

Spatiotemporal distribution of microbial communities in a coastal, sandy aquifer system (Doñana, SW Spain)

SERGIO VELASCO^{1,2}, MARÍA DEL CARMEN GUERRERO¹, CARLOS MONTES¹ AND ANA ISABEL LÓPEZ-ARCHILLA¹

¹Departamento de Ecología, Facultad de Ciencias, Universidad Autónoma de Madrid, Cantoblanco, 28049 Madrid, Spain

²Dirección de Estudios, Agua y Medio Ambiente, Área de Medio Ambiente Hidrico, Centro de Estudios Hidrográficos, CEDEX, Ministerio de Fomento, 28005 Madrid, Spain

ABSTRACT

The aquifer system of Doñana (SW Spain) represents the most important freshwater source in the Doñana Natural Area. Its spatiotemporal dynamics favours the hydrological connection between surface and subsurface ecosystems, and promotes matter fluxes among the different terrestrial and aquatic systems present here. This aquifer has been intensively studied from a hydrogeological point of view but little is known from an ecological perspective. In order to understand the ecological roles played by microbial communities in this system, we conducted a long-term seasonal study of bacterial abundance, cell biomass, bacterial biomass and functional activities over a 2-year period. Bacterial abundance ranged between $2.11 \pm 1.79 \times 10^5$ and $8.58 \pm 6.99 \times 10^7$ **bact mL⁻¹** groundwater, average cell biomass was estimated to be 77.01 ± 31.56 fgC and bacterial biomass varied between $8.99 \pm 4.10 \times 10^{-2}$ and 5.65 ± 0.70 μ gC mL⁻¹. Iron-related bacteria showed the highest activities among the functional groups studied. Moreover, among the variables that usually control spatial distributions of microbial communities in aquifer systems, depth did not have a relevant effect on this aquifer, at least in the range of depths studied, but grain size, probably due to its direct effects on hydrogeological parameters, such as permeability or porosity, appeared to exert moderate control, principally in terms of bacterial abundance. Finally, significant seasonal differences in the means of these microbiological variables were also observed; temperature seems to be the main factor controlling the temporal distribution of microbial communities in this aquifer system.

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Corresponding author: Sergio Velasco, Tel.: xxxxxxxxxx; fax: xxxxxxxxxx; e-mail: sergio.velasco@uam.es.

INTRODUCTION

The paradigm in the study of aquifer systems has changed over the last few decades from a pure hydrogeological point of view to a more ecological one (Danielopol, 1989; Baker *et al.*, 2000). As a consequence, aquifer systems are currently considered to constitute a heterogeneous assemblage of discrete macro- and microscale habitats, providing a variety of living conditions (Goldscheider *et al.*, 2006).

From an ecological standpoint, aquifer systems should be regarded as open systems interchanging materials and energy with other aquatic and terrestrial systems located in the vicinity (Danielopol, 1989; Chapelle, 2000b; Danielopol *et al.*, 2003). The recognition of interactions occurring within and among surrounding environments over a range of scales will allow for a better understanding of the ecological roles played by

microbial communities in aquifer systems (Brockman & Murray, 1997; Bennett *et al.*, 2000; Musslewhite *et al.*, 2003; Hancock *et al.*, 2005). However, the identification of such interactions requires long-term spatiotemporal studies, very rare to date. Multidisciplinary studies would be of use in this identification only if all the approximations share similar spatiotemporal scales (Brockman & Murray, 1997; Musslewhite *et al.*, 2003).

Microbial ecology studies of sedimentary, relatively shallow aquifer systems are relatively abundant (Marxsen, 1988; Alfrieder *et al.*, 1997; Martino *et al.*, 1998; Griebler *et al.*, 2002; Velasco *et al.*, 2008; in press), but very few exist with large spatial and temporal scales. Some of these studies have shown aquifers as more stable ecosystems, with less spatiotemporal variation in their ecological processes, than other aquatic systems. The main goal of the present study, conducted in an area of approximately

1 100 km² over a 2-year period, is to enhance our knowledge of
 2 the microbial communities existing in the aquifer system of
 3 Doñana, an ecosystem in which there have been only a few
 4 studies based on a biological or an ecological approach. This
 5 aquifer is becoming to be considered as part of a great ecosystem,
 6 called the *hydroecosystem*, made up of several aquatic and
 7 terrestrial systems, and located in the Doñana Natural Area
 8 (Montes *et al.*, 1998; Manzano & Custodio, 2007; Manzano
 9 *et al.*, 2007). All these ecosystems in the Doñana Natural Area
 10 make it unique in many senses: it is a major stepping-stone in
 11 the migration route of birds moving between Europe and Africa,
 12 it is home to the most endangered mammals in the world, as
 13 well as many endemic, threatened or ecologically interesting
 14 species, and it contains what is possibly Europe's most
 15 significant wetland. Consequently, there has been an increasing
 16 number of interdisciplinary studies in the last few years,
 17 and groups of limnologists, microbiologists, hydrogeologists,
 18 economists and sociologists are working together in order
 19 to obtain a general overview of the ecological functioning of
 20 Doñana's hydroecosystem (Manzano *et al.*, 2007).

21 Bearing in mind that the most important freshwater source
 22 in the Doñana Natural Area is the groundwater, and that the
 23 aquifer controls the hydrological regime of all other ecosystems
 24 located in this area, it is important to understand the role played
 25 by microbial communities present in the aquifer system. To
 26 this end, the first step is to define these microbial communities
 27 in terms of bacterial abundance and cell biomass (Murphy &
 28 Schramke, 1998), because this information will enable the
 29 potential activity of the population to be identified (Bratbak,
 30 1993). Knowledge of factors, such as depth, grain size or
 31 temperature, controlling spatial and temporal patterns in
 32 bacterial abundances, cell biomasses and microbial activities
 33 are also important to characterize the distribution and dynamics
 34 of these communities. The results of this paper complement
 35 and consolidate others resulting from a parallel, intensive study,
 36 in which microbial communities present in the groundwater
 37 located in the surroundings of four very productive shallow
 38 lakes were studied by sampling 13 piezometers, different from
 39 those employed in this paper (Velasco *et al.*, 2008; in press).

41 MATERIALS AND METHODS

43 Site description

45 The Doñana aquifer system is included in the Doñana Natural
 46 Area and located in the SW Atlantic coast of Spain. The aquifer
 47 has a surface area of around 3000 km² and hosts the
 48 Guadalquivir river marshes and Doñana National and Natural
 49 Parks, two protected natural areas of international relevance
 50 comprising approximately 1100 km² (Fig. 1). They were
 51 declared Biosphere Reserves in 1981, RAMSAR Sites in 1982
 52 and Natural World Heritage Sites in 1994. The climate is
 53 Mediterranean subhumid with Atlantic influence: dry summers
 54 and wet winters. Mean rainfall, mainly concentrated from

October to March, is 500–600 mm, but has a high interannual
 variability. Mean yearly temperature is around 17 °C near the
 coast and 18 °C in the centre of the Natural Area (Manzano
et al., 2007).

The aquifer system consists of detrital, unconsolidated
 Plio-Quaternary sediments overlapping impervious Miocene
 marine marls. The Pliocene materials are impermeable marls,
 silts and sandy silts. The Quaternary materials consist of deltaic
 and alluvial silts, sands and gravels to the north, and on littoral,
 alluvial and aeolian sands to the west. They mainly comprise
 amorphous silica grains. Carbonates may be present either as
 detrital grains or as shell remains, except in the upper part of
 the aeolian sand layers of the western sector (Manzano *et al.*,
 2007). The Quaternary layers thicken from N to S and from
 W to SE. To the SE, the coarse sediments are covered by a
 thick (50–80 m) sequence of estuarine and marshy clays. The
 aquifer system varies in thickness, ranging from 20 m inland to
 over 150 m at the coast line. At regional scale, the aquifer
 system presents two lithological domains: a sandy one to the
 N and W of the marshes, which extensive areas of aeolian sands,
 which roughly behaves as an unconfined aquifer, and a clayey
 one in the marsh area, under which a large confined aquifer is
 found (Manzano *et al.*, 2007). In the aeolian sands, there are
 two different lithological subdomains (called upper and lower
 units) that allow for the presence of two different hydrodynamic
 units. The thick and fine to medium sand deposits of the upper
 unit conform with a relatively homogeneous phreatic aquifer
 that contains the water table and overlies a lower, less homo-
 geneous and semiconfined aquifer composed of coarse sands and
 gravels (Trick & Custodio, 2004). The hydraulic transmissivity
 of the lower, thinner aquifer is higher than that of the upper
 one. Between the upper and the lower units there is an inter-
 mediate layer of grey clays and fine to medium clayed sands
 containing iron oxide minerals. Recharge occurs via direct rain
 infiltration in the aeolian sands (Trick & Custodio, 2004).
 Groundwater mainly flows eastward from aeolian sands in the
 west to the ecotone and the marsh in the east (Manzano *et al.*,
 2007) (Fig. 1). Natural discharge takes place to the ocean,
 rivers and ravines, to many small phreatic shallow lakes
 situated above the aeolian mantles (Coletto, 2003), and through
 phreatic evapotranspiration (Manzano & Custodio, 2007). In
 general, there are vertical, descending flows in recharge areas
 and vertical, ascending flows in discharge areas and ground-
 water extraction points (Trick & Custodio, 2004).

Sampling procedure: physical and chemical variables

Groundwater samples were collected seasonally (winter 2003
 to winter 2005) from 17 piezometers located in an area
 encompassing approximately 100 km² (Fig. 1). Except well piso
 (installed and monitored by the Universidad Autónoma de
 Madrid, UAM), all boreholes were installed and are monitored
 by the Spanish Geological Survey and the Guadalquivir River
 Basin Authority. Screen depth of piezometers ranges from

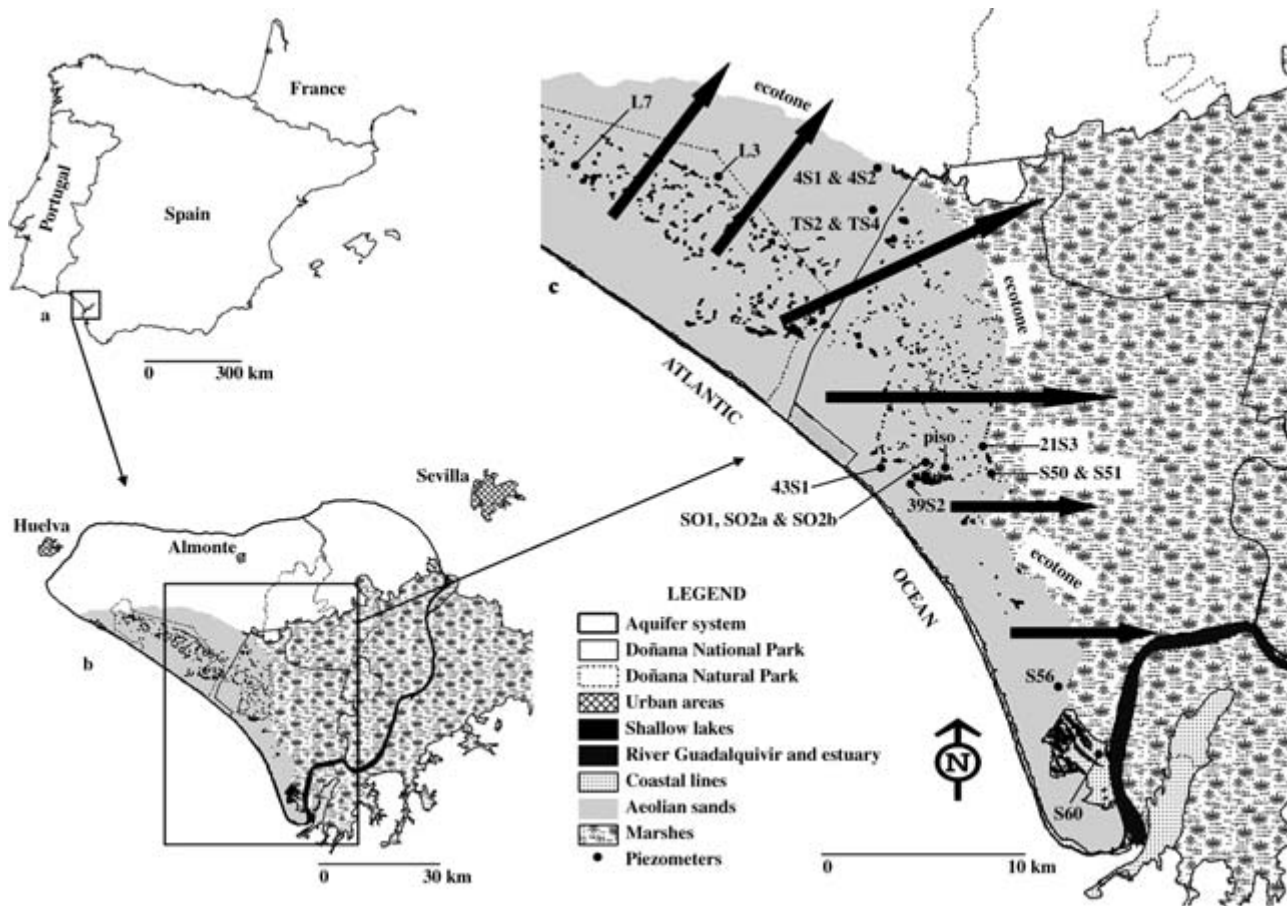


Fig. 1 Geographical location of the Doñana Natural Area on the south-west coast of the Iberian Peninsula (A). The figure also shows the boundary of the aquifer system of Doñana, the boundaries of the Doñana National and Natural Parks, as well as the most important ecosystems in the Doñana Natural Area (B). Both shallow lakes and the 17 studied piezometers are also shown. Arrows indicate the general direction of the regional groundwater flow from east (aeolian sands) to west (marshes) (C).

3.00 to 80.00 meters below land surface (mbls) and all are located in the aeolian sands (both in the upper and in the lower unit) (Table 1).

Groundwater samples were collected following chemical (Dunlap *et al.*, 1977) and microbiological (Fredrickson & Phelps, 1997) standard procedures. A special pneumatic pump was employed to avoid mixing processes between groundwater and retained water in the piezometer (Danielopol & Niederreiter, 1987). Groundwater was pumped from each well until temperature, dissolved oxygen (DO), pH and electric conductivity readings stabilized (Manzano *et al.*, 2007). All physical parameters were measured with a WTW 340i handheld multiparameter device. Chemical parameters (alkalinity, ammonium, nitrate, nitrite, soluble reactive phosphorus (SRP) and total phosphorus (TP)) were estimated by means of standard methods during the 10 following days (APHA *et al.*, 1987); alkalinity was analysed during the same sampling day (APHA *et al.*, 1987). Ferrous and ferric iron concentrations, as well as total iron, were determined by the ferrozine colorimetric method (Viollier *et al.*, 2000).

Microbiological variables

Groundwater samples for microbiological variables were collected from each well in triplicate, stored in 100 mL polyurethane bottles (prewashed in 5% HCl and distilled water), and fixed with formaldehyde (2% v/v final concentration). Samples were stored in the dark at 4 °C until the filtration process. Bacterial abundance was determined by epifluorescence microscopy after staining with DAPI (4',6-diamidino-2-phenylindole-dihydrochloride) 1 µg mL⁻¹ final concentration (Fry, 1990). The solution was then filtered through a 0.2-µm pore size GTBP 25 mm Ø Millipore filter (Millipore Inc., Billerica, MA, USA). Filters were transferred to a labelled microscope slide and kept frozen until samples could be counted (Bölter *et al.*, 2002). Three different filters were prepared for each sampled well per season, and 20 different fields were viewed and counted for each filter (Kirc *et al.*, 1982) with an Olympus IX50 inverted microscope. The results of the counts for bacterial abundance are presented as bact mL⁻¹ groundwater. Length and width measurements of bacterial cells under a magnification

Table 1 Geographical, lithological and hydrogeological features of the selected piezometers

Piezometer	UTM X (29)	UTM Y (29)	Altitude (masl)	Screen Depth (mbls)	Hydrogeological Unit*	Screen Lithology	Transmissivity (m ² day ⁻¹) [†]	Permeability (m day ⁻¹) [†]	Contamination [†]
Piso	724598	4095905	5.50	2.00–2.25	U	Fine to medium sands			
S51	727730	4097026	2.00	3.00–7.00	U	Fine sands	1.13	0.28	
21S3	727969	4101313	4.00	5.40–8.20	U	Fine sands			
4S1	722485	4111933	4.38	8.00–10.00	U	Fine to medium sands			Nitrate
L3	711081	4111414	43.00	8.00–10.00	U	Fine to medium sands	0.20	0.10	
L7	700031	4113890	69.00	8.00–10.00	U	Coarse sands			Nitrate
39S2	723848	4095698	5.70	8.50–11.40	U	Fine to medium sands			
TS4	719101	4112389	16.30	10.00–11.00	U	Fine to medium sands	3.00	3.00	Nitrate
43S1	722031	4096458	11.30	11.50–14.30	U	Fine to medium sands	0.13	0.05	
S60	734318	4080575	3.00	16.00–17.00	U	Fine sands			
TS2	719101	4112389	16.30	18.00–19.00	INT	Clays	0.50	0.50	Nitrate
SO2a	724189	4096032	6.00	44.00–46.00	U	Fine to medium sands			
4S2	722485	4111933	4.57	36.50–43.50	L	Clays and fine sands			Nitrate
SO2b	724189	4096032	6.00	44.00–46.00	U	Fine to medium sands			
S50	727730	4097026	3.00	52.00–60.00	L	Gravels and coarse sands			
S56	733010	4087500	2.00	74.00–80.00	L	Gravels and coarse sands			
SO1	724188	4096038	6.00	67.00–72.00	U	Fine to medium sands			

*Location of wells in the hydrogeological units (U, upper; L, lower; INT, intermediate).

[†]Data from Trick 1998.

masl, metres above sea level; mbls, metres below land surface.

of $\times 1000$ were used to determine the cell biovolume; 60 cells were measured in each filter and were classified as cocci, small and large rods, and filamentous bacteria. Biovolumes of bacterial cells were calculated assuming that the shapes of the cells were either perfect spheres or cylinders with hemispheres on both sides (Fry, 1990). Linear dimensions were converted to volumetric units using geometric formulas (Bölter *et al.*, 2002). Cell biomass was calculated from the cell biovolume using a conversion factor based on the allometric model with the formula $C = 120 \times V^{0.7}$, where C = cell biomass in fgC and V = cell biovolume in μm^3 (Psenner, 1993). Cell biomass is presented as fgC. The product of the bacterial abundance and the cell biomass is the bacterial or population biomass, which is shown as $\mu\text{gC mL}^{-1}$ groundwater.

Evidence of microbial activity for some functional groups of bacteria (nitrifying (NB), denitrifying (DNB), iron related (IRB), and sulphate-reducing bacteria (SRB)) was provided by commercial biological activity reaction tests (BARTTM) (Cullimore, 1993). A BARTTM test is a simple and effective method for detecting the presence and the relative potential activity of a specific functional group of microorganisms. Briefly, three 10 mL replicates of groundwater were incubated during 10 days at room temperature for each functional group. The first day following incubation in which the activity was detected, was selected as the start of activity by a specific functional group. The more time required for the detection of a specific activity, the less activity the functional group showed. Assays were carried out during winters and summers (years 2003 and 2004).

Hydrogeological variables

Transmissivity and permeability values for some piezometers were previously measured (Trick & Custodio, 2004). Rainfall data were obtained with permission (Spanish Meteorological Service, INM) from a sampling weather station located in Doñana National Park.

Statistical analyses

Differences in the means of bacterial abundance, cell biomass and bacterial biomass among wells were tested, for each sampling campaign, using a one-way MANOVA test. Temporal differences in the means of the same microbiological variables for each well sampled more than three times during the study period were also tested by means of a one-way MANOVA test. Two different MANOVAs had to be performed to test the effect of one out of the two different factors (wells or seasons) because the data matrix was not regular. In other words, not all the piezometers were sampled during the same sampling campaigns, or at least the same three sampling campaigns. However, all piezometers were taken into account for statistical purposes when spatial differences were searching. Univariate *F*-tests were also performed after MANOVA testing. Tukey's honestly significant difference test (HSD test) was employed, as an *a posteriori* method, to compare pairs of means after MANOVA testing. Differences in the means for microbial activities of functional groups were tested, for each sampling campaign, using a one-way ANOVA test. Temporal differences in the means

of microbial activities during both years for each functional group were tested using a one-way ANOVA test. Tukey's HSD test was employed, as an *a posteriori* method, to compare pairs of means after ANOVA testing. Homogeneity of variances was tested with the Levene test (Zar, 1998). Relationships among measured variables were explored using Pearson product moment correlations or Spearman rank order correlations, depending on whether the dataset in question was parametric or nonparametric in nature. Normality was examined using the Kolmogorov–Smirnov test, and variables were transformed when necessary and when possible. Relationships between physicochemical (temperature, DO, pH, electric conductivity, ammonium, nitrate, SRP, TP, ferric iron, ferrous iron, total iron and alkalinity) and microbiological (bacterial abundance, cell biomass and bacterial biomass) variables were explored throughout a canonical correlation analysis; Bartlett's χ^2 test was used for testing the significance of the correlations between any pairs of the canonical variates (Quinn & Keough, 2002). A principal component analysis (PCA), with seasons as the grouping variable, was performed as a multivariate ordination method to compare different seasons and to detect temporal patterns. Data were standardized for PCA (Legendre & Legendre, 1998). A *P*-value of 0.05 was set as the significant threshold for statistical analyses. All statistical analyses were performed with Statistica 6.0 for Windows (Statsoft, Inc., Tulsa, OK, USA).

RESULTS

Physicochemical variables

Groundwater temperature showed mean values of approximately 19 °C; these values increased throughout the year during both years (Table 2). Dissolved oxygen exhibited mean values of 2 mg L⁻¹, although with a different temporal pattern, with higher winter and spring values than those for summer or autumn. Therefore, temperature and DO negatively correlated ($r = -0.341$, $P = 0.004$, $n = 71$). The pH was very constant and ranged from 6 to 7 in most of the wells (Table 2). Electric conductivity (EC) values were measured around 300–400 $\mu\text{S cm}^{-1}$; however, in some piezometers located in the ecotone, close to the marsh (Fig. 1), conductivities were higher.

Among the inorganic nitrogen forms, nitrate concentrations (mg L⁻¹ N-NO₃⁻) were higher than ammonium (mg L⁻¹ N-NH₄⁺) or nitrite concentrations (mg L⁻¹ N-NO₂⁻; data not shown) in most of the boreholes (Table 3). In some wells, nitrate concentrations were very high (Table 3). Soluble reactive phosphorus (mg L⁻¹ P-PO₄³⁻) and TP (mg L⁻¹ P) showed seasonal concentrations of approximately 0.05 mg L⁻¹ and 0.08 mg L⁻¹, respectively (Table 3). Ferric iron usually displayed higher concentrations than ferrous iron. Total iron concentration ranged from 1 to 2 mg L⁻¹ in most of the wells, and reached maximum values of approximately 20 mg L⁻¹ (Table 3). Total alkalinity values were around 1 meq L⁻¹ (Table 2).

Microbiological variables

Only prokaryotic cells were observed in microscope counts, with dominance of rod-shaped bacteria. No differences among cell morphologies were observed in samples from different depths or from different sites. Average bacterial abundance was $1.70 \times 10^7 \pm 1.99 \times 10^6$ bact mL⁻¹ groundwater. Significant differences for bacterial abundance among piezometers were observed during the first three sampling campaigns in 2003 ($P \leq 0.039$). Seasonal differences for bacterial abundance in wells sampled more than three times were observed ($P \leq 0.047$) except in boreholes 39S2, 43S1, SO2a and SO2b ($P \geq 0.156$) (Fig. 2A,B). Bacterial abundances were statistically higher during summer or autumn 2003 than during winter or spring 2003 ($P \leq 0.042$), except for wells 43S1, SO2a, SO2b and SO1 ($P \geq 0.143$). During 2004, no statistical differences were observed among seasons ($P \geq 0.256$). This variable positively correlated with temperature ($r = 0.386$, $P = 0.001$, $n = 71$), nitrate ($r = 0.430$, $P = 0.000$, $n = 70$) and cell biomass ($r = 0.335$, $P = 0.004$, $n = 71$), whereas it negatively correlated with DO ($r = -0.237$, $P = 0.047$, $n = 71$), ferrous iron ($r = -0.316$, $P = 0.034$, $n = 41$) and total iron ($r = -0.265$, $P = 0.036$, $n = 63$). Bacterial abundances in wells S51, SO2a, SO2b and SO1 negatively correlated with rainfall from 2 months prior to sampling ($P \leq 0.046$). No correlations among bacterial abundance, screen depth, permeability and transmissivity were found, except during spring 2003, when bacterial abundance and depth positively correlated ($r = 0.890$, $P = 0.009$, $n = 8$).

Mean cell biomass was determined to be 77 ± 32 fgC (Fig. 2C,D). Cell biomass showed significant differences among piezometers during all seasons ($P \leq 0.048$), except in winter 2005 ($P \geq 0.109$). Significant seasonal differences for cell biomass were observed in piezometers sampled more than three times during the study period ($P = 0.000$), except in well TS4 ($P = 0.204$). A significant increase in cell biomass during 2003 was observed toward the warmer seasons in all piezometers ($P \leq 0.004$), except in wells TS4 and SO2a ($P \geq 0.654$). A significant, although less evident, increase in cell biomass throughout 2004 was observed in wells S51, 4S1, SO2a, SO2b and SO1, with statistical differences between winter or spring and summer or autumn ($P \leq 0.023$). Cell biomass positively correlated with temperature ($r = 0.487$, $P = 0.000$, $n = 71$) and negatively with DO ($r = -0.242$, $P = 0.037$, $n = 71$). In wells S51 and SO1 cell biomass negatively correlated with rainfall from 2 months prior to sampling ($P \leq 0.028$). No significant correlations were found among cell biomass, depth, permeability and transmissivity in any season.

Average bacterial biomass was 1.40 ± 0.57 $\mu\text{gC mL}^{-1}$. Differences in the means of bacterial biomass among piezometers were statistically significant in all seasons ($P = 0.000$), but more evident during 2003 than during 2004. Seasonal differences were observed in all piezometers sampled more than three times throughout the study period ($P = 0.000$) (Fig. 2E,F).

Table 2 Physical variables and alkalinity seasonally measured in the different wells (mean \pm standard deviation) (T, temperature; DO, dissolved oxygen; EC, electric conductivity; win, winter; spr, spring; sum, summer; aut, autumn)

Piezometer	Season	T ($^{\circ}$ C)	DO (mg L $^{-1}$)	pH	EC (μ S cm $^{-1}$)	Alkalinity (meq L $^{-1}$)
Piso	sum-03	19.90 \pm 0.26	2.30 \pm 0.10	6.76 \pm 0.24	202.20 \pm 0.26	0.61 \pm 0.12
	aut-03	20.63 \pm 0.35	1.83 \pm 0.06	7.16 \pm 0.15	172.52 \pm 1.41	0.50 \pm 0.01
	sum-04	22.53 \pm 0.15	2.57 \pm 0.05	6.39 \pm 0.01	263.67 \pm 2.08	0.57 \pm 0.06
	aut-04	21.30 \pm 0.10	2.16 \pm 0.00	6.51 \pm 0.02	210.67 \pm 3.21	0.53 \pm 0.06
	win-05	15.90 \pm 0.20	1.97 \pm 0.01	6.78 \pm 0.07	235.67 \pm 7.37	0.27 \pm 0.11
S51	win-03	14.90	5.48	8.30	366.00	
	spr-03	19.70 \pm 0.28	1.90 \pm 0.49	7.89 \pm 0.01	303.67 \pm 0.09	1.92 \pm 0.01
	aut-03	21.07 \pm 0.41	1.07 \pm 0.04	8.14 \pm 0.11	276.00 \pm 2.64	1.95 \pm 0.06
	spr-04	18.03 \pm 0.15	1.97 \pm 0.07	8.15 \pm 0.13	246.37 \pm 0.57	1.43 \pm 0.08
	aut-04	20.17 \pm 0.05	1.26 \pm 0.00	8.21 \pm 0.01	339.67 \pm 17.95	1.60 \pm 0.00
	win-05	18.70 \pm 0.10	1.86 \pm 0.02	8.04 \pm 0.00	303.67 \pm 3.06	1.27 \pm 0.11
21S3	win-03	17.40	1.12	7.33	184.10	
	spr-03	18.65 \pm 0.63	1.49 \pm 0.48	7.15 \pm 0.21	177.95 \pm 7.70	0.87 \pm 0.01
	sum-03	22.23 \pm 0.05	1.40 \pm 0.17	7.67 \pm 0.01	169.27 \pm 1.25	1.22 \pm 0.10
	aut-03	21.97 \pm 0.40	0.97 \pm 0.15	7.50 \pm 0.12	168.30 \pm 0.98	0.97 \pm 0.08
4S1	spr-03	19.30 \pm 0.04	1.75 \pm 0.07	7.30 \pm 0.16	290.50 \pm 0.26	0.45 \pm 0.00
	sum-03	20.07 \pm 0.05	0.77 \pm 0.15	7.12 \pm 0.14	326.70 \pm 8.46	2.31 \pm 0.28
	aut-03	20.03 \pm 0.40	0.47 \pm 0.12	7.65 \pm 0.41	313.00 \pm 6.92	2.05 \pm 0.05
	aut-04	18.17 \pm 0.15	0.92 \pm 0.02	7.83 \pm 0.00	250.00 \pm 1.02	2.08 \pm 0.08
	sum-04	20.50 \pm 0.19	1.26 \pm 0.04	7.08 \pm 0.01	300.00 \pm 2.64	1.40 \pm 0.20
	aut-04	18.83 \pm 0.37	2.57 \pm 0.00	7.31 \pm 0.00	299.33 \pm 1.15	1.60 \pm 0.00
	win-05	18.30 \pm 0.40	2.59 \pm 0.01	7.09 \pm 0.01	264.00 \pm 0.00	1.07 \pm 0.23
L3	sum-03	19.45 \pm 0.07	3.55 \pm 0.04	6.08 \pm 0.07	74.42 \pm 3.79	0.40 \pm 0.00
	spr-04	19.33 \pm 0.05	4.36 \pm 0.07	5.87 \pm 0.01	66.33 \pm 1.52	0.55 \pm 0.02
L7	win-03	17.30	6.20	6.03	796.00	
	sum-03	19.36 \pm 0.20	2.40 \pm 0.09	6.54 \pm 0.07	760.50 \pm 6.50	1.46 \pm 0.24
	sum-04	22.10 \pm 0.09	1.49 \pm 0.00	6.90 \pm 0.03	463.33 \pm 1.52	1.27 \pm 0.12
	aut-04	19.43 \pm 0.11	1.56 \pm 0.00	6.51 \pm 0.00	474.33 \pm 0.57	1.30 \pm 0.10
39S2	win-04	17.33 \pm 0.15	2.74 \pm 0.10	6.62 \pm 0.01	136.67 \pm 4.72	0.72 \pm 0.17
	spr-04	19.27 \pm 0.05	4.89 \pm 0.04	6.78 \pm 0.02	125.67 \pm 0.57	0.52 \pm 0.5
	sum-04	20.27 \pm 0.15	1.86 \pm 0.07	6.44 \pm 0.00	134.67 \pm 0.57	0.60 \pm 0.00
	aut-04	20.53 \pm 0.15	3.29 \pm 0.00	6.64 \pm 0.01	169.33 \pm 1.52	0.47 \pm 0.06
	win-05	17.80 \pm 0.20	2.89 \pm 0.01	6.70 \pm 0.02	166.67 \pm 0.58	0.40 \pm 0.00
TS4	win-03	18.80	5.75	6.19	228.00	
	sum-03	20.63 \pm 0.75	4.50 \pm 0.17	5.67 \pm 0.06	225.00 \pm 3.35	0.55 \pm 0.01
	aut-03	19.50 \pm 0.09	2.70 \pm 0.05	6.02 \pm 0.07	214.67 \pm 8.38	0.54 \pm 0.05
	win-04	18.27 \pm 0.05	1.57 \pm 0.05	7.64 \pm 0.06	233.33 \pm 3.05	0.61 \pm 0.06
43S1	win-03	17.30	3.54	6.70	950.00	
	spr-03	20.55 \pm 0.35	3.08 \pm 0.05	6.29 \pm 0.01	374.66 \pm 1.06	0.53 \pm 0.00
	sum-03	19.57 \pm 0.05	0.67 \pm 0.48	6.16 \pm 0.05	232.67 \pm 1.15	1.42 \pm 0.13
	aut-03	19.23 \pm 0.15	3.00 \pm 0.34	7.09 \pm 0.21	147.67 \pm 10.96	0.53 \pm 0.04
S60	win-03	18.20	2.68	7.82	1668.00	
	win-04	18.33 \pm 0.15	1.63 \pm 0.05	7.55 \pm 0.06	7020.00 \pm 30.69	19.97 \pm 0.06
	win-05	19.60 \pm 0.00	2.19 \pm 0.01	7.84 \pm 0.01	16400.00 \pm 10.00	10.00 \pm 0.00
TS2	win-03	18.80	4.73	5.88	278.00	
	sum-03	20.77 \pm 0.25	2.77 \pm 0.15	5.78 \pm 0.01	275.00 \pm 0.61	0.64 \pm 0.04
	aut-03	19.27 \pm 0.05	1.46 \pm 0.13	6.35 \pm 0.14	279.00 \pm 1.25	0.44 \pm 0.12
	win-04	17.03 \pm 0.32	3.93 \pm 0.15	5.94 \pm 0.06	170.33 \pm 0.70	1.91 \pm 0.08
SO2a	spr-03	18.70 \pm 0.14	2.05 \pm 0.07	6.62 \pm 0.03	148.67 \pm 0.05	0.53 \pm 0.00
	aut-03	21.40 \pm 0.14	1.71 \pm 0.00	7.02 \pm 0.01	188.33 \pm 1.52	1.05 \pm 0.05
	spr-04	17.77 \pm 0.15	2.51 \pm 0.02	6.92 \pm 0.01	161.33 \pm 1.98	0.99 \pm 0.08
	sum-04	19.70 \pm 0.09	2.11 \pm 0.01	6.71 \pm 0.01	244.67 \pm 8.02	0.67 \pm 0.12
	aut-04	19.17 \pm 0.20	1.93 \pm 0.05	6.88 \pm 0.00	202.00 \pm 2.56	0.53 \pm 0.12
	win-05	18.60 \pm 0.10	2.48 \pm 0.05	6.68 \pm 0.01	198.67 \pm 0.58	0.53 \pm 0.11

Table 2 Continued

Piezometer	Season	T (°C)	DO (mg L ⁻¹)	pH	EC (µS cm ⁻¹)	Alkalinity (meq L ⁻¹)
4S2	win-03	18.10	3.36	7.69	328.00	
SO2b	spr-03	18.75 ± 0.35	1.50 ± 0.28	6.51 ± 0.07	183.00 ± 0.26	0.52 ± 0.00
	aut-03	20.60 ± 0.01	1.11 ± 0.00	6.90 ± 0.03	233.00 ± 2.56	1.05 ± 0.04
	spr-04	18.57 ± 0.05	2.10 ± 0.25	6.74 ± 0.02	210.00 ± 3.58	0.86 ± 0.26
	sum-04	19.20 ± 0.33	1.44 ± 0.00	6.78 ± 0.02	310.00 ± 4.21	0.73 ± 0.12
	aut-04	18.50 ± 0.17	1.51 ± 0.01	6.70 ± 0.00	261.00 ± 1.25	0.47 ± 0.12
	win-05	18.60 ± 0.10	1.60 ± 0.02	6.56 ± 0.01	243.00 ± 1.00	0.60 ± 0.00
S50	sum-03	19.67 ± 0.05	1.80 ± 0.10	6.73 ± 0.06	178.27 ± 0.05	1.41 ± 0.14
	sum-04	19.57 ± 0.15	1.38 ± 0.05	7.14 ± 0.02	152.33 ± 0.57	1.25 ± 0.04
S56	sum-04	22.07 ± 0.11	0.86 ± 0.00	7.45 ± 0.03	21 010.00 ± 149.33	6.00 ± 0.00
SO1	spr-03	18.05 ± 0.19	2.90 ± 0.00	6.94 ± 0.07	91.50 ± 0.01	0.49 ± 0.00
	sum-03	20.33 ± 0.15	2.33 ± 0.20	7.28 ± 0.09	153.00 ± 0.75	1.00 ± 0.09
	aut-03	18.63 ± 0.05	2.45 ± 0.06	7.36 ± 0.12	126.00 ± 0.02	0.54 ± 0.04
	spr-04	18.93 ± 0.05	2.10 ± 0.08	7.12 ± 0.02	99.00 ± 2.36	0.63 ± 0.04
	sum-04	14.60 ± 0.10	2.13 ± 0.01	6.87 ± 0.00	163.00 ± 3.69	0.63 ± 0.06
	sum-04	18.87 ± 0.15	1.79 ± 0.01	7.11 ± 0.01	135.00 ± 2.56	0.60 ± 0.00
	win-05	18.50 ± 0.20	2.20 ± 0.02	6.67 ± 0.01	136.00 ± 0.00	0.47 ± 0.11

Table 3 Chemical variables seasonally measured in the piezometers (mean ± SD) (SRP, soluble reactive phosphorus; win, winter; spr, spring; sum, summer; aut, autumn)

Piezometer	Season	Ammonium (mg L ⁻¹ N-NH ₄ ⁺)	Nitrate (mg L ⁻¹ N-NO ₃ ⁻)	SRP (mg L ⁻¹ P-PO ₄ ³⁻)	Total P (mg L ⁻¹ P)	Ferric iron (mg L ⁻¹ Fe ²⁺)	Ferrous iron (mg L ⁻¹ Fe ³⁺)	Fe (mg L ⁻¹ Fe)
Piso	sum-03	0.014 ± 0.008	1.009 ± 0.050	0.002 ± 0.001	0.057 ± 0.009	0.328 ± 0.198	0.917 ± 0.460	1.245 ± 0.654
	aut-03	0.048 ± 0.001	0.728 ± 0.032	0.016 ± 0.007	0.036 ± 0.007	0.082 ± 0.082	0.267 ± 0.065	0.349 ± 0.029
	sum-04	0.032 ± 0.013	1.313 ± 0.159	0.018 ± 0.004	0.019 ± 0.009	0.000 ± 0.000	0.349 ± 0.078	0.349 ± 0.078
	aut-04	0.493 ± 0.161	0.783 ± 0.072	0.013 ± 0.002	0.031 ± 0.002	0.000 ± 0.000	0.133 ± 0.027	0.133 ± 0.017
	win-05	0.024 ± 0.012	1.060 ± 0.108	0.011 ± 0.003	0.020 ± 0.006	0.073 ± 0.122	1.095 ± 0.914	1.167 ± 0.867
S51	win-03	0.152 ± 0.012	0.008 ± 0.001	0.047 ± 0.008	0.165 ± 0.003			
	spr-03	0.009 ± 0.000	0.059 ± 0.060	0.059 ± 0.004	0.286 ± 0.042	3.183 ± 0.746	2.510 ± 2.063	5.693 ± 5.810
	aut-03	0.065 ± 0.001	0.149 ± 0.007	0.046 ± 0.008	0.189 ± 0.096	0.272 ± 0.471	4.510 ± 1.666	4.327 ± 1.199
	spr-04	0.064 ± 0.022	0.118 ± 0.012	0.093 ± 0.008	0.353 ± 0.042	0.000 ± 0.000	1.954 ± 0.649	1.495 ± 0.649
	aut-04	0.045 ± 0.031	0.000 ± 0.000	0.120 ± 0.025	0.130 ± 0.017	0.000 ± 0.000	5.614 ± 1.329	5.614 ± 1.329
	win-05	0.006 ± 0.003	0.192 ± 0.007	0.239 ± 0.144	0.247 ± 0.025	0.080 ± 0.078	2.083 ± 1.948	2.163 ± 1.811
21S3	win-03	0.160 ± 0.103	0.008 ± 0.001	0.008 ± 0.000	0.042 ± 0.015			
	spr-03	0.010 ± 0.013	0.001 ± 0.004	0.000 ± 0.000	0.030 ± 0.001	0.700 ± 0.008	0.592 ± 0.129	1.292 ± 0.137
	sum-03	0.005 ± 0.004	0.054 ± 0.003	0.012 ± 0.006	0.040 ± 0.001	0.645 ± 0.037	0.509 ± 0.187	1.153 ± 0.533
	aut-03	0.044 ± 0.019	0.107 ± 0.003	0.011 ± 0.001	0.042 ± 0.006	0.048 ± 0.031	1.663 ± 0.205	1.712 ± 0.224
4S1	spr-03	0.016 ± 0.011	0.024 ± 0.033	0.001 ± 0.002	0.024 ± 0.005	2.030 ± 0.004	18.604 ± 2.200	20.635 ± 2.160
	sum-03	0.019 ± 0.012	0.031 ± 0.003	0.000 ± 0.000	0.043 ± 0.004	0.122 ± 0.040	0.654 ± 0.383	0.776 ± 0.343
	aut-03	0.047 ± 0.005	0.108 ± 0.004	0.003 ± 0.002	0.025 ± 0.008	0.000 ± 0.000	0.888 ± 0.158	0.888 ± 0.158
	aut-04	0.058 ± 0.002	0.248 ± 0.001	0.013 ± 0.002	0.062 ± 0.021	0.000 ± 0.000	1.647 ± 0.308	1.647 ± 0.308
	sum-04	0.038 ± 0.013	0.495 ± 0.025	0.006 ± 0.002	0.019 ± 0.011	0.119 ± 0.022	0.853 ± 0.046	0.972 ± 0.067
	aut-04	0.053 ± 0.037	0.004 ± 0.006	0.006 ± 0.001	0.028 ± 0.001	0.000 ± 0.000	1.655 ± 0.723	1.655 ± 0.723
win-05	win-05	0.019 ± 0.018	0.346 ± 0.011	0.006 ± 0.001	0.007 ± 0.004	0.079 ± 0.068	2.928 ± 3.624	3.007 ± 0.004
L3	sum-03	0.029 ± 0.041	0.170 ± 0.158	0.004 ± 0.001	0.027 ± 0.000	1.024 ± 0.169	1.692 ± 1.575	2.715 ± 1.744
	spr-04	0.049 ± 0.014	0.112 ± 0.008	0.009 ± 0.005	0.083 ± 0.044	0.000 ± 0.000	1.702 ± 0.984	1.702 ± 0.984
L7	win-03	0.005 ± 0.007	0.069 ± 0.007	0.008 ± 0.001	0.031 ± 0.005			
	sum-03	0.000 ± 0.000	0.203 ± 0.016	0.007 ± 0.004	0.040 ± 0.017	0.658 ± 0.622	0.693 ± 0.600	1.351 ± 1.222
	sum-04	0.458 ± 0.016	0.204 ± 0.023	0.016 ± 0.007	0.087 ± 0.020	1.047 ± 0.753	12.910 ± 3.470	13.957 ± 2.784
	aut-04	0.309 ± 0.120	0.384 ± 0.114	0.026 ± 0.021	0.085 ± 0.007	0.000 ± 0.000	6.355 ± 1.105	6.255 ± 1.105
39S2	win-04	0.025 ± 0.009	1.678 ± 0.023	0.101 ± 0.004	0.144 ± 0.011	0.211 ± 0.366	0.806 ± 0.217	1.018 ± 0.254
	spr-04	0.064 ± 0.002	1.233 ± 0.132	0.041 ± 0.005	0.073 ± 0.003	0.002 ± 0.000	2.546 ± 1.647	2.567 ± 1.463
	sum-04	0.265 ± 0.035	2.077 ± 0.238	0.144 ± 0.054	0.156 ± 0.001	0.006 ± 0.002	0.186 ± 0.035	0.192 ± 0.032

Table 3 Continued

Piezometer	Season	Ammonium (mg L ⁻¹ N-NH ₄ ⁺)	Nitrate (mg L ⁻¹ N-NO ₃ ⁻)	SRP (mg L ⁻¹ P-PO ₄ ³⁻)	Total P (mg L ⁻¹ P)	Ferric iron (mg L ⁻¹ Fe ²⁺)	Ferrous iron (mg L ⁻¹ Fe ³⁺)	Fe (mg L ⁻¹ Fe)
TS4	aut-04	0.043 ± 0.015	1.028 ± 0.152	0.154 ± 0.071	0.155 ± 0.010	0.002 ± 0.003	0.884 ± 0.215	0.886 ± 0.212
	win-05	0.025 ± 0.005	1.112 ± 0.444	0.061 ± 0.008	0.075 ± 0.007	0.000 ± 0.000	2.114 ± 1.719	2.114 ± 1.719
	win-03	0.156 ± 0.006	11.657 ± 0.931	0.003 ± 0.000	0.022 ± 0.009			
	sum-03	0.002 ± 0.000	9.245 ± 0.676	0.012 ± 0.006	0.013 ± 0.003	0.066 ± 0.032	0.364 ± 0.285	0.430 ± 0.317
	aut-03	0.000 ± 0.000	12.568 ± 1.989	0.016 ± 0.006	0.022 ± 0.003	0.000 ± 0.000	0.602 ± 0.670	0.602 ± 0.671
43S1	win-04	0.001 ± 0.003	11.645 ± 0.493	0.014 ± 0.006	0.059 ± 0.009	0.130 ± 0.125	0.376 ± 0.076	0.507 ± 0.245
	win-03	0.078 ± 0.054	0.374 ± 0.054	0.016 ± 0.000	2.064 ± 0.063			
	spr-03	0.019 ± 0.000	0.866 ± 0.781	0.001 ± 0.001	0.040 ± 0.006	0.164 ± 0.039	1.185 ± 0.725	1.350 ± 0.764
	sum-03	0.003 ± 0.000	0.135 ± 0.008	0.006 ± 0.001	0.061 ± 0.031	0.357 ± 0.057	1.414 ± 0.899	1.771 ± 0.906
S60	aut-03	0.025 ± 0.017	0.464 ± 0.053	0.011 ± 0.003	0.062 ± 0.008	0.000 ± 0.000	2.392 ± 1.102	2.392 ± 1.102
	win-03	4.613 ± 0.019	0.060 ± 0.000	1.813 ± 0.014	1.814 ± 0.023			
	win-04	2.215 ± 0.411	0.348 ± 0.061	0.012 ± 0.007	0.073 ± 0.015	1.016 ± 1.001	0.747 ± 0.978	1.762 ± 2.086
	win-05	7.308 ± 1.175	0.160 ± 0.022	1.614 ± 0.062	1.640 ± 0.107	0.254 ± 0.298	1.066 ± 0.226	1.319 ± 0.511
TS2	win-03	0.136 ± 0.017	9.591 ± 2.141	0.028 ± 0.017	0.067 ± 0.041			
	sum-03	0.034 ± 0.037	12.226 ± 1.308	0.020 ± 0.004	0.065 ± 0.024	0.266 ± 0.175	0.741 ± 0.242	1.007 ± 0.139
	aut-03	0.031 ± 0.014	19.220 ± 0.284	0.008 ± 0.006	0.051 ± 0.016	0.000 ± 0.000	1.010 ± 0.297	1.010 ± 0.297
	win-04	0.000 ± 0.000	4.565 ± 0.294	0.041 ± 0.007	0.093 ± 0.011	0.136 ± 0.035	0.392 ± 0.068	0.528 ± 0.251
SO2a	spr-03	0.007 ± 0.001	0.780 ± 0.703	0.023 ± 0.003	0.024 ± 0.003	0.040 ± 0.021	0.361 ± 0.170	0.401 ± 0.191
	aut-03	0.010 ± 0.010	0.515 ± 0.065	0.038 ± 0.005	0.079 ± 0.012	0.003 ± 0.004	0.424 ± 0.082	0.427 ± 0.081
	spr-04	0.039 ± 0.013	0.717 ± 0.009	0.031 ± 0.002	0.058 ± 0.012	0.000 ± 0.000	1.018 ± 0.696	1.018 ± 0.696
	sum-04	0.000 ± 0.000	1.114 ± 0.095	0.033 ± 0.001	0.040 ± 0.008	0.005 ± 0.005	1.343 ± 0.696	1.348 ± 0.218
	aut-04	0.000 ± 0.000	0.411 ± 0.043	0.037 ± 0.002	0.053 ± 0.002	0.000 ± 0.000	0.263 ± 0.134	0.263 ± 0.134
	win-05	0.000 ± 0.000	0.535 ± 0.090	0.035 ± 0.001	0.037 ± 0.000	0.030 ± 0.029	0.620 ± 0.610	0.650 ± 0.591
4S2	win-03	0.273 ± 0.014	0.009 ± 0.000	0.009 ± 0.009	0.085 ± 0.009			
SO2b	spr-03	0.000 ± 0.000	0.671 ± 0.605	0.008 ± 0.001	0.015 ± 0.000	0.030 ± 0.005	0.328 ± 0.181	0.331 ± 0.176
	aut-03	0.017 ± 0.003	0.199 ± 0.005	0.016 ± 0.000	0.048 ± 0.009	0.000 ± 0.000	0.613 ± 0.571	0.613 ± 0.791
	spr-04	0.023 ± 0.011	0.334 ± 0.038	0.017 ± 0.003	0.022 ± 0.004	0.000 ± 0.000	0.503 ± 0.361	0.503 ± 0.361
	sum-04	0.027 ± 0.004	0.999 ± 0.011	0.015 ± 0.002	0.020 ± 0.005	0.004 ± 0.005	0.239 ± 0.080	0.242 ± 0.075
	aut-04	0.000 ± 0.000	0.222 ± 0.039	0.013 ± 0.002	0.044 ± 0.007	0.000 ± 0.000	0.077 ± 0.036	0.077 ± 0.036
	win-05	0.000 ± 0.000	0.250 ± 0.162	0.014 ± 0.000	0.015 ± 0.001	0.040 ± 0.054	0.546 ± 0.454	0.586 ± 0.437
S50	sum-03	0.000 ± 0.000	0.134 ± 0.001	0.022 ± 0.002	0.038 ± 0.011	0.029 ± 0.007	0.166 ± 0.030	0.195 ± 0.029
	sum-04	0.015 ± 0.001	0.261 ± 0.017	0.029 ± 0.000	0.033 ± 0.006	0.001 ± 0.000	0.408 ± 0.168	0.408 ± 0.168
S56	sum-04	7.728 ± 1.385	0.431 ± 0.035	0.423 ± 0.041	0.521 ± 0.041	0.123 ± 0.083	1.317 ± 0.725	1.440 ± 0.649
SO1	spr-03	0.000 ± 0.000	0.622 ± 0.562	0.007 ± 0.000	0.026 ± 0.004	0.000 ± 0.000	0.232 ± 0.067	0.232 ± 0.067
	sum-03	0.034 ± 0.029	0.223 ± 0.019	0.028 ± 0.003	0.032 ± 0.005	0.425 ± 0.197	1.177 ± 0.655	1.602 ± 0.725
	aut-03	0.031 ± 0.007	0.571 ± 0.020	0.023 ± 0.002	0.040 ± 0.014	0.000 ± 0.000	5.832 ± 4.963	5.832 ± 8.963
	spr-04	0.038 ± 0.012	0.466 ± 0.049	0.019 ± 0.002	0.031 ± 0.011	0.000 ± 0.000	0.318 ± 0.178	0.318 ± 0.178
	sum-04	0.000 ± 0.000	0.879 ± 0.083	0.025 ± 0.001	0.035 ± 0.007	0.011 ± 0.005	0.158 ± 0.018	0.169 ± 0.023
	sum-04	0.023 ± 0.018	0.540 ± 0.024	0.027 ± 0.009	0.045 ± 0.008	0.000 ± 0.000	0.227 ± 0.026	0.227 ± 0.026
	win-05	0.000 ± 0.000	0.151 ± 0.009	0.024 ± 0.002	0.034 ± 0.003	0.018 ± 0.032	0.478 ± 0.441	0.496 ± 0.426

A significant increase in bacterial biomass was seasonally observed during 2003 in all wells, with higher values during summer or autumn than during winter or spring ($P \leq 0.008$), except in borehole SO2a ($P \geq 0.378$). During 2004, a similar temporal pattern was observed ($P \leq 0.023$), except in well SO2a ($P \geq 0.567$). Bacterial biomass positively correlated with temperature ($r = 0.471$, $P = 0.000$, $n = 71$) and nitrate ($r = 0.423$, $P = 0.000$, $n = 71$), and negatively with DO ($r = -0.276$, $P = 0.021$, $n = 71$). In wells S51, SO2b and SO1 bacterial biomass negatively correlated with rainfall from 2 months prior to sampling ($P \leq 0.035$). This variable

did not correlate with depth, permeability or transmissivity in any season.

Microbial activities of functional groups, except those for NB, were found in this aquifer system. Significant differences for the activities of different functional groups, measured as the mean of the first day after incubation in which activity was detected, were observed in all seasons ($F \geq 5.530$, $P \leq 0.009$) (Table 4). During 2003 and winter 2004, IRB showed statistically higher activities than SRB and DN ($P \leq 0.001$), whose activities were not significantly different ($P \geq 0.099$). In summer 2004, IRB and SRB showed statistically similar

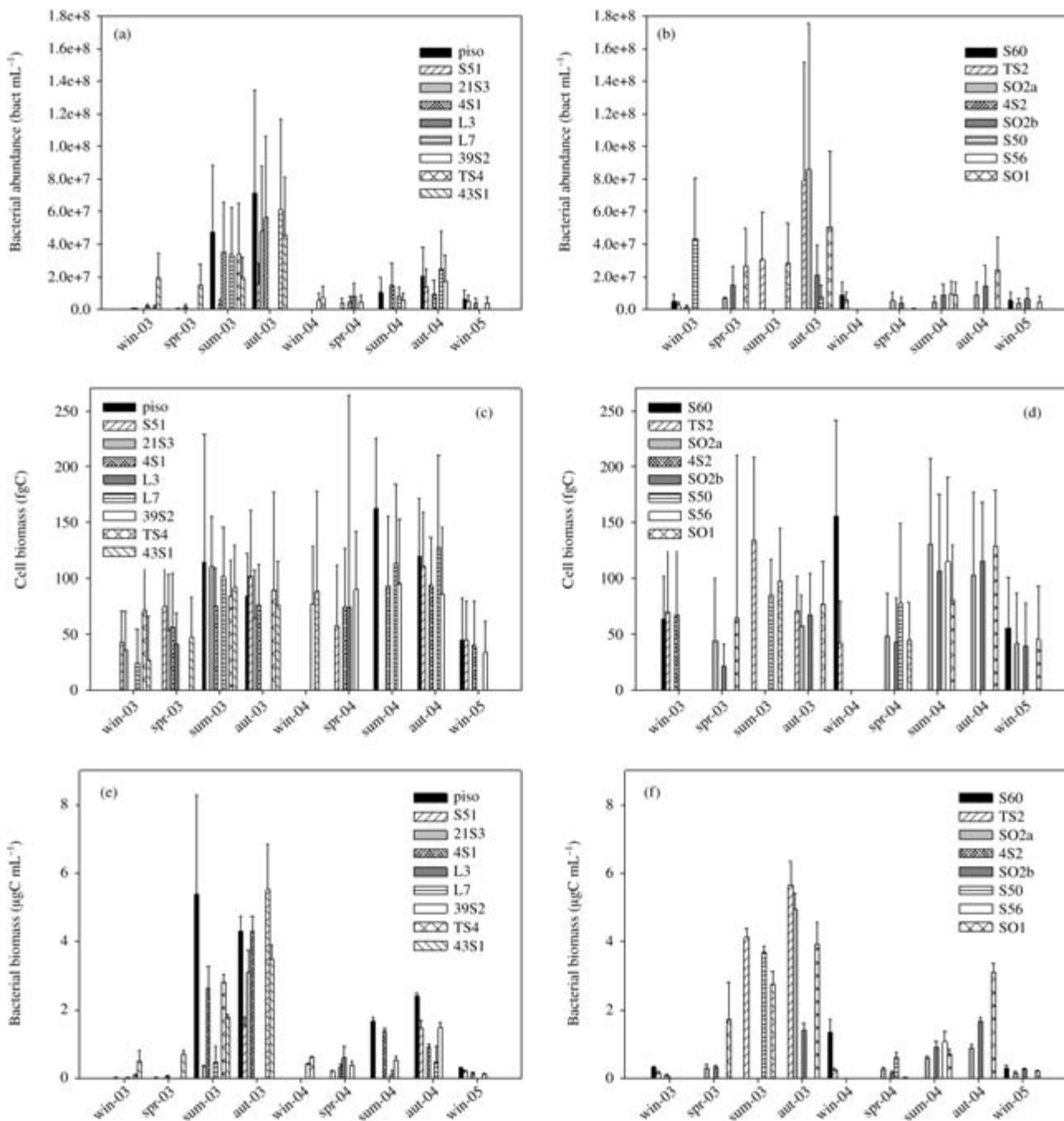


Fig. 2 Seasonal changes in microbiological variables during the study period: a and b, bacterial abundance, c and d, cell biomass, e and f, bacterial biomass (win, winter; spr, spring; sum, summer; aut, autumn; solid bars denote standard deviation).

activities ($P \geq 0.123$) and significantly higher than DN ($P \leq 0.009$). During 2003, higher activities in summer than in winter were found in all cases, although they were only significant for IRB and SRB activities in some wells (21S3, L7 and TS2; $P \leq 0.035$). In general, there were not significant differences in the means of microbial activities between summer and winter during 2004. No significant correlations were found among microbial activities, depth, permeability and transmissivity.

Exploratory statistical analyses

Bartlett's χ^2 test showed that the two sets of variables (microbiological and physicochemical) in the canonical correlation analysis were not independent ($\chi^2 = 60.985$, d.f. = 39, $P = 0.014$); the first two canonical variate pairs were significantly correlated (Table 5). The strong correlation observed for the first canonical variate pair symbolizes a correlation between a variate that

Table 4 Summary of microbial activities for different functional groups provided by BARTTM-tests (DN, denitrifying bacteria; IRB, iron-related bacteria; SRB, sulphate-reducing bacteria; win, winter; sum, summer). Numbers indicate first day in which bacterial activity was recorded

Piezometer	DNB				IRB				SRB			
	win-03	sum-03	win-04	sum-04	win-03	sum-03	win-04	sum-04	win-03	sum-03	win-04	sum-04
Piso		10		3		4		2		4		9
S51	6				3				10			
21S3	5	3			10	4			10	10		
4S1		9		2		2		3		5		3
L7	10	10		2	4	3		2	7	5		7
39S2			10	10			3	2			9	4
TS4	8	4	4		4	3	4		10	6	10	
43S1		10				4				6		
S60	5		4		1		1		3		4	
TS2	4	4	4		3	1	4		8	5	8	
SO2a				3				3				6
4S2	7				4				10			
SO2b				3				2				4
S50		6				2				8		
S56				1				2				5
SO1		10		6		3		10		5		3

Table 5 Results after Bartlett's χ^2 test with successive variate pair removed for the canonical correlation analysis

Variate pair removed	Canonical R	Canonical R ²	χ^2	d.f.	P
0	0.710	0.504	80.709	39	0.000
1	0.557	0.310	37.534	24	0.039
2	0.461	0.212	14.673	11	0.198

combines temperature, DO, nitrate, ferrous iron and total iron, and another variate that integrates bacterial abundance, cell biomass and bacterial biomass (Fig. 3). Bacterial abundances, and mainly, cell and bacterial biomasses increase with higher temperatures and nitrate concentrations but, with higher DO, ferrous ion and total iron concentrations, bacterial abundances, cell biomasses and bacterial biomasses show a decrease. The correlation observed for the second canonical variate pair was less significant (Table 5). The variance explained by the first three factors included in the PCA of the correlation matrix performed was 70.21%. Microbiological variables, temperature and nitrate concentration defined the principal component I. Samples were classified into two major groups in relation to axis I: one on the positive side (winter/spring 2003, winter/spring 2004 and winter 2005) and another on the negative side (summer/autumn 2003, and summer/autumn 2004) (Fig. 4).

DISCUSSION

Physicochemical variables

As surface waters, whose temperature and DO values display temporal patterns (Coletto, 2003), groundwater temperature

and DO also exhibited temporal patterns in the aquifer system of Doñana. In other aquifer systems, no significant seasonal differences for temperature, at least between winter and summer, were observed (Balkwill *et al.*, 1989; Pedersen *et al.*, 1996). This aquifer system therefore appears to be very different to other subsurface systems considered to be highly stable (Vorobyova *et al.*, 1997). The decrease in groundwater DO from winter to autumn during both years might be due to inorganic reactions involving reduced metals, microbial utilization of reduced organic matter, or both (Phelps *et al.*, 1994; Brugger *et al.*, 2001; López-Archilla *et al.*, 2007). Organic matter concentration in the Doñana aquifer is moderately high (Coletto, 2003); as a result, low DO concentrations found in autumns might be due to microbial consumption (Griebler *et al.*, 2002). Electric conductivity was higher in wells located close to the marsh because there is a clear influence of brackish water. pH was close to neutral and, due to the absence of correlations between this chemical variable and the microbiological variables, we suggest that pH was not a key factor controlling the spatiotemporal distribution of microbial communities present in this aquifer system, as has been previously pointed out (Gounot, 1996); however, pH was a central factor controlling the diversity and the taxonomic composition of microbial communities in Doñana's groundwater (López-Archilla *et al.*, 2007).

Although aquifers have often been considered to be oligotrophic environments (Mikell *et al.*, 1996) more similar to open marine systems than to lakes or shallow lakes, and with bacterial densities close to those observed for oceans (Pedersen & Ekendahl, 1990), we consider that these ideas can neither be generalized nor extrapolated. Doñana's aquifer system, at least its upper unit, appears to be meso- or eutrophic if concentrations of nitrogen and phosphorus inorganic chemical

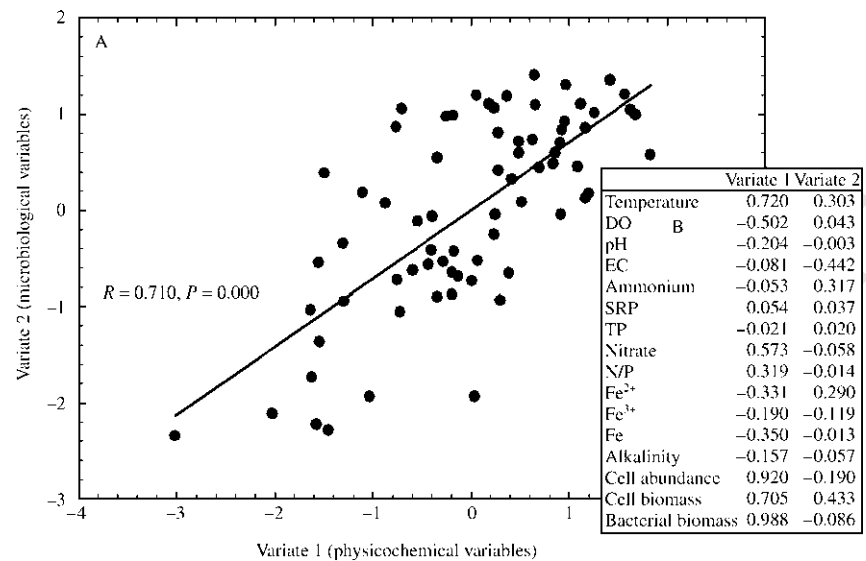


Fig. 3 Canonical correlation analysis showing physicochemical variables versus microbiological variables form the first variate pair (A). Contributions of the descriptors to the first variate pair (B) (boldface type indicates the major canonical loadings in the first variate pair). DO, dissolved oxygen; EC, electric conductivity; SRP, soluble reactive phosphorus; TP, total phosphorus.

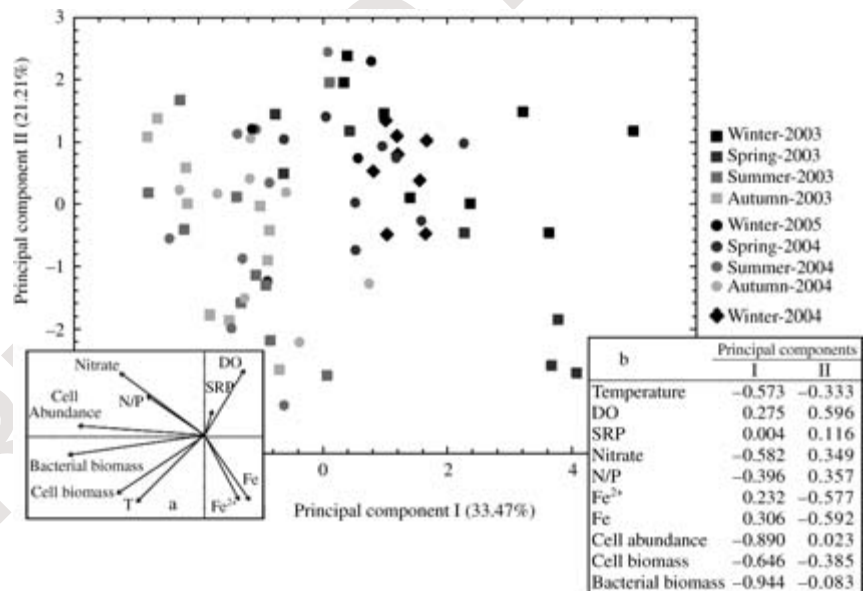


Fig. 4 Positions of the 71 seasonal samples plotted in the reduced space of the first two principal components, showing both the 10 descriptors or the variables projected on the plane determined by the first two principal axes (box a) and the factor loadings of these descriptors on each of the first two axes (box b) (boldface type indicates the major factor loadings on each axis). DO, dissolved oxygen; SRP, soluble reactive phosphorus.

forms are considered (Table 3). Although several wells were used in this study, and spatiotemporal variability is notable, ammonium, nitrate, SRP and TP concentrations were higher than, or at least similar to, those values reported for other aquifer systems (Alfreider *et al.*, 1997; Murphy & Schramke, 1998; Trojan *et al.*, 2003; Mehnert *et al.*, 2007). Most of the wells in this study showed higher concentrations for nitrate than for ammonium, probably because most of them were well oxygenated during the study period (Tables 2 and 3). Nitrate concentrations are usually low in groundwaters, unless there is a pollution source from agricultural fertilizers (Gounot, 1996). In this study, high nitrate concentrations found in wells TS2 and TS4 are explained by the presence of intensive strawberry

crops (Manzano *et al.*, 2007). A high iron concentration in the groundwater is explained by the presence of an intermediate layer with iron oxide minerals (Trick & Custodio, 2004). High total iron concentrations, as well as ferrous and ferric iron concentrations, had a considerable influence on the type of microorganisms found in this ecosystem (López-Archilla *et al.*, 2007). At the same time, high concentrations of organic matter, that can act as a source of electrons for microorganisms, were also determined in this aquifer system (Coletto, 2003). Both ferric iron and nitrate can act as electron acceptors when DO is depleted. In fact, IRB have displayed the highest activities, highlighting the relative importance of iron (and its chemical forms) in the general microbial metabolism of this aquifer

system. Although high nitrate concentrations were found in this aquifer system, however, DNB exhibited lower activity levels than IRB and SRB, demonstrating that there are no reasons to consider linear relationships among certain chemical forms, used as electron acceptors by microorganisms, and functional groups (Mauck & Roberts, 2007), because aquifer heterogeneity results in a heterogeneous distribution of the microbial communities and their activities (Goldscheider *et al.*, 2006). In any case, the activity of microbial communities is probably influencing the geochemistry of this aquifer system, as has been observed elsewhere (Bennett *et al.*, 2000; Chapelle, 2000a; Penny *et al.*, 2003; Dassonville *et al.*, 2004; Haack *et al.*, 2004; Roadcap *et al.*, 2006).

Microbiological variables

Prokaryotes appear to dominate the microbial communities in this aquifer system. Eukaryotes were not detected in microscopic counts. This is not surprising because, although the presence of algae, protozoa and fungi could be important in some aquifer ecosystems, prokaryotes represent, by far, the most abundant and diverse microbial group in aquifers, at least in the phreatic zone (Balkwill, 1989; Sinclair & Ghiorse, 1989; Whitman *et al.*, 1998).

Short rod-shaped bacteria have some advantages over large rod-shaped and filamentous bacteria for transport through sandy sediments (Harvey *et al.*, 1984). Data in this study confirm previous ones (Velasco *et al.*, 2008; in press) and demonstrate that mean bacterial abundance observed in this aquifer system was, at least, one order of magnitude higher than that found in other sedimentary and relatively similar aquifer ecosystems (Harvey *et al.*, 1984; Kölbl-Boelke *et al.*, 1988; Marxsen, 1988; Hirsch & Rades-Rohkohl, 1990; Hazen *et al.*, 1991; Alfreider *et al.*, 1997; Griebler *et al.*, 2002). Groundwater mean bacterial abundance found in this study was also higher than planktonic bacterial densities found in granite (Eydal & Pedersen, 2007) or rock aquifer systems (Lehman *et al.*, 2004). Indeed, planktonic bacterial abundance in the Doñana aquifer was close to values reported for attached bacteria in other sedimentary aquifer systems (Alfreider *et al.*, 1997; Kieft *et al.*, 1998; Martino *et al.*, 1998). Several comparative studies have shown that attached bacteria exhibit more density than unattached bacteria in sedimentary aquifers (Alfreider *et al.*, 1997), as well as in rock aquifers (Lehman *et al.*, 2004). Bearing in mind that our data only show the density of planktonic bacteria, the real bacterial abundance of Doñana's aquifer system might well be higher. However, the abundance of unattached bacteria can be significant in shallow, eutrophic and sedimentary aquifer systems (Harvey *et al.*, 1984; Harvey & George, 1987; Bengtsson, 1989; Griebler *et al.*, 2002).

Average cell biomass values were similar to those found in other sandy sediments (Bone & Balkwill, 1988), but were slightly higher than a value reported for a sedimentary, sandy and very similar aquifer system (Marxsen, 1988). Bacterial

biomass values were in the same order of magnitude as those found in other sandy, aquifer systems (Marxsen, 1988).

Distribution of microbial communities in the aquifer system

Differences in bacterial abundance among wells sampled during the same season were not significant in most sampling campaigns, as has been observed elsewhere (Kölbl-Boelke *et al.*, 1988). Moreover, differences for cell and bacterial biomass among wells were not great during some seasons. In sandy aquifer systems, spatial heterogeneity can be more important for sediments than for groundwaters on a small scale (Fredrickson *et al.*, 1991; Brockman & Murray, 1997), in spite of the homogeneity of lithologies that can occur on a greater scale (Zhou *et al.*, 2004). Consequently, microbiological variables could present fewer and less significant differences among groundwater samples than among sediment core samples (Kölbl-Boelke *et al.*, 1988). Moreover, differences among replicates of the same aquifer sample point can be higher than differences among different aquifer sample points (Brockman & Murray, 1997). In other studies, no differences in bacterial abundance were found, either in groundwaters (Pedersen & Ekendahl, 1990) or in sediment core samples (Hazen *et al.*, 1991). Finally, it should be borne in mind that field samples represent the summation of a complex series of environmental interactions acting over vastly different temporal and spatial scales, being difficult to find clear spatiotemporal patterns in microbial communities (Shi *et al.*, 1999).

Although it was presumed that bacterial abundance decreases with depth, in general, no obvious correlations between these variables have been found in shallow, sedimentary aquifer systems (Beloin *et al.*, 1988; Sinclair & Ghiorse, 1989; Phelps *et al.*, 1994; Fredrickson *et al.*, 1997; Martino *et al.*, 1998). Negative relationships have been observed, however, between these two variables in the vadose zone of some low recharge aquifer systems (Fredrickson *et al.*, 1997; Kieft *et al.*, 1998). In Doñana's aquifer system, no significant correlations were detected between depth and bacterial abundance during any season, with exceptions in some deep wells. Moreover, no significant correlations were found between cell biomass and depth during any season in the Doñana aquifer system. Consequently, depth does not seem to be a key factor controlling microbial communities in terms of abundance and biomass in this aquifer system, at least in the range of depths studied. Furthermore, no significant correlations were found between microbial functional groups and depth, although conclusions should be established with care because the number of samples was low ($n \leq 8$).

Grain size has often been considered to be one of the most important factors controlling microbial abundance and activity in aquifer systems (Musslewhite *et al.*, 2003). Layers with higher clay content usually exhibit lower attached bacterial abundances than sandy layers (Fredrickson *et al.*, 1997; Musslewhite *et al.*, 2003), although positive correlations between

sediment clay content and bacterial density have also been observed (Balkwill, 1989), showing that we should emphasize the need to consider site-specific environmental factors in order to understand microbial distribution and activity (Kieft *et al.*, 1998). In spite of the fact that subsurface sediments and groundwaters represent different milieus (Madsen & Ghiorse, 1993), grain size might also affect the planktonic microbial communities because there is a permanent cell exchange between suspended and attached bacteria (Hirsch & Rades-Rohkohl, 1990; Madsen & Ghiorse, 1993). In the aquifer system of Doñana, piezometers with the finest materials (high clay contents) in the screen region (TS2 and 4S2) (Table 1) showed lower bacterial abundances than boreholes with coarser materials (medium sands or coarse sands), although no significant differences were found. Moreover, wells 21S3 and S51, with fine sands in the screen region, showed lower bacterial densities, often with significant differences, than other piezometers with coarser lithologies (Fig. 2 A,B) and borehole L7, with coarse sands in the screen region, displayed high bacterial abundances during three sampling seasons. Consequently, a clearer relationship between grain size and bacterial abundance than between depth and bacterial abundance was observed in the Doñana aquifer system. Nonetheless, differences for cell biomasses between wells with fine materials and wells with coarse materials were less evident. Finally, no significant or clear patterns were found between the microbial activities of functional groups and grain size, although conclusions should be reached with care because only a few samples were considered ($n \leq 5$).

The apparent control of grain size over microbial communities might be due to some hydrogeological parameters, such as permeability, porosity or transmissivity (Brockman & Murray, 1997; Musslewhite *et al.*, 2003). Areas that show higher hydraulic conductivities tend to display higher bacterial biomasses (Fredrickson *et al.*, 2004) and activities (Chapelle & Lovley, 1990), because this variable determines the hydrological flows and, consequently, the nutrient supplies to bacteria (Lehman *et al.*, 2001; Zhou *et al.*, 2004) and the movement of cells through the aquifer system (Balkwill *et al.*, 1998). It seems that clay content, *per se*, may not directly control microbial population densities in the subsurface, but rather the influence of clay on microbial populations in the subsurface may be due to the effect that clay has on hydraulic conductivity or water activity (Fredrickson *et al.*, 1991). However, no correlations between microbiological and hydrogeological variables (permeability and transmissivity) were observed in this study. Considering the grain size homogeneity reported at least for the upper unit of this aquifer system (Trick & Custodio, 2004), where most of the sampled piezometers are located, a detailed hydrogeological study would contribute to clarifying the spatial distribution of their microbial communities. A common project between microbiologists and hydrogeologists is desirable, but only if both share the same spatiotemporal scale (Brockman & Murray, 1997).

Moreover, and taking into account the correlations found between microbiological variables and rainfall, hydrology,

determined by rainfall, could exert an important control over the microbial communities of this aquifer system, mainly if rainfall is considered to be the only source of freshwater in the aquifer system and, as a result, largely determines hydrogeological flows and phreatic levels (Trick & Custodio, 2004). Bacterial abundance and cell biomass negatively correlated with rainfall 2 months prior to sampling in wells SO1, SO2a, SO2b and S51. Curiously, these wells are located close to the lower unit, where important hydrological horizontal flows occur (SO1, SO2a and SO2b), or close to the ecotone, the most important discharge area of the aquifer system (S51). As a consequence, it seems that hydrology has a relatively significant influence over microbial communities in areas where regional hydrogeological flows are significant. However, other wells located in the ecotone (21S3) or close to other important discharge areas of the aquifer (4S1, 4S2 and L3) did not correlate with rainfall, which suggests that there are other variables, probably also related to hydrogeology, controlling the distribution of microbial communities in this aquifer system.

Significant correlations between microbiological and physicochemical variables in sedimentary aquifers (Martino *et al.*, 1998; Musslewhite *et al.*, 2003; Santoro *et al.*, 2006) are scarce, because aquifer microbial communities are controlled by environmental attributes that are more difficult to quantify, such as microscale spatial heterogeneity and temporal variability of geochemical parameters (Kölb-Boelke *et al.*, 1988; Fredrickson *et al.*, 1991; Brockman & Murray, 1997; Santoro *et al.*, 2006; Mauck & Roberts, 2007). However, some correlations have been observed between groundwater physicochemical variables and microbial communities in crystalline rock aquifers (Pedersen & Ekendahl, 1990). In the present study, a canonical correlation analysis showed a strong correlation between a variate integrating nitrate, ferrous iron and total iron and a variate combining bacterial abundance and both cell and bacterial biomass (Fig. 3). Nitrate concentrations are usually low in aquifers unless a source from agricultural fertilizers exists (Gounot, 1996; Brugger *et al.*, 2001), and this is the case observed for some piezometers (Table 1) located in the vicinity of strawberry crops in Doñana. Indeed, three of five piezometers affected by high nitrate concentrations (TS2, TS4 and L7) showed high or the highest bacterial abundance and cell biomass values during some seasons. Relationships among microbiological variables, ferrous iron and total iron were not so clear, but the ferric iron might probably be used as an electron acceptor when DO is depleted (McLean *et al.*, 2006).

Temporal pattern

A temporal pattern was observed in the microbial communities of this aquifer system, mainly during 2003, but also during 2004, with highest bacterial abundances, cell biomasses and bacterial biomasses during the warmest months of the year (Fig. 4). Similar results were observed in a parallel study (Velasco *et al.*, 2008; in press). Temperature correlated with these three microbiological variables and seemed to be the

most important physical factor controlling them throughout the year. Light increases of this variable triggered microbiological processes, including functional groups. However, the temporal pattern was not clear in deep wells (SO1, SO2a and SO2b). As a consequence, in deep areas of Doñana's aquifer system there are probably other factors, perhaps more related to hydrogeology than to temperature, controlling the temporal distribution of the microbial communities. Seasonal variations of microbial communities in other aquifer systems have also been described (Balkwill & Ghiorse, 1985; Beloin *et al.*, 1988; Bone & Balkwill, 1988). Bacterial abundance also correlated with cell biomass in this study, demonstrating a close relationship between the increase in bacterial abundance and the amount of accumulated carbon in cells (Kieft *et al.*, 1998).

CONCLUSIONS

Although it would be incorrect to depict general conclusions if only a limited number of samples in a large system were analysed, this study has demonstrated the presence of important and active microbial communities, in terms of bacterial abundance, cell biomass and functional groups, in an area encompassing approximately 100 km² of the aquifer system located in Doñana, corroborating parallel results obtained in another study conducted in a smaller area. Due to the homogeneity, not only for the groundwater but also for the lithologies, the spatial pattern of the unattached community was difficult to explain. Among the abiotic factors that usually control the spatial distribution, depth did not play any role, at least in the studied range, and grain size seemed to exert a moderate control on bacterial abundance, although less clear on cell biomass. It is, however, plausible that hydrogeology, through some other related variables such as permeability, porosity or transmissivity, plays a more important role, controlling the spatial pattern of microbial communities, although unfortunately, there are no hydrogeological models with adequate scales for groundwater microbiology studies. Moreover, nitrate seemed to play an important role in controlling bacterial abundance and biomass, because the higher the nitrate concentration, the higher the bacterial abundance, cell biomass and bacterial biomass. On the other hand, all boreholes (except wells SO1, SO2a and SO2b) sampled more than three times throughout the study period showed a clear temporal pattern, mainly during 2003, with higher bacterial abundances, cell biomasses and bacterial biomasses during summers and autumns than during springs and winters, showing the microbial communities of this aquifer system to be highly reactive. Temperature was the most important factor controlling this temporal pattern, triggering not only abundance and carbon content but also activity, mainly IRB activity. As a result, when referring to microbial ecology in the aquifer system of Doñana, there is a need to talk of a dynamic system, probably more similar to the surface aquatic systems located in the same area than to other aquifers. Finally, hydrogeological

models jointly developed between microbiologists and hydrogeologists will provide an understanding of the control exerted by hydrology (rainfall, hydrogeological flows, hydrological relationships between groundwater and surface aquatic systems) on the microbial communities of this aquifer system, a phenomenon that has been partially described in this study.

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