J. Microbiol. Biotechnol. (2014), 24(6), 843–853
http://dx.doi.org/10.4014/jmb.1312.12010

jmb

Multi-Bioindicators to Assess Soil Microbial Activity in the Context of an Artificial Groundwater Recharge with Treated Wastewater: A Large-Scale Pilot Experiment

Caroline Michel[1*], Catherine Joulian[1], Patrick Ollivier[1], Audrey Nyteij[1], Rémi Cote[1], Nicolas Surdyk[1], Jennifer Hellal[1], Joel Casanova[1], Katia Besnard[2], Nicolas Rampnoux[2], and Francis Garrido[1]

[1]*BRGM (Bureau de Recherches Géologiques et Minières), BP 36009, 45060 Orléans, France*
[2]*VERI (Veolia Environment Recherche et Innovation), 95500 Rueil-Malmaison, France*

Received: December 6, 2013
Revised: February 28, 2014
Accepted: March 7, 2014

First published online
March 10, 2014

*Corresponding author
Phone: +33 (0)2 38 64 47 22;
Fax: +33 (0)2 38 64 36 52;
E-mail: c.michel@brgm.fr

pISSN 1017-7825, eISSN 1738-8872

In the context of artificial groundwater recharge, a reactive soil column at pilot-scale (4.5 m depth and 3 m in diameter) fed by treated wastewater was designed to evaluate soil filtration ability. Here, as a part of this project, the impact of treated wastewater filtration on soil bacterial communities and the soil's biological ability for wastewater treatment as well as the relevance of the use of multi-bioindicators were studied as a function of depth and time. Biomass; bacterial 16S rRNA gene diversity fingerprints; potential nitrifying, denitrifying, and sulfate-reducing activities; and functional gene (*amo*, *nir*, *nar*, and *dsr*) detection were analyzed to highlight the real and potential microbial activity and diversity within the soil column. These bioindicators show that topsoil (0 to 20 cm depth) was the more active and the more impacted by treated wastewater filtration. Nitrification was the main activity in the pilot. No sulfate-reducing activity or *dsr* genes were detected during the first 6 months of wastewater application. Denitrification was also absent, but genes of denitrifying bacteria were detected, suggesting that the denitrifying process may occur rapidly if adequate chemical conditions are favored within the soil column. Results also underline that a dry period (20 days without any wastewater supply) significantly impacted soil bacterial diversity, leading to a decrease of enzyme activities and biomass. Finally, our work shows that treated wastewater filtration leads to a modification of the bacterial genetic and functional structures in topsoil.

Keywords: Bioindicators, soil enzyme activities, 16S rRNA gene CE-SSCP fingerprints, microbial biomass, treated wastewater, groundwater recharge

Introduction

The demands for drinking water are forever growing while the water resource is decreasing owing to intensive use and chemical and microbial contaminations [23, 44, 53, 54]. More than half of the world's population lives in coastal areas, where water stress problems are exacerbated by climate change [38]. Declining groundwater levels associated with sea-level rising due to climate change may lead to saline water intrusion into groundwater aquifers and thus may decrease and/or degrade the available groundwater resources. In this context, artificial recharge of aquifers using treated wastewater has been suggested as a solution to replenish overdrafted aquifers and provide sustainable water supplies, and to protect underground freshwater in coastal aquifers by pushing back the sea-born slats [34]. The soil aquifer treatment (SAT) method has been proposed as a low-cost wastewater reclamation method [9]. This method involves using soils for the final treatment of municipal and industrial wastewaters. Treated wastewater is thus purified by physical, chemical, and biological processes in the soil and in the aquifer, removing microorganisms and suspended materials, and potentially toxic inorganic constituents such as heavy metals and trace

oxyanions [8, 22, 51]. However, these processes may strongly affect both the soil quality and soil biology [56].

Microbial communities in SAT play an important role in the attenuation of organic pollutants (*e.g.*, organic matter) and are involved in biogeochemical processes (*e.g.*, nitrification, denitrification, reductive dissolution of Mn and Fe) occurring within the soil, which may govern the sorption and/or release of trace elements (*e.g.*, As, Mn, Fe, Ni). Studies of soil quality and microbial activity are thus required in SAT. However, the dynamics and the role of microbial communities during SAT as well as the impact of wastewater on microbial communities remain relatively unknown [56]. Recently, it has been suggested to use microbial biomass and some soil enzyme activities (*e.g.*, phosphatase, dehydrogenase, β-glucosidase) as bioindicators to highlight the impacts of wastewater use on the soil microbial component [1, 32]. Moreover, it has been shown that microbial biomass and soil enzyme activities may be greatly affected by pollution and physical and chemical conditions [7, 17, 19]. The degree of inhibition or activation of enzymes is thus commonly used as a bioindicator of soil health [3, 25, 27, 49]. Activities targeting enzymes related to N, C, S, and P cycles are mostly studied. Microbial diversity is another powerful bioindicator. It has proved to be a suitable tool for the study of soil health and the monitoring of microbial resilience [4]. Finally, molecular approaches such as gene cloning and diversity fingerprints have been widely used to assess the impact of physical and chemical perturbations on biodiversity and detect community shifts over time and under different environmental conditions [6, 14]. These techniques enable to target the total bacterial diversity (when working on the 16S rRNA gene) or bacteria belonging to a metabolic group (*e.g.*, using functional genes such as *amo* gene for ammonia-oxidizing bacteria [36] or the *dsr* gene for sulfate-reducing bacteria [55]). Coupling several bioindicators has been widely demonstrated to be a good tool to provide evidence of soil function and health and monitor the impact of a potential perturbation (*e.g.*, pollution, temperature and pH variations, organic matter, nutrients) on soil [3, 5, 6, 17, 18, 27, 37, 52]. In the context of SAT, the coupling of microbial biomass and biodiversity, soil enzyme activities, and molecular approaches may thus be relevant to monitor the activity/dynamics/reactivity of the soil microbial community and the impact of wastewater.

A large-scale pilot study (25 m^3) has been carried out to better understand the effect of treated wastewater infiltration throughout the reactive soil column on the quality of water exported from the pilot and the geochemical evolution of soil and water [39]. As a part of this project, our work is dedicated to better understand (i) the reactivity of the soil microbial community and (ii) its potential (*i.e.*, maximal activity in batch experiments in optimal conditions) and *in situ* activity as a function of the soil depth in the context of the SAT. The effect of treated wastewater infiltration throughout the reactive soil column on the microbial community (*e.g.*, reactivity and activity) was investigated during the first 6 months of operation, by looking at microbial biomass and diversity and soil enzyme activities. This is essential to understand the evolution of biogeochemical processes within the soil that are responsible for trace element release and hence for the SAT and groundwater management under an artificial recharge regime.

Materials and Methods

Pilot Description

A pilot (reactive soil column of 4.5 m depth and 3 m in diameter) containing a soil-quartz mixture was implemented in the wastewater treatment plant of Amphora in the city of La Garde (France, Var). Physical and chemical characteristics of the soil-quartz mixture as well as the operating mode of wastewater application are described in detail in Ollivier *et al*. [39]. Briefly, soil was collected in a clay horizon close to the city of Carmoule (France), mixed with inert quartz sand (99% pure silica (Holcim 0.4/0.8)) in a proportion soil:sand of 45:55, and was then packed into the cylinder. The pilot was fed with secondary treated wastewater from the municipal wastewater treatment plant of Amphora. The properties/composition of the treated wastewater and the output water (in terms of pH, electrical conductivity, redox potential, dissolved oxygen, dissolved major cations and anions, dissolved trace elements, dissolved inorganic carbon (DIC), dissolved organic carbon (DOC), and total organic carbon (TOC)) were analyzed during the experiment (Tables 1 and 2; [39]). Treated wastewater (0.25 m^3) was applied by spraying (a few minutes only) above the soil infiltrative surface (SIS) at constant time intervals of 6 h in order to achieve an unsaturated flow regime in the pilot (saturated-unsaturated cycles). The pilot had an open top to allow soil to be affected by local climate conditions (temperature, evaporation, rainwater) to simulate realistic operating conditions. Water contribution from rain input was measured *in situ* using an automatic precipitation sampler, and was in good agreement with the values provided by Météo France at the meteorological station of Toulon-Hyères International Airport (~12 km from the pilot). The total rainfall collected at the site was 202 mm (mean value of 1.1 mm/day) during the study period. Six significant rain events (>14 mm/day, *i.e.*, 10% of the daily treated wastewater input) occurred. One of them (in April) contributed 28% of the daily treated wastewater input [39]. Daily evapotranspiration data were provided by Météo France. It was 4.9 mm/day during the study period. It increased from 0.8 to 9 mm/day from April to July and then decreased to reach

Table 1. Temperature (°C), pH, redox potential (Eh), electrical conductivity (EC; in µS/cm), dissolved oxygen (O_2; in mg/l), dissolved cations (Ca, Na, Mg, K, and NH_4), anions (Cl, NO_3, NO_2, SO_4, and PO_4), dissolved and total organic carbon, inorganic carbon (IC) and trace elements (Li, Mn, Fe, Ni, As, and Ba) concentrations in treated wastewaters.

Date	Temp	pH	Eh (mv)	EC	O_2	Ca	Na	Mg	K	NH_4	Cl	NO_3
03/06/09	23.6	7.28	297.6	1,855	6.6	146.7	138.8	29.4	19.0	50.2	242.0	30.2
02/07/09	25.7	7.36	312.1	1,757	6.9	132.4	148.3	30.3	20.1	36.5	243.0	46.6
26/08/09	26.8	7.14	312.6	1,799	7.5	128.5	129.0	28.8	18.3	27.9	259.0	61.0
14/10/09	22.8	7.28	316.7	1,824	7.9	121.8	148.1	25.2	18.9	44.7	264.0	52.5
Mean (period)	24.7 ± 1.8	7.27 ± 0.1	309.7 ± 8.3	1,809 ± 41	7.2 ± 0.6	132.4 ± 10.5	141.1 ± 9.2	28.4 ± 2.2	19.1 ± 0.7	39.8 ± 9.7	252.0 ± 11.2	47.6 ± 13
Mean (18 months)	21.1 ± 3.9	7.24 ± 0.2	349.2 ± 45	1,632 ± 446	7.6 ± 0.6	126.0 ± 18.6	128.5 ± 38.9	26.3 ± 6.1	16.8 ± 4.9	30.0 ± 16.3	232.3 ± 69.2	52.4 ± 19.2

	NO_2	SO_4	PO_4	DOC	TOC	IC	Li	Mn	Fe	Ni	As	Ba
03/06/09		165.0	< 0.01	15.5	18.1	75.2	19.3	71.3	0.33	2.70	0.87	16.0
02/07/09	0.59	152.0	1.0	18.4	19.6	91.0	13.4	79.5	0.49	2.80	1.02	10.8
26/08/09	0.43	144.0	1.3	11.3	11.7	72.2	15.0	112.8	0.36	2.60	1.12	8.0
14/10/09	0.59	156.0	< 0.01	12.4	13.7	12.4	18.6	56.1	0.25	1.90	0.53	12.8
Mean (period)	0.54 ± 0.1	154.3 ± 8.7	0.58 ± 0.67	14.4 ± 3.2	15.8 ± 3.7	62.7 ± 34.5	16.6 ± 2.8	79.9 ± 24	0.36 ± 0.1	2.50 ± 0.41	0.89 ± 0.26	11.9 ± 3.4
Mean (18 months)	0.73 ± 0.73	154.5 ± 28	0.36 ± 0.45	12.3 ± 4.1	14.5 ± 6.8	70.1 ± 24.6	19.4 ± 3.9	71.3 ± 23	0.24 ± 0.11	2.23 ± 0.48	0.71 ± 0.3	16.3 ± 6.5

Mean values (± σ) for the study period (April to October 2009) and for a period of 18 months (April 2009 to December 2010) are also reported. Cation and anions are in mg/l. Trace elements are in µg/l except for Fe, which is in mg/l. Blank means not measured (data from [39]).

Table 2. Temperature (°C), pH, redox potential (Eh), electrical conductivity (EC; in µS/cm), dissolved oxygen (O_2; in mg/l), dissolved cations (Ca, Na, Mg, K, and NH_4), anions (Cl, NO_3, NO_2, SO_4, and PO_4), dissolved and total organic carbon, inorganic carbon (IC), and trace elements (Li, Mn, Fe, Ni, As, and Ba) concentrations in the output water.

Date	Temp	pH	Eh (mv)	EC	O_2	Ca	Na	Mg	K	NH_4	Cl	NO_3
03/06/09	26.3	7.15	301.6	1,570	0.8	162.2	111.1	22.2	11.4	4.4	187.0	62.4
02/07/09	25.7	7.04	399.4	1,828	4.6	204.5	131.7	30.3	14.3	1.7	227.0	205.0
26/08/09	27.9	6.96	413.9	1,823	2.6	157.9	123.7	26.1	15.8	< 0.09	219.0	146.0
14/10/09	20.9	7.14	446.3	1,542	2.1	164.8	117.3	27.5	14.8	0.3	220.0	132.0
Mean (period)	25.2 ± 3	7.07 ± 0.09	390.3 ± 62.3	1,691 ± 156	2.5 ± 1.5	172.4 ± 21.6	121 ± 8.8	26.5 ± 3.4	14.1 ± 1.9	1.6 ± 2	213.3 ± 17.9	136.4 ± 59

	NO_2	SO_4	PO_4	DOC	TOC	IC	Li	Mn	Fe	Ni	As	Ba
03/06/09	< 5 x 10^{-4}	172.0	< 0.01	4.9	5.2	112.7	5.0	19.0	< 1.1	2.2	0.61	187.0
02/07/09	5.05	169.0	< 0.01	3.5	3.6	62.1	3.0	14.9	< 1.1	2.5	0.51	258.9
26/08/09	0.03	148.0	< 0.01	2.5	3.4	63.9	3.2	0.5	< 1.1	1.4	0.46	208.7
14/10/09	0.01	139.0	< 0.01	3.6	2.9	3.4	3.4	25.2	< 1.1	2.2	0.39	181.2
Mean (period)	1.3 ± 2.5	157 ± 16.1	< 0.01	3.6 ± 1	3.8 ± 1	60.5 ± 44.7	3.7 ± 0.9	14.9 ± 10.5	< 1.1	2.1 ± 0.5	0.49 ± 0.09	209 ± 35.3

Cation and anions are in mg/l. Trace elements are in µg/l (data from [39]).

1.5 mm/day in October [39]. Soil was bare and was manually kept vegetation-free over the study period (no use of herbicides).

Soil Samples

Soil samples were collected at depths of 10, 20, 30, 50, 100, 150, 200, 250, and 350 cm below SIS, in April 2009 (0 month; *i.e.*, before the beginning of wastewater application), June 2009 (2 months of operation), July 2009 (3 months), and October 2009 (6 months, after 20 days without irrigation) using augers, from sealed access ports of 5 cm in diameter. Samples were then sieved through a

2 mm mesh screen using a sterile sieve, and stored in sterile bags at 4°C for transport and until further processing on the following day. To obtain a representative sample, for each depth, three soil samples (100 g, wet weight) were collected and pooled.

Soil Microcosms and Potential Microbial Activities (Nitrification, Denitrification, and Sulfate Reduction)

Potential nitrifying, denitrifying and sulfate-reducing activities were estimated by soil incubations in batch experiments (for all soil samples except those collected 10 cm below SIS). Twelve grams of moist soil was incubated in 250 ml flasks and the following conditions: for nitrifying activity measurements, 120 ml of a 1 mM ammonium sulfate solution and 12 ml of a 1.5 M NaCl solution under aerobic conditions; for denitrifying activity measurements, 120 ml of a 1 mM potassium nitrate solution and 12 ml of a 1.5 M NaCl solution under anaerobic conditions; and for sulfate-reducing activity measurements, 120 ml of a 1 mM ammonium sulfate solution under anaerobic conditions. For anaerobic incubations (denitrification and sulfate reduction), air was removed from the flasks with a vacuum pump during three successive cycles (20 min), and then replaced with sterile N_2 before incubation. The internal flask's pressure was equilibrated at $+0.5 \times 10^5$ Pa above atmospheric pressure. During incubation, the pressure was monitored and sterile N_2 was added as needed to prevent a pressure decrease in the flasks. To ensure the reproducibility of experiments, triplicates were done. A control experiment was also run: soil was sterilized three times (at 24 h intervals) by autoclaving for 1 h at 105°C. Flasks were incubated during 28 days at 25°C in the dark with shaking at 175 rpm. Potential activities were calculated by measuring nitrate production (nitrification), nitrate consumption (denitrification), and sulfate consumption (sulfate reduction), and were expressed on the bases of sample dry weights. NH_4^+, NO_3^-, and SO_4^{2-} concentrations were determined using the Merck Spectroquant kits 1.00683.0001, 1.09713.0001, and 1.14799.0001, respectively.

Soil Microbial DNA Extraction and Quantification

Soil microbial DNA was extracted directly from approximately 1 g of moist soil sampled in the pilot, using the Fast DNA Spin Kit for soil (Bio101, USA). Extracted soil DNA was quantified by SYBR Green I dye according to the protocol previously described [30], and used as an estimation of microbial biomass [43].

CE-SSCP Microbial Diversity Fingerprints and PCR Amplification of 16S rRNA Genes

The semi-automated structural analysis CE-SSCP (capillary electrophoresis-single strand conformational polymorphism) technique, which allows the discrimination of single-strand DNA fragments of the same length but with divergent nucleotide sequences, was applied to generate diversity fingerprints of 16S rRNA genes. About 200 bp of the V3 region of 16S rRNA genes of members of the Bacteria domain were amplified from DNA extracts with the forward primer w49 (5′-ACGGTCCAGACTCCT ACGGG-3′) and the reverse primer w34 (5′-TTACCGCGGCTG CTGGCAC-3′), 5′ end-labeled with the fluorescent dye FAM. PCR was performed in a total volume of 15 µl containing 1 µl of DNA, 8.6 µl of nuclease-free H_2O, 3 µl of buffer 5× (GoTaq Flexi Buffer, Promega), 1.5 µl of $MgCl_2$ (25 mM), 0.15 µl of dNTP (10 mM each), 0.3 µl of each primer (20 pmol/µl), and 0.15 µl of GoTaq Flexi DNA Polymerase (Promega). The cycling conditions of the PCR were an initial denaturation step at 95°C (2 min), followed by 29 cycles consisting of denaturation at 95°C (30 sec), annealing at 61°C (30 sec), and elongation at 72°C (30 sec). Amplification was completed by a final elongation step at 72°C for 5 min.

One microliter of diluted (10- to 70-fold in nuclease-free water) PCR product was added in a mixture of 18.6 µl of deionized formamide and 0.4 µl of Genescan-600 LIZ internal standard (Applied Biosystems). To obtain single-strand DNA, samples were heat denaturated for 5 min at 95°C, and immediately cooled on ice. CE-SSCP analyses were performed on an ABI Prism 310 genetic analyzer using a 47 cm length capillary, a non-denaturating 5.6% CAP polymer (Applied Biosystems), and the following electrophoresis conditions: run temperature 32°C, sample injection for 5 sec at 15 kV, data collection for 32 min at 12 kV.

PCR Amplification of *amoA*, *narG*, *nirS*, *nirK*, and *dsrAB* Genes

PCRs were performed in a total volume of 20 µl containing 1 µl of DNA, 12.2 µl of nuclease-free H_2O, 4 µl of buffer 5× (Green GoTaq Flexi Buffer, Promega), 2 µl of $MgCl_2$ (25 mM), 0.2 µl of dNTP (10 mM each), 0.2 µl of each primer (50 pmol/µl), and 0.2 µl of GoTaq Flexi DNA Polymerase (Promega). Primers used for PCR were AmoA-1F-GC/AmoA-2R-TC for *amoA* [36], narG-F/narG-R for *narG* [11], nirS1F/nirS3R for *nirS* [10], nirK876/nirK1040 for *nirK* [24], and DSR1Fdeg/DSR4Rdeg for *dsrAB* [28].

The cycling conditions of the PCR were an initial denaturation step at 95°C (2 min), followed by 35 cycles consisting of denaturation at 95°C (30 sec), annealing for 30 sec at 57°C (*amoA*), 55°C (*nirS*), 58°C (*nirK*), and for 1 min at 54°C (*dsrAB*), and elongation at 72°C for 30 sec (*nirS* and *nirK*), 1 min (*amoA*), or 3 min (*dsr*). Amplification was completed by a final elongation step at 72°C for 5 min (10 min for *dsrAB* gene amplification).

For *narG* gene amplification, a touchdown protocol was used: an initial denaturation step at 95°C (2 min), followed by six cycles consisting of denaturation at 95°C for 30 sec, a touchdown-annealing step for 30 sec, and elongation at 72°C for 30 sec. This was followed by another 40 cycles of denaturation at 95°C for 30 sec, annealing at 58°C for 30 sec, and elongation at 72°C for 30 sec. Amplification was completed by a final elongation step at 72°C for 5 min.

Positive controls on purified DNA from reference strains were included in all PCR experiments along with negative controls (no DNA added).

Statistical Analyses

CE-SSCP fingerprints were analyzed using the StatFingerprints software [33]. More than 700 peaks were detected within each soil

sample. Peaks intensities were converted into a matrix (soil samples as lines and peaks as columns), subjected to principal component analysis (PCA) providing an ordination of bacterial communities in a factorial map based on the scores of the first two principal components. PCA was performed on the final data matrix using ADE-4, an R software [42, 50].

After checking normal data distribution with a Shapiro-Wilk normality using XLSTAT (ver. 2013.2.07), differences between nitrification potentials were determined with an analysis of variance (ANOVA) followed by a *post hoc* Tukey test.

The ANOVA test was used to determine if there were significant differences between groups. The Tukey test was used to determine which groups were significantly different. Practically, a first test was carried out between soil depths showing no significant differences, and then separate tests were carried out for each depth to identify an effect of time on nitrification potentials.

A similar approach was used to analyze microbial biomass data, except that as only one replicate was carried out for each condition, comparisons could only be carried out with all the data between sampling times and between depths at all sampling times.

The correlation between microbial biomass and potential nitrifying activity was calculated using Microsoft XLSTAT (ver. 2013.2.07).

Results

Microbial Biomass

Total microbial biomass was evaluated by quantifying the total soil DNA. Whatever the time of operation, the highest amounts of DNA were found in the topsoil (*i.e.*, from 0 to 20 cm) and were significantly higher than the amounts found in all the soils lower than 50 cm (p-value < 0.05). They also appeared higher in the topsoil, by a factor of 1.5 to 3, than in the initial soil (before the wastewater application) (Fig. 1), although no significant differences between incubations times were identified (p-value = 0.444). By contrast, DNA amounts measured in samples collected below 20 cm (*i.e.*, from 30 to 350 cm) were generally similar to those obtained in the initial soil, with an exception concerning samples collected after 2 months of operation between 50 and 250 cm, which showed amounts of DNA lower by a factor of up to 2 compared with those measured in the initial soil.

Bacterial Diversity

The bacterial diversity within the soil column was studied by CE-SSCP as a function of depth and time. All samples were characterized by an important bacterial diversity as demonstrated by the large number of peaks on the CE-SSCP profiles (data not shown).

In detail, PCA of CE-SSCP profiles indicated that

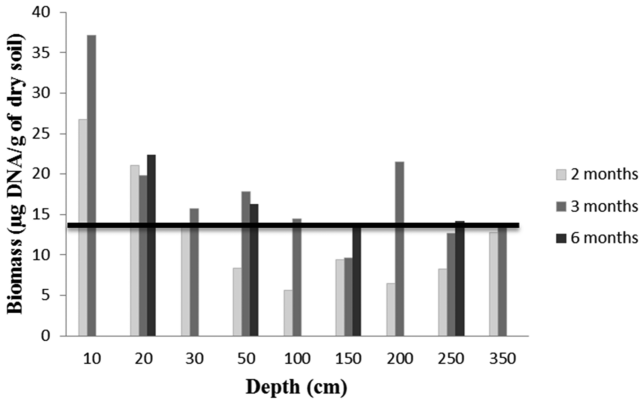

Fig. 1. Total microbial biomass in the soil column as a function of depth and time.

Extracted soil microbial DNA was quantified by SYBR Green I dye and used as an estimation of microbial biomass. The dark horizontal line indicates the biomass measured at 0 month (initial soil-quartz mixture, see text for detail). After 6 months of wastewater application, soils were collected at 20, 50, 150, and 250 cm depth only.

biodiversity was quite homogeneous within the soil column after 2 months of operation, but different from that obtained at T_0 (Fig. 2A). After 3 months, biodiversity was quite similar to that obtained after 2 months except in the topsoil samples (*i.e.*, from 0 to 30 cm; samples 3m-30, 3m-20, and 3m-10 on Fig. 2A) for which biodiversity was clearly different, with the PC1 axis explaining 52.8% of the variability. After 6 months operating, results of PCA highlight that biodiversity was rather homogeneous within the soil column, but it differed, on the PC2 axis, from that previously observed (Fig. 2A). Biodiversity in topsoil (0–30 cm) and subsoil (below 30 cm) thus evolved differently. The PCA performed for topsoil (Fig. 2B) highlights the evolution of the bacterial community with time. For the subsoil, the biodiversity evolved clearly from T_0, being quite homogenous at 2 months but very heterogeneous at 3 months (Fig. 2C). Then the diversity was again quite homogeneous but clearly different from the diversity of the precedent months.

Presence of Nitrifying, Denitrifying, and Sulfate-Reducing Bacteria in Soil: Detection of *amoA*, *narG*, *nirK*, *nirS*, and *dsrAB* genes

As bacteria present in the soil column can be non-active and thus non-detectable by soil enzyme activity tests, the presence of *amoA* (for nitrification), *narG*, *nirK*, and *nirS* (for denitrification) and *dsrAB* (for sulfate-reduction) genes was studied in soil samples. It is noticeable that *amoA*, *nirS*, *nirK*, and *narG* genes were found in all samples, showing the presence of nitrifying and denitrifying bacteria along

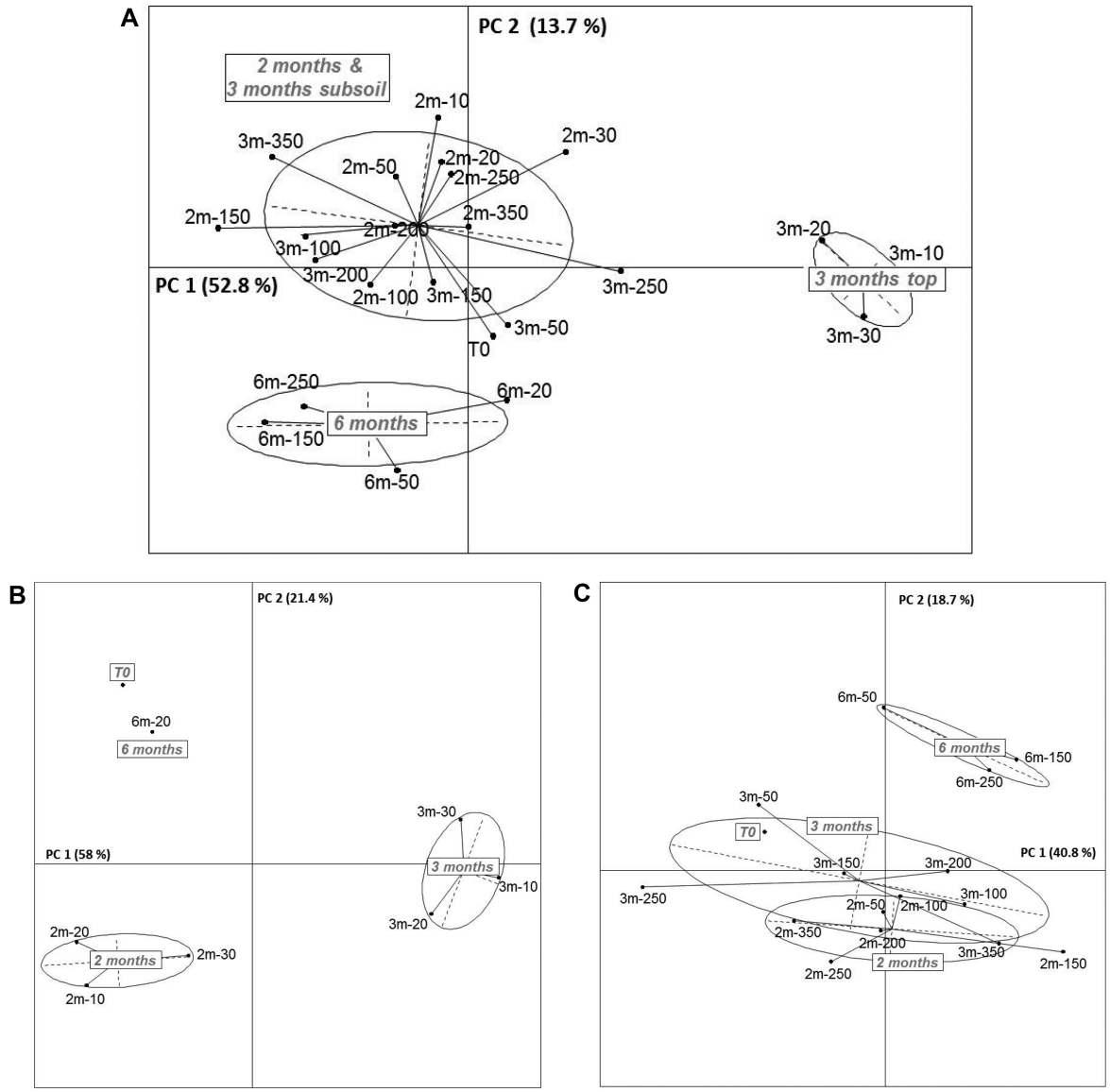

Fig. 2. Principal component (PC1 × PC2) analysis (PCA) ordination of the bacterial genetic structure generated from CE-SSCP fingerprints along the soil column after 0 (T0), 2 (2 m), 3 (3 m), and 6 (6 m) months of wastewater application for samples collected at depths of 10, 20, 30, 50, 100, 150, 200, 250, and 350 cm.

At 6 months, samples were only collected at depths of 20, 50, 150, and 250 cm. The percentages of explained variations for the first two axes are indicated within the figure. (**A**) PCA obtained for all samples. (**B**) PCA obtained for topsoil samples (from 0 to 30 cm). (**C**) PCA obtained for subsoil samples (from 50 to 350 cm).

the soil column, whatever the duration of wastewater application. In contrast, no amplification of *dsrAB* genes was obtained, suggesting that sulfate-reducing bacteria were not numerous enough to be detected by PCR.

Potential Activities in Soil: Batch Experiments

The potential nitrifying, denitrifying, and sulfate-reducing activities in the initial soil and in soil samples collected in the column after 2, 3, and 6 months of wastewater application were estimated by soil incubation in batch experiments, as described above. Results showed a significant potential nitrifying activity for all soil samples, which significantly varied during the 6-month-long experiment (Fig. 3). After 2 months of wastewater application, the potential activity increased all along the soil column with no significant differences between soil depths (ANOVA, *P*-

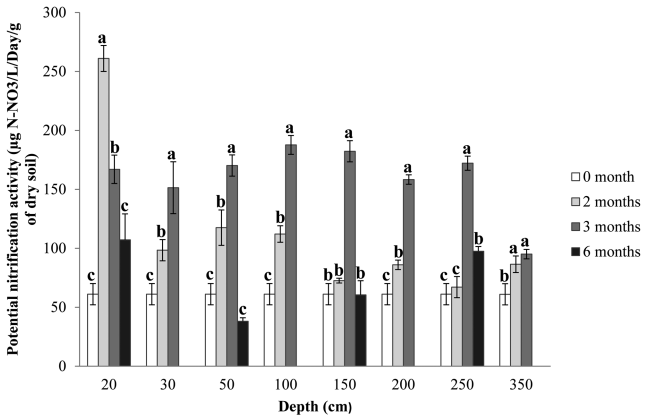

Fig. 3. Potential nitrifying activity within the soil column as a function of depth and time.

The potential activity was measured in laboratory batch experiments by incubating soils sampled in the pilot under optimal conditions for nitrification (see text for details). After 6 months of wastewater application, soils were collected at 20, 50, 150, and 250 cm depth only. Standard deviations are displayed. Statistical differences analyzed by ANOVA and Tukey tests between different sampling times for each depth are indicated by the letters above the bars.

value = 0.277). After 3 months, the potential nitrification activity carried on increasing in all soil samples except for the topsoil sample where it decreased (ANOVA P-value < 0.001). Activity was almost homogeneously distributed within the soil column, with values higher by a factor of 2 to 3 compared with those measured in the initial soil. After 6 months, potential nitrification activity significantly decreased in all the samples where it was measured (*i.e.*, 20, 50, 150, and 250 cm depth; ANOVA, P-value < 0.001). No correlation was observed between biomass and nitrification activity (r = 0.16; P-value > 0.001) (data not shown).

Soil incubation experiments for denitrifying and sulfate-reducing activities did not show any measurable activity whatever the location of samples in the pilot and even after 6 months operating.

Discussion

The topsoil (0 to 20 cm depth) was very reactive to wastewater application, as shown by the modification of biodiversity and the significant increase of both total biomass and microbial activity compared with samples collected deeper in the soil column. Indeed, results showed that the biodiversity in the topsoil differed from that in other samples after 3 months, while total microbial biomass and potential nitrifying activity in the topsoil increased by a factor of 2 and 5, respectively, after only 2 months of wastewater application (Figs. 1 and 2). This is in agreement with previous studies that showed that soil irrigation with treated wastewaters leads to the development of microorganisms in the first top centimeters of soil [16, 31, 41]. This increase may be explained by the supply of easily decomposable organic matter and nutrients contained in treated wastewater. It is well known that the addition of organic carbon supports microbial growth and its associated activities [1, 16, 41]. In our study, TOC content in the topsoil at the end of the experiment (3.8 g/kg) was 3 times higher than that measured in the initial soil (1.1 g/kg), but it decreased sharply from 2 cm of soil depth and was not different from that in the initial soil below 10 cm depth [39]. Biodiversity modification (*i.e.*, the presence/absence of the microbial species in soil along time) in the topsoil after 3 months of wastewater application may also be explained by the excessive electron donor and high nutrient input by the treated wastewater ([39]) favoring the development of multiples microbes. Fierer *et al*. [19] suggested that soil resource availability (as organic carbon) is the main factor responsible for the modification of the microbial community (in terms of species composition) through the soil profiles. Blume *et al*. [7] also implied that it is the low level in C that may account for lower biomass in deep zones. Soil pH, temperature, and texture would thus have fewer impacts in the differentiation of microbial communities [19]. Another potential explanation for the microbial diversity in the topsoil is the potential accumulation of microorganisms coming from the treated wastewater, as discussed in previous studies [31]. Concerning microbial activities, potential nitrification was the main activity in the soil column and increased strongly after 2 months of operation. This is in good agreement with Ollivier *et al*. [39], who showed an increase of NO_3 concomitant with the decrease of NH_4 in the topsoil a few days only after the beginning of the treated wastewater application. Nitrifying processes may be promoted by the frequency of treated wastewater application (at constant time intervals of 6 h) associated with the relatively high infiltration rates that allowed the topsoil to be well aerated. Therefore, all bioindicators highlight a rapid response of the microbial community to treated wastewater application. It is interesting to note that even if the soil used in the pilot is a composite soil that was initially homogeneous, the biomass, biodiversity, and activity distribution along the soil column rapidly evolved (after only a few weeks of treated wastewater irrigation) as for a natural soil (forest soil, sandy loam, clay loam…) [2, 7, 19].

After 6 months of treated wastewater application, bioindicators suggest that significant changes occurred in the soil column. Potential nitrifying activity strongly decreased to reach levels similar to those measured in the initial soil, and bacterial diversity became homogeneous along the soil column. The influence of seasons and temperature has been demonstrated in previous work to explain such a variation [5, 7, 17, 52]. In our study, the decrease of the mean daily temperature between July (3 months) and October (6 months) was about 8°C to 10°C. This decrease alone is probably not sufficient to explain the decrease of enzyme activity observed in the soil column [39]. Soil biology changes are probably related to the operating mode of the pilot. Indeed, after 6 months operating, soil samples were collected after a long interruption (about 20 days) of the treated wastewater application owing to maintenance. It is known that water dynamics play an important role in microbial respiration, nutrient cycles, and terminal electron availability [13, 45, 47]. Soil moisture affects O_2 availability and redox potential, imparting a strong control on microbial metabolism and activity [26, 40, 48]. Bacteria need to adapt to the change in water content and oxygen, and this adaptation time may vary within the range of minutes to days [12, 46]. Our results suggest that changes in operating conditions of wastewater application significantly and rapidly affect the soil microbiology.

Another interesting result is the difference observed between activities measured *in situ* (NH_4, NO_3, and NO_2, and SO_4 contents measured in the pilot, as reported by Ollivier *et al*. [39]) and the potential activities detected in batch experiments or by functional gene research. Soil incubations (batch experiments) and microbial functional gene detection highlight that potential nitrifying activity was present all along the soil column, while water chemical data issued from the pilot showed that nitrification was limited to the upper soil horizon [39]. This difference is probably directly related to the strong ammonium ion sorption associated with the rapid consumption of molecular oxygen in the topsoil, limiting nitrification in deeper horizons. Indeed, the capacity of soil to adsorb NH_4 during wastewater application is a well-known feature [20, 21, 29, 35]. Moreover, Miller *et al*. [35] reported that dissolved oxygen present in percolating water was rapidly consumed in the infiltration interface. Batch experiments show that denitrification did not occur after 28 days of soil incubation under anaerobic conditions, while the presence of genes involved in denitrification (*nirS*, *nirK*, and *narG*) was detected, and nitrate reduction was observed in the deep part of the pilot in the early months of treated wastewater application. All the (bio)indicators (gene detection, batch experiments, and *in situ* chemical analysis) suggest, however, that no sulfate reduction occurred in the soil column: genes involved in sulfate reduction (*dsrAB*) were not detected, batch experiments showed no activity, and concentrations of SO_4 changed little as wastewater passed through the soil column during the first 6 months of treated wastewater application [39]. All these (bio)indicators do not carry the same information on soil microbial properties and abilities (microbial metabolisms presence or expression). However, they are complementary for the microbial characterization of soils and its potential evolution over time. As shown above, the potential microbial activities may be present but not expressed in the pilot. The microbial potential for treated wastewater treatment within the soil column is, therefore, greater than that actually observed during the experiment. Modifications of chemical and physical parameters such as wastewater composition, temperature, and water precipitation due to seasons, punctual climatic events (*e.g.*, extreme rain events), as well as the evolution of physical and chemical conditions in the soil column, may lead to the expression of the potential activities detected by bioindicators (*e.g.*, denitrification) [5, 17, 52]. During the 6 months of pilot functioning, no extreme modification (in terms of rain events, temperature variation…) occurred [39]; this could explain that no significant modification of microbial activities was detected in the pilot. From a bioremediation point of view, it is thus quite important to determine the physical, chemical, and microbiological properties of soil as well as its biogeochemical potential activities to take benefit of all soil (bio)properties. Changes in soil conditions may significantly modify the SAT performance. For example, it has been shown that the decrease of infiltration rates due to soil clogging in the uppermost soil layer favored anaerobic conditions below the infiltrative surface and the development of biogeochemical processes such as reductive dissolution of Mn and Fe, resulting in release of trace elements (Mn, Fe, As, Ni) [39]; on the other hand, the interruption of treated wastewater supply can lead to dry periods, nitrification (release of NO_3), and hydric stress for microbes. Monitoring of bioindicators such as total microbial biomass, bacterial diversity, and soil enzyme activities is thus essential to improve the management of groundwater under artificial recharge regime.

To conclude, this study highlights the rapid reactivity of microbial communities in a soil fed by treated wastewater. All the biological (biomass, gene presence, biodiversity, soil enzyme activities) and non-biological (chemical analysis)

parameters used to characterize biological and chemical modifications occurring in the soil column bring evidence enabling to better constraint both the soil biology and soil quality. This underlines that the use of various monitoring approaches gives a good insight of soil microbiology, and that soil enzyme activities, microbial biomass, and biodiversity analyses are good tools to study soil microbial response to an environmental stress. Our results suggest that the biological potential of soil for wastewater treatment can be present but not expressed. Thus, by identifying and then modifying the physical and chemical parameters that specifically impact/enhance soil biological activities, it should be possible to optimize the bioremediation properties of soil during the SAT.

Acknowledgments

This work was supported by VERI and BRGM (PDR10EAU47/BIO Project). We are especially indebted to André Burnol for valuable discussions on the interpretation of the results. We also thank Mickael Beaulieu, Pierre Galle-Cavalloni, and Stéphanie Coulon for technical support. We are grateful to two anonymous reviewers and Dr. Kaizhi Xie for their detailed and thorough reviews.

References

1. Adrover M, Farrús E, Moyà G, Vadell J. 2012. Chemical properties and biological activity of Mallorca following twenty years of treated wastewater irrigation. *J. Environ. Manage.* **95:** S188-S192.
2. Agnelli A, Ascher J, Corti G, Ceccherini MT, Nannipieri P, Pitramellara G. 2004. Distribution of microbial communities in a forest soil profile investigated by microbial biomass, soil respiration and DDGE of total and extracellular DNA. *Soil Biol. Biochem.* **36:** 859-868.
3. Alkorta I, Aizpurua A, Riga P, Albizu I, Amézaga I, Garbisu C. 2003. Soil enzyme activities as biological indicators of soil health. *Rev. Environ. Health* **18:** 65-73.
4. Alkorta I, Amezaga I, Albizu I, Aizpurua A, Onaindia M, Buchner V, Garbisu C. 2003. Molecular microbial biodiversity assessment: a biological indicator of soil health. *Rev. Environ. Health* **18:** 131-151.
5. Antunes S, Pereira R, Marques SM, Castro BB, Gonçalves F. 2011. Impaired microbial activity caused by metal pollution: a field study in a deactivated uranium mining area. *Sci. Total Environ.* **410:** 87-95.
6. Arias M, González-Pérez J, González-Vila F, Ball A. 2005. Soil health – a new challenge for microbiologists and chemists. *Int. Microbiol.* **8:** 13-21.
7. Blume E, Bischoff M, Reichert JM, Moorman T, Konopka A, Turco RF. 2002. Surface and subsurface microbial biomass, community structure and metabolic activity as a function of soil depth and season. *Appl. Soil Ecol.* **20:** 171-181.
8. Bouwer H. 1985. Renovation of wastewater with rapid-infiltration land treatment systems, pp. 249-282. *In* Asano T (ed.). *Artificial Recharge of Groundwater*. Butterworth Publishers, Boston.
9. Bouwer H, Pyne G, Goodrich J. 1990. Recharging groundwater. *Civil Eng.* **60:** 63-66.
10. Braker G, Fesefeldt A, Witzel K-P. 1998. Development of PCR primer systems for amplification of nitrite reductase genes (*nirK* and *nirS*) to detect denitrifying bacteria in environmental samples. *Appl. Environ. Microbiol.* **64:** 3769-3775.
11. Bru D, Sarr A, Philippot L. 2007. Relative abundances of proteobacterial membrane-bound and periplasmic nitrate reductases in selected environments. *Appl. Environ. Microbiol.* **73:** 5971-5974.
12. Burger M, Jackson LE, Lundquist EJ, Louie DT, Miller RL, Rolston DE, Scow KM. 2005. Microbial responses and nitrous oxide emissions during wetting and drying of organically and conventionally managed soil under tomatoes. *Biol. Fertil. Soils* **42:** 109-118.
13. Burgin AJ, Yang WH, Hamilton SK, Silver WL. 2011. Beyond carbon and nitrogen: how the microbial energy economy couples elemental cycles in diverse ecosystems. *Front. Ecol. Environ.* **9:** 44-52.
14. Chen Z, Liu Z, Wu M, Xie X, Wu J, Wei W. 2012. Differentiated response of denitrifying communities to fertilization regime in paddy soil. *Microb. Ecol.* **63:** 446-459.
15. Dequiedt S, Saby NPA, Lelievre M, Jolivet C, Thioulouse J, Toutain B, *et al*. 2011. Biogeographical patterns of soil molecular biomass as influenced by soil characteristics and management. *Global Ecol. Biogeogr.* **20:** 641-652.
16. Deshmukh S, Singh A, Datta S, Annapurna K. 2011. Impact of long-term wastewater application on microbiological properties of vadose zone. *Environ. Monit. Assess.* **175:** 601-612.
17. Dick R. 1997. Soil enzyme activities as integrative indicators of soil health, pp. 121-156. *In* Pankhurst CE, Doube BM, Gupta VVSR (eds.). *Biological Indicators of Soil Health*. CAB International, New York, USA.
18. Dick A, Cheng L, Wang P. 2000. Soil acid and alkaline phosphatase activity as pH adjustment indicators. *Soil Biol. Biochem.* **32:** 1915-1919.
19. Fierer N, Schimel JP, Holden P. 2003. Variations in microbial community composition through two soil depth profiles. *Soil Biol. Biochem.* **35:** 167-176.
20. Foppen JWA. 2002. Impact of high-strength wastewater infiltration on groundwater quality and drinking water supply: the case of Sana'a, Yemen. *J. Hydrol.* **263:** 198-216.
21. Foppen JWA, van Herwerden M, Kebtie M, Noman A, Schijven JF, Stuyfzand PJ, Uhlenbrook S. 2008. Transport of

Escherichia coli and solutes during waste water infiltration in an urban alluvial aquifer. *J. Contam. Hydrol.* **95:** 1-16.
22. Fox P, Narayanaswamy K, Genz A, Drewes J. 2001. Water quality transformations during soil aquifer treatment at the Mesa Northwest water reclamation plant, USA. *Water Sci. Technol.* **43:** 343-350.
23. Gleeson T, VanderSteen J, Sophocleous MA, Taniguchi M, Alley WM, Allen DM, Zhou Y. 2010. Groundwater sustainability strategies. *Nat. Geosci.* **3:** 378-379.
24. Henry S, Baudoin E, Lopez-Gutierrez J, Martin-Laurent F, Baumann A, Philippot L. 2004. Quantification of denitrifying bacteria in soils by *nirK* gene targeted real-time PCR. *J. Microbiol. Methods* **59:** 327-335.
25. Hinojosa MB, García-Ruíz, Viñegla B, Carreira J. 2004. Microbiological rates and enzyme activities as indicators of functionality in soils affected by the Aznalcóllar toxic spill. *Soil Biol. Biochem.* **36:** 1637-1644.
26. Hunt JR, Holden PA, Firestone MK. 1995. Coupling transport and biodegradation of VOCs in surface and subsurface soils. *Environ. Health Perspect.* **103:** 75-78.
27. Khan S, El-Latif Hesham A, Qiao M, Rehman S, He J-Z. 2010. Effects of Cd and Pb on soil microbial community structure and activities. *Environ. Sci. Pollut. Res.* **17:** 288-296.
28. Klein M, Friedrich M, Roger AJ, Hugenholtz P, Fishbain S, Abicht H, *et al*. 2001. Multiple lateral transfers of dissimilatory sulfite reductase genes between major lineages of sulfate-reducing bacteria. *J. Bacteriol.* **183:** 6028-6035.
29. Lance JC, Whisler FD. 1972. Nitrogen balance in soil columns intermittently flood with secondary sewage effluent. *J. Environ. Qual*. **1:** 180-186.
30. Leggate J, Allain R, Isaac L. 2006. Microplate fluorescence assay for the quantification of double stranded DNA using SYBR Green I dye. *Biotechnol. Lett.* **28:** 1587-1594.
31. Malkawi H, Mohammad M. 2003. Survival and accumulation of microorganisms in soils irrigated with secondary treated wastewater. *J. Basic Microbiol.* **43:** 47-55.
32. Masto R, Chhonkar P, Singh D, Patra A. 2009. Changes in soil quality indicators under long-term sewage irrigation in a sub-tropical environment. *Environ. Geol.* **56:** 1237-1243.
33. Michelland R, Dejean S, Combes S, Fortun-Lamothe L, Cauquil L. 2009. StatFingerprints: a friendly graphical interface program for processing and analysis of microbial fingerprint profiles. *Mol. Ecol. Resour.* **9:** 1359-1363.
34. Miller GW. 2006. Integrated concepts in water reuse: managing global water needs. *Desalination* **187:** 65-75.
35. Miller JH, Ela WP, Lansey KE, Chipello PL, Arnold RG. 2006. Nitrogen transformation during soil aquifer treatment of wastewater effluent-oxygen effects in field studies. *J. Environ. Eng.* **132:** 1298-1306.
36. Nicolaisen M, Ramsing N. 2002. Denaturing gradient gel electrophoresis (DGGE) approaches to study the diversity of ammonia-oxidizing bacteria. *J. Microbiol. Methods* **50:** 189-203.
37. Nielsen MN, Winding A. 2002. Microorganisms as indicators of soil health. *In: Technical Report No. 388*. National Environmental Research Institute, Denmark.
38. Oki T, Kanae S. 2006. Global hydrological cycles and world water resources. *Science* **313:** 1068-1071.
39. Ollivier P, Surdyk N, Azaroual M, Besnard K, Casanova J, Rampnoux N. 2013. Linking water quality changes to geochemical processes occurring in reactive soil column during treated wastewater infiltration using large-scale pilot experiment: insights into Mn behavior. *Chem. Geol*. **356:** 109-125.
40. Or D, Smets BF, Wraith J, Dechesne A, Friedman S. 2007. Physical constraints affecting bacterial habitats and activity in unsaturated porous media - a review. *Adv. Water Resour.* **30:** 1505-1527.
41. Pavelic P, Dillon PJ, Mucha M, Nakai T, Barry KE, Bestland E. 2011. Laboratory assessment of factors affecting soil clogging of soil aquifer treatment systems. *Water Res.* **45:** 3153-3163.
42. R Development Core Team. 2004. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL http://www.R-project.org.
43. Rajeb AB, Kallali H, Aissa NB, Bouzaiene O, Jellali S, Jedidi N, Hassen A. 2009. Soil microbial growth and biofilm expansion assessment under wastewater infiltration percolation treatment process: column experiments. *Desalination* **246:** 514-525.
44. Rodell M, Velicogna I, Famiglietti JS. 2009. Satellite based estimates of groundwater depletion in India. *Nature* **460:** 999-1002.
45. Rubol S, Silver WL, Bellin A. 2012. Hydrologic control on redox and nitrogen dynamics in a peatland soil. *Sci. Tot. Environ*. **432:** 37-46.
46. Rudaz AO, Davidson EA, Firestone MK. 1991. Sources of nitrous-oxide production following wetting of dry soil. *FEMS Microbiol. Ecol*. **85:** 117-124.
47. Schimel J, Balser TC, Wallenstein M. 2007. Microbial stress-response physiology and its implications for ecosystem function. *Ecology* **88:** 1386-1394.
48. Silver WL, Lugo AE, Keller M. 1999. Soil oxygen availability and biogeochemistry along rainfall and topographic gradients in upland wet tropical forest soils. *Biogeochemistry* **44:** 301-328.
49. Srinivasulu M, Jaffer Mohiddin G, Subramanyam K, Rangaswamy V. 2012. Effect of insecticides alone and in combination with fungicides on nitrification and phosphatase activity in two groundnut (*Arachis hypogeae* L.) soils. *Environ. Geochem. Health* **34:** 365-374.
50. Thioulouse J, Chessel D, Dolédec S, Olivier JM. 1997. ADE-4: a multivariate analysis and graphical display software. *Stat. Comput*. **7:** 75-83.
51. Toze S, Hanna J, Smith T, Edmonds L, McCrow A. 2004.

Determination of water quality improvements due to the artificial recharge of treated effluent, pp 53-60. *In* Steenvoorden J, Endreny T (eds.). *International Symposium on Wastewater Re-Use and Groundwater Quality*. International Association of Hydrological Sciences, Wallingford, UK.

52. Turner B, Baxter R, Whitton B. 2002. Seasonal phosphatase activity in three characteristic soils of the English uplands polluted by long-term atmospheric nitrogen deposition. *Environ. Pollut.* **120:** 313-317.
53. Wada Y, van Beek LPH, van Kempen CM, Reckman JWTM, Vasak S, Bierkens MFP. 2010. Global depletion of groundwater resources. *Geophys. Res. Lett.* **37:** L20402, DOI: 10.1029/2010GL044571.
54. Wada Y, van Beek LPH, Bierkens MFP. 2012. Nonsustainable groundwater sustaining irrigation: a global assessment. *Water Resour. Res.* **48:** W00L06, DOI: 10.1029/2011WR010562.
55. Wagner M, Roger A, Flax J, Brusseau G, Stahl D. 1998. Phylogeny of dissimilatory sulfite reductases supports an early origin of sulfate respiration. *J. Bacteriol.* **180:** 2975-2982.
56. Zhang X, Zhao X, Zhang M. 2012. Functional diversity changes of microbial communities along a soil aquifer for reclaimed water recharge. *FEMS Microbiol. Ecol.* **80:** 9-18.