak residues and treated with terbuthylazine., in prep.

Changes in microbial activity in a soil amended with wood amendments and treated with linuron herbicide

Abstract

This work studies the effects of wood amendments on soil microbial community functioning and on the potential of this community for linuron degradation. For this purpose, soil dehydrogenase activity and the number of live bacteria, which represent broad scale measurements of the activity and viability of soil organisms, were assessed in soil treated with linuron and either amended with pine or oak wood or unamended (sterilized and non-sterilized). The overall results show that the microbial community had a significant role in linuron degradation. The linuron half-life values indicated a slower degradation rate in pine and oak amended soils than in unamended ones. This is attributed both to the higher sorption of linuron by these soils compared to the unamended ones and a consequent lower bioavailability of the herbicide for microbial degradation, and to the use of the pine and oak as an alternative carbon source by degrading microorganisms. Linuron did not affect the microbial community in terms of dehydrogenase activity and number of live bacteria, presumably because it had adapted to the herbicide. However, the dehydrogenase activity was significantly higher in the soils amended with pine or oak than in the non-amended ones, indicating that the presence of a carbon source favoured the overall bacterial community.

Introduction

Linuron (N'-(3,4-dichlorophenyl)-N-methoxy-N-methylurea) is a substituted phenylurea herbicide used widely to selectively control newly established broadleaf weeds and grasses in fruit and field crops, cereals and shelter belts. Chemical degradation of phenylurea herbicides is of minor importance in most agricultural soils compared to biodegradation (Caux et al., 1998; Sørensen et al., 2003). Linuron and some of its major metabolites are suspected of being endocrine disruptors (Lintelmann et al., 2003) and of exerting toxic effects on aquatic and soil organisms (Caux et al., 1998). Microorganisms capable of degrading linuron through metabolic and co-metabolic pathways have been isolated (Caux et al., 1998; El-Fantroussi et al., 2000; Dejonghe et al., 2003; Sørensen et al., 2005; Breugelmans et al., 2007). However, degradation beyond the aniline-based metabolites is not frequently found and the mineralisation process is reported to occur slowly in soil (Rasmussen et al., 2005). Several studies suggest the involvement in degradation of a bacterial consortium rather than a single strain (El-Fantroussi et al., 2000; Sørensen et al., 2003). Dejonghe et al. (2003) isolated a single strain capable of degrading linuron, but it was stimulated by a synergistic interaction with other strains. The degradation data reported are quite variable, with DT_{50} values in the range of 38-135 days in laboratory studies and 13–82 days in field ones (Caux et al., 1998; Rodríguez-Cruz et al., 2001; Rasmussen et al., 2005; FOOTPRINT Pesticide Properties

Database), and indicate linuron can be moderately persistent and moderately mobile. Linuron has been categorized as a transitional herbicide between potential leachers and non-leachers (Caux et al., 1998), and it raises concerns about the possibility of leaching widespread contamination from soil to groundwater.

Organic matter has pointed out as the main soil parameter controlling linuron mobility (Sánchez-Camazano et al., 2000). Recently, point sources of pesticides, such as spills from the devices used to apply them and uncontrolled disposal in soil of waste and equipment-washing water, have been identified as causes of soil and water pollution which can be more significant than that due to agricultural practice (Fait et al., 2007). The use of organic materials has been proposed to prevent the mobility of pesticides coming from these point sources of contamination (Rodríguez-Cruz et al., 2007a). In particular, adsorbent wood residues, such as oak and pine, have recently been investigated as biomaterials for the immobilization of several pesticides in soil, including linuron (Rodríguez-Cruz et al., 2007b). However, any organic matter and nutrients added to soil can strongly affect the structure and activity of bacterial and fungal populations as a result of their increased metabolism of these readily available nutrients (Briceño et al., 2007) and can consequently affect pesticide biodegradation. Some organic amendments may stimulate biodegradation, but others can reduce it (Moorman et al., 2001; Briceño et al., 2007). However, the effects of wood amendments on the soil microbial community and on the potential of this community for linuron degradation were not investigated in the past.

As a result, the aim of this work was to assess how an agricultural soil bacterial community was influenced by the presence of pine and oak amendments both in its general functions, such as dehydrogenase activity and viability (broad scale processes, Bending et al., 2007), and in its specific linuron degradation capability (narrow niche soil process, Girvan et al., 2005). For this purpose, the number of live bacteria and soil dehydrogenase activity were assessed in soil treated with linuron and either amended with pine or oak residues or unamended. The ability of microbiologically active soils to degrade the herbicide linuron was evaluated by comparing the half-lives ($t_{1/2}$) in the various scenarios to those in sterile soil.

Materials and methods

Soil and wood samples

Soil samples were collected from the surface layer (0–15 cm depth) of an agricultural field located in Aldearrubia (Salamanca, Spain) cropped with corn and potato, in which several pesticides had been intensively used for several years. Soil was left to dry at room temperature and then sieved (<2 mm). The soil was sandy-loam (11.8% clay, 13.6% silt and 74.5% sand), with 0.72% of organic carbon content, a pH of 6.3, and a cation exchange capacity of 4.8 cmol kg^{-1} (Rodríguez-Cruz et al., 2007a).

Wood samples consisting of pine and oak residues were selected because of their different Freundlich adsorption constant (Kf) values (74.4 in the case of oak and 96.2 in that of pine, which are related to their lignin content of 18.2% and 24.4%, respectively) found in a

previous work (Rodríguez-Cruz et al., 2007b). The pine and oak residues were obtained from a local company in Salamanca (Spain), the <1 mm fraction was selected as the organic soil amendment as described in Rodríguez-Cruz et al. (2007b). The amended soils were prepared by uniformly mixing soil with oak or pine (5%, w/w).

Sub-samples were analysed to assess both the total organic carbon (TOC) content, by using an elemental carbon analyser (Wosthoff Carmograph 12 H Omega, Bochum, Germany), and the soluble carbon, by using a Shimadzu 5050 Carbon analyser (Shimadzu, Columbia, MD). The carbon content in the soil amended with pine (SP) or oak (SO) sawdust was about 4-fold greater than that in unamended soil (S). Moreover, the most soluble carbon content was found in SP (Table 1).

Table 1 - Total organic carbon (TOC %), soluble carbon (%) and pH of unamended (S) and amended soils with pine (SP) or oak (SO).

Sample	TOC (%)	Soluble C (%)	pH
S	0.72	0.008	6.3
SP	2.89	0.047	6.6
SO	2.79	0.037	5.9

Chemicals

Linuron (N'-(3,4-dichlorophenyl)-N-methoxy-N-methylurea) was supplied by Riëdel de Haën (Hannover, Germany) (>99% purity). The linuron metabolites (99.5% purity) N-(3,4-dichlorophenyl)- N'-methylurea, N-(3,4-dichlorophenyl)-N'-methoxyurea, N-(3,4-dichlorophenyl)urea and 3,4-dichloroaniline were supplied by Höechst AG (Germany).

Laboratory degradation experiments with amended and unamended soils

The herbicide degradation experiment was conducted in duplicate in accordance with SETAC guidelines (Lynch, 1995) and previous experiments (Barra Caracciolo et al., 2005a, b, c). A linuron stock solution (1 mg mL^{-1}) was prepared by dissolving the standard compound in acetone and was then diluted in sterile water. The water solution was added to soil (200 g) to obtain a final herbicide concentration of 1 mg kg^{-1}, which corresponds to an agricultural rate. Some soil samples were first sterilized (autoclaved 120 ±2 °C, 20 min on 2 consecutive days) and then treated with linuron (SSL); other samples of soils were only treated with linuron (SL); others were treated with both linuron and pine (SPL) or oak (SOL) sawdust; and, finally, microbiological control soils were prepared with only water (S), with water and with pine sawdust (SP) and with water and oak sawdust (SO). All soils were thoroughly stirred with a sterilized spatula and the water added was in all cases sterilized by filtration (0.22 µm). The final moisture content was adjusted to 60% of the maximum soil water holding capacity.

Soils were maintained in beakers closed with a sterilized cotton plug wrapped in gauze to allow air exchange (Figure1).

Figure 1. Experimental set with soil microcosms.

The soil moisture was kept constant during the entire period of the experiments by periodically weighing and replacing any losses with sterile water. Samples were incubated at 20 ±0.5 °C in the dark. Solutions and instruments were sterilized and all steps were performed in a sterile cabinet. Sampling was performed at different times (0, 2, 7, 14, 30, 50 and 66 days) for both chemical and microbiological analysis.

Chemical analysis: $t_{1/2}$ in microbiologically active vs. sterile soil

The ability of microbial populations to degrade the herbicide linuron was evaluated in terms of half-lives ($t_{1/2}$) in the unamended and amended soils for an incubation period of 66 days and compared with the $t_{1/2}$ value found in the sterile soil. Two soil replicates (1 g) were taken from each microcosm and shaken with 5 mL of methanol for 24 h at 20°C for residue analysis. Samples were centrifuged and 4 mL of each supernatant were evaporated under air stream and re-dissolved in 0.5 mL of methanol for analysis. Quantitative determination of linuron and its metabolites was performed by HPLC–DAD–MS in a Waters chromatograph (Waters Assoc., Milford, MA) using the conditions described elsewhere (Rodríguez-Cruz et al., 2007a). Detection by HPLC/MS to confirm the identity of these compounds was carried out by monitoring the positive molecular ion (*m*/*z*) 249.1 for linuron, 219.1 for N-(3,4-dichlorophenyl)-N'-methylurea, 235.1 for N-(3,4-dichlorophenyl)- N'-methoxyurea, 205.1 for N-(3,4-dichlorophenyl)urea and 162.1 for 3,4-dichloroaniline. Recovery values for linuron in the unamended and amended soil ranged between 95% and 98%. The detection limits for linuron and its metabolites were <10 ng mL^{-1}.

Soil dehydrogenase activity, total cell number and cell viability

Soil dehydrogenase activity (two sub-sample replicates for each microcosm) was determined using 6 g of soil and the reduction of 2,3,5-triphenyltetrazolium chloride (TTC) solution to

triphenylformazan (TPF) was measured using the method reported by Tabatabai (1994) and modified by Bending et al. (2007). Soil dehydrogenase activity was expressed as mg TPF g^{-1} dry soil. The total cell number (No. bacteria g^{-1} dry soil) was assessed (three sub-sample replicates) in 1 g of fixed soil with the epifluorescence direct count method, using DAPI (4',6'-diamidino-2-phenylindole) as the DNA fluorescent agent, as reported in detail in previous works (Barra Caracciolo et al., 2005a,b). Cell viability was measured (three sub-sample replicates) in 1 g of fresh soil in order to estimate cell viability (% live cells/ live+dead), using two fluorescent dyes, SYBR Green II and propidium iodide (Sigma–Aldrich, Germany) in order to distinguish between viable (green) and dead (red) cells under a fluorescence microscope (Leica DM 4000B Leica Microsystems GmbH, Wetzlar, Germany), as reported in a previous work (Amalfitano et al., 2008).

Statistical analysis

Analysis of variance (one-way analysis of variance) was used to determine the significant differences in dehydrogenase activity and number of live bacteria among the different soil treatments, using the Statistical software SIGMASTAT (version 3.0).

Results

The decrease (expressed in percentages of linuron initially applied) of the linuron concentrations in unamended soil (SL), un-amended and sterile soil (SSL) and soils amended with pine (SPL) or oak (SOL) over a period of 66 days is shown in Figure 2.

The degradation patterns of linuron in all scenarios fitted first-order kinetics. The half-life ($t_{1/2}$) values were 14.1 ±2 days (r = 0.95) in SL, 18.7 ±1 days (r = 0.99) in SOL and 42.5 ±0.2 days (r = 0.97) in SPL (Table 2). In the SSL condition about 80% of the initial herbicide concentration still persisted at the end of the experiment (66 days) and the theoretical $t_{1/2}$ value calculated from the regression curve was 158 ±22.4 days (r = 0.92) (Figure 2). The latter result shows that the soil microbial community had an active role in linuron degradation.

The curves representing the linuron concentrations vs. time in the soil treated with pine or oak residues are always above the curve tracing linuron in unamended soil. This indicates a higher residual amount of compound in amended soils, i.e. a higher linuron persistence in wood residue-treated soils.

Monitoring of linuron metabolites in different soils was also carried out. N-(3,4-dichlorophenyl)-N'-methoxyurea was the only metabolite found in the non-sterilized soils.

Table 2 shows the metabolite concentrations (μg kg^{-1} soil) detected at different sampling times. The highest amount detected was 53.6 μg kg^{-1} soil in SL after 7 days. The concentration then decreased and at 50 days 12.9 μg kg^{-1} soil of this metabolite was found, suggesting its further degradation. Lower concentrations of this metabolite were found in SPL (22–27 μg kg^{-1} soil) and in SOL (20–26 μg kg^{-1} soil) in line with a lower parent compound degradation in these soils.

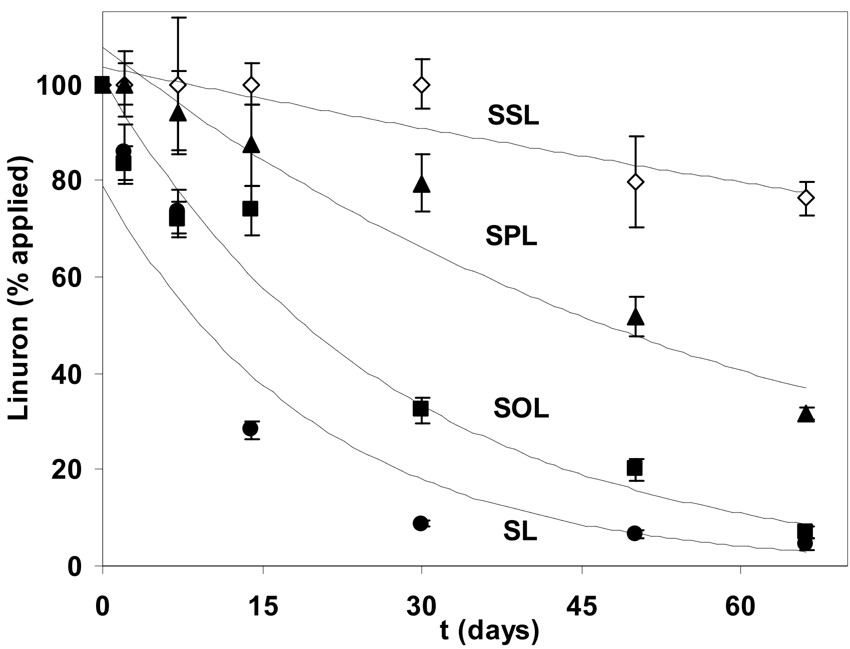

Figure 2. Percentages of linuron in unamended soil (sterile, SSL; non-sterile, SL) and amended soil with pine (SPL) and oak (SOL) residues as a function of time. Symbols are measured average data and lines are exponential models. Bars represent standard errors.

Table 2 - Half-life ($t_{1/2}$) for linuron degradation and linuron metabolite (N-(3,4-dichlorophenyl)-N´-methoxyurea) concentration (μg kg^{-1} soil) in unamended and amended soils.

	Linuron $t_{1/2}$ **(d)**	**Linuron metabolite** (μg kg^{-1} soil)				
		7 days	14 days	30 days	50 days	66 days
SSL	158.0 ±22.4	nd	nd	nd	nd	nd
SL	14.1 ±2.0	53.6 ±3.4	33.9 ±3.1	19.6 ±0.4	12.9 ±2.6	nd
SPL	42.5 ±0.2	24.7 ±4.2	27.4 ±7.3	22.5 ±0.9	nd	nd
SOL	18.7 ±1.0	23.8 ±6.4	nd	25.7 ±0.0	nd	19.8 ±1.9

SSL: sterile + linuron; SL: linuron; SPL: linuron + pine; SOL: linuron+ oak
nd, no detected

The herbicide effect on dehydrogenase activity and number of live bacteria was studied in all herbicide-treated soils (SPL, SOL, SL) and compared with non-treated ones (SP, SO, S).

Figure 3 shows dehydrogenase activity (μg TPF g^{-1} dry soil) vs. time in the treated soils (A) and in the control ones (B).

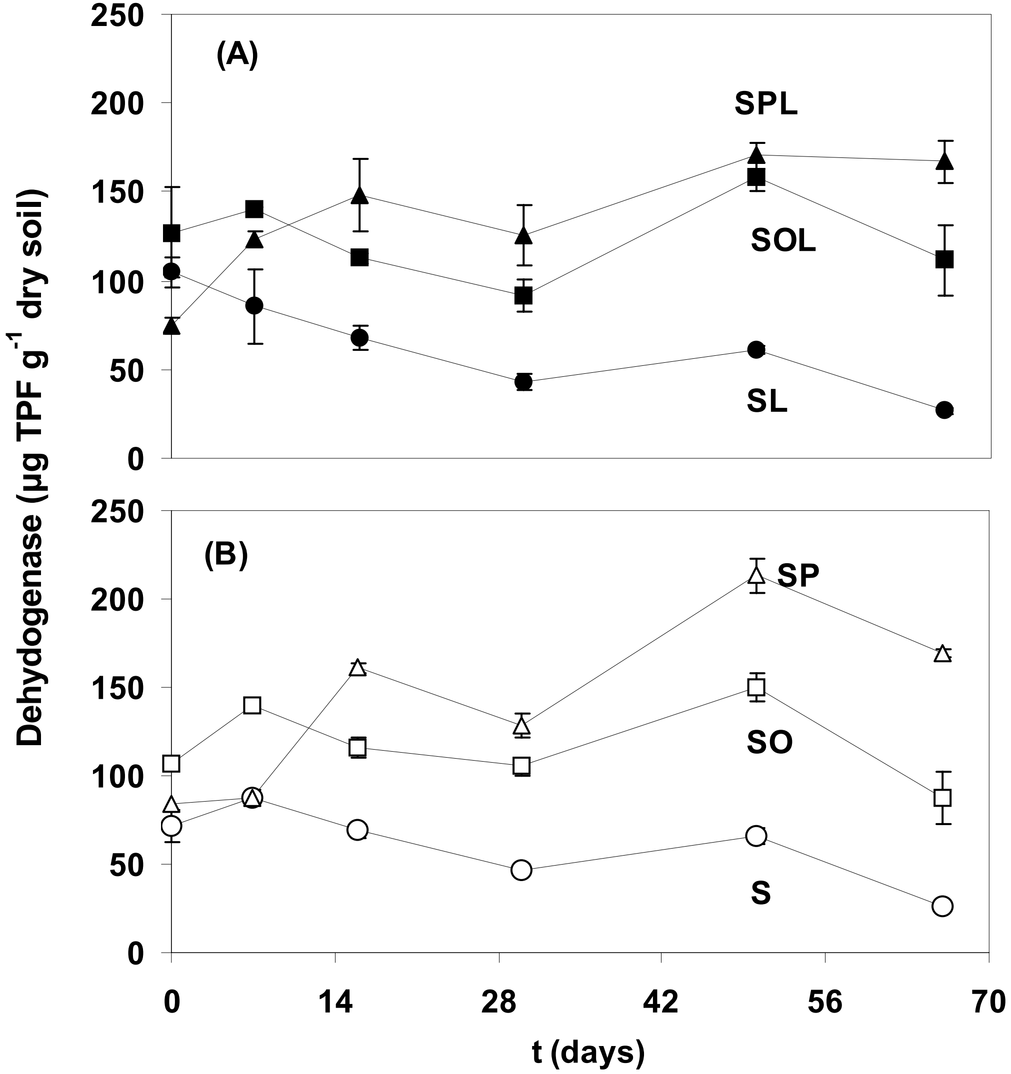

Figure 3. Dehydrogenase activity (µg TPF g^{-1} dry soil) detected over time in the linuron-treated soils (A) and in the control ones (B). Bars represent standard errors. SPL, linuron + pine; SOL, linuron + oak; SL, linuron; SP, amended with pine; SO, amended with oak; S, unamended.

A significant difference in dehydrogenase activity among the different soil treatments was observed (one-way analysis of variance, $F = 7.21$, $P < 0.0002$). Dehydrogenase activity was significantly higher (*t*-tests, $p < 0.01$) in all the soils amended with pine or oak (SPL, SOL, SP, SO) than in non-amended ones (SL, S). After 66 days, the pine (SPL, SP) or oak (SOL, SO) amendment led to an increase of dehydrogenase activity of about 7-fold and 3- fold, respectively, compared to the unamended soil (S, SL). The presence of amendments therefore, stimulated soil dehydrogenase activity, during the experimental period. On the contrary, the presence of the herbicide linuron did not significantly affect this microbial activity.

We calculated the number of viable cells (No. live bacteria g^{-1} dry soil), from the total cell number, obtained by DAPI counts, multiplied by viability (expressed as % live cells/live+dead), detected by using concurrently the two SYBR Green II and propidium iodide

fluorescent dyes (Figure 4 A and B). The number of live bacteria was generally higher (*t*-tests, $p < 0.01$) in the amended soils than in the unamended ones, except in the case of SOL. That is to say that, in line with dehydrogenase activity data, the presence of a carbon source (pine and oak) favoured the overall bacterial community.

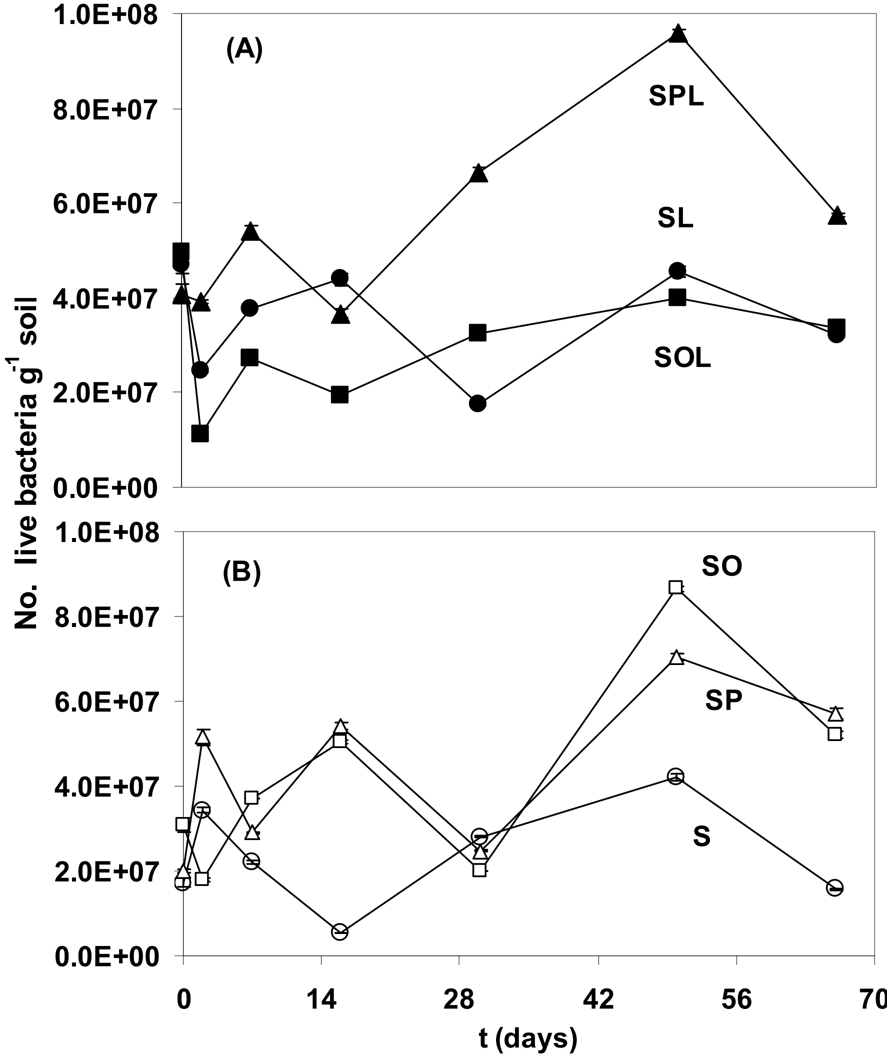

Figure 4. Number of live bacteria (No. live bacteria g^{-1} soil) detected over time in the treated soils (A) and in the control ones (B). Bars represent standard errors. SPL, linuron + pine; SOL, linuron + oak; SL, linuron; SP, amended with pine; SO, amended with oak; S, unamended.

Discussion

The half-life for linuron (14 days) found in SL suggests the presence in our soil of an active microbial community able to transform this herbicide, supporting the key role of biotic processes in phenylurea degradation (Barra Caracciolo et al., 2005c; Sørensen et al., 2003). The half-life values reported for linuron in other experiments are highly variable, with values ranging from a few days up to several years (Wauchope et al., 1992; Di et al., 1998; Rodriguez-Cruz et al., 2001, 2003). However, the soil pH and carbon content are reported to

influence the biodegradation, by controlling the metabolic activity of linuron and other phenylurea degrading microorganisms (Bending et al., 2003; Rasmussen et al., 2005; Bending & Rodríguez-Cruz, 2007). The higher $t_{1/2}$ values in the soil amended with pine (43 days) and oak (19 days) can be ascribed to the presence of the latter, since they increased the soil organic carbon content, and promoted its adsorption capacity. In fact, the high linuron adsorption by pine and oak (Kf = 96.2 and Kf = 74.4, respectively; Rodríguez-Cruz et al., 2007b) decreased the bioavailability of the herbicide and consequently its biodegradation. A lower availability of herbicides for degrading soil microorganisms, due to the binding to organic matter, was found by other authors (Benoit and Barriuso, 1999; Rodríguez-Cruz et al., 2001; Johannesen et al., 2003). Moreover, in the presence of a higher organic carbon content, as in the amended soils, the degrading populations were less stimulated to metabolize the herbicide as a carbon source. This was particularly evident in SPL, where the organic carbon content was higher than in SOL. A negative correlation between natural carbon presence and linuron degradation was found by other authors (Rasmussen et al., 2005).

Although the transformation products searched for were N-(3,4-dichlorophenyl)-N'-methylurea, N-(3,4-dichlorophenyl)-N'-methoxyurea, N-(3,4-dichlorophenyl)urea and 3,4-dichloroaniline (3,4-DCA), we only detected N-(3,4-dichlorophenyl)-N'-methoxyurea. This is in fact the most common transformation product found owing to the presence of several soil microorganisms able to perform the N-demethylation (Sørensen et al., 2003). The absence of the other metabolites may be ascribed to their rapid formation and further transformation into non-detectable concentrations. However, since other authors found that 3,4-DCA can be irreversibly sorbed to soil (Li and Lee, 1999), we cannot exclude that this phenomenon might have also occurred during our experiment.

The results of the microbiological analysis indicated that linuron did not affect the activity of bacterial populations (in terms of viable cell number and dehydrogenase activity) and therefore did not have any toxic effect on these functions, presumably because they were adapted to its presence. On the contrary, the presence of pine and oak had a positive effect on it, as shown by the significant increase of dehydrogenase activity in all amended soils. The stimulation of microbial activity by the carbon readily bioavailable from organic amendments, has been found by other authors (Felsot and Dzantor, 1995; Moorman et al., 2001). The 'driven force' in the soil microbial community studied was therefore the organic matter and its quality; in fact bacterial community activity was favoured (SPL ≈ SP > SOL ≈ SO > SL ≈ S) by a more soluble carbon (0.047% in pine, 0.037% in oak vs. 0.008% in unamended soil) and lignin presence (24.4% in pine and 18.2% in oak) in the amended soils. Since dehydrogenase activity was always higher in the presence of the amendments, it can be considered a good indicator of soil quality at the broad scale process level (Filip, 2002). However, the broad scale approach to soil microbial analysis provides an indication of net community response and we cannot exclude any specific community changes, which may be more subtle but nonetheless of ecological significance. In particular, the broad scale approach to measuring pesticide impacts does not take account of microbial diversity, which may be a

crucial contributor to soil quality, by controlling long-term sustainability and resistance to perturbations (Lynch et al., 2004). Further studies of this aspect are in progress.

Conclusions

The overall results show that the microbial community had a significant role in linuron degradation, as shown by the degradation results in the sterile soil vs. microbiologically active soils. The presence of the pine and oak amendments had a positive effect on the entire bacterial community during the experiment, as shown by the increase in their number and their dehydrogenase activity. During this short-term study, however, the specific activity of the linuron degrading populations was less stimulated by the amendments. This was ascribable both to a lower bioavailability of the herbicide for degradation, due to the linuron adsorption, and to the use of pine and oak as an alternative carbon source by soil microorganisms.

The results indicate that the use of wood amendments can be effective in limiting the mobility of linuron in soils, although the capacity of amended soils to adsorb it should be taken into account since this can modify the persistence of the herbicide.

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Changes in microbial activity in an agricultural soil amended with pine and oak residues and treated with terbuthylazine herbicide

Abstract

The effects of wood amendments (pine and oak residues) on soil microbial community functioning and on the potential of this community for terbuthylazine degradation were studied. For this purpose, degradation kinetics, soil dehydrogenase activity and the number of live bacteria were assessed in soil treated with terbuthylazine and either amended with pine or oak wood or unamended (sterilized and non-sterilized). At day 65, 85% of the applied herbicide still persisted in the sterile soil, 73% in the pine-amended one, and 63% in the oak-amended and unamended one.

Pine and oak residues increased the sorption of terbuthylazine to soil. The Kf value for the sorption of terbuthylazine by pine (Kf = 52.5) was much higher than by oak (Kf = 4.44). This higher sorption capacity by pine could be due to its higher lignin (hydrophobic wood component) content. Pine residues, owing to a high soil sorption of terbuthylazine and a decrease in the bioavailability of the herbicide, hampered the microbial degradation. On the contrary, in the presence of oak residues the herbicide sorption did not increase enough to hamper the microbial degradation. The overall results confirm that the active role of the soil microbial community in terbuthylazine degradation, even if the herbicide was found to be quite persistent in this soil, and show that terbuthylazine persistence in soil is highly variable. The formation of desethyl-terbuthylazine metabolite (DET) was detected during the degradation process. Higher concentrations of this metabolite were found in oak-amended and unamended soil in line with the higher terbuthylazine degradation in these soils. Dehydrogenase activity was not significantly affected by the herbicide presence and it was generally higher in the presence of pine and oak soils, indicating that the amendments had a generally positive effect on overall bacterial activity during the experimental period.

Introduction

Terbuthylazine is an *s*-triazine herbicide widely used in agriculture to control grass and broad-leaved weeds in a variety of crops. In Italy terbuthylazine is used in maize and sorghum (Fait et al., 2010) and in Spain is used also in olive tree cultures (Cabrera et al., 2007). Organic matter (OM) is the main soil constituent controlling adsorption of terbuthylazine (Dolaptsoglou et al., 2007). The major degradation products of terbuthylazine are produced by dealkylation (desethyl-terbuthylazine) and hydroxylation (2-hydroxydesethyl-terbuthylazine), with eventual ring cleavage. Terbuthylazine and its metabolite desethyl-terbuthylazine have been frequently monitored in surface water and groundwater at levels above the 0.1 μg L^{-1},

which is the limit established in the EU for individual pesticides in drinking water (Azevedo et al., 2001; Guzzella et al., 2006; Hildebrandt et al., 2008). Biodegradation and mineralization of terbuthylazine have been shown to be carried out by bacterial consortia and by strains isolated from contaminated soil and groundwater (Grenni et al., 2009a; Barra Caracciolo et al., 2010). However, terbuthylazine biodegradation in soil is modulated by abiotic factors such as organic matter content, pH, temperature, water content, presence of exogenous nitrogen, like urea and so on (Di Corcia et al., 1999; Barra Caracciolo et al., 2001; Barra Caracciolo et al., 2005; Barra Caracciolo et al., 2010).

In many cases, the water pollution by pesticides arising from point sources (spills, uncontrolled disposal, equipment washing water, etc.) can be more important than that due to agricultural practice (De Wilde et al., 2007; Fait et al., 2007; Müller et al., 2002). The use of organic materials or wastes may prevent the mobility of pesticides coming from these point sources of contamination and enhance their biodegradation (Rodriguez-Cruz et al., 2007a). In recent years different low-cost sorbent systems (biobed, biomassbed, biofilter, etc.) have been developed to minimize point sources of pesticide pollution. These systems consist of a mixture of different organic biomaterials and soil which can retain and degrade pesticides (Castillo et al., 2008; De Wilde et al., 2007; Fait et al., 2007). In particular, the use of wood residues as low-cost sorbents has recently been developed as a new technology for the immobilization of heavy metals, dyes, pesticides, other organic compounds, etc., in soil (Gupta et al., 2009; Rodríguez-Cruz et al., 2007b; Shukla et al., 2002). Organic sorbents such as pine and oak wood residues are being investigated as biomaterials for the immobilization of pesticides in soil (Rodriguez-Cruz et al., 2007b). Freundlich sorption constants (Kf) of herbicides by oak and pine were related to the lignin content of these woods (18.2% and 24.4%, respectively). However, the influence of the addition of wood residues on the degradation of pesticides in soils has been studied less (Grenni et al., 2009b). The addition of organic amendments to soil can affect the biodegradation of pesticides due to the application of an additional source of organic matter and sometimes microorganisms (Briceño et al., 2007) with the results of accelerating the degradation of pesticides. In other cases, the addition of an organic residue to soil, by decreasing the bioavailability of pesticides owing to their increased sorption capacity, can lead to a decrease in pesticide degradation. (Briceño et al., 2007; Grenni et al., 2009b; Moorman et al., 2001).

The effects of wood amendments (pine and oak residues) on terbuthylazine degradation and the soil microbial community were studied. For this purpose, degradation kinetics, soil dehydrogenase activity and the number of live bacteria, which represent measurements of soil microorganisms activity and viability, were assessed in soil treated with terbuthylazine and either amended with pine or oak wood or unamended (sterilized and non-sterilized). The findings of this study should improve our knowledge of the effects on soil of wood amendments if used as a tool for preventing contamination by terbuthylazine.

Material and methods

Soil and wood samples

Soil samples were collected from the surface horizon (0-20 cm depth) of an agricultural field located in central Italy (Assisi, Perugia) with previous terbuthylazine application. Soil was left to dry at room temperature and then sieved (<2 mm) and analyzed. The soil texture was classified as silty-clay (USDA), the organic carbon and nitrogen content were 0.46% and 0.13%, respectively, and the pH 7.7

Pine and oak wood samples (<1mm) were selected as the organic soil amendments because of their different lignin content of 18.2% and 24.4%, respectively (Rodríguez-Cruz et al., 2007b). The pine and oak residues were obtained from a local company in Salamanca (Spain). The amended soils were prepared by uniformly mixing soil with oak and pine (5% w/w). Organic carbon (OC) was analyzed in sub-samples by using a Total Organic Carbon Analyser (Shimadzu model TOC500A-ASI-5000A). The organic carbon in amended sample soils was found to increase (to 1.97% in pine-amended and 1.89% in oak amend soil, respectively).

Chemicals

Terbuthylazine (N^2-tert-butyl-6-chloro-N^4-ethyl-1,3,5-triazine-2,4-diamine) and its main metabolites such as desethyl-terbuthylazine (DET), desethyl-debutyl-terbuthylazine or desisopropyl-atrazine (DIA), were supplied by Dr. Ehrenstorfer GmbH (Augsburg, Germany) ($>$ 98.0% purity). Terbuthylazine is a colourless powder with a water solubility of 8.5 mg L^{-1} at 20°C and log K_{ow} of 3.21 (Tomlin, 2003).

Sorption studies

Sorption of terbuthylazine by woods and soils was determined using a batch equilibrium method described elsewhere (Rodriguez-Cruz et al., 2007b). Briefly, duplicate 100 mg wood samples or duplicate 5 g unamended and wood-amended (5%) soils were equilibrated with 10 mL of an aqueous solution of terbuthylazine at concentrations between 0.5 and 8 mg L^{-1}. The pesticide amount adsorbed was considered to be the different between that initially present in solution and that remaining after equilibration with the wood. Sorption data were fitted to the Freundlich equation. The linearized form of this adsorption equation is

$$\log Cs = \log Kf + nf \log Ce$$

where Cs (mg kg^{-1}) is the amount of sorbed pesticide, Ce (mg L^{-1}) is the equilibrium concentration of pesticide solution and Kf (mg^{1-nf} mL^{nf} kg^{-1}) and nf are the Freundlich affinity and nonlinearity coefficients, respectively.

Laboratory degradation experiments with amended and unamended soils

The herbicide degradation experiment was conducted in duplicate in accordance with SETAC guidelines (Lynch, 1995) and previous experiments (Barra Caracciolo et al., 2005; Grenni et

al., 2009b). Terbuthylazine was added to unamended or amended soil (200 g) to obtain a final concentration of 1.5 mg kg^{-1}. Some samples were first sterilized (autoclaved 120 ±2 °C, 20 min on 2 consecutive days) and then treated with terbuthylazine (SST); other samples of soil were only treated with terbuthylazine (ST); others were treated with both terbuthylazine and pine (SPT) or oak (SOT) sawdust; and finally, microbiological control soils (S) were prepared with only water and with water and pine or oak sawdust. All soils were thoroughly stirred with a sterilized spatula and the water added was in all cases sterilized by filtration (0.22 µm). The final moisture content was adjusted to 60% of the maximum soil water holding capacity.

Soils were maintained in beakers closed with a sterilized cotton plug wrapped in gauze to allow air exchange. The soil moisture was kept constant during the entire period of the experiments by periodically weighing and replacing any losses with sterile water. Samples were incubated at 20 ±2 °C in the dark. Solutions and instruments were sterilized and all steps were performed in a sterile cabinet.

Sampling was performed at different times (0, 6, 12, 20, 33, 49, and 64 days) for both chemical and microbiological analysis.

Herbicide extraction and analysis

Soil sub-samples (1 g) were taken from each microcosm and shaken with 6 mL of methanol for 24 h at 20°C for residue analysis. Samples were centrifuged and 4 mL of each supernatant were evaporated at 30°C under nitrogen stream (Concentrator EVA VLM-EC-2V-130, Germany) and re-dissolved in 0.5 mL of methanol for analysis.

Quantitative determination of terbuthylazine and its metabolites (DET and DIA) was performed by GC-MS in a 7890A Agilent gas chromatograph coupled to a 5975C Agilent mass spectrometer (Agilent Technologies, Avondale, USA) with an Agilent 7683 autosampler. Chromatographic separation was performed on a 30m×0.25mm I.D, 0.25µm film thickness HP-5MS capillary column (J&W, Folsom, USA). A volume of 1.0 µL was injected in the splitless mode at 250ºC. The carrier gas was ultra-pure helium at a flow of 1 mL min^{-1}. The following temperature program was used: temperature was increased from 100ºC to 150ºC at 50ºC min^{-1} and maintained for 1 min, then at 5ºC min^{-1} to 200ºC and finally increased to 290 ºC at 30ºC min^{-1} and maintained for 1 min. The quadrupole mass spectrometer was operated in electron impact ionization (EI) mode at 70 eV. The transfer line and the injector were set up at 250ºC and the source and the quadrupole were at 230 and 150ºC, respectively. Measurements in the GC–MS were performed in the single-ion monitoring (SIM) mode. The more abundant ions were chosen for quantification (Terbuthylazine m/z 214, DET m/z 186 and DIA m/z 173). The quantification was carried out by double injection. Recoveries for terbuthylazine, DET, DIA were 90%, 80%, 72% respectively. Samples were extracted and analyzed in duplicate.

Soil dehydrogenase activity, total cell number and cell viability

Soil dehydrogenase activity (DHA), a measure of overall microbial activity, at 0, 6, 12, 20, 33, 49, and 64 days after herbicide application was determined following a modified version of the method of Tabatabai (1994). The method is based on extraction and colorimetric determination of the intensely coloured 2,3,5-triphenyl formazan (TPF) produced from the reduction of colourless 2,3,5-Triphenyltetrazolium chloride (TTC) in soils after an 24 h incubation at 37°C in the dark. Results were expressed as μg TPF g^{-1} dry soil. Measurements were performed in duplicate for each microcosm.

The total cell number (No. bacteria g^{-1} dry soil) was assessed (in triplicate for each microcosm) in 1 g of fixed soil with the epifluorescence direct count method, using 4′,6′-diamidino-2-phenylindole (DAPI) as the DNA fluorescent agent, as reported in detail in previous works (Barra Caracciolo et al., 2005).

Cell viability was measured (in triplicate for each microcosm) in 1 g of fresh soil in order to estimate cell viability (% live cells/ live+dead), using two fluorescent dyes, SYBR Green II and propidium iodide (Sigma-Aldrich, Germany) in order to distinguish between viable (green) and dead (red) cells under a fluorescence microscope (Leica DM 4000B Leica Microsystems GmbH, Wetzlar, Germany), as reported in a previous work (Amalfitano et al., 2008).

Statistical analysis

Analysis of variance (one-way analysis of variance) was used to determine the significant differences in dehydrogenase activity and number of live bacteria among the different soil treatments, using the Statistical software SIGMASTAT (version 3.0).

Results and discussion

Sorption of terbuthylazine by wood residues and soil

Sorption isotherms of terbuthylazine by the woods used and the unamended and wood-amended soils were obtained. Table 1 shows the sorption coefficients, Kf and nf, obtained as a result of fitting all the isotherms to the Freundlich equation with r values > 0.99.

The Kf value for the sorption of terbuthylazine by pine was much higher than by oak. This higher sorption capacity by pine could be due to its higher lignin (hydrophobic wood component) content (24.4%). These Kf constants are higher than those reported by Rodriguez-Cruz et al. (2007b) for the adsorption of other non-ionic pesticides (linuron, alachor and metalaxyl) by pine and oak and are in agreement with the higher hydrophobicity of terbuthylazine.

For the sorption of terbuthylazine by soils, the lowest Kf value corresponded to sorption by unamended soil, followed of soil amended with oak, and the highest one to sorption by soil amended with pine. Sorption capacity increased by 2-fold and 27-fold for soils amended with oak and pine, respectively. These results are in agreement with the higher lignin content of

pine (24.4%) than that of oak (18.2%) and the lower dissolved organic carbon content of pine (67.2 mg L^{-1}) than that of oak (264 mg L^{-1}). Lignin content and dissolved organic carbon of woods were significantly related with the sorption constants (Kf) of other non-ionic pesticides (Rodriguez-Cruz et al., 2007b). Capacity of pine-amended soil to adsorb terbuthylazine was higher than that of soils amended with other organic residues (Cabrera et al., 2007; Dolaptsoglou et al., 2007).

Table 1. Sorption constants of terbuthylazine by pine and oak residues and by unamended soil and pine-amended and oak-amended soils.

	Kf	**nf**	**r^2**	**%A**
Pine	1663 ±13.6	0.94 ±0.04	0.99	94.5 ±0.33
Oak	86.3 ±4.87	0.82 ±0.03	0.99	41.3 ±0.97
Soil	1.98 ±0.04	0.99 ±0.21	0.99	52.2 ±7.34
Soil + Pine	52.5 ±6.47	1.00 ±0.04	0.99	96.7 ±0.25
Soil + Oak	4.44 ±0.10	1.20 ±0.01	0.99	71.8 ±6.45

Degradation kinetics of terbuthylazine and metabolite formation

The decrease (expressed in percentages of terbuthylazine initially applied) of the herbicide concentrations in unamended soil (ST), unamended and sterile soil (SST) and soils amended with pine (SPT) or oak (SOT) over a period of 64 days is shown in Figure 1. Degradation of terbuthylazine fitted first-order kinetics: $C = C_0 e^{-kt.}$, where C is the concentration at time t, C_0 is the initial concentration at t=0, and k is the first-order rate constant. The theoretical DT_{50} values calculated from the exponential equations, obtained from the regressions between concentration and time were: sterilized unamended soil (SST): 257 d < pine amended soil (SPT): 161 d < unamended soil (ST): 105 d < oak amended soil (SOT): 95 d.

These theoretical values suggest that the herbicide is quite persistent in the soil studied. However, the DT_{50} was not reached during the experimental time and at day 64 the lowest herbicide concentrations observed was in the unamended (ST) and oak-amended (SOT) soils. At this time about 40% of the initial concentration was degraded and we cannot exclude that the degradation was faster than the theoretical calculations.

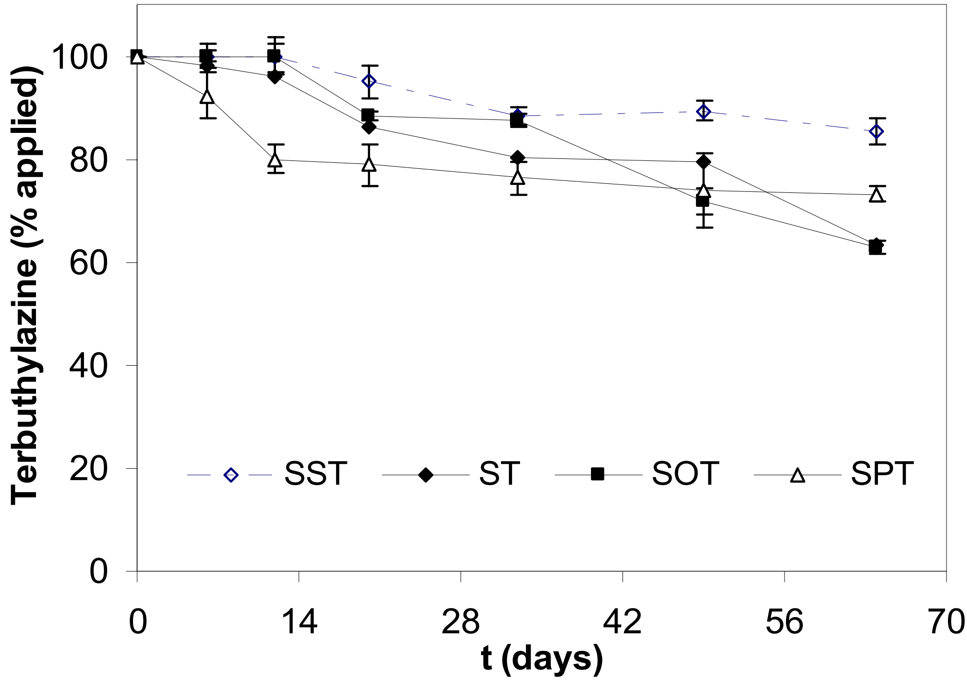

Figure 1. Terbuthylazine degradation kinetics in unamended and amended soil with pine and oak residues as a function of time.

The results confirm that terbuthylazine degradation is mainly due to microorganisms and that its rate is highly variable (Ma et al., 2000) and depends not only on temperature, moisture and organic carbon content, and pH, but also on the soil intrinsic characteristics which can significantly modulate the degradation process. In fact, in our previous microcosm studies, performed in similar laboratory conditions (temperature, absence of light and so on), we observed a DT_{50} of 30 days and 22 days in sandy-loam and silty-loam soil, respectively (Barra Caracciolo et al., 2001; Barra Caracciolo et al., 2005). The relatively high DT_{50} values (95-105 days) found in this silty-clay soil can be ascribed to the structure of its clay component. In fact, it was possible that soil water, being partially incorporated in the mineral structure of soil clay, was not completely available for biotic and abiotic degradation processes. Our soil was in fact quite heterogeneous and lumpy when the water was added, so that the latter was not fully available for degradation and the space between soil pores was reduced substantially and did not permit efficient degradation.

The occurrence of clays can strongly affect both the transport and the degradation of soil contaminants and consequently makes it difficult to forecast their fate.

The decrease in terbuthylazine concentration in the sterile condition was presumably due to terbuthylazine dehalogenation to its hydroxylated form, which can occur through chemical hydrolysis (Di Corcia et al., 1999; Barra Caracciolo et al., 2005; Barra Caracciolo et al., 2010). The detection of the hydroxylated metabolites was not performed in this experiment,

because it requires HPLC analysis and we performed only GC-MS ones. DET was not detected in sterile soil in line with the fact that its formation is reported to occur exclusively via biotic transformation (Di Corcia et al., 1999, Barra Caracciolo et al., 2005).

Formation of the DET metabolite was detected in microbiologically active soils during the degradation process and the amounts of this metabolite generally increased over time, as shown in Figure 2.

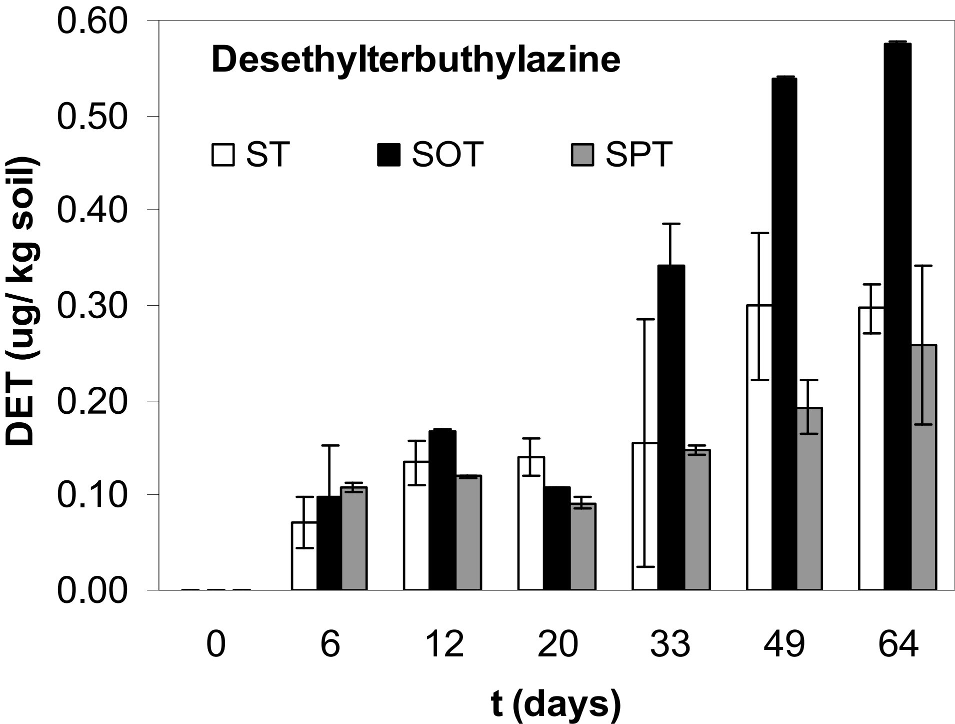

Figure 2**.** Formation of terbuthylazine metabolite DET in unamended and amended soils during the experimental time.

After 64 days the maximum amounts of DET were 0.30 in unamended soil (ST), 0.58 µg kg^{-1} in soil amended with oak (SOT) and 0.26 in soil amended with pine (SPT). DET was found as the main metabolite in similar studies (Dousset et al., 1997; Navarro et al., 2003; Dolaptsoglou et al., 2007) and was detected in higher concentrations in oak-amended soil, in line with a higher terbuthylazine degradation observed in this condition until day 64. The slower degradation in pine-amended soil than in unamended and oak-amended ones can be ascribable to the high herbicide sorption to soil (Table 1), which reduced its bioavailability for microbial degradation. This is in agreement with previous studies where the addition of organic amendments (urban sewage sludge, poultry compost and alperujo) to soil retarded the degradation of terbuthylazine by increasing its sorption (Navarro et al., 2003; Cabrera et al.,

2007; Dolaptsoglou et al., 2007). On the contrary, in presence of oak amendments the herbicide sorption did not increase significantly to hamper the biodegradation and the activity of the overall bacterial community was favoured by the presence of an additional source of organic carbon provided with the amendment.

Microbiological analysis

The herbicide effect on soil dehydrogenase activity and number of live bacteria was studied in all herbicide-treated soils and compared with non-treated ones. Figure 3 shows soil dehydrogenase activity (µg TPF g^{-1} dry soil) vs. time in the treated soils (A) and in the control ones (B).

A significant difference in dehydrogenase activity was observed among the different soil treatments. Dehydrogenase activity was significantly higher in all the soils amended with pine or oak than in unamended ones. After 64 days, the pine and oak amendment led to an increase of dehydrogenase activity of up to 5-fold and 10-fold, respectively, compared to the unamended soil. The presence of amendments, rich in labile carbon fractions, therefore, stimulated soil dehydrogenase activity during the experimental period. Some authors have noted the positive influence of organic amendments on the dehydrogenase activity of the microbial community (Moorman et al., 2001; Delgado-Moreno and Peña, 2007; Grenni et al., 2009b). Terbuthylazine did not negatively affect the bacterial community functioning in terms of dehydrogenase activity. On the contrary, in the case of oak-amended soil, a positive effect was observed at day 49 (Figure 3). A stimulatory effect on dehydrogenase activity by pesticides was reported in previous works (Hussain et al., 2009).

The bacterial number obtained by DAPI counts was not significantly different among the different conditions and treatments (data not shown). The cell viability values (Live/Live+Dead) are reported in Figure 4. In the presence of the herbicide there was a transient decrease of viability at day 6 and then the values increased in all conditions. The bacterial viability trend can be linked to the activation of bacterial populations involved in the herbicide degradation and this is particularly evident in the ST. In fact, in the control soil (non-terbuthylazine treated soil) the overall cell viability tended to decrease during the experimental period.

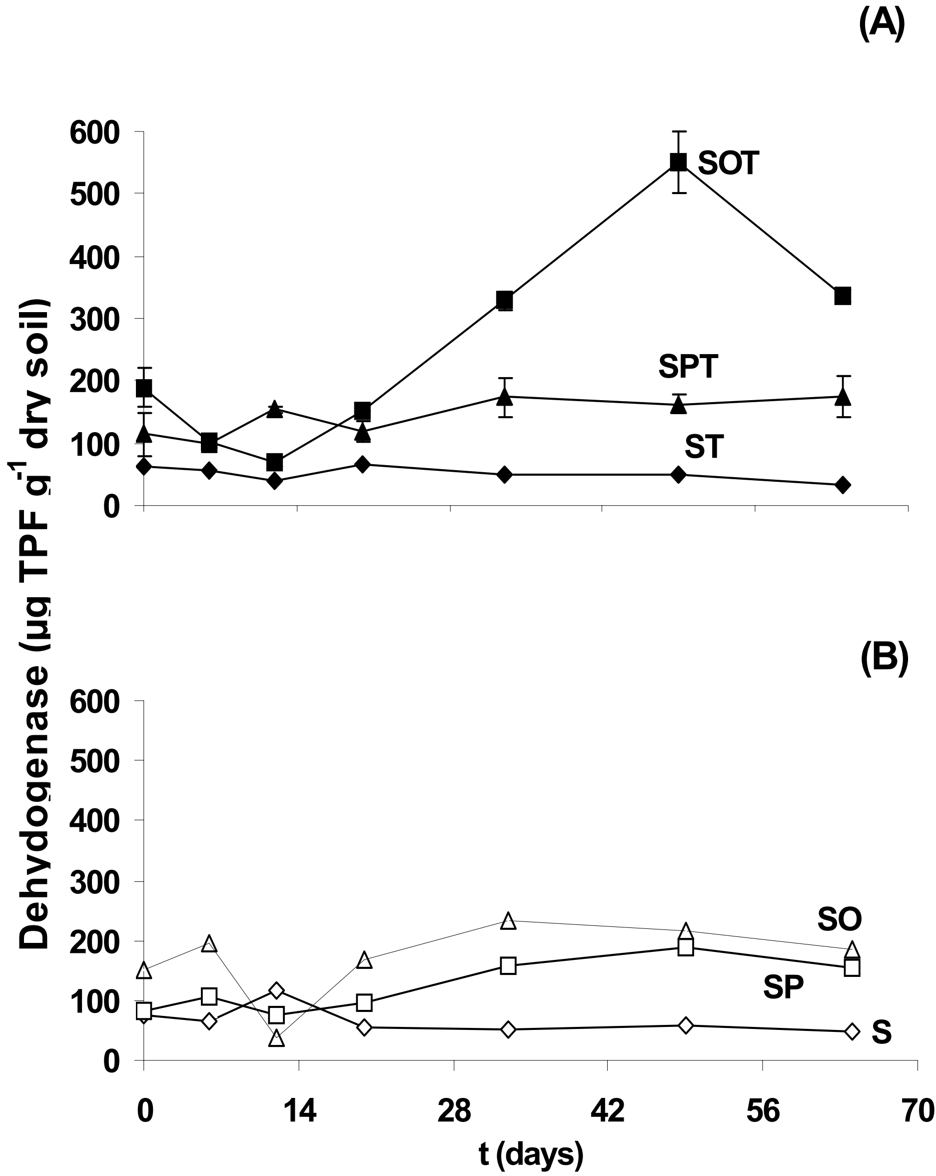

Figure 3. Soil dehydrogenase activity (µg TPF g^{-1} dry soil) detected over time in the soils treated with terbuthylazine (A) and in the control ones (B). Bars represent standard errors. Unamended soil (S), soil amended with oak (S+O) and with pine (S+P). Soil treated with terbuthylazine (ST), treated with terbuthylazine and amended with pine (SPT) or oak (SOT).

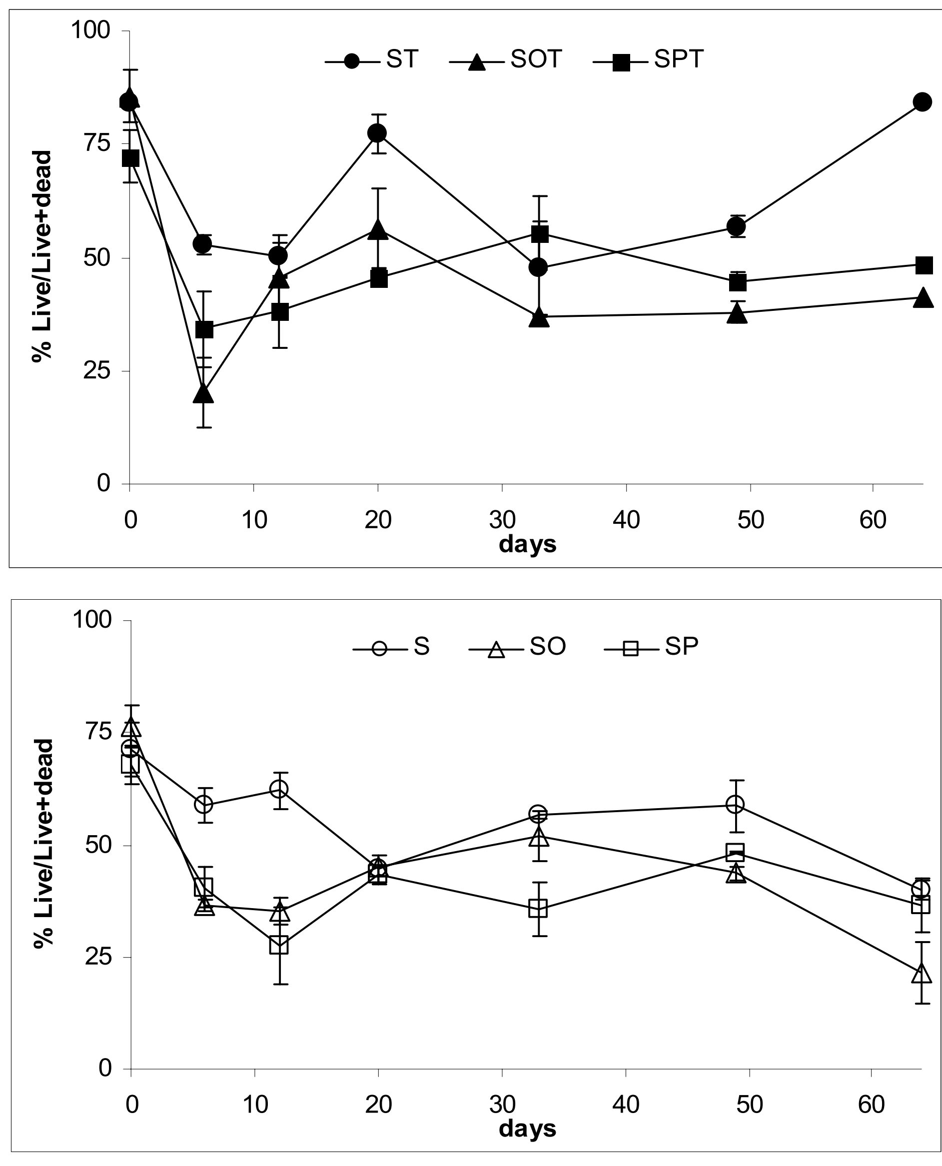

Figure 4. Cell viability vs. time in the soils treated with Terbuthylazine (ST, SOT, SPT) and in the control ones (S, SO, SP). Bars represent standard errors.

Conclusions

In conclusion this short-term study shows:

The microbial community had a significant role in the terbuthylazine degradation, as shown when comparing the degradation results for sterile soil and microbiologically active soil. However the persistence of the herbicide was quite high if compared to other agricultural soils and this can be ascribed to the intrinsic soil characteristics (in particular high clay content) which could have limited bacterial activity.

The presence of pine significantly increased the terbuthylazine sorption to soil, as shown by the high Kf value, and this fact reduced the herbicide bioavailability and consequently its degradation. Although pine did not have any detrimental effect on the soil bacterial community, since it significantly affects herbicide biodegradation by hampering its bioavailability, it is not suitable for application on soil for pesticide mobility prevention.

On the contrary, in the presence of oak amendments the herbicide sorption did not increase enough to hamper the biodegradation and the activity of the overall bacterial community was favoured by the presence of an additional source of organic carbon provided by the amendment. Oak amendments can be used as an effective tool to prevent terbuthylazine mobility in soil.

The knowledge of the fate of pesticides in the presence of wood amendments can contribute to achieving sustainable agricultural practices which minimize soil and water pollution.

This research activity was performed at IRSA-CNR in Rome (Paola Grenni, Anna Barra Caracciolo and Francesca Falconi) in collaboration with the Spanish CSIC - Institute of Natural Resources and Agrobiology of Salamanca (M.S. Rodríguez-Cruz, E. Herrero-Hernández, M. Marín-Benito, M.J. Sánchez-Martín).

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2nd PART: Research regarding the effects of antibiotic and antiviral drugs on soil and water bacterial communities

Pharmaceuticals in the environment, identified as emerging contaminants, are becoming a subject of global concern, with potential environmental consequences and are ubiquitously present in water resources and in soil. Such compounds are released more or less continuously from multiple sources and enter water and soil ecosystems. The effects of pharmaceuticals continuously released into the environment should not be underestimated and certainly investigated carefully before widespread use of a drug is encouraged. Further knowledge of the causes, occurrence and effects of drugs as environmental pollutants is necessary for a better understanding of this ecological issue, as well as to improve abatement strategies and mitigate subtle environmental consequences. Environmental contamination might affect microbial communities, with consequent changing in bacterial populations which have a key role in fundamental ecosystem processes such as nutrient transformations and biomass decomposition.

This section deals with the evaluation of soil and water contamination by pharmaceuticals using a microbial ecology approach.

The effects of pharmaceutical waste disposal from a factory producing antibiotics (josamycin, erythromycin) on bacterial communities in soil and groundwater were studied and the soil and groundwater quality state evaluated.

The environmental fate of the antiviral drug Tamiflu (oseltamivir carboxylate), used as an influenza A H5N1 and H1N1 virus prophylaxis, was studied in a surface water ecosystem. Moreover, the bacterial community structure was analysed by using fluorescence *in situ* hybridization.

Finally, it is report a collection of data about the application of Fluorescence *in situ* hybridization in soil and water ecosystems as a useful method for studying the effects of xenobiotics (pesticide and pharmaceuticals) on bacterial community structure.

The results of these studies are reported in the following papers:

- Barra Caracciolo A., Grenni P., Mascolo G., Caputo M.C., Uricchio V., 2011. Pharmaceutical waste disposal in a disused open quarry: assessment of its effects on bacterial communities in soil and groundwater. *Chemistry and Ecology*, 27: 43-51.
- Barra Caracciolo A., Grenni P., Saccà M.L., 2010. Effect of the Antiviral Drug Oseltamivir (Tamiflu) on the Bacterial Community Structure of a Surface Water Ecosystem Analysed using Fluorescence *In Situ* Hybridization. *Bulletin of Environmental Contamination and Toxicology*, 85: 443-446.
- Barra Caracciolo A., Grenni P., 2010. Microbial ecology methods for assessing the effects of xenobiotics in water and soil ecosystems. *Comparative Biochemistry and Physiology*, Part A Molecular & Integrative Physiology 157 S1–S2: S56.

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Assessment of the effects of pharmaceutical waste disposal on bacterial communities in soil and groundwater

Abstract

A preliminary ecological characterisation of an open quarry that had been used for the disposal of pharmaceutical wastes from a factory producing antibiotics was performed. Pharmaceutical wastes and groundwater samples were collected and analysed in order to assess both the bacterial community structure and functioning, and the contamination by organic compounds, including antibiotics. Bacterial abundance measured using the epifluorescence direct count method, cell viability measured by using two fluorescent dyes, species diversity measured by assessing the bacterial community structure using fluorescence in situ hybridisation (FISH) and soil microbial activity based on dehydrogenase activity were used as microbiological indicators to evaluate the 'quality state' of the area studied. The overall results show that groundwater has a low-quality state in terms of bacterial viability, activity and diversity, associated with trace contamination by antibiotics and chlorinated volatile organics.

Introduction

Studying soil using an ecological approach is a necessary prerequisite for improving the understanding of its structure (biodiversity) and functioning (Bardgett, 2002; Widinga et al., 2005). Soil and the water located beneath its surface, groundwater, have to be considered a single ecosystem which needs to be protected against infiltration by pollutants.

Microorganisms have a key role in ecosystem functioning (Atlas and Bartha 1997). They are the main mediators in the detritus-based food web, making it possible for the energy contained in dead organic matter to be used by detritivores, are responsible for the complete mineralisation of organic matter and recycling of nutrients and, finally, are capable of performing a homeostatic action with exogenous molecules (Tiunov and Scheu 2004; Altieri 1999; De Long and Pace, 2001; Iker et al., 2010). Recovery from contamination is only possible if the quantity and toxicity of the molecules do not hamper or inhibit their activity. The presence of an abundant and varied microbial community is a necessary prerequisite for an immediate and effective response to the various natural and anthropic disturbances that can affect an ecosystem (Topp, 2003). Microorganisms are essential constituents of the soil purification processes associated with groundwater quality. In particular, soil enzyme activity determines the biodegradation of organic compounds passing through the soil profile (Shultz et al., 2010). However, only a small fraction of bacteria in soils and groundwater is amenable to culturing in the laboratory, which limits our ability to study these organisms (Bakken, 1997; Topp, 2003).

The study of microbial communities is highly dependent on the availability of appropriate methods for identifying their structure (e.g. number and diversity of species) and their function (e.g. bacterial activities) without a need for isolation and cultivation. In the present study we apply some microbial ecology methods to soil and groundwater samples collected from a quarry in order to evaluate their quality state. The open quarry was used for waste disposal by a pharmaceutical company producing antibiotics.

The results reported here are part of an eco-diagnosis study with broader aims: (i) to evaluate the possible presence of contamination in the area of the study; (ii) to provide a description of the geological and hydro-geological features of the quarry, in order to establish the true situation in view of the conflicting information produced by previous reports, just obtained by local Government; (iii) to encourage the involvement of the local community in both the problem analysis and project phases.

Materials and Methods

Area studied

The area studied is a disused open calcarenite quarry near Brindisi (Southern Italy), which was used for about 10 years (from 1980 to 1990) for waste disposal by a pharmaceutical company producing antibiotics (in particular erythromycin, one of the most commonly used macrolide antibiotics in human medicine) using fermentative processes and subsequent chemical transformations. In particular the waste included: a. some exhausted mycelium, produced during the antibiotic production process, which was mixed with the soil; b. biologically stabilized sludge coming from the activated sludge treatment plant into which all the waste from production departments and liquid residues from all other parts of the factory flowed.

The disused quarry was chosen for the definitive digestion of the sludge after ferric chloride treatment, partial dehydration and stabilization with hydrated lime.

The local geology consists of Cretaceous bedrock formed from dolomitic limestone and limestone, unconformably overlain with Plio-Pleistocene calcarenites. The oldest formations contain a deep, confined aquifer characterized by a water table with a depth below ground surface ranging from 70 m to 80 m depth. In the Plio-Pleistocenic calcarenites geophysical measurements detected a shallow aquifer with a water table about 25 m below ground surface and about 12 m below the bottom of the quarry.

Field infiltrometer tests were carried out on the calcarenite outcrop in the bottom of the quarry at three different times (July, September, and November). The mean infiltration rate value obtained was about 0.03 m h^{-1} in the saturated condition and in the unsaturated one it was one order of magnitude greater (Caputo et al., 2011). These results, combined with the relatively small depth of the vadose zone, make this shallow aquifer particularly vulnerable to contamination.

Collection of soil and groundwater samples

Two vertical coring samplings (S1 in the most inner part and S2 in a fringe area of the quarry, (Figure 1) were carried out at 8 m depth.

Figure 1. Sampling points.

The material sampled consisted of soil mixed with pharmaceutical waste. Each core was split into several sub-samples (see Table 1).

Table 1. Collection of soil samples (geographic coordinates and sub-sample depth)

Geographic coordinates (GPS references) of sampling	
S1= 40°25'50.3'' N 17°48'40.0'' E	**S2**= 40°25'49.5'' N 17°48'38.8'' E
Sub-sample depth (m)	
S1 (2.5 – 4.0)	**S2** (1.5 – 2.0)
S1 (4.0 – 5.0)	**S2** (4.0 – 4.5)
S1 (5.0 – 6.5)	–

Moreover some samples (consisting of a mixture of soil with aged pharmaceutical waste) were collected manually in 2 different points (named S12 and S22 respectively) from the superficial layer (0-20 cm) close to the S1 coring sampling point.

Groundwater samples were collected with a sterile bailer from three different piezometers (named S1, S2 and S2bis, being close to the coring points) at different depths. S1 (51 m depth) was located a few hundred meters north of the area with pharmaceutical waste material while the other two (23 and 46 m depth, respectively) were to the south of it. The organic carbon (OC) was determined in both soil and groundwater samples as described in previous

works, with a CHN analyser and a TOC analyser respectively, as described previously (Grenni et al., 2009a; Grenni et al., 2009b).

Each chemical and microbiological analysis of soil and groundwater samples was performed at least in triplicates using 3 sub-samples.

Chemical analysis

Determination of extractable organic halogens (EOX) was performed using the EPA 9023 method. Determination of antibiotics was performed by liquid chromatography/tandem mass spectrometry (HPLC/MS-MS) using an Acquity chromatographic system equipped with a diode array detector (Waters) interfaced to an API 5000 mass spectrometer (Applied Biosystem/MSD Sciex) by means of a turbo-ionspray interface (positive ion). Samples (5 µl) were injected by the Acquity autosampler equipped with a Rheodyne valve and a 10 µL loop, and eluted at 0.35 mL min^{-1} through a Supelco Ascentis analytical column (150 x 2.1 mm inner diameter and 2.7 µm) with a water/methanol (with 0.1 % formic acid in each solvent) gradient from 95/5 to 0/100 in 9 min. Determinations in solid samples were performed after a previous extraction with acetonitrile and a filtration with PTFE-filters.

Determination of volatile organic compounds was performed by solid phase micro extraction/gas chromatography/mass spectrometry (SPME/GC/MS) using a Varian Saturn 2200 GC/MS system (electron impact ion source) equipped with a 8200 autosampler and a SPME syringe (Supelco) with a 100 µm (non-bonded) polydimethylsiloxane fibre. Aqueous samples (0.8 mL) were placed into 2mL vials equipped with silicone/Teflon septa and the SPME fibre was exposed to the vapour phase for 30 min in order to adsorb the volatile organics. The SPME syringe was then automatically introduced into the injector of the GC/MS system in order to desorb and analyse the compounds.

Microbiological analysis

Microbiological analyses were performed on both soil (1 g) and groundwater (10 mL) sub-samples. Bacterial abundances were measured using the epifluorescence direct count method reported in detail elsewhere (Grenni et al., 2009b; Di Corcia et al., 1999; Barra Caracciolo et al., 2005a; Barra Caracciolo et al., 2005b), using DAPI (4’,6’-diamidino-2-phenylindole) as the DNA fluorescent agent. Cell viability was measured using two fluorescent dyes, SYBR Green II and propidium iodide (Sigma-Aldrich, Germany), to distinguish between viable (green) and dead (red) cells under a Leica fluorescence microscope, as reported in previously (Amalfitano et al., 2008; Grenni et al., 2009b).

Soil dehydrogenase activity was determined using the reduction of 2,3,5-triphenyltetrazolium chloride (TTC) solution to triphenylformazan (TPF), measured by the method reported in Grenni *et al*. (2009b). Finally, bacterial community phylogenetic composition was analysed using Fluorescence *In Situ* Hybridization (FISH) (Barra Caracciolo et al., 2005a; Barra Caracciolo et al., 2005b). Bacterial groups identified by FISH and their corresponding Cy3-labelled probes were: *Bacteria* (EUB338I-III); α-*Proteobacteria* (ALF1b); β-*Proteobacteria* (BET42a); γ-*Proteobacteria* (Gam42a); *Planctomycetes* (Pla46

and Pla866), *Cytophaga–Flaviobacterium* cluster phylum CFB (CF319a); Sulfate-Reducing Bacteria (SRB385); and Sulfur-Reducing heterotrophic Epsilon (EPS710) (Loy et al., 2007).

The application of FISH to soil samples was possible after a cell extraction procedure described in detail in Barra Caracciolo *et al*. (2005b).

Results and Discussion

Soil sampling at different depths

The superficial layer (0-20 cm) was analysed at 2 different points (S12 and S22) in triplicates; at S22, where more organic carbon was found than at S12 (Table 2), a higher bacterial activity, both in terms of dehydrogenase (Figure 2) and % of *Bacteria* detected by FISH (Figure 3), was observed (t tests significant, $p < 0.01$).

Organic matter and carbon are among the most important parameters in defining soil quality and microbial activity is strictly dependent on their amounts (Chen et al., 2003). In undisturbed ecosystems bioactive soil OC is a direct and stable reservoir of energy and nutrients consisting of living and dead organic material subject to rapid biological decomposition.

Table 2. Percentages (%) of Total Carbon (C_{Tot}), Organic Carbon (OC) and Total Nitrogen (N_{Tot}) analysed by Elemental Analyser CHNS; bacterial abundance (No. Bacteria g^{-1}), Cell viability (% Viability) and No. Live Bacteria g^{-1} analysed by epifluorescence microscope methods at different depths.

Soil depth (m)	C_{Tot} (%)	OC (%)	N_{Tot} (%)	No. Bacteria g^{-1}	Viability (%)	No. Live Bacteria g^{-1}
S12 (0 - 0.2)	5.0	3.2	0.31	8.0×10^7	73.3	5.8×10^7
S22 (0 - 0.2)	10.9	9.8	0.32	5.9E $\times 10^7$	77.3	4.6×10^7
S1 (2.5 - 4.0)	16.0	13.8	1.01	3.2×10^8	14.0	4.3×10^7
S1 (4.0 - 5.0)	18.0	15.7	1.12	1.9×10^8	23.0	4.3×10^7
S1 (5.0 - 6.5)	14.8	13.7	0.87	8.3×10^8	15.0	1.2×10^8
S2 (1.5 - 2.0)	13.3	9.4	0.96	1.5×10^8	18.0	2.6×10^7
S2 (4.0 - 5.0)	13.9	12.6	0.75	1.6×10^8	42.0	6.6×10^7

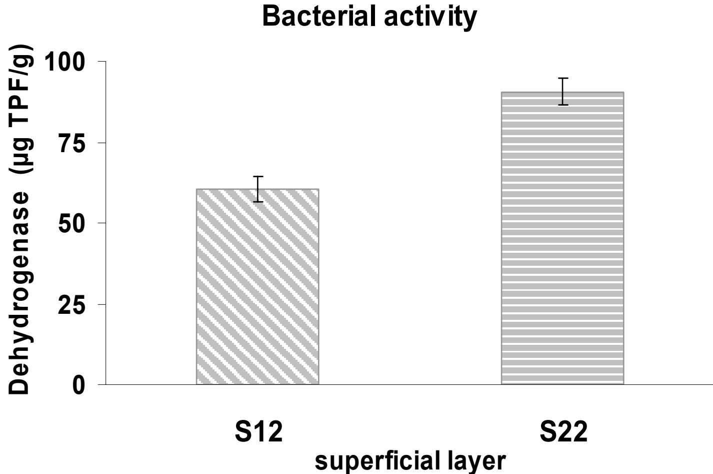

Figure 2. Dehydrogenase activity (µg TPF/g) measured by triphenyl tetrazolium assay in superficial (0-20 cm) samples S12 and S22. The error bars indicate SE.

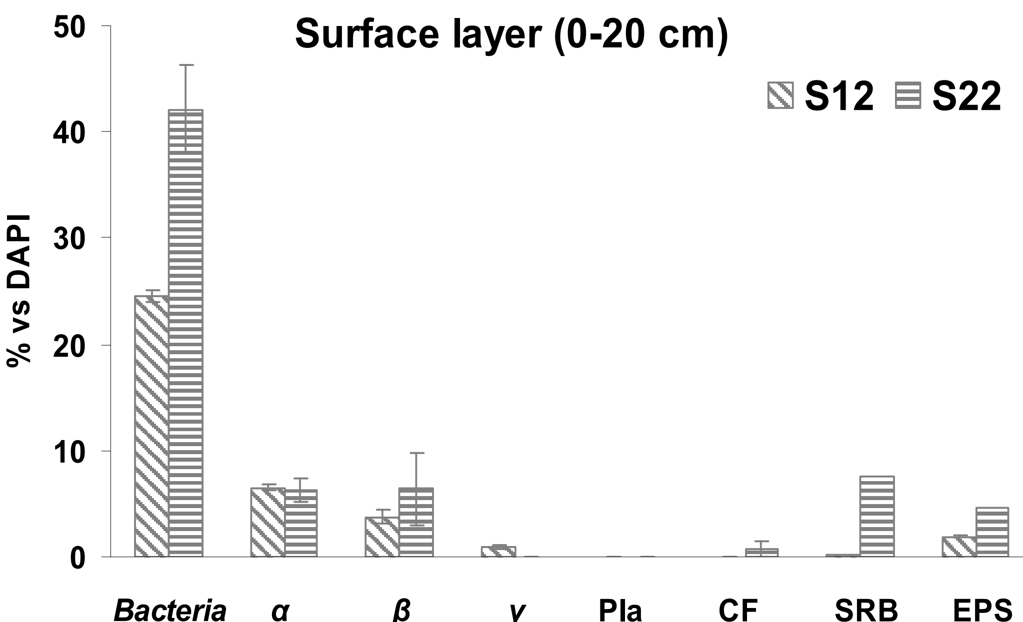

Figure 3. Bacterial community structure detected by Fluorescence *In Situ* Hybridization (FISH) in superficial samples (S12 and S22). The value of the cells binding each different probe is expressed as a percentage (%) of total DAPI-positive cells. The error bars indicate SE.
α = α-*Proteobacteria*; β = β-*Proteobacteria*; γ = γ-*Proteobacteria*; Pla = *Planctomycetes*; CF = Cytophaga–Flaviobacterium cluster phylum CFB; SRB = Sulfate-Reducing Bacteria; EPS = Sulfur-Reducing heterotrophic Epsilon.

Consequently, the presence in soil of a high amount of OC should promote cell viability and bacterial activity (Grenni et al., 2009b; Chen et al., 2003). However, although the organic carbon content in the S12 and S22 samples was much higher than that generally found in another works (e.g. 0.72% in a sandy-loam soil, 2.79-2.89% in the same soil with wood amendments), the dehydrogenase and viability were similar to the lowest values found in soil

in presence of low OC content (Grenni et al., 2009b; Pandey and Singh, 2006; Bending et al., 2007).

The FISH analysis, which is able to identify exclusively metabolically active populations (Demters et al., 2004, Allison and Martiny, 2008) was in line with the dehydrogenase and viability results. In fact the percentage of all bacterial groups detected was quite low, pointing definitely to a poor quality OC. Moreover anaerobic bacteria, such as Sulfate-Reducing Bacteria (SRB) and Sulfur-Reducing heterotrophic Epsilon (EPS) were detected. These results are in line with the relatively high percentage of OC (9.8%) found as this will have promoted the consumption of oxygen and thus the presence of anaerobic bacterial groups (Barton and Fauque, 2009; Engel et al., 2003). The chemical analysis did not find any particular contamination either by antibiotics or by other organic contaminants. These results suggest that in 19 years (the quarry had not been used for waste disposal since 1990) the soil had been partially decontaminated of toxic compounds and the pharmaceutical waste had been transported towards the deeper soil layers owing to the intrinsic vulnerability of the vadose zone because there were some fractures, water flow was likely to have easily reached the deepest layers of the subsoil. To confirm this hypothesis, we found consistently higher OC concentrations in all the deeper S1 and S2 soil layers than in the surface one (Table 2).

Such a high OC concentration is much unexpected in depth layers where the OC concentration is generally very low and less than 1% (Di Corcia et al., 1999; Barra Caracciolo et al., 2005c).

Although in S1 and S2 and at all the depths analysed, organic carbon content was very high (ranging from 9.4% to 15.7%), cell viability values were quite low (14% to 42%) (Figure 4, Table 2). These results indicate not only that the organic carbon was of poor quality and did not have any positive effect on the overall bacterial populations, but also that the presence of antibiotic residues (< 0.01 mg kg^{-1} of erythromycin and josamycin) and other organic contaminants (such as dimethylsulfur, toluene and derivatives, and phenol derivatives) found in all the soil samples analysed, presumably had a negative effect on the bacterial community.

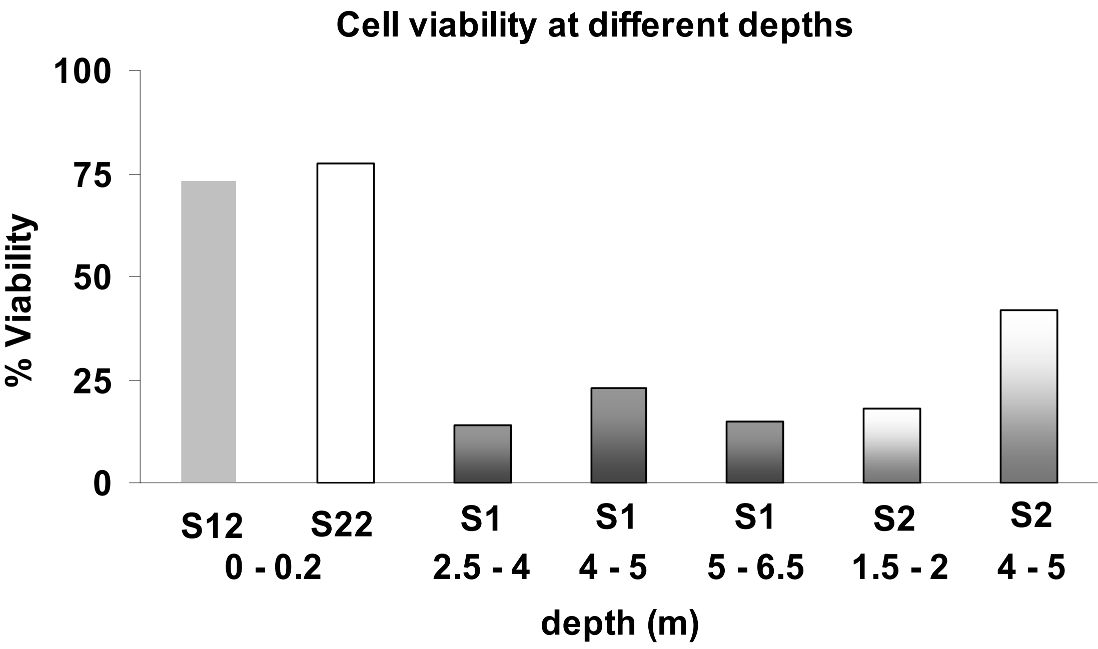

Figure 4. Bacterial cell viability of samples at different depths. S12 and S22 surface layer; S1 and S2 deeper layers.

Groundwater samples

The number of live bacteria (No. live bacteria mL^{-1}) detected at 3 different sampling points and at 3 different depths (S2: 23 m, S2bis: 46 m and S1: 51 m) shows that it was not inversely related either to the depth or to the high dissolved organic carbon content (Figure 5A and B). A low organic carbon content is a factor limiting the growth of bacterial communities in groundwater (Barra Caracciolo et al., 2001; de Lipthay et al., 2003) and the DOC values normally found range from 0.40 to 2 mg L^{-1} in the case of a very shallow aquifer (Pabich et al., 2001) on the contrary, in our samples, we observed that the lowest cell viability (20.8%) at S2 23 m was associated with the highest DOC value (8.37 mg L^{-1}).

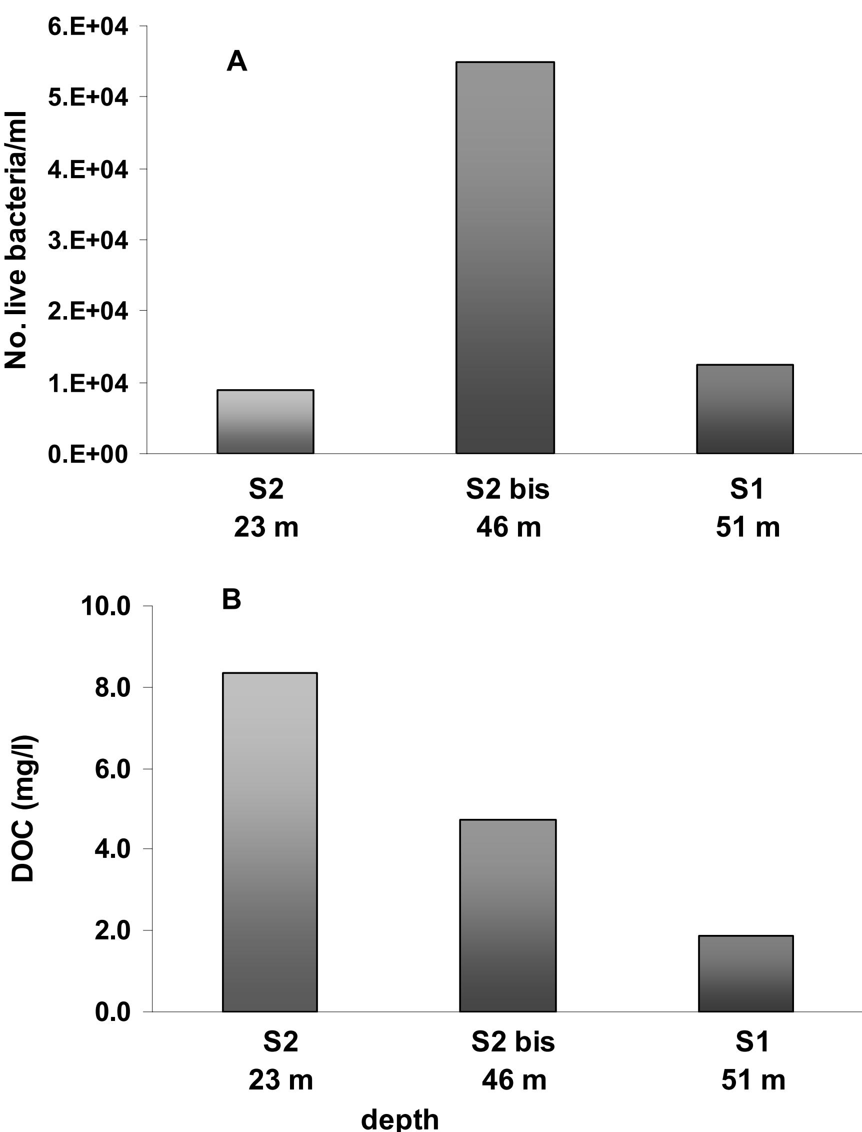

Figure 5. Groundwater analysis. **A**. Number of live bacteria (No. live bacteria/mL) detected under the epifluorescence microscope and **B**. Dissolved Organic Carbon (DOC).

This result would suggest both an allocthonous origin for the organic carbon found and its negative effect on the bacterial community. This supposition is supported by the volatile organic compound contamination (chloroform, ethylbenzene, *o*-, *p*-xilene, *m*-xilene and toluene) and in particular the chloroform (0.9-51.7 µg L^{-1}) found in all the groundwater analysed and the specific contamination by the antibiotic josamycin (0.15 µg L^{-1}) found at S2 23 m (Table 3); in fact it is well known that soil microorganisms are killed both by chloroform (Hu and van Bruggem 1998; Eberhardt et al., 1996) and antibiotics such as josamycin (Kim and Cermiglia 2005; Alighardashi et al., 2009).

Finally, when the FISH method was applied to the latter groundwater samples, only a few bacterial populations (β-*Proteobacteria* and Sulfur-Reducing heterotrophic Epsilon) were

successfully identified, which can be ascribed to both low bacterial community diversity and low cell viability and activity, which may have limited probe hybridization. In fact, the detection and quantification of phylogenetic groups by FISH in environmental samples depend on cellular rRNA content, which is itself linked to cellular metabolic activity (Detmers et al., 2004).

Table 3. Concentrations of volatile organics in groundwater samples S1, S2, S3 and corresponding legal limits (D.lgs 152/2006; D.M. 471/1999) in the last column

	Compound concentration (μg L^{-1})			
sample	S1	S2	S2bis	Legal limit (polluted sites)
Chloroform	51.7	0.9	1.8	0.15
Ethylbenzene	1.0	2.0	2.0	50
o-, *p*-xilene	1.5	3.0	3.0	10 (*p*-xilene)
m-xilene	0.5	1.0	1.0	-
toluene	0.3	0.6	0.6	15

Conclusions

The results show that an inappropriate use of the quarry for the disposal of pharmaceutical waste had caused soil and groundwater contamination. Although the latter is at the present limited to some deeper samples analysed it has to be considered the fact that the quarry was abandoned 20 years ago and that the sampling points were relatively few.

The current occurrence of organic contaminants and antibiotic residues in the sampling points would therefore suggest a previous diffuse contamination on the surface.

The decision to use this disused quarry to store sludge waste was based on the fact that the deep aquifer was locally confined and thus not subject to contamination. However, it was wrong in that the high vulnerability of the shallow aquifer was not considered. This kind of inappropriate land use is a result of incorrect land management and demonstrates how important it is to know the hydrogeological characteristics of a specific area before deciding its use.

Although the chemical analysis of organic contaminants in groundwater samples showed that Italian legal limits (D.lgs 152/2006; D.M. 471/99) were exceeded only in the case of chloroform, we cannot consider this groundwater and the soil above it to be not polluted. The presence of the antibiotics and the high organic carbon concentration found in sub-soil and groundwater samples suggest the waste had been transported from the surface layer to groundwater. Although antibiotics are not considered in the laws currently in force, they are emerging environmental contaminants (Zuccato et al., 2006) and they have the potential to cause health risks through drinking water exposure (US EPA, 2009) and induce resistance

genes in the natural environment especially at residual concentrations (Martinez, 2008; Allen et al., 2010). There therefore needs to be particular concern if they are present in groundwater both because it may be used as drinking water (Hirsch et al., 1999) and because of its naturally slow remediation capacity. Finally, the bacterial community analysis indicated that the quality state of both the soil and groundwater analysed was poor in terms of bacterial viability and activity and microbial diversity, and in view of the presence of anaerobic bacterial populations, which are typical of contaminated environments such as those containing industrial waste and waste water. The overall results suggest the usefulness of bacterial structure and functioning studies as microbiological indicators for assessing soil and groundwater quality states.

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Effect of the antiviral drug Tamiflu on the bacterial community structure of a surface water ecosystem analysed using fluorescence *in situ* hybridization

Abstract

The antiviral drug Tamiflu is receiving particular attention because of its recommended use against the influenza A H5N1 and H1N1 viruses. Hundreds of millions of courses of the pro-drug Oseltamivir Phosphate (chemical name (3*R*,4*R*,5*S*)-4-acetylamino-5-amino-3-(1-ethylpropoxy)-1-cyclohexene-1-carboxylic acid ethyl ester) have been deployed worldwide. Given its resistance to degradation and its hydrophilicity the active metabolite, Oseltamivir Carboxylate (OC), is expected to enter the aquatic ecosystem from sewage treatment plants. The present work deals with the bacterial community characterization of surface water samples treated with OC (1.5 mg L^{-1}) in microcosm experiments, using the Fluorescence *In Situ* Hybridization (FISH) method. Since some bacterial groups increased in OC-treated vs. non-treated water samples, their active role in its degradation is reported

Introduction

The recent influenza A H1N1 virus pandemic has forced health agencies to adopt strategies to contain them. Although vaccination is the primary step in prevention, the World Health Organization (WHO, 2005) has recommended the treatment of people at risk with the antiviral drug Tamiflu (Oseltamivir Phosphate). After its administration, the pro-drug Oseltamivir Phosphate is hydrolysed to its active metabolite, Oseltamivir Carboxylate (OC). Pharmacological studies have demonstrated that over 80% of the oral dose of OC is excreted renally and fecally (Ward et al., 2005). Fick et al. (2007) highlighted that OC is not completely removed in sewage treatment plants, and considering its low sorption into sewage sludge (low Log P), its high water solubility (Straub, 2009) and its negligible degradation in river water in the absence of an active bacterial community (Accinelli et al. 2007; Saccà et al. 2009; Accinelli et al. 2010), it may be considered a potential contaminant of aquatic ecosystems. Widespread use of Tamiflu could lead to considerable pollution of surface water, leading to the development of OC-resistant strains of the viruses (Fick et al. 2007; Singer et al. 2007), and a mass administration of Tamiflu could pose a risk for drinking water safety and ecological health.

Previous studies indicated that the fate of OC in surface water is mainly governed by microbial and photochemical processes (Accinelli et al., 2007; Bartels and von Tümpling, 2008).

The main objective of this study was to assess the effect of OC on the bacterial community structure of a surface water ecosystem (an irrigation canal in Northern Italy) by applying the

main fluorescent oligonucleotide probes with which it is possible to identify most known freshwater species (Zwart et al., 2002).

Materials and Methods

Samples were collected from a 150-km-long irrigation canal which receives water from the Po river (Figure 1).

The main physic-chemical water characteristics were: pH 8, 13.1 mg L^{-1} dissolved oxygen (DO), and 4.7 mg L^{-1} dissolved organic carbon (DOC). The canal provides water to a 3,000 km^2 area for agricultural, urban and industrial uses. Sampling operations were conducted manually in April by immersing 2-L sterile glass bottles approximately 10 cm below the water surface. The water from the canal had never been exposed previously to OC.

Figure 1. Sampling site: surface water from an irrigation canal in northern Italy (Canale Emiliano Romagnolo)

Microcosms (2 replicates for each condition, Figure 2) were set up by transferring samples (80 mL) into 250-mL sterile Erlenmeyer flasks under aseptic conditions, and treating them with oseltamivir carboxylate (OC) dissolved in 50 mM NaH_2PO_4 to give a final concentration of 1.5 mg mL^{-1}. This concentration was chosen because it was comparable with the Predicted Environmental Concentration (PEC) of common use drugs (Singer et al., 2007).

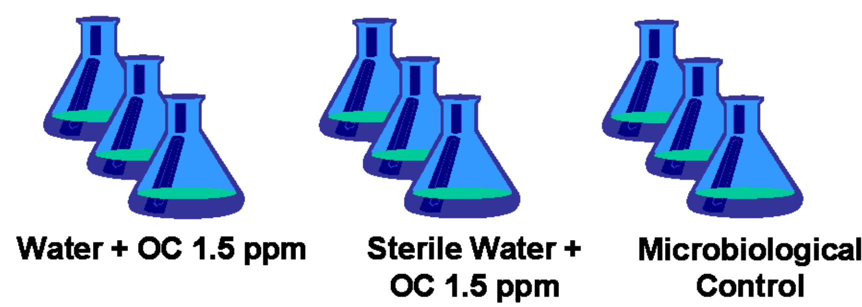

Figure 2. Microcosms set up.

Table 1. Chemical structure and main chemical-physical characteristics of Tamiflu (OP) and of its active form OC.

Pharmaceutical:	**Pro-drug Tamiflu (OP)**	**Active form OC**
	OP	OC
Molecular weight	312.41	284.35
Solubility (water)	> 200 mg L^{-1}	> 500 mg L^{-1}
Melting point	~193°C (OP-PO_3)	
Vapour pressure	~≤1.4 x 10^{-3}	~≤7.3 x 10^{-10} Pa
Solubility (water)	>200	>500 mg L^{-1}

Moreover, control microcosms were set up with the same amount of no-treated water. Flasks were sealed and incubated at 20°C on an orbital shaker (125 rpm) in the dark, as described in detail in a previous work (Accinelli et al., 2007).

The main chemical-physical characteristics of Tamiflu (OP) and its active metabolite OC are shown in Table 1.

In order to investigate if microbial populations can be affected by the antiviral drug and if they can be involved in its degradation, Fluorescence *In Situ* Hybridization (FISH) was performed on OC-treated (1.5 mg L^{-1}) and untreated sub-samples collected from degradation microcosms.

The phylogenetic composition of the OC-treated and control samples was analysed at different sampling times (0, 14, 21 and 36 days). For each condition four sub-samples (1 mL each) were fixed (1:1) with a solution composed of phosphate-buffered saline: 130 mM NaCl; 7 mM Na_2HPO_4, 3 mM NaH_2PO_4; 2% formaldehyde; 0.5% Tween 20 and 100 mM Sodium Pyrophosphate.

In order to separate the bacterial aggregates found in this water, a gentle sonication (10 sec, 15 W using a Microson XL2000 ultrasonic liquid processor) was performed on each sub-sample, which was then filtered on a 0.2 µm polycarbonate membrane. The filters were stored at -20°C until further processing.

Fluorescence *In Situ* Hybridization (FISH) of the harvested cells, counterstained with DAPI, was performed using fluorescent probes for the identification, under the epifluorescence microscope, of the major bacterial divisions found in freshwater (Zwart et al., 2001), such as *α-Proteobacteria*, *β-Proteobacteria*, *γ-Proteobacteria*, *Planctomycetes*, the *Cytophaga-Flaviobacterium* cluster of the *Cytophaga-Flavobacter-Bacteroides* phylum, and Gram-positive low G+C-content bacteria. For this purpose the following Cy3-labelled oligonucleotide probes were applied: EUB338, EUB338 II, EUB338 III (for *Bacteria*) and inside this domain ALF1b, BET42a, GAM42a, PLA46 together with PLA886, CF319a and LGC354a. Further details of these probes are available at http://www.microbial-ecology.net/probebase (Loy et al., 2007).

Averages of the cells binding the bacterial probes were calculated as a percentage 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 mounted with a drop of Vectashield Mounting Medium and the preparation examined and counted with a Leica DM 4000B epifluorescence microscope at 1,000× magnification.

The protocols for both FISH and bacterial abundance by DAPI counts are described in detail in our previous works (Barra Caracciolo et al., 2009; Grenni et al., 2009). The FISH experimental data are reported as the number of each bacterial group obtained from the mean of four sub-samples. We calculated this number (expressed as No. cells mL^{-1}) by multiplying the total cell abundance (previously determined by DAPI direct counts) and the percentage of cells detected by each specific probe.

Moreover, in order to know the number of actually viable bacteria, we assessed cell viability (% of live cells/live+dead), at each sampling time and for each experimental condition assessed by FISH (in two replicates), using a two-dye fluorescence bacterial viability kit (Kit Live/Dead® Bacterial Viability Kit, BacLight™), which distinguishes between viable (green) and dead (red) cells under a fluorescence microscope (Alonso et al., 2002). Then we calculated live cell abundance (No. live bacteria mL^{-1}) from the total cell abundance, obtained by DAPI counts, multiplied by % of live cells/live+dead.

Statistical analysis of the overall bacterial group data was done using Kruskal-Wallis One Way Analysis of Variance on Ranks, with significant differences at the $p < 0.01$ level. The comparison of data for each *Bacteria* group at 21 days was done using the Mann-Whitney Rank Sum Test. The PC Program used was SIGMASTAT Statistical software.

Results and Discussion

The addition of OC led to an initial decrease in the number of *Bacteria* cells detected by the EUB probes. However, at day 21 a significant peak ($p < 0.01$) in the *Bacteria* cell number was

observed in the OC-treated samples (Figure 3A). This trend was observed not only with the general probes for *Bacteria*, but also inside this domain ($p < 0.01$) for *α-Proteobacteria*, *β-Proteobacteria* and *γ-Proteobacteria* (Figure 3B; C, D).
In particular, the *β-Proteobacteria* group was the most abundant and constituted 40% of the *Bacteria* domain, suggesting an active role in the OC degradation. These results are in line with the transient decrease in live cell abundance (No. live bacteria mL^{-1}) at day 14, followed by a significant increase at day 21 in OC-treated samples (Figure 4).

The other bacterial groups investigated by FISH were not significantly affected by the presence of OC, and represented about 1-2% of the *Bacteria* domain; in particular *Cytophaga-Flaviobacterium* was the relatively most abundant group (the average number mL^{-1} during the experimental period was about $1.39 \times 10^4 \pm 4.02 \times 10^3$) followed by Gram-positive low G+C-content bacteria (about $2.8 \times 10^3 \pm 1.67 \times 10^3$) and finally *Planctomycetes* (about $1.22 \times 10^4 \pm 7.3 \times 10^3$).

The results of the analysis of the bacterial community show that the decrease at day 14 in bacterial abundance of the main phylogenetic groups in the presence of OC was transient; this initial negative effect was subsequently more than offset by a significant increase in their presence and presumably activity.

This hypothesis is confirmed by the fact that in the presence of the bacterial community about 65% of the OC applied was degraded in 36 days, as shown in a previous work using the same water (Accinelli et al., 2007, Singer et al., 2008), while in the same experimental time just 5% of it was degraded in the sterilized water.

Consequently the overall results not only confirm the key role of the bacterial community in OC degradation, but also suggest which bacterial groups, i.e. *α-Proteobacteria*, *γ-Proteobacteria* and above all *β-Proteobacteria* could be directly involved. The presence of OC in the environment is a relatively recent issue, and to our knowledge no OC-degrading bacteria belonging to the *β-Proteobacteria* have been identified until now.

These results encourage the performance of further studies to better investigate the bacterial metabolism (metabolism and/or co-metabolism) of this compound and the formation of its transformation products prior to its possible mineralisation.
The presence/absence of bacterial populations with a natural attenuation capacity versus pharmaceuticals is a crucial factor in assessing their actual environmental fate in aquatic ecosystems.

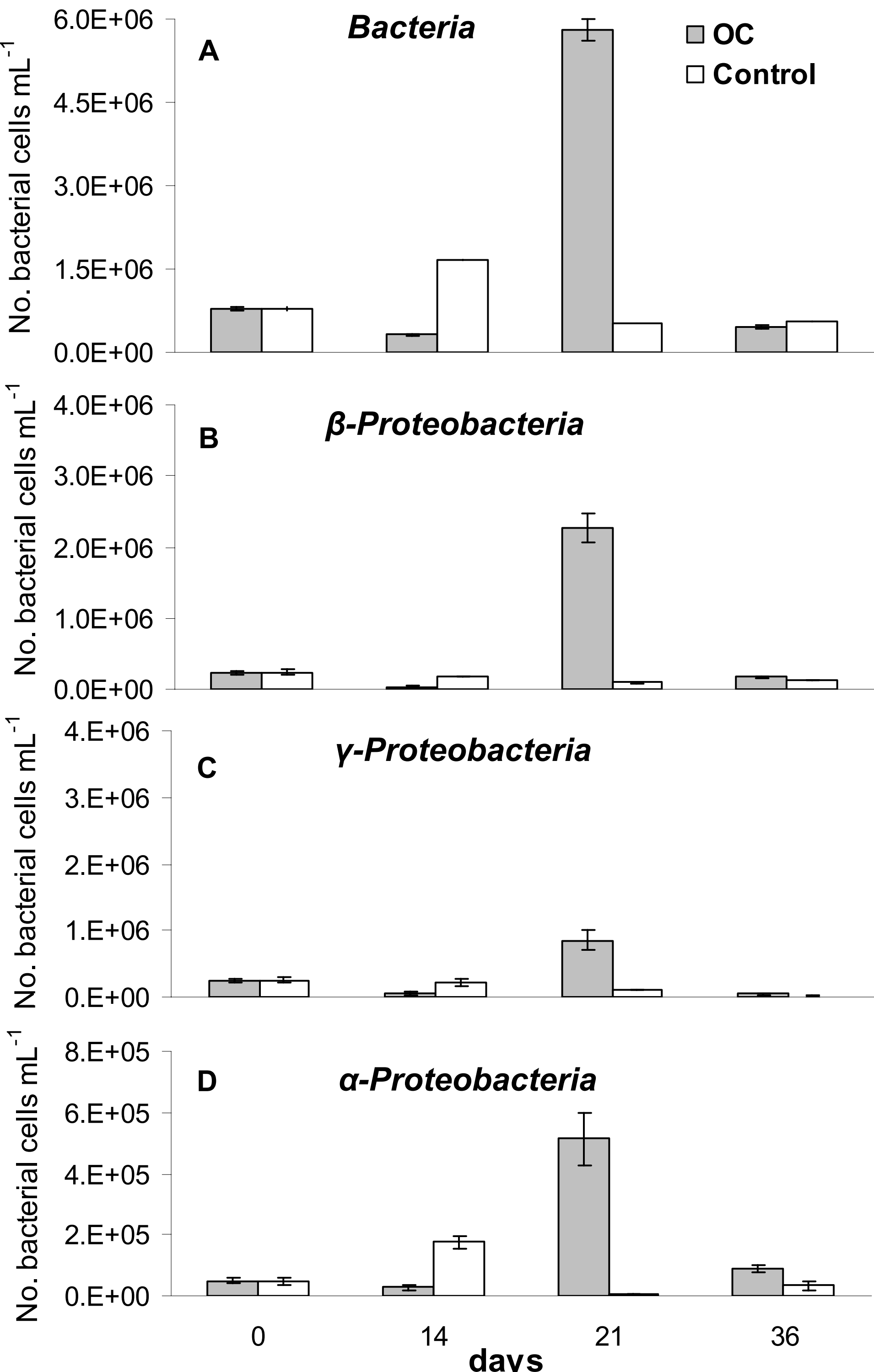

Figure 3**.** Bacterial community structure detected by FISH in surface water at different times: 0, 14, 21 and 36 days, in presence of Oseltamivir Carboxylate (OC) and Control. Vertical bar represents standard errors. **A**: *Bacteria*; **B**: *β-Proteobacteria*; **C**: *γ-Proteobacteria* **D**: *α-Proteobacteria*

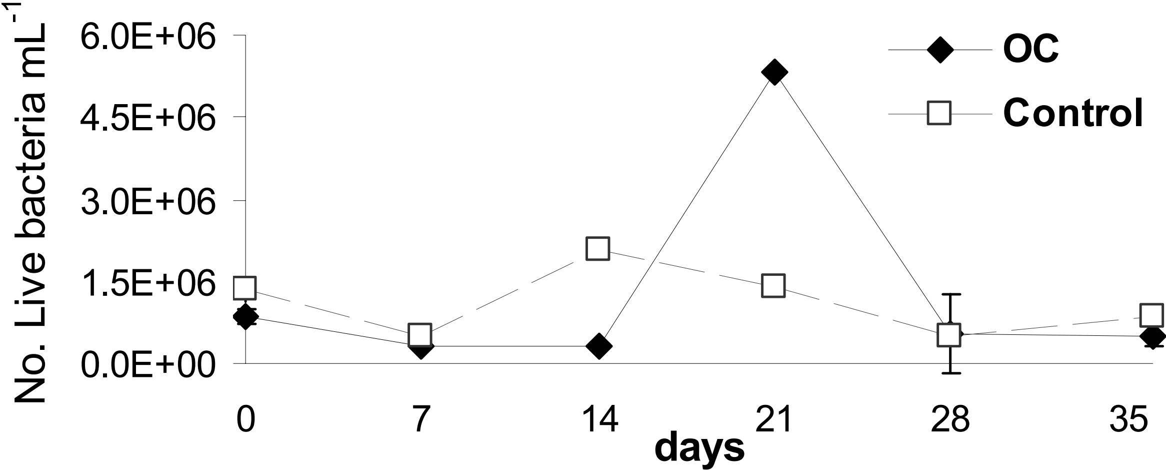

Figure 4. Live cell abundances (No. live bacteria mL^{-1}) at different sampling times in OC-treated and Control in surface water samples. Vertical bar represents standard errors.

This research activity was performed during collaboration between Water Research Institute-CNR Rome department (A. Barra Caracciolo, P. Grenni F. Falconi) and University of Bologna, (M.L. Saccà, C. Accinelli).

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Fluorescence *in situ* hybridization applied in soil and water ecosystems contaminated by xenobiotics: a useful method for studying their effect of on bacterial community structure

Abstract

The ability of soil and groundwater ecosystems to recover from chemical contamination is primarily dependent on the presence of a microbial community which has the ability to remove it. Nevertheless, there has been a little research into these communities because it is strictly dependent on methods capable of identifying and characterizing their community structure and functioning. The use of molecular methods makes it possible to overcome this kind of identification limitation. In this work, we applied the fluorescence *in situ* hybridization (FISH) method to different samples, such as soil and groundwater contaminated with *s*-triazine herbicides (simazine or terbuthylazine) and surface water treated with the pharmaceutical oseltamivir carboxylate (Tamiflu). We compared the bacterial community structure in the presence/absence of these xenobiotics. The use of 16S rRNA-targeted oligonucleotide probes, designed specifically for the main phylogenetic levels (*Archaea, Bacteria, α-, β-, γ-, ε*-subdivision of *Proteobacteria, Planctomycetes*, Gram-positive bacteria with a high or low DNA G+C content, *Cytophaga-Flavobacter-Bacteroides* phylum, and sulfate-reducing bacteria), and a DAPI stain made it possible to assess the structure of the bacterial community and its changes in the presence of these xenobiotics in all the ecosystems studied.

Introduction

The environmental fate of xenobiotics (pesticides, biocides, and pharmaceuticals) depends on their degradation at the input point (mainly soil) and on their potential transport to other compartments. The degradation depends on chemical and above all biological processes. However, only microbial metabolism can completely remove it from the environment. The more a xenobiotic is degraded in soil, the less the likelihood of it being leached to groundwater or run off to surface water. Several bacteria able to use xenobiotics as a carbon and/or nitrogen source have been isolated, although it has been estimated that less than 1% of the bacterial species in soils are currently known (Torsvik and Øvreås 2007). This means that much of the potential of bacterial metabolism to degrade dangerous chemicals has still to be investigated and exploited for remediation purposes. Moreover, morphological and physiological properties are not enough for classifying bacteria from a phylogenetic point of view and only a minority of soil microorganisms can be characterized by conventional methods such as cultivation (Curtis and Sloan 2005).

Consequently, their definitive characterization requires molecular methods, which allow their phylogenetic identification, in order to avoid losing information about the microbial component which, although viable and active, is not capable of duplicating in a culture

medium. The phylogenetic identification is based on the sequencing of the gene codifying for bacterial 16S rRNA (Schleifer and Stackebrandt 1983; Ludwig and Schleifer 1994). In this context, fluorescence *in situ* hybridization (FISH) is a technique that in recent years has been found to be very promising, thanks to its speed and sensitivity for studying microorganisms in their natural environment. The technique is considered a powerful tool for phylogenetic, ecological, diagnostic and environmental research in microbiology (Bottari et al. 2006). In fact the FISH method consists of the *in situ* identification (without cultivation) of bacteria using fluorescent labelled 16S rRNA-targeted oligonucleotide probes. A molecular probe consists of 15–30 nucleotides, labelled with a fluorochrome, able to hybridize with a specific complementary sequence of 16S rRNA (Amann, Krumholz, and Stahl 1990). Once a molecular probe is introduced into a cell, it can hybridize exclusively with a complementary rRNA sequence and the hybridization can be visualized under the microscope as a fluorescent signal. FISH has been successfully used in environmental studies for identifying bacteria at different phylogenetic levels (from domain to species) in activated sludge and wastewater by using rRNA-targeted oligonucleotide probes (Amann, Ludwig, and Schleifer 1995).

However, the detection of FISH-stained cells is generally hampered by strong autofluorescence from the background of soil or sediment particles and, compared to other environments, FISH has not been frequently applied in soil and groundwater (Eickhorst and Tippkotter 2008).

In microbial ecology, the knowledge of the composition and distribution of microorganisms in natural habitats can be interesting for ecological reasons.

Applications of FISH to soil, surface water, and groundwater samples treated or untreated with xenobiotics of concern for human and ecosystem health (i.e., the herbicides simazine and terbuthylazine and the antiviral drug oseltamivir carboxylate) are presented in this work. Here, we show the versatility and sensitivity of this technique, using the optimized protocols described for each different matrix (soil, surface water, and groundwater).

Materials and methods

Soil sampling and characterization

Bulk soil samples were collected from the surface layer A (0–20 cm depth) of two different agricultural fields from the Po plain (Lodi, Northern Italy) and Henares plain (Alcalà de Henares, Spain). The texture class of both soils was loam, and the organic carbon, the nitrogen content, and the pH were 0.8% and 0.5%, 0.1%, and 0.06%, 5.9% and 8.2% in the Lodi and Alcalà soils, respectively.

Two different experiments were carried out to assess the persistence of the herbicide simazine (6-chloro-N,N’-diethyl-1,3,5-triazine-2,4-diamine), in the two soils treated with the herbicide at the concentration of 1.5 mg kg^{-1}, as described in detail in Barra Caracciolo et al. (2005a). Simazine is one of the most commonly used pesticides and belongs to the *s*-triazine family of chemical compounds (six-member ring containing three carbon and nitrogen atoms), which are used to control broad-leaf weeds and annual grasses in crop fields such as fruit orchards.

The main results of the Lodi soil have been previously reported (Barra Caracciolo et al. 2005a), while new results regarding Alcalà soil are described here.

Soil sample fixation and pre-treatment for FISH analysis

The phylogenetic composition of the Lodi and Alcalà soil samples was analysed at the sampling time (0 day) and at selected times (7, 14, 30, and 70 days) during the degradation experiments.

Each analysis consisted of two replicates of each sub-sample (1 g each) fixed in a test tube containing 9 mL of a fixing solution (composed of phosphate-buffered saline: 130 mM NaCl; 7 mM Na_2HPO_4, 3 mM NaH_2PO_4; 2% formaldehyde; 0.5% Tween 20, and 100 mM sodium pyrophosphate). After mixing of the tube content (400 rpm in an orbital shaker), an aliquot of slurry was transferred to a centrifuge tube and a density gradient centrifugation was carried out in order to separate and extract the detachable bacteria from the soil particles by high-speed centrifugation with the non-ionic density gradient medium Nycodenz, as described in detail in Barra Caracciolo et al. (2005b). Volumes of 100 µL of the cell layer, located just above the Nycodenz cushion, were then filtered through a 0.2 µm polycarbonate membrane (45 mm diameter) using a gentle vacuum (50.2 bar) and air-dried. The filters were stored at -20°C until further processing. FISH of the harvested cells counterstained with DAPI was performed according to published protocols (Barra Caracciolo et al. 2005a, 2005b), using the probes for identifying the following bacterial groups*: α-, β-, γ-Proteobacteria, Planctomycetes*, Gram-positive bacteria with high DNA G+C content.

Surface water sampling and characterization

A degradation experiment was carried out to assess the persistence of the active antiviral drug oseltamivir carboxylate (Tamiflu) in surface water, and the active role of microbial populations in its degradation was shown (Accinelli, Barra Caracciolo, and Grenni 2007). Water samples were collected from an irrigation canal located in the River Po plain (Italy). The main physic-chemical properties were pH 8, dissolved oxygen (DO) 13.1 mg L^{-1}, and dissolved organic carbon (DOC) 4.7 mg L^{-1}. In order to better investigate the microbial populations involved in the Tamiflu degradation, FISH was performed on water sub-samples from the same degradation experiments and original results of this analysis are reported here.

Surface water samples fixation for FISH analysis

The phylogenetic composition of the water samples was analysed at different sampling times (0, 14, 21, and 36 days) in two replicates (1 mL each) fixed (1:1) with the solution described above for soil samples. In order to separate the bacterial aggregates found in the water, we performed a gentle sonication (10 s, 15W using a Microson XL2000 ultrasonic liquid processor), and then we filtered the samples on polycarbonate filters as described above. The filters were stored at -20°C until further processing. FISH of the harvested cells was performed using the same probes mentioned above for soil samples and a further two probes

covering the *Cytophaga-Flaviobacterium* cluster of the *Cytophaga-Flavobacter-Bacteroides* phylum and Gram-positive bacteria with a low G+C content.

Groundwater sampling and characterization

Groundwater samples were collected from two phreatic aquifers, but with different hydrogeological settings (the first one volcanic and the second alluvial), and were selected in order to compare two different land uses corresponding, respectively, to non-use and intensive use of agrochemicals. In fact in the contaminated aquifer, *s*-triazines such as terbuthylazine (N^2-tert-butyl-6-chloro-N^4-ethyl-1,3,5-triazine-2,4-diamine) and its metabolite desethyl-terbuthylazine (at concentrations >0.1 mg L^{-1}) and fertilizers (nitrate concentrations >100 mg L^{-1}) are always found in the Umbria Regional Environmental Agency's monitoring surveys.

Groundwater samples were collected from two wells in, respectively, the non-contaminated (BIO1) and contaminated (L2) aquifer, using sterile bailer samplers, and placed into sterile polyethylene bottles to avoid any bacterial contamination. Field parameters were measured on site (pH: 7.4 and 7.1; temperature: 15°C and 14°C; O2: 8.07 mg L^{-1} and 9.01 mg L^{-1}, respectively, for BIO1 and L2), while the DOC, measured in the laboratory using a Shimadzu ASI-5000A Total Organic Carbon Analyser, was of 0.23 and 0.56 mg L^{-1}, respectively, for BIO1 and L2. The main results of the L2 aquifer have been previously reported (Barra Caracciolo et al. 2010), while new results regarding BIO1 one are described here.

Groundwater sample fixation for FISH analysis

The phylogenetic composition of the groundwater bacterioplancton was analysed in four replicates of ethanol-fixed sub-samples (5 mL each). Each groundwater sub-sample was directly filtered through a 0.2 µm polycarbonate membrane using a gentle vacuum (50.2 bar), followed by a 70%, 90%, and 95% (v/v) ethanol series for 10 min each at room temperature, and then air-dried. Since in the case of groundwater, owing to the low bacterial abundance, it is necessary to sample a greater amount of water than in that of surface water, it is very important to fix the samples directly in ethanol instead of adding formaldehyde. This is because it would be necessary to use a greater amount of the latter and this can hinder subsequent cell visualization. The filters were stored at -20°C until further processing.

FISH of the harvested cells, counterstained with DAPI, was performed according to published protocols (Pernthaler et al. 2001; Grenni et al. 2009; Barra Caracciolo et al. 2010) using the same probes listed for soil and surface water samples, which cover most known bacterial species (Zwart et al. 2002) and probes for two further groups, i.e., *ε-Proteobacteria* and sulfate-reducing bacteria, found in contaminated groundwater (Amann et al. 1990; Engel et al. 2003).

FISH of soil, surface water, and groundwater samples

Each filter (soil, surface water, and groundwater) was cut into about 16 sections and two filter sections were hybridized with the same probe. The hybridizations (except for the *Archaea*

domain) were performed using simultaneously the general fluorescein-labelled EUB probes and a specific cyanine-labelled oligonucleotide probe. The Cy3-labeled BET42a probe was always used together with its competitor, the unlabelled GAM42a; similarly the Cy3-labeled GAM42a probe was used together with its competitor, the unlabelled BET42a. All probes were synthesized by MWG AG Biotech, Germany. Probe sequences, target sites and the proportion of formamide used are given in Table 1.

Table 1. Probe names, sequences, target sites and % of formamide (stringency) used.

Name, Taxa and Reference	Sequence from 5' to 3'	Target rRNA and position	Stringency %
ARCH915 *Archaea* [1]	GTG CTC CCC CGC CAA TTC CT	16S (915-934)	20
EUB338 *Bacteria* [2]	GCT GCC TCC CGT AGG AGT	16S (338-355)	20
EUB338 II *Bacteria* [3]	GCA GCC ACC CGT AGG TGT	16S (338-355)	20
EUB338 III *Bacteria* [3]	CTG CCA CCC GTA GGT GT	16S (338-355)	20
ALF1b *α-Proteobacteria* [4]	CGT TCG YTC TGA GCC AG	16S (19-35)	20
BET42a *β-Proteobacteria* [4]	GCC TTC CCA CTT CGT TT	23S (1027-1043)	35
GAM42a *γ-Proteobacteria* [4]	GCC TTC CCA CAT CGT TT	23S (1027-1043)	35
EPS710 *Epsilonproteobacteria* [5]	AGT ATC ATC CCA GCA GA	16S (710-726)	30
PLA46 *Planctomycetes* [6]	GACT TGC ATG CCT AAT CC	16S (46-63)	30
PLA886 *Planctomycetes* [6]	GCC TTG CGA CCA TAC TCCC	16S (886-904)	35
CF319a *Cytophaga-Flaviobacterium* [7]	TGG TCC GTG TCT CAG TAC	16S (319-336)	35
HGC69A Gram+ bacteria with high DNA G+C content [8]	TAT AGT TAC CAC CGC CGT	23S (1901-1918)	25
LGC354a Gram+ bacteria with low G+C content [9]	TGG AAG ATT CCC TAC TGC	16S (354-371)	35
SRB385 Sulfate-Reducing Bacteria [2]	CGG CGT CGC TGC GTC AGG	16S (385-402)	35

Notes of the Table 1: [1] Stahl and Amann (1991); [2] Amann et al. (1990); [3] Daims et al. (1999); [4] Manz et al. (1992); [5] Engel et al. (2003); [6] Neef et al. (1998); [7] Manz et al. (1996); [8] Roller et al. (1994); [9] Meier et al. (1999)

Further details about these probes are on-line in probeBase (http://www.microbial-ecology.net/probebase) (Loy et al. 2007).

A hybridization buffer (5 M NaCl, 1 M Tris/HCl, 10% sodium dodecyl sulfate and a probe-specific amount of formamide) containing a 50 ng μL^{-1} Cy3-labeled probe and a DAPI solution (1:10) was put on each piece of filter, which had been previously placed on a slide.

The DAPI is a fluorescent dye able to stain all the cells in the sample independently of their physiological state and metabolic activity and for this reason is suitable for total counts. The slides were kept in a humidified hybridization chamber for 90 min at 46°C. After hybridization, the filters were washed in a buffer at 48°C for 15 min, rinsed with distilled water and air-dried. The slides were mounted with drops of Vectashield Mounting Medium (H-1000; Vector Lab., Burlingame, CA, USA) and then the bacteria were examined and counted with a Leica DM LB 30 epifluorescence microscope at 1000× magnification, counting a minimum of 300 cells per section.

The estimation of the cells binding the fluorescent probes was calculated as a proportion of the total DAPI-positive cells (reported as % DAPI positive cells).

Results and discussion

Soil samples

At the start of the simazine degradation experiments, the hybridization with the probes for the Bacteria domain detected about 60% of DAPI-stained cells (2.40×10^7 cells g^{-1} soil) in Lodi and 80% (3.20×10^7 cells g^{-1} soil) in Alcalá. The percentage of *Bacteria* cells increased in both simazine-treated soils (Lodi and Alcalá) from day 0, reaching peaks of 80–90% between 7 and 14 days (data not shown). Moreover, *in situ* hybridization with other probes within the *Bacteria* domain detected different percentages of positive cells to the specific probes between the treated (simazine) and untreated (control) soils. In particular, in Lodi soil *β-Proteobacteria* increased and maintained a high number in the treated soil during the experimental period; *α-Proteobacteria* increased between 0 and 30 days and then at the end of the experiment their presence was similar to that in the control soil (Figure 1).

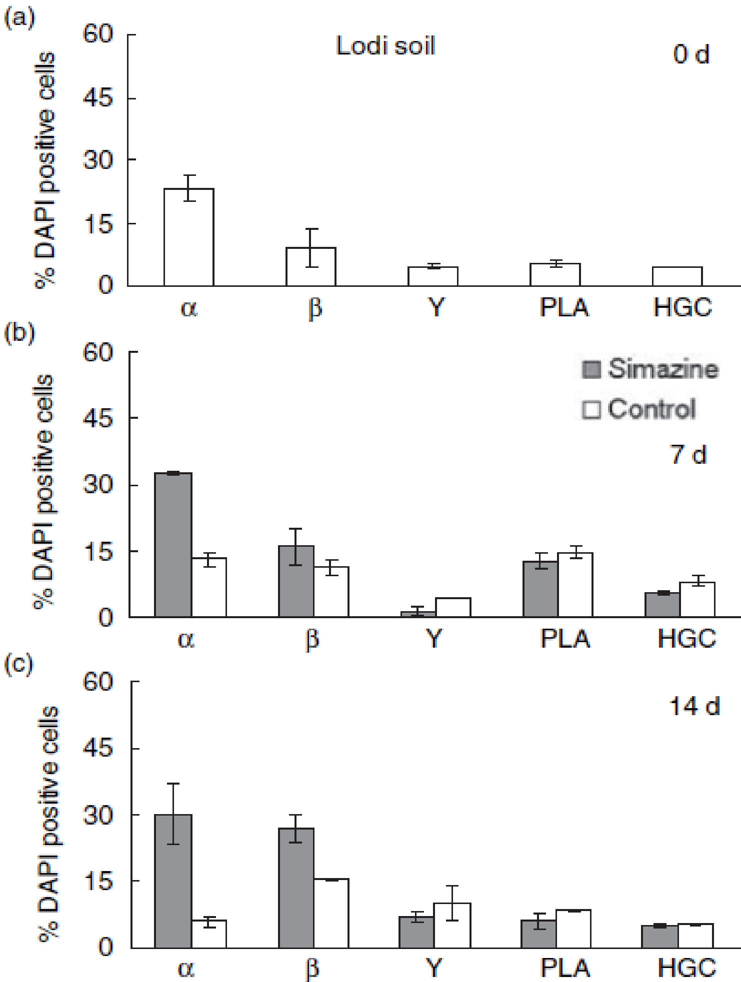

Figure 1 (a-c). Bacterial community structure detected by FISH of the simazine degradation experiment in Lodi soil at different times: 0 (a), 7 (b) and 14 days (c). Notes: Vertical bar: standard errors. α: α-*Proteobacteria;* β: β- *Proteobacteria*; γ: γ-*Proteobacteria*; PLA: *Planctomycetes*; HGC: Gram+ bacteria with high DNA G+C content.

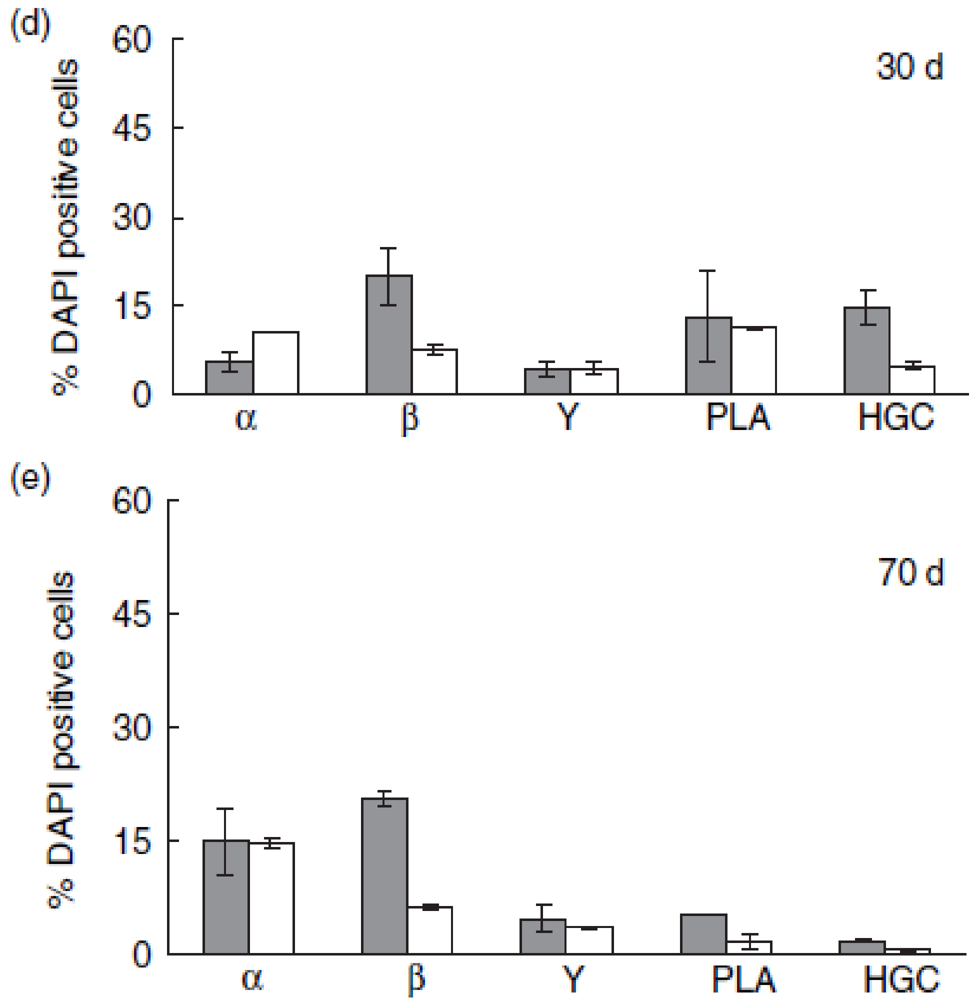

Figure 1 (d-e). Bacterial community structure detected by FISH of the simazine degradation experiment in Lodi soil at different times: 30 (d) and 70 days (e). Notes: Vertical bar: standard errors. α: α-*Proteobacteria;* β: β- *Proteobacteria*; γ: γ-*Proteobacteria*; PLA: *Planctomycetes*; HGC: Gram+ bacteria with high DNA G+C content.

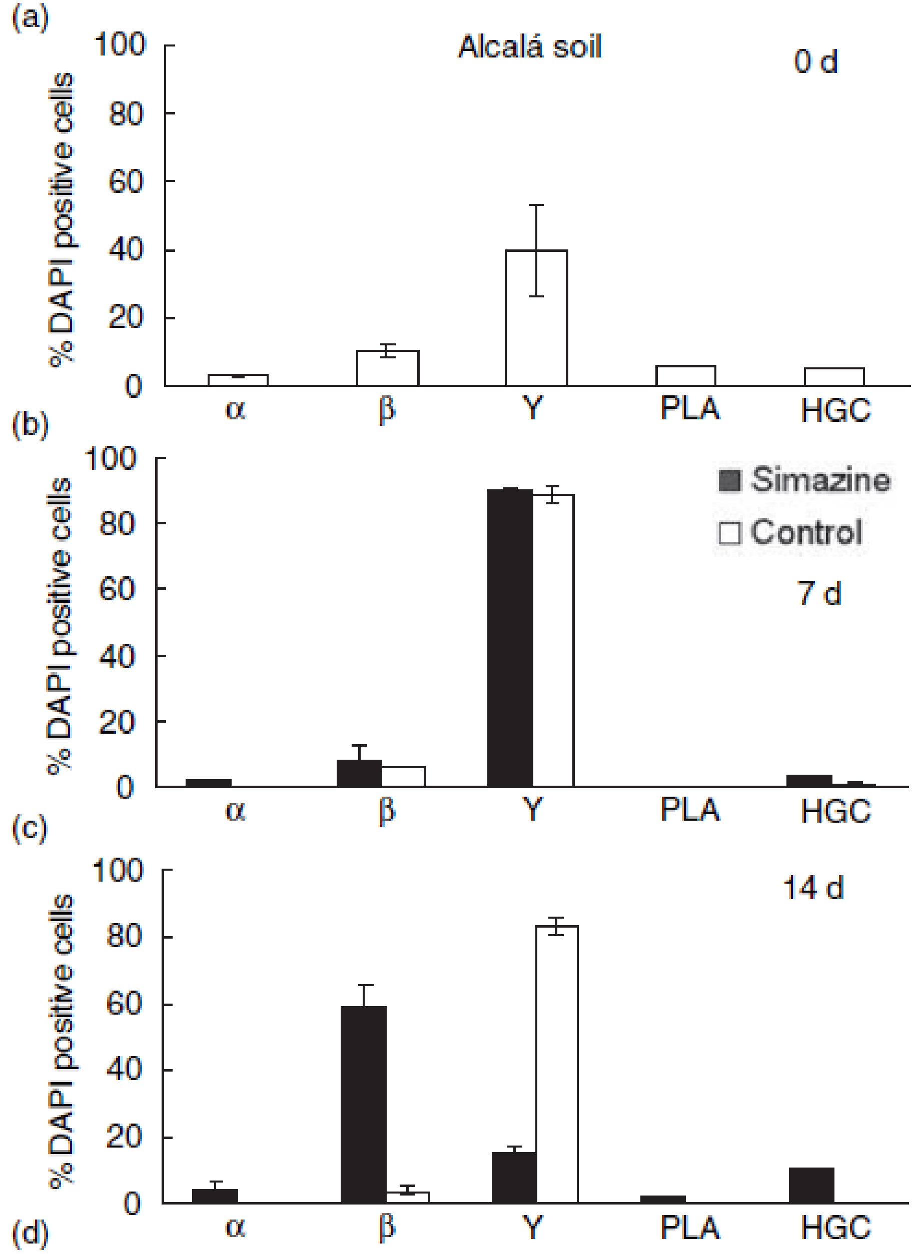

Figure 2 (a-c). Bacterial community structure detected by FISH of the simazine degradation experiment in Alcalá soil at different times: 0 (a), 7 (b) and 14 days (c). Notes: Vertical bar: standard errors. α: α-*Proteobacteria*; β: β-*Proteobacteria*; γ: γ-*Proteobacteria*; PLA: *Planctomycetes*; HGC: Gram+ bacteria with high DNA G+C content.

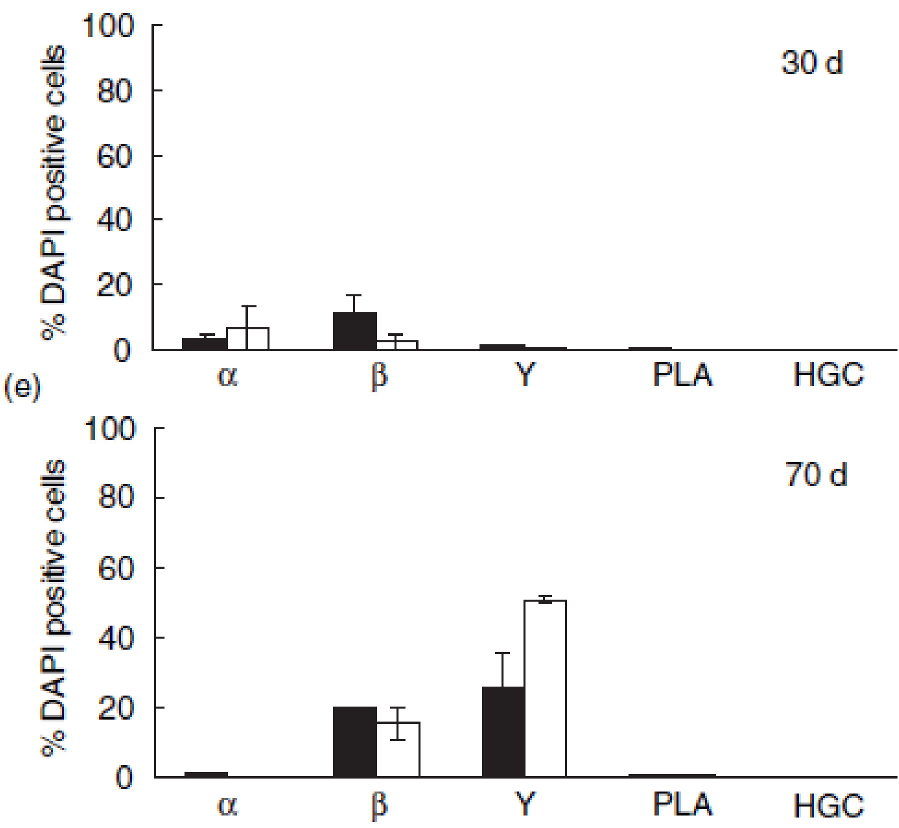

Figure 2 (d-e). Bacterial community structure detected by FISH of the simazine degradation experiment in Alcalá soil at different times: 30 (d) and 70 days (e). Notes: Vertical bar: standard errors. α: α-*Proteobacteria*; β: β-*Proteobacteria*; γ: γ-*Proteobacteria*; PLA: *Planctomycetes*; HGC: Gram+ bacteria with high DNA G+C content.

The other bacterial groups (*γ-Proteobacteria* and *Planctomycetes*) seem to be not significantly affected by the presence of the herbicide, except for the case of Gram-positive bacteria with high G+C content (HGC), which increased in number at day 30 (Figure 1d).

In the Alcalá soil (Figure 2), the initial predominant group was *γ-Proteobacteria* (40% of DAPI positive cells) and during the experimental period its percentage remained quite high in the control soil, while in the treated soil its percentage diminished drastically from day 14.

β-Proteobacteria was positively affected by the presence of the herbicide in this soil as well, particularly at day 14, and their percentage remained higher than that of the control until the end of the experiment (Figure 2c–e). *α-Proteobacteria* and *Planctomycetes* were not significantly affected by the presence of the herbicide, while HGC increased in number at day 14 (Figure 2c). The overall results suggest that the percentage increases in β-Proteobacteria during the experimental period and that in HGC (at day 30 in Lodi and at day 14 in Alcalà) could be related to the role of these two bacterial groups in simazine degradation. These results are in accordance with another work in which *β-Proteobacteria* were found to increase in soil treated with the herbicide simazine (Morán et al. 2006).

Moreover, a bacterial strain belonging to HGC, *Rhodococcus wratislaviensis* FPA1, and, for example, two *β-Proteobacteria* species (*Advenella incenata* and *Janthinium lividum*) capable of using *s*-triazines (including simazine) were recently isolated (Grenni et al. 2009; Barra Caracciolo et al. 2010).

Surface water

The percentage of *Bacteria* cells detected by EUB probes was similar until day 14. In the microcosms treated with the antiviral drug oseltamivir carboxylate (Tamiflu) a higher percentage (69% of DAPI positive cells) than in control ones (28% of DAPI positive cells) was observed at day 21 (data not shown). Moreover, at this sampling time, a percentage increase in the *α-Proteobacteria* and *β-Proteobacteria* groups was also observed (Figure 3). These results are in accordance with our previous results, which found the bacterial number (5.50×10^6 cells mL^{-1} water) to be similar until day 21 in both treated and untreated water, followed by a significant increase at day 21 in the Tamiflu condition. Moreover, the increase in bacterial number was found to be related to the degradation of 50% of the initial Tamiflu concentration (Accinelli, Barra Caracciolo, and Grenni 2007).

The FISH results reported here, therefore, not only confirm the role of the bacterial community in the Tamiflu degradation, but also suggest the specific involvement of the *α-* and *β-Proteobacteria* groups in this process.

Further studies are in progress to investigate the bacterial metabolism (metabolism and/or co-metabolism) of this compound and the formation of its transformation products until its possible mineralisation.

Groundwater samples

The use of 16S rRNA-targeted oligonucleotide probes made it possible to determine the structure of the autochthonous bacterial communities at the phylogenetic level in the two different aquifers. About 80% of the cells detected by DAPI in L2 belonged to the Bacteria domain and about 4% belonged to the *Archaea* domain; while in BIO1 the cell numbers positive to both the *Bacteria* and *Archaea* probes were much lower (46% and 2.9%, respectively). The FISH results within the Bacteria domain are shown in Figure 4. In L2, a greater bacterial abundance ($8.73 \times 10^3 \pm 1 \times 10^3$) than in BIO1 ($2.82 \times 10^4 \pm 2 \times 10^3$) was associated with a greater percentage of cells positive to more specific probes (such as α-, *β-* and *γ-Proteobacteria*, *Planctomycetes*, and HGC) inside this domain.

Moreover, the dominant bacterial group was *β-Proteobacteria* in the case of L2 and α-*Proteobacteria* in that of BIO1.

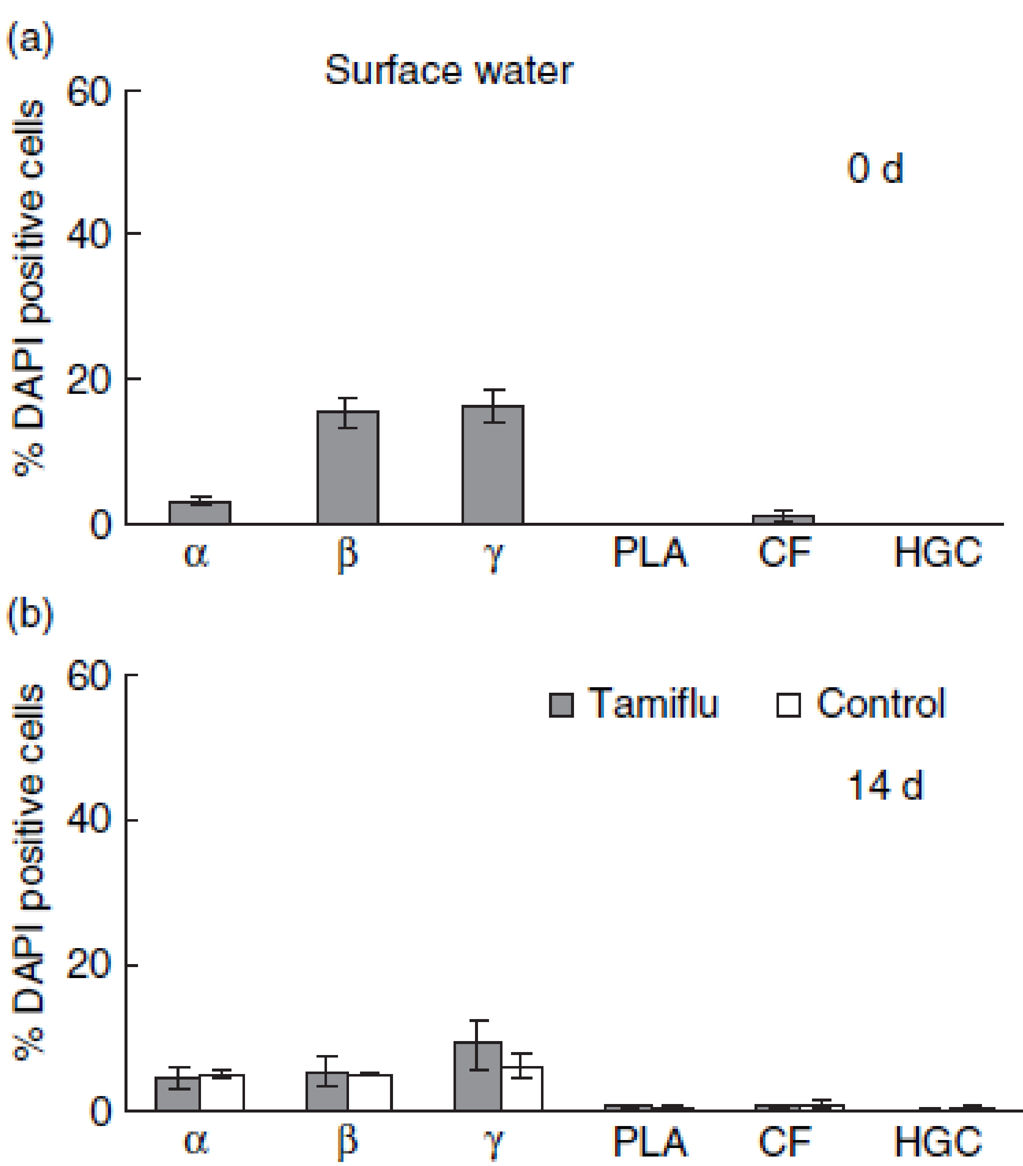

Figure 3 (a-b). Bacterial community structure detected by FISH in the degradation experiment of the pharmaceutical oseltamivir carboxylate (Tamiflu) in surface water at different times: 0 (a) and 14 days (b). Notes: Vertical bar: standard errors. α: *α-Proteobacteria;* β: *β-Proteobacteria*; γ: *γ-Proteobacteria*; PLA: *Planctomycetes*; CF: *Cytophaga-Flaviobacterium* cluster of the *Cytophaga-Flavobacter-Bacteroides* (CFB) phylum.

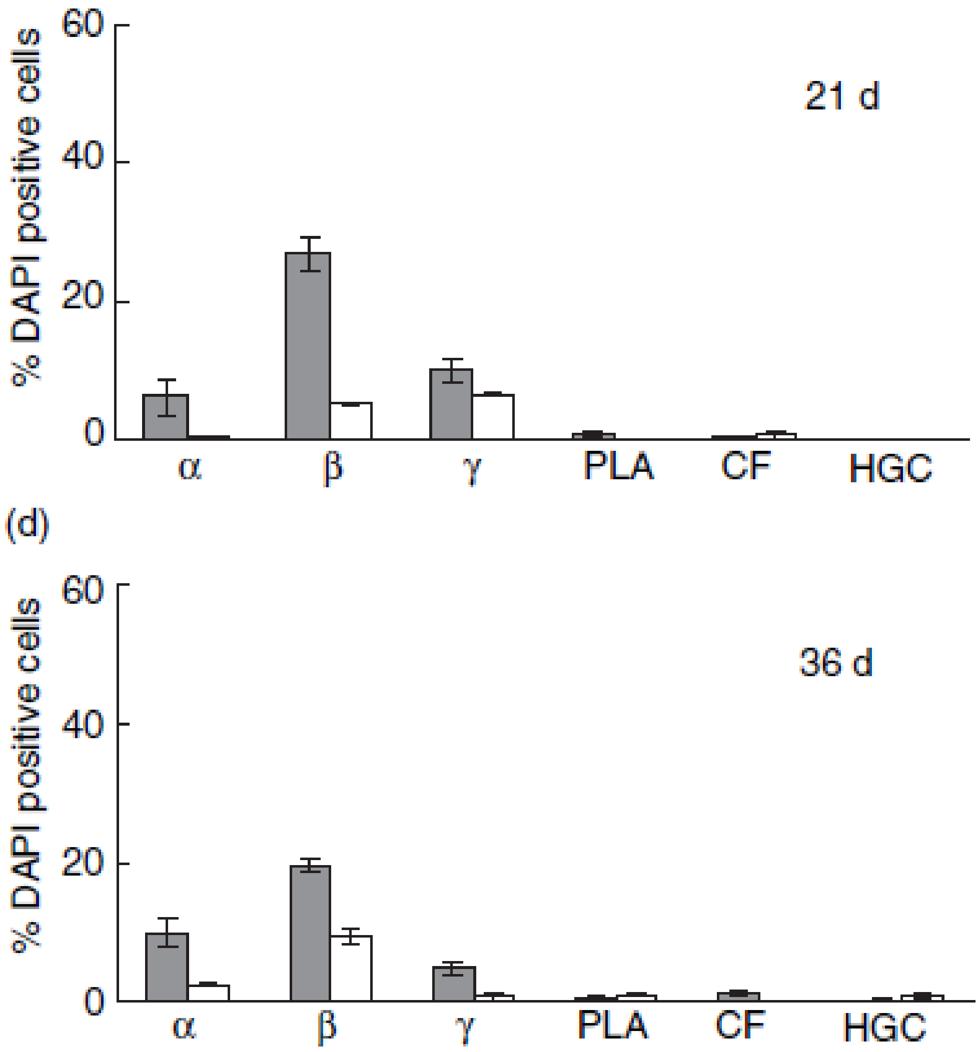

Figure 3 (c-d). Bacterial community structure detected by FISH in the degradation experiment of the pharmaceutical oseltamivir carboxylate (Tamiflu) in surface water at different times: 21 (c) and 36 days (d). Notes: Vertical bar: standard errors. α: *α-Proteobacteria;* β: *β-Proteobacteria*; γ: *γ-Proteobacteria*; PLA: *Planctomycetes*; CF: *Cytophaga-Flaviobacterium* cluster of the *Cytophaga-Flavobacter-Bacteroides* (CFB) phylum.

We initially selected two different aquifers, one contaminated (L2) and the other non-contaminated (BIO1) by *s*-triazines, in order to evaluate the applicability of the FISH technique to an ecosystem of which for a long time only the chemical and physical characteristics had been considered, while its active autochthonous microbial community, capable of responding to anthropic contamination, had been completely ignored. The fact that we used FISH successfully with both groundwater samples and that we detected all the bacterial groups belonging to the probes applied, but with differences in percentages and dominance between the contaminated and non-contaminated ones, shows the usefulness and sensitiveness of this method in the assessment of bacterial communities in groundwater ecosystems.

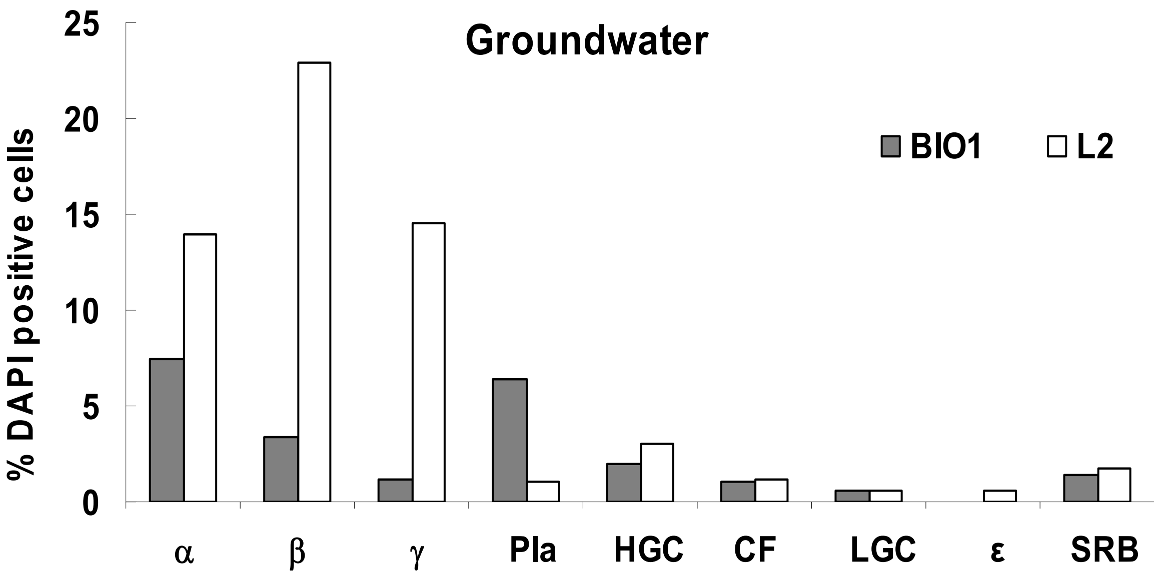

Figure 4. Bacterial community structure detected by FISH in natural groundwater ecosystems, one (BIO1) non-contaminated and the other (L2) contaminated by *s*-triazines and nitrates. Notes: Vertical bar represents standard errors. α – *α-Proteobacteria; β– β-Proteobacteria; γ – γ-Proteobacteria*; PLA – *Planctomycetes,* HGC – Gram-positive bacteria with high DNA G+C content; CF – *Cytophaga-Flaviobacterium* cluster of the *Cytophaga-Flavobacter-Bacteroides* (CFB) phylum; LGC – Gram-positive bacteria with low G+C content; ε – ε-*Proteobacteria*; SRB – sulfate-reducing bacteria

Since the ability of groundwater to recover from herbicide contamination is primarily dependent on the presence (or not) of a bacterial community able to degrade the chemicals, the method provides new opportunities for the study of this naturally oligotrophic ecosystem and for the assessment of its bacterial community structure under different anthropic impact conditions. With regard to FISH results, we found *β-Proteobacteria* to be the dominant group in L2 (chronically contaminated by *s*-triazines) and we can hypothesize that this group was favoured by the presence of triazines. To confirm this hypothesis, a recent study (Barra Caracciolo et al. 2010) showed the presence of *β-Proteobacteria* species able to degrade the herbicide terbuthylazine in this aquifer.

Conclusions

In all the experiments our application of FISH was successful thanks to the use of suitable pre-treatment of the soil (by using a cell extraction method), surface water (by using sonication), and groundwater (by fixing the cells immediately in ethanol) in order to obtain, after the oligonucleotide probe hybridization, a good visualization and quantification of the bacteria under microscope.

The overall results show that this method was able to assess the changes in the bacterial community structure in terms of dominance and relative percentage of some bacterial groups (e.g., *β-Proteobacteria*) in presence/absence of the applied contaminants, such as the *s*-

triazine herbicides and the pharmaceutical Tamiflu. Consequently, FISH was found to be a useful tool for following the dynamics of individual microbial populations in the soil ecosystem and highlighted the presence of particular groups presumably involved in chemical degradation.

This research activity was performed during collaboration between Water Research Institute- CNR Rome department (A. Barra Caracciolo, P. Grenni F. Falconi and M.L. Saccà) and Istituto Superiore di Sanità (P. Bottoni).

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CONCLUSIONS

Microbial communities are valuable indicators of the occurrence of disturbances due to exogenous physic-chemical stressors. The presence of an abundant and varied microbial community is a necessary prerequisite for an immediate and effective homeostatic response to the various xenobiotics that can negatively affect an ecosystem. The assessment of variations in microbial community structure and functioning are of basic importance for evaluating the impact of a chemical on an ecosystem. This thesis focuses on environmental contamination caused by some classes of pesticide (terbuthylazine, simazine and linuron) and pharmaceuticals (antiviral Tamiflu and two macrolide antibiotics) on several soil, surface and groundwater microbial communities. The homeostatic capabilities were evaluated in terms of degradation (DT_{50}) of the selected contaminants in presence/absence of the natural microbial community. The formation of transformation products and biodegradation pathways was also studied. The effects of the xenobiotics on microbial communities were evaluated in terms of structure (bacterial abundance and phylogenetic diversity) and functioning (cell viability and activity). Innovative molecular techniques, such as fluorescence *in situ* hybridization (FISH), were applied in order to identify which microbial populations were really affected by chemicals in their natural environments.

The results of the research on the *role of a groundwater bacterial community in terbuthylazine degradation* highlighted the ability of the autochthonous bacterial community to degrade the triazines in an ecosystem considered to have been lacking in life for a long period. This ability was presumably acquired through chronic exposure to herbicide contamination due to intensive agriculture and the high permeability of the aquifer. The fact that the bacterial community played a role in terbuthylazine degradation was confirmed by the changes in its functioning (viability and carbon production) and structure (abundance and phylogenetic structure determined by fluorescence *in situ* hybridization, FISH). The results of the phylogenetic characterization by FISH supported the hypothesis that the herbicide exerted a selective pressure on the bacterial community, promoting the dominance of some bacterial groups involved in the triazine degradation, such as the *β-Proteobacteria* and Gram-positive bacteria with high G+C content groups. The consequent isolation on enrichment cultures and characterization, by 16S rRNA gene PCR amplification, of *Janthinobacterium lividum* and *Advenella incenata*, belonging to the *Betaproteobacteria* group and *Rhodococcus wratislaviensis* strain FPA1 belonging to Gram-positive bacteria with high G+C content definitely confirmed the occurrence of microbial populations with homeostatic capabilities in natural groundwater. In fact, the selected strains contained on their plasmid the *atz*A and *atz*B genes involved in triazine degradation. The results show the potential use of bioremediation strategies in the treatment of aquifers polluted with triazine herbicides, such as using the bacterial strains isolated for bioaugmentation or bioremediation purposes. Finally, the use of

the new RhLu fluorescent oligonucleotide probe (for *in situ* detection of *s*-triazine-degrading *Rhodococcus wratislaviensis* in contaminated groundwater and soil samples) can be useful for bio-monitoring the natural attenuation capacity of terbuthylazine-contaminated groundwater. To the best of our knowledge, these are the first results showing the isolation, from groundwater, of a bacterial strain able to degrade and mineralise *s*-triazines.

The results of the research on the *effects of phenylurea and triazine herbicides in the co-presence of pine and oak amendments* on soil bacterial communities showed that the amendments did not negatively affect the overall bacterial community functioning in terms of dehydrogenase activity.

Pine and oak residues effectively increased the sorption of the herbicides to soil. However the sorption of linuron and terbuthylazine by pine was much higher than by oak and this was particularly evident in the terbuthylazine experiment. This higher sorption capacity for pine could be due to its higher lignin (hydrophobic wood component) content. Pine residues, owing to a high soil sorption of linuron and terbuthylazine and a decrease in the bioavailability of the herbicides, hampered microbial degradation. On the contrary, in the presence of oak residues, the herbicide sorption did not increase enough to hamper the microbial degradation. The overall results indicate that the use of these amendments can be effective in limiting the mobility of herbicide in soils, although the capacity of amended soils to adsorb it should be taken into account since this can modify the persistence of the herbicide.

The results of the study *effects of pharmaceutical waste disposal on bacterial communities in soil and groundwater* reported here are part of an eco-diagnosis study with the broader aims of evaluating the possible presence of contamination in the area of the study and providing a description of the geological and hydro-geological features of the quarry, in order to establish the true situation in view of the conflicting information produced by previous reports, obtained previously from the local authorities. Although the results of the chemical analysis of organic contaminants in the groundwater samples showed that Italian legal limits (DM 471/99, D.lgs 152/2006) were exceeded only for chloroform, we cannot consider this groundwater and the soil above it to be not polluted. The detection of the antibiotics and the high organic carbon concentration found in sub-soil and groundwater samples suggest the waste had been transported from the surface layer to groundwater. Although antibiotics are not considered in the laws currently in force, they are emerging environmental contaminants and have the potential to cause health risks through drinking water exposure and induce resistance genes in the natural environment especially at residual concentrations. The bacterial community analysis indicated that the quality state of both the soil and the groundwater analysed was poor in terms of bacterial viability and activity, and this was confirmed by the presence of anaerobic bacterial populations, which are typical of contaminated environments such as those containing industrial waste and waste water. The overall results suggest the usefulness of bacterial structure and functioning studies as microbiological indicators for assessing soil and groundwater quality states. Studying soil using an ecosystem approach,

taking into account the structure and functioning of the microbial community, is a necessary prerequisite for improving the understanding and management of its biodiversity and for developing a solid reserve of tools for evaluating and monitoring its state.

The results of the *assessment of the effects of the antiviral drug Tamiflu* (recommended for the treatment of cases of avian and swine influenza) on the bacterial community of a surface ecosystem confirm the key role of the bacterial community in its degradation. Moreover, the identification by FISH of some bacterial groups (such as *α-Proteobacteria*, *γ-Proteobacteria* and *β-Proteobacteria*), which increased in number when the pharmaceutical halved, show the usefulness of this method for detecting and quantifying the bacterial populations really active in the degradation of chemicals, including pharmaceuticals.

Finally, several applications in experiments of fluorescence *in situ* hybridization to soil, surface-water and groundwater experiments were discussed. They aimed to evaluate the *variations of bacterial community structure in the presence of herbicides or pharmaceuticals*.

In all the experiments our application of fluorescence *in situ* hybridization (FISH) was successful thanks to a suitable pre-treatment of the soil (by using a cell extraction method), surface water (by using sonication), and groundwater (by fixing the cells immediately in ethanol) in order to obtain, after the oligonucleotide probe hybridization, a good visualization and quantification of the bacteria under the microscope.

The overall results show that this method is able to assess changes in bacterial community structure in terms of dominance and relative percentages of some bacterial groups (e.g., *β-Proteobacteria*) in the presence/absence of the contaminants applied. Consequently, FISH is a useful tool for following the dynamics of individual microbial populations in soil and water ecosystems and highlighting the presence of particular groups presumably involved in chemical degradation.

In conclusion, microbial communities are valuable indicators of the occurrence of disturbances due to exogenous physic-chemical stressors. The assessment of variations in microbial community structure is of basic importance, since it contributes to evaluating the impact of an environmental stressor. Complex microbial communities may serve as ideal and ecologically significant toxicity indicators. The number of techniques for studying microbial communities, in particular culture-independent methods (such as molecular biological techniques), has changed our view of microbial diversity. Among these techniques epifluorescence microscopy techniques, such as direct count of bacterial abundance (DAPI count), viability (Live/Dead cell viability assay) and Fluorescence *In Situ* Hybridization (FISH), are very useful for studying microbial communities directly in their natural environments.

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