IN SITU ANALYSIS OF AUTOCHTONOUS BACTERIAL COMMUNITIES IN GROUNDWATER: A NEW APPROACH TO STUDY THE EFFECT OF PESTICIDE ON MICROBIAL COMMUNITIES

Barra Caracciolo A°, Grenni P°, Preziosi E°, Falconi F°, Martin M*, Nande M*, Garbi C*

°Water Research Institute - National Research Council - Via Reno, 1 00198 Rome -Italy caraccio@irsa.cnr.it

*Complutense University - Avenida Puerta de Hierro, 28040 Madrid – Spain

INTRODUCTION

Groundwater quality and pesticide contamination of aquifers have become of great concern in all countries in which populations rely on this resource for drinking water. The transport of pesticides from agricultural fields to groundwater bodies is mainly due to water infiltration through soil. The ability of groundwater to recover from pesticide contamination is primarily dependent on the presence of a microbial community able to degrade the pesticides. Although once considered sterile, the subsurface environment is now known to harbour a wide variety and numbers of bacteria. Microbial processes and degradation in the subsurface environment occur at different rates and with different limiting factors compared to surface environments. Nevertheless, there is little research into these microrganisms because it is strictly dependent on methods able to identify and characterize their community structure and functioning. The use of molecular methods makes it possible to overcome this kind of identification limitation. In the present study we applied the Fluorescence In situ Hybridization (FISH) method to groundwater samples from phreatic aquifers, one contaminated by s- triazines and the other not, in order to identify and compare the structure and functioning of the autochthonous bacterial communities.

MATERIAL AND METHODS

Areas studied and main characteristics of the aquifers

Two phreatic aquifers, but with different hydrogeological settings (one volcanic and the second one alluvial) were selected in Central Italy in order to compare different environmental situations. Groundwater samples were collected from two private wells (BIO1 and L2) used for domestic purposes with daily frequency. The main characteristics of the aquifers, measured at the sampled wells, are shown in Table 1.

A preliminary estimation of the vulnerability of the aquifers (1) based on the combination of water table depth and infiltration capability of the rocks (the so-called "Protection Capability of the Unsaturated zone" (2), which can be considered an approximation of vulnerability) was performed.

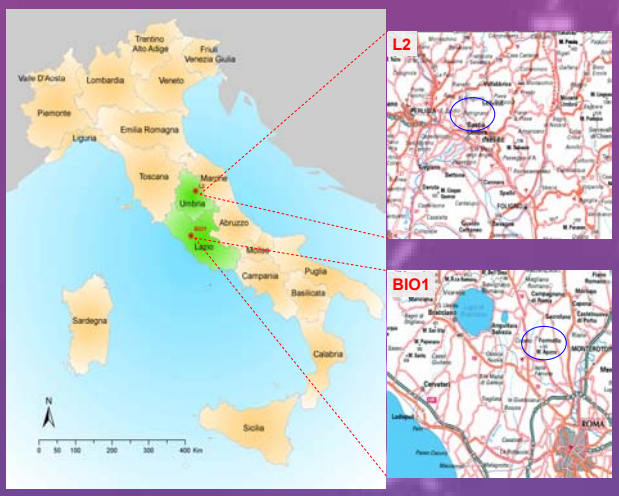
The alluvial aquifer (L2), in which water table depth doesn't exceed 30 m, has been given a "medium to high" vulnerability estimation. Moreover agriculture is highly developed and manure spreading is a common practice. Conversely, in the volcanic aquifer (BIO1) water table depth is generally higher (several tens of meters) and vulnerability has been assessed as "medium". Moreover BIO1 is located in a rural area where agriculture is not intensive.

Groundwater collection

Groundwater samples were collected by bailer and put in sterile polyethylene bottles. Some samples were fixed or treated immediately for different purposes. Field parameters were determined at the sampling point (Tab. 1).

Table 1 - Main characteristics of the aquifers

	BIO1	L2
Lithology	Volcanites (0-95) Ancient alluvial deposits (95-100)	Alluvial sands, gravels and clays
Depth (m)	100	40
Water table depth (m)	80	12
Pump depth (m)	90	36
Elevation (m a.s.l.)	173	217
Geochemical facies	Earth-alcaline bicarbonate	Alcaline-bicarbonate
Vulnerability	Medium	Medium-High
Land use	Agriculture, pasture	Intensive agriculture
Temperature °C	15.03	14.06
pH	7.4	7.1
Eh (mV)	268	210
Electric Cond. (µS/cm)	348	930
O_2 (mg/L)	8.07	9.01
s-triazine contamination	no	Yes, >0.1 µg/L
Nitrate contamination	<10 mg/L	Yes, > 100 mg/L



Geochemical analysis

Inorganic anions (Cl, PO_4, NO_3, $N0_2$, SO_4) were determined by ionic chromatography (IC); major elements (Na, K, Ca, Mg) were analyzed in inductive coupled plasma mass spectrometry (ICP-MS).

FISH with AtzB1

AtzB1, a specific probe for the plasmidic DNA of the *atz*B gene sequence, recently designed at the University of Madrid (7, 8), was also applied to L2 and BIO1 water samples in order to verify the presence of the specific enzyme involved in the hydrolytic deamination of s-triazines. AtzB1 is a FAM-labeled oligonucleotide probe and the sequence is:

5'-GGA GAGCACCGATACTTTTCTT-3'.

Bacterial community structure: abundance, live/dead and Fluorescence In Situ Hybridization (FISH)

Microbial abundance (N. bacteria/mL water) was measured by the epifluorescence direct count method using DAPI as the fluorescent agent (3). Moreover, a two dye fluorescent bacterial viability kit (live/dead) was used to distinguish viable and dead cells in each water sample (4). Community structure was analyzed by FISH, using Cy3-labeled oligonucleotide probes (5, 6). Probe sequence, target sites for the probe and the proportion of formamide used are given in Table 2.

Table 2 - Probe sequences, target sites and % formamide

Name	Sequence form 5' to 3'	Target molecule and position	Stringency (%)
ARCH915 Archaea	GTG CTC CCC CGC CAA TTC CT	16S rRNA 915 - 934	20
EUB338 Most Bacteria	GCT GCC TCC CGT AGG AGT	16S rRNA 338-355	20
EUB338 II Planctomycetales	GCA GCC ACC CGT AGG TGT	16S rRNA 338-355	20
EUB338 III Verrucomicrobiales	GCT GCC ACC CGT AGG TGT	16S rRNA 338-355	20
ALF1B α-Subdivision of Proteobacteria	CGT TCG (CT) TC TGA GCC AG	16S rRNA 19-36	20
BET42a β-Subdivision of Proteobacteria	GCC TTC CCA CTT CGT TT	23S rRNA 1027-1043	35
GAM42a γ-Subdivision of Proteobacteria	GCC TTC CCA CAT CGT TT	23S rRNA 1027-1043	35
PLA46 Planctomycetes	GAC TTG CAT GCC TAA TCC	16S rRNA 46-63	35
PLA886 Planctomycetes	GCC TTG CGA CCA TAC TCC C	16S rRNA 886-904	35
HGC69A Gram-positive bacteria with high DNA G+C content	TAT AGT TAC CAC CGC CGT	23S rRNA 1901-1918	25
CF319a Cytophaga-Flavobacterium cluster of CFB phylum	TGG TCC GTG TCT CAG TAC	16S rRNA 319 - 336	35
LGC354A Firmicutes (Gram-positive bacteria with low G+C content)	CGG AAG ATT CCC TAC TGC	16S rRNA 354 - 371	35
EPS710 Epsilonbacteria	AGTATCATCCCAGCAGA	16S	30
SRB385 Sulfate-reducing bacteria (δ), other δ and Gram-positive bacteria	CGG CGT CGC TGC GTC AGG	16S rRNA 385 - 402	35

RESULTS AND DISCUSSION

Geochemical analysis

Nitrate (111 mg/L) and herbicide (> 1 µg/L) occurrence (see Tab. 3) shows L2 to be a polluted groundwater, confirming the vulnerability (medium-high) estimated by the model used. The intensive agricultural practices, but also cattle breeding and uncontrolled civil or industrial effluents, together with the intrinsic vulnerability of the L2 aquifer, contribute to its pollution risk.

Table 3 - Geochemical and microbiological analyses. The microbial abundance (N/mL) and the dissolved organic carbon (DOC) detected in BIO1 were lower than L2, indicating the latter to be a more active ecosystem.

	BIO1	L2
N. bacteria/mL	8.73E+03	2.82E+04
live/dead %	70%	72%
AtzB%	n.d.	yes
DOC mg/L	0.23	0.56
DES ug/L	n.d.	0.13*; 0.15**
TBA µg/L	n.d.	0.06*
NO_3 mg/L	9.19	111
NO_2 mg/L	0.06	0.005
Cl mg/L	40	31
PO_4 mg/L	0.2	0.004
SO_4 mg/L	9.17	49
HCO_3 mg/L	177	370
Na mg/L	29.8	19
Mg mg/L	6.3	14
K mg/L	39.4	1.5
Ca mg/L	33	154

n.d. (Not detected); *detected in 2003 ; **detected in 2005

Bacterial community structure: BIO1 vs L2

The use of 16S rRNA-targeted oligonucleotide probes designed specifically for the main phylogenetic levels, and DAPI stains made it possible to determine the structure of the bacterial communities studied and highlight quantitative and qualitative differences between the two groundwaters analyzed (Fig. 1 and 2). The bacterial abundance, the percentage of live/dead cells and the dissolved organic carbon detected in BIO1 and L2 GW samples are in Tab. 3.

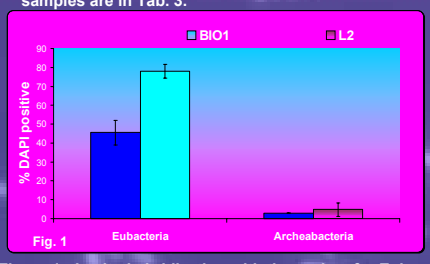


Fig. 1

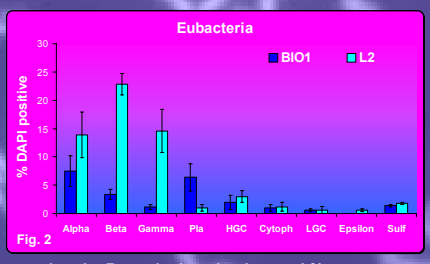
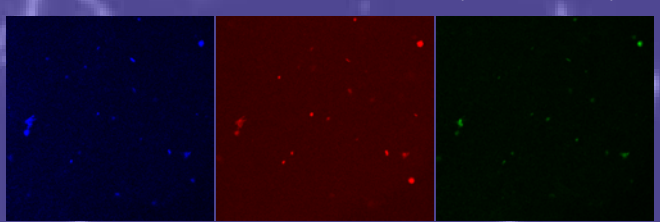


Fig. 2

Figure 1 - In situ hybridization with the probes for Eubacteria, targeting the Bacteria domain, detected % of DAPI-stained cells averaging 45.5% in BIO1 and 78% in L2 respectively. Moreover, even if in a lower percentage, Archeabacteria were detected at both sites and with similar values (2.9% BIO1, 4.8% L2).

Figure 2 - FISH with the other probes for Eubacteria subgroups made it possible to detect DAPI-stained cells at both sites, although in significantly different and generally greater percentages in L2 than in BIO1.

Figure 3 Images at the confocal microscope of bacteria detectable by DAPI-staining (blue colour), by EUB338 (red, Cy3-labeled) and by the specific AtzB1 probe (green, FAM-labeled) in L2 groundwater samples.



Bacterial community functioning: AtzB1

The L2 groundwater samples were positive (6%) to the specific AtzB1 probe (Fig. 3, green colour). The detection of the plasmidic gene *atz*B indicates that some bacteria populations among the autochthonous community have the potential capability to degrade the s-triazines by performing their hydrolytic deamination. The fact that herbicide contamination has not been increasing for the last two years (terbuthylazine was detected in 2003, but not in 2005, and desethyl-terbuthylazine concentration did not increase from 2003 to 2005) seems to confirm that herbicide degradation actually occurs.

ACKNOWLEDGMENTS: We thank the Regional Environmental Agency (ARPA-UMBRIA) for its helpful during sampling collection and field measurements and for s-triazine data availability.

CONCLUSIONS

This study is the first characterization, using the FISH method, of the structure (phylogenetic identification) and the functioning (potential for s-triazine degradation) of groundwater microbial communities contaminated and not-contaminated by s-triazines. The results are very promising because they show the FISH method to be a valuable and new tool for characterizing and comparing groundwater ecosystems under different anthropic impact conditions.

REFERENCES

1. Vrba J, Zaporozec A, 1994. Guidebook on mapping groundwater vulnerability, 16 Verlag-Heinz-Heise
2. Preziosi et al, 2005. Aquifer Vulnerability and Risk. 4th Congress Protection and Management of Groundwater – Parma, Italy: 1-11.
3. Fava L et al, 2001. Pest Manag Sci 57: 1135-41.
4. Quirin NV et al, 2004. J Microbiol Methods 57: 351-367
5. Barra Caracciolo A et al, 2005. Pest Manag Sci 61: 917-921
6. Barra Caracciolo, A et al, 2005. FEMS Microbiol Lett 253: 55-58.
7. Martinez-Iñigo M.J. et al 2003. Applicability of fluorescence in situ hybridization to monitor target bacteria in soil samples. Proc XI Symp Pesticide Chemistry, Piacenza, Italy: 609-615.
8. Martin M, Gibello A, Martinez-Iñigo M.J, Lobo MC, Nande M, Garbi C, Barra Caracciolo A, Grenni P, 2006. *Proposal for a Natural Attenuation Coefficient for simazine- contaminated soils based on fluorescence in situ hybridization. Submitted to* Soil Biology and Biochemistry.