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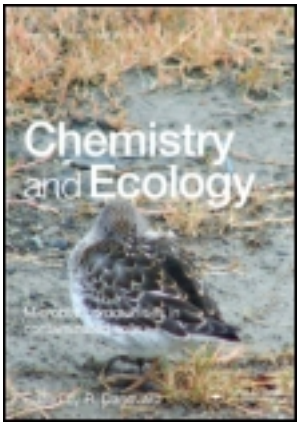

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Pharmaceutical waste disposal: assessment of its effects on bacterial communities in soil and groundwater

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Pharmaceutical waste disposal: assessment of its effects on bacterial communities in soil and groundwater

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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.

Keywords: pharmaceutical waste; erythromycin; CAS 114-07-8; soil and groundwater contamination; bacterial communities

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1. Introduction

Studying soil using an ecological approach is a necessary prerequisite for improving the understanding of its structure (biodiversity) and functioning [1,2]. Soil and the water located beneath its surface, groundwater, have to be considered as a single ecosystem which needs to be protected against infiltration by pollutants.

Microorganisms have a key role in ecosystem functioning [3]. 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, they are responsible for the complete mineralisation of organic matter and recycling of nutrients and, finally, they are capable of performing a homeostatic action with exogenous molecules [4–7]. Recovery from contamination is possible only if the quantity and toxicity of the molecules do not hamper or inhibit microbial activity. The presence of an abundant and varied microbial community is a necessary prerequisite for an immediate and

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effective response to the various natural and anthropic disturbances that can affect an ecosystem. [8,9] 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 [10]. However, only a small fraction of the bacteria in soils and groundwater is amenable to culturing in the laboratory, which limits our ability to study these organisms [8,9]. 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 function (e.g. bacterial activities) without the need for isolation and cultivation. In this 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: (1) to evaluate the possible presence of contamination in the area of the study; (2) to provide a description of the geological and hydrogeological 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; and (3) to encourage the involvement of the local community in both the problem analysis and project phases.

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2. Materials and methods

2.1. *Area studied*

The area studied is a disused, open calcarenite quarry near Brindisi (southern Italy), which was used for ~10 years (from 1980 to 1990) for waste disposal by a pharmaceutical company that produced antibiotics (particularly erythromycin, one of the most commonly used macrolide antibiotics in human medicine) using fermentative processes and subsequent chemical transformations. In particular, the waste included: (1) some exhausted mycelium, produced during the antibiotic production process, which was mixed with the soil; and (2) biologically stabilised sludge from an 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 following ferric chloride treatment, partial dehydration and stabilisation with hydrated lime.

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

Field infiltrometer tests were carried out on the calcarenite outcrop in the bottom of the quarry on three different occasions (July, September, November). The mean infiltration rate value obtained was ~0.03 $m \cdot h^{-1}$ in the saturated condition and one order of magnitude greater in the unsaturated condition [11]. These results, combined with the relatively small depth of the vadose zone, make this shallow aquifer particularly vulnerable to contamination.

2.2. *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) were carried out at 8 m depth. The material sampled consisted of soil mixed with pharmaceutical waste. Each core was split into several subsamples (Table 1).

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

	S1	S2
Geographic coordinates (GPS references) of sampling	40° 25′ 50.3″N, 17° 48′ 40.0″E	40° 25′ 49.5″N, 17° 48′ 38.8″E
Subsample depth (m)	(2.5–4.0) (4.0–5.0) (5.0–6.5)	(1.5–2.0) (4.0–4.5) –

Moreover, some samples (consisting of a mixture of soil with aged pharmaceutical waste) were collected manually in two different points (S12 and S22) 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 (S1, S2 and S2bis, being close to the coring points) at different depths. S1 (51 m depth) was located a few hundred metres north of the area with pharmaceutical waste material, whereas 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 using a CHN analyser and a TOC analyser, respectively, as described previously [12,13].

Each chemical and microbiological analysis of soil and groundwater samples was performed at least in triplicate using three subsamples.

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2.3. *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 turboionspray interface (positive ion). Samples (5 μL) were injected using 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 × 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 previous extraction with acetonitrile and filtration with PTFE-filters.

Determination of volatile organic compounds was performed by solid-phase microextraction/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 2 mL 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.

2.4. *Microbiological analysis*

Microbiological analyses were performed on both soil (1 g) and groundwater (10 mL) subsamples. Bacterial abundances were measured using the epifluorescence direct count method, reported in detail elsewhere [13–16], using 4′,6′-diamidino-2-phenylindole (DAPI) 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 previously [13,17]. Soil dehydrogenase activity was determined using the reduction of 2,3,5-triphenyltetrazolium chloride (TTC) solution to triphenylformazan (TPF), measured using the method reported in Grenni et al. [13]. Finally, bacterial community phylogenetic composition was analysed using fluorescence *in situ* hybridisation (FISH) [15,16]. 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), sulphate-reducing bacteria (SRB385) and sulphur-reducing heterotrophic epsilon (EPS710) [18].

The application of FISH to soil samples was possible after a cell extraction procedure described in detail in Barra Caracciolo et al. [16].

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3. Results and discussion

3.1. *Soil sampling at different depths*

The superficial layer (0–20 cm) was analysed at two different points (S12 and S22) in triplicate; at S22, where more OC was found than at S12 (Table 2), a higher bacterial activity, both in terms of dehydrogenase (Figure 1) and percantage of bacteria detected by FISH (Figure 2), was observed (t-tests significant, $p < 0.01$). Organic matter and OC are among the most important parameters

Table 2. Percentages (%) of total carbon (C_{Tot}), organic carbon (OC) and total nitrogen (N_{Tot}) analysed by a CHN elemental analyser; 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	$8.0 \cdot 10^7$	73.3	$5.8 \cdot 10^7$
S22 (0–0.2)	10.9	9.8	0.32	$5.9 \cdot 10^7$	77.3	$4.6 \cdot 10^7$
S1 (2.5–4.0)	16.0	13.8	1.01	$3.2 \cdot 10^8$	14.0	$4.3 \cdot 10^7$
S1 (4.0–5.0)	18.0	15.7	1.12	$1.9 \cdot 10^8$	23.0	$4.3 \cdot 10^7$
S1 (5.0–6.5)	14.8	13.7	0.87	$8.3 \cdot 10^8$	15.0	$1.2 \cdot 10^8$
S2 (1.5–2.0)	13.3	9.4	0.96	$1.5 \cdot 10^8$	18.0	$2.6 \cdot 10^7$
S2 (4.0–5.0)	13.9	12.6	0.75	$1.6 \cdot 10^8$	42.0	$6.6 \cdot 10^7$

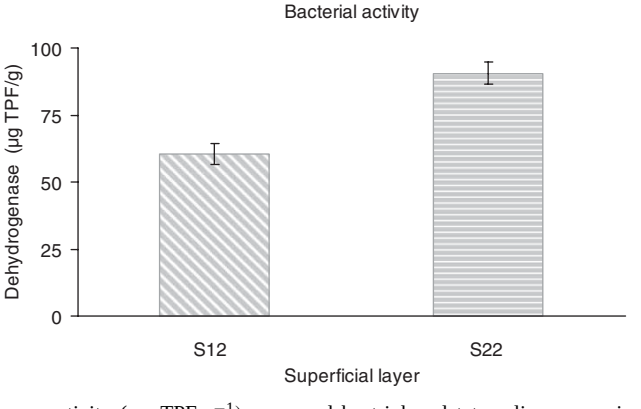

Figure 1. Dehydrogenase activity (µg TPF·g^{-1}) measured by triphenyl tetrazolium assay in superficial (0–20 cm) samples S12 and S22. Error bars indicate SE.

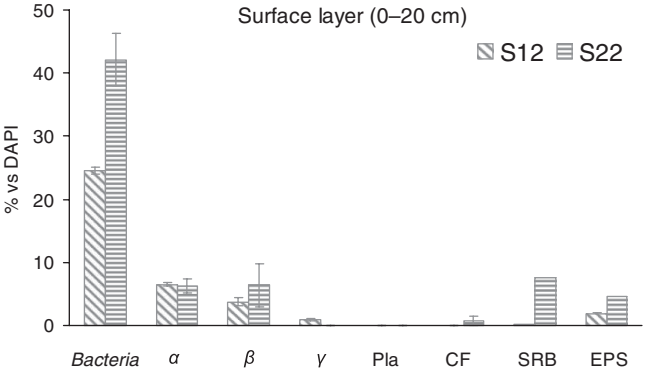

Figure 2. Bacterial community structure detected by fluorescence *in situ* hybridisation (FISH) in superficial samples (S12 and S22). The value of the cells binding each different probe is expressed as a percentage (%) of total DAPI-positive cells. Error bars indicate SE. α, *α-Proteobacteria*; β, *β-Proteobacteria*; γ, *γ-Proteobacteria*; Pla, *Planctomycetes*; CF, Cytophaga–Flaviobacterium cluster phylum CFB; SRB, sulphate-reducing bacteria; EPS, sulphur-reducing heterotrophic epsilon.

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in defining soil quality and microbial activity is strictly dependent on their amounts [19]. 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. Consequently, the presence in soil of a high amount of OC should promote cell viability and bacterial activity [13,19].

However, although the OC content in the S12 and S22 samples was much higher than that generally found in an other 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 [13,20,21].

The FISH analysis, which is able to identify exclusively metabolically active populations [22], 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 sulphate-reducing bacteria (SRB) and sulphur-reducing heterotrophic epsilon (EPS) were detected. These results are in line with the relatively high percentage of OC (9.8%) found because this will have promoted the consumption of oxygen and thus the presence of anaerobic bacterial groups [23,24]. 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 deeper soil layers owing to the intrinsic vulnerability of the vadose zone. Moreover, 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 very unexpected in depth layers where the OC concentration is generally very low and $<1\%$ [14,25].

Although in S1 and S2 and at all the depths analysed, OC content was very high (9.4–15.7%), cell viability values were quite low (14–42%) (Figure 3 and Table 2). These results indicate not only that the OC 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 dimethylsulphur, toluene and derivatives, and phenol derivatives) found in all the soil samples analysed, presumably had a negative effect on the bacterial community.

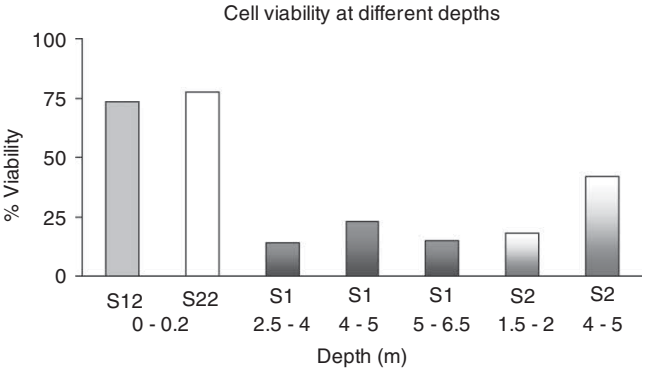

Figure 3. Bacterial cell viability of samples at different depths. S12 and S22 surface layers; S1 and S2 deeper layers.

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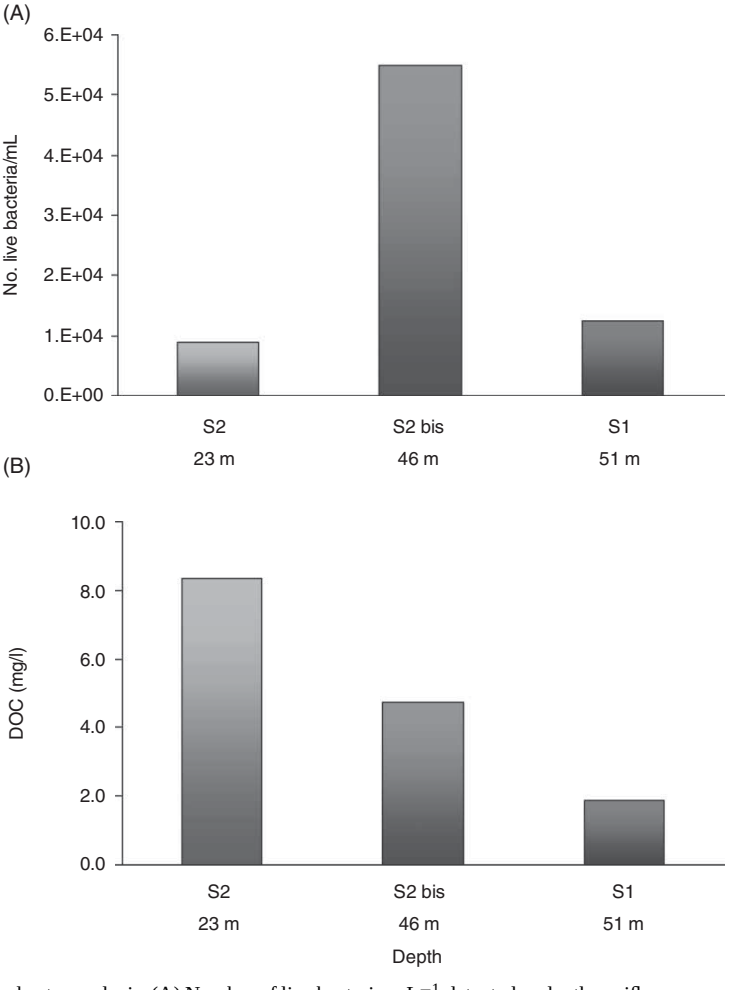

Figure 4. Groundwater analysis. (A) Number of live bacteria·mL^{-1} detected under the epifluorescence microscope and (B) dissolved organic carbon (DOC).

Table 3. Concentrations of volatile organics in groundwater samples S1, S2, S3 and corresponding legal limits [34,35].

	Concentration ($\mu g \cdot L^{-1}$)			Legal limit ($\mu g \cdot L^{-1}$) (polluted sites)
	S1	S2	S2bis	
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

3.2. *Groundwater samples*

The number of live bacteria (no. live bacteria$\cdot mL^{-1}$) detected at three different sampling points and at three different depths (S2, 23 m; S2bis, 46 m; S1, 51 m) shows that it was not inversely related to either the depth or the high dissolved organic carbon (DOC) content (Figure 4A, B). A low OC content is a factor limiting the growth of bacterial communities in groundwater [26,27] and the DOC values normally found range from 0.40 to 2 $mg \cdot L^{-1}$ in the case of a very shallow aquifer [13,28]; by contrast, 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 $mg \cdot L^{-1}$). This result suggests both an allocthonous origin for the OC 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 $\mu g \cdot L^{-1}$) found in all the groundwater analysed and the specific contamination by the antibiotic josamycin (0.15 $\mu g \cdot L^{-1}$) found at S2 23 m (Table 3); in fact, it is well known that soil microorganisms are killed both by chloroform [29,30] and antibiotics such as josamycin [31,32].

Finally, when the FISH method was applied to the latter groundwater samples, only a few bacterial populations (*β-Proteobacteria* and sulphur-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 hybridisation. 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 [33].

4. 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 present limited to some deeper samples analysed, it has to be considered 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 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 type 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 [34,35] were exceeded only in the case of chloroform, we cannot consider this groundwater and the soil above it as unpolluted. The presence of antibiotics and the high OC

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concentration found in subsoil 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 [36] and have the potential to cause health risks through drinking water exposure [37] and induce resistance genes in the natural environment, especially at residual concentrations [38,39]. There therefore needs to be particular concern if they are present in groundwater, both because it may be used as drinking water [40] 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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