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Quantitative PCR Monitoring of Antibiotic Resistance Genes and Bacterial Pathogens in Three European Artificial Groundwater Recharge Systems▽†

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Aquifer recharge presents advantages for integrated water management in the anthropic cycle, namely, advanced treatment of reclaimed water and additional dilution of pollutants due to mixing with natural groundwater. Nevertheless, this practice represents a health and environmental hazard because of the presence of pathogenic microorganisms and chemical contaminants. To assess the quality of water extracted from recharged aquifers, the groundwater recharge systems in Torreele, Belgium, Sabadell, Spain, and Nardò, Italy, were investigated for fecal-contamination indicators, bacterial pathogens, and antibiotic resistance genes over the period of 1 year. Real-time quantitative PCR assays for *Helicobacter pylori*, *Yersinia enterocolitica*, and *Mycobacterium avium* subsp. *paratuberculosis*, human pathogens with long-time survival capacity in water, and for the resistance genes *ermB*, *mecA*, *blaSHV-5*, *ampC*, *tetO*, and *vanA* were adapted or developed for water samples differing in pollutant content. The resistance genes and pathogen concentrations were determined at five or six sampling points for each recharge system. In drinking and irrigation water, none of the pathogens were detected. *tetO* and *ermB* were found frequently in reclaimed water from Sabadell and Nardò. *mecA* was detected only once in reclaimed water from Sabadell. The three aquifer recharge systems demonstrated different capacities for removal of fecal contaminators and antibiotic resistance genes. Ultrafiltration and reverse osmosis in the Torreele plant proved to be very efficient barriers for the elimination of both contaminant types, whereas aquifer passage followed by UV treatment and chlorination at Sabadell and the fractured and permeable aquifer at Nardò posed only partial barriers for bacterial contaminants.

Artificial recharge has for a long time provided means to mitigate depletion of groundwater levels, protect coastal aquifers from saltwater intrusion, and store surface water for future use (10, 19, 28). Aquifer storage has some general advantages over surface water reservoirs, which might be more costly and have a high environmental footprint. Major concerns about the safety of aquifer recharge arise from the potential contamination with pathogenic and drug-resistant bacteria. Antibiotics are released daily into the natural environment with treated wastewater effluent and through use in animal husbandry (7). Significant amounts of antibiotics are excreted unaltered or as metabolites (up to 75%), which presents a major source of antibiotic input to the environment. Many of these compounds can now be detected easily in water resources (21, 24, 48), leading to increasing concerns with regard to their contribution to the abundance and persistence of antibiotic resistance in populations of pathogenic, commensal, and nonpathogenic microorganisms (7, 31). These concerns are derived partly from a lack of critical information with respect to the transfer of resistance genes within and between bacterial populations in the environment (18, 35). The input of antibiotic-resistant bacteria into the environment seems to be an important source of resistance in the natural environment. Koike et al. monitored the tetracycline resistance genes *tetM*, *tetO*, *tetQ*, *tetW*, *tetC*, *tetH*, and *tetZ* in lagoons and groundwater adjacent to swine production facilities by real-time quantitative PCR (qPCR) (20). They found elevated concentrations of the resistance genes in groundwater affected by two swine confinement operations. However, they also detected tetracycline resistance genes in background control wells, albeit at lower concentrations (20). These genes could be incorporated by indigenous environmental bacteria by horizontal gene transfer. However, the extent of the environmental impact of resistant bacteria is not yet known. To understand the ecology of antibiotic resistance, it is required to characterize the occurrence, fate, and transport of antibiotic-resistant microorganisms. A first step in this process is the identification of major sources of antibiotics

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and antibiotic resistance in the environment. For instance, wastewater treatment plant (WWTP) effluents can be an important source of pathogenic bacteria and antibiotics in surface waters (7, 48).

To monitor and guarantee the quality of reclaimed water, the possible track of pathogens and antibiotic resistance genes in the water reclamation and distribution system from the effluent of the WWTP to the end user needs to be investigated; thus, the number of pathogenic bacteria has to be measured at various stages of the process. However, the traditional detection methods for pathogenic bacteria, especially those for intracellular pathogens, show many drawbacks with regard to very special culture requirements and long cultivation time. For routine analysis of alternative water sources and for analysis in case of waterborne disease outbreak, rapid, sensitive, and specific techniques for identification and quantification of the pathogen under suspicion are required.

In a previous study of subsurface flow constructed wetlands, PCR-based methods for rapid detection of pathogenic bacteria, such as *Campylobacter* spp. and *Yersinia enterocolitica*, in treated wastewater were developed and applied (2, 3). In the present work, (i) the presence of six different antibiotic resistance genes conferring resistance to antibiotics with different mechanisms of action, (ii) the presence of three intracellular human pathogens, and (iii) fecal-contamination indicators were investigated in three artificial groundwater recharge systems. The work was conducted through the interdisciplinary project Reclaim Water from the Sixth European Union Research Framework Programme, contract number 018309. The resistance genes were selected due to their abundance and the persisting increase of resistant microorganisms in the environment (e.g., see references 7, 12, 15, 16, 22, 37, and 40): *ampC* (ampicillin resistance), *mecA* (methicillin resistance), *blaSHV-5* (extended β-lactam resistance conferring broad resistance to penicillins and cephalosporins), *ermB* (erythromycin resistance), *tetO* (tetracycline resistance), and *vanA* (vancomycin resistance). Three pathogens were selected for the analysis: *Yersinia enterocolitica*, *Helicobacter pylori*, and *Mycobacterium avium* subsp. *paratuberculosis*. For all of these pathogens, surviving long-term in aquatic systems and causing waterborne diseases are well documented (6, 8, 23, 29, 30, 33, 43, 45). Additionally, the ability of *Y. enterocolitica* (39), *H. pylori* (1, 29, 32), and *M. avium* (5, 34, 46) to persist for a long time in a dormant or viable but nonculturable state in aquatic environments has been reported. Therefore, culture-independent, rapid, and specific tests are required for the detection of these potential water pollutants. The fate of *Y. enterocolitica*, *H. pylori*, and *M. avium* subsp. *paratuberculosis* was monitored quantitatively. For this purpose, quantitative molecular assays for the six antibiotic resistance genes and the three pathogens were developed. The technique consists of sample concentration, mechanical cell disruption, and total DNA extraction followed by real-time qPCR. The procedure was adapted to water samples with different concentrations of organic pollutants and was applied in a hygienic survey of three European artificial groundwater recharge systems differing in construction and operation.

MATERIALS AND METHODS

Sampling sites. (i) Sabadell. Sabadell, a city of about 200,000 inhabitants, is located 20.6 km north of Barcelona, Spain. The average rainfall is 600 to 700 mm per year. The artificial recharge system is based on the Ripoll River, whose water naturally infiltrates through the riverbed and reaches the alluvial aquifer. The amount of water carried by the river is increased by the discharge of the effluent of one of the two WWTPs of the city. The wastewater is pretreated by a settling tank, followed by physicochemical primary treatment and activated sludge secondary treatment with nutrient removal. The secondary effluent is discharged into the Ripoll River at two different points (Colobrers Stream and Torrella Mill) and during heavy rain periods at a third one (Sant Oleguer River), resulting in a more balanced flow. After recharge, the water from the alluvial aquifer is recovered in a mine, UV disinfected, chlorinated, and used for irrigation of an urban park and for street cleaning.

(ii) Nardò. Nardò is a town of about 30,000 inhabitants located 50 km south of Lecce, Italy. The average rainfall is 500 to 550 mm per year. The artificial recharge method is based upon injection in a sinkhole, located in a karst area. Water used for recharge is made up of effluents from a municipal treatment plant (Galatone WWTP) and the surface draining water from its surrounding area. The water is collected and transported in an open channel (Asso channel, 140 to 200 liters/s) to the sinkhole. The injection rate is 12,000 to 17,000 m^3/day. The reclaimed water is used for irrigation purposes.

(iii) Torreele. Torreele, the intermunicipal water company of the Veurne region, supplies drinking water to the western part of the coastal region of Flanders, Belgium. The number of consumers grows from 60,000 to 250,000 in the summer season. The average rainfall is about 700 mm per year. The Torreele facility treats tertiary wastewater effluent by ultrafiltration and reverse osmosis. This water is then recharged into the dunes of the St-André water catchment via an infiltration pond and subsequently abstracted using 112 wells sited at least 35 m inland from the pond. Abstracted water is given conventional drinking water treatment, aeration, and rapid sand filtration, followed by UV disinfection prior to distribution. The combination of water reuse and groundwater recharge results in sustainable groundwater management of the St-André water catchment.

Sample processing. Seven-liter water samples were taken from the WWTP effluents and 40-liter samples from all other sampling points. Six sampling points were probed at Torreele and five at Nardò and Sabadell. All samples were concentrated by filtration through one to three 0.45-μm cellulose acetate filters (14.2 cm in diameter) with a stainless steel in-line filter holder (Sartorius, Göttingen, Germany). The filter holder was washed twice with 70% ethanol before use. In order to prevent early clogging of the filters, highly polluted samples were prefiltered through sterile standard paper filters. Samples were taken in three or four sampling campaigns: in Torreele in January, July, and October 2007; in Nardò in February, May, and September 2007; and in Sabadell in January, March, June, and October 2007. In Torreele, samples were taken from six sampling points: (i) the effluent from the WWTP, (ii) water after ultrafiltration, (iii) the infiltration water before transport, (iv) the infiltration pond, (v) groundwater after infiltration, and (vi) drinking water. In Nardò, samples were taken from five different sampling points: (i) secondary effluent from the Galatone WWTP, (ii) the sinkhole (collecting Galatone WWTP secondary effluent and surface water flows), for delivery to the fractured aquifer (at a 27-m depth), (iii) a well at a 350-m distance from the sinkhole, (iv) a well at a 500-m distance from the sinkhole, and (v) reference groundwater. Groundwater samples were collected by a submerged pump 1 m below the water table. In Sabadell, samples were taken from five different sampling points: (i) secondary effluent from the Ripoll River WWTP, (ii) a Ripoll River reference point (sample of the river water before WWTP effluent discharge), (iii) infiltration water (sample of the river water after WWTP effluent discharge), (iv) recovered groundwater, and (v) disinfected groundwater ready to use (park irrigation and street cleaning). All filters were stored separately at 4°C and were analyzed within 1 week after sampling. The cell material was removed from the filter with a plastic scraper and resuspended in 5 to 20 ml phosphate-buffered saline buffer (8.0 g/liter NaCl, 0.2 g/liter KCl, 1.44 g/liter Na_2HPO_4, 0.24 g/liter KH_2PO_4, pH 7.4). The volume of the phosphate-buffered saline buffer was adjusted to the amount of cell material and then concentrated by centrifugation for 5 min at 10,000 × *g*, yielding a final cell suspension volume of 500 μl to 1,500 μl.

Determination of fecal-contamination indicators. Heterotrophic plate counts were determined by the APAT-CNR-IRSA, 7050, 2003 method (Nardò); APHA 9215 D (4) (Sabadell); and ISO 6222 (Torreele). Fecal coliforms were measured by APAT-CNR-IRSA, 7020, 2003 (Nardò); EPA accepted 40, CFR part 136 (Sabadell); and AFNOR BRD-07/1-07/93 (Torreele). *Escherichia coli* cells were quantified by application of most probable number (MPN)/100 ml (Environment Agency 2002: Methods for the Examination of Waters and Associated Materials, part 4

TABLE 1. Primers used to produce real-time qPCR standards

Primer name	Target gene	Primer sequence (5′–3′)	Amplicon size (bp)
ampCstF	*ampC*	GTGACCAGATACTGGCCACA	822
ampCstR	*ampC*	TTACTGTAGCGCCTCGAGGA	
ermBstF	*ermB*	GCATTTAACGACGAAACTGGCT	573
ermBstR	*ermB*	GACAATACTTGCTCATAAGTAATGGT	
mecAstF	*mecA*	TAATAGTTGTAGTTGTCGGGTTTG	733
mecAstR	*mecA*	TAACCTAATAGATGTGAAGTCGCT	
SHVstF	*blaSHV-5*	TGTTAGCCACCCTGCCGCT	825
SHVstR	*blaSHV-5*	GTTGCCAGTGCTCGATCAG	
tetOstF	*tetO*	GGATGGCATACAGGCACAGA	738
tetOstR	*tetO*	GTTTGGATCATAGGGAGAGGAT	
vanAstF	*vanA*	GAAATCAACCATGTTGATGTAGCA	572
vanAstR	*vanA*	TTTGCCGTTTCCTGTATCCGT	
YestF	16S rRNA gene from *Y. enterocolitica*	AGATTGAACGCTGGCGGCA	639
YestR	16S rRNA gene from *Y. enterocolitica*	CTACAAGACTCTAGCTTGCCA	
MapF2	IS*900* sequence from *M. avium* subsp. *paratuberculosis*	AATGACGGTTACGGAGGTGGT	76
MapR2	IS*900* sequence from *M. avium* subsp. *paratuberculosis*	GCAGTAATGGTCGGCCTTACC	
HpF	16S rRNA gene from *H. pylori*	TGCGAAGTGGAGCCAATCTT	118
HpR	16S rRNA gene from *H. pylori*	GGAACGTATTCACCGCAACA	

[http://www.environment-agency.gov.uk/]) (Nardò); by EPA accepted 40, CFR part 136 (Sabadell); and by the Colilert-18 technique (Torreele). Enterococci were determined by following the APAT-CNR-IRSA, 7040, 2003 method (Nardò); APHA 9230 C (4) (Sabadell); and ISO 7899/2 (Torreele). *Clostridium* spores were measured by determination of CFU/100 ml in sulfite-polymyxin-sulfadiazine agar (9a) (Nardò) and by a membrane filtration method derived from ISO 7937 (Sabadell and Torreele).

pH and electrical conductivity measurement. pH was determined by the APHA 4500-H^+ B electrometric method (4) (Nardò and Sabadell) and by following ISO 10523 (Torreele). Electrical conductivity was measured by APHA 2520 B (4) (Nardò), APHA 2510 B (4) (Sabadell), and ISO 7888 (Torreele).

Reference strains, plasmids, and culture techniques. *Yersinia enterocolitica* (DSM 11502), *Mycobacterium avium* subsp. *paratuberculosis* (DSM 44133), and *Helicobacter pylori* (DSM 4867) were obtained from the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen). *Mycobacterium avium* subsp. *paratuberculosis* and *Helicobacter pylori* were obtained as actively growing cultures from the DSMZ. A plasmid containing *tetO* was obtained from Marilyn Smith (University of Kansas Medical Center). Plasmid pIP501 (DSM 8629) containing *ermB*, *Enterobacter cloacae* (DSM 46348) containing *ampC*, *Enterococcus faecium* (DSM 17050) containing *vanA*, *Klebsiella pneumoniae* (DSM 16609) containing *blaSHV-5* (for sulfhydryl variable, extended-spectrum β-lactamase), and *Staphylococcus aureus* subsp. *aureus* (DSM 13661) containing *mecA* were obtained from the DSMZ. *Yersinia enterocolitica* and the bacteria harboring the resistance genes were cultured overnight in tryptone soy broth at room temperature with shaking.

DNA extraction. DNA was extracted from bacterial cultures and wastewater samples with a FastDNA spin kit for soil (MP Biomedicals Europe, Heidelberg, Germany) according to the manufacturer's instruction, with minor modifications. The spin filter centrifugation step with 500 μl salt-ethanol wash solution (SEWS-M buffer; MP Biomedicals Europe, Heidelberg, Germany) was performed at 14,000 × *g* for 2 min. DNA was eluted with 100 μl distilled water. The concentration and integrity of extracted DNA were verified by electrophoresis on 0.7% agarose gels. Depending on the DNA concentration, different serial dilutions of DNA in Tris-EDTA buffer containing 10 μg/ml herring sperm DNA were applied to the real-time qPCR assay.

Standards and internal amplification control for real-time qPCR assays. Absolute quantifications were performed with the real-time qPCR assays for the detection of *ampC*, *ermB*, *mecA*, *blaSHV-5*, *tetO*, and *vanA* with serial diluted exogenous standards that consisted of purified PCR products and for the detection of *Yersinia enterocolitica*, *Helicobacter pylori*, and *Mycobacterium avium* subsp. *paratuberculosis* with serial diluted exogenous standards consisting of purified chromosomal DNA. The standards were generated by PCR with DNA from the corresponding reference strains and plasmids. The primers listed in Table 1 amplify PCR products that harbor the complete target sequences of the appropriate real-time qPCR assays. Each 50-μl reaction mixture contained 1.0 μM of each primer, 0.2 mM deoxynucleoside triphosphates, 2.5 U *Taq* DNA polymerase (GenScript, New Jersey), 1× reaction buffer [10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris-HCl, 2.0 mM $MgSO_4$, 0.1% Triton X-100, pH 9.0, at 25°C], and template DNA. PCR was carried out with a Primus 96 plus thermocycler (MWG-Biotech, Ebersberg, Germany) with the following three segments: (i) initial denaturation at 95°C for 2 min; (ii) 35 cycles of 95°C for 30 s, 60°C for 45 s, and 72°C for 1 min; and (iii) final elongation at 72°C for 7 min. PCR products were purified with a QIAquick PCR purification kit (Qiagen, Hilden, Germany), analyzed on 2% agarose gels, and quantified with an AlphaImager instrument (Biozym, Hessisch Oldendorf, Germany). Serial dilutions of the standards were made with Tris-EDTA buffer containing 10 μg/ml herring sperm DNA. A VIC-labeled internal activity control included in a TaqMan exogenous internal positive control reagent VIC probe kit (Applied Biosystems, Foster City, CA) was applied to each real-time qPCR assay.

Primers and probes. All primers (Tables 1 and 2) and probes (Table 2) were synthesized by TIB Molbiol (Berlin, Germany). The following sequences were used to design the oligonucleotides: GenBank accession no. AJ005633 (*ampC*), U00453 (*ermB*), X52593 (*mecA*), X55640 (*blaSHV-5*), M18896 (*tetO*), AF516335 (*vanA*), Z49830 (16S rRNA genes of *Y. enterocolitica)*, NC002944 (*M. avium* subsp. *paratuberculosis*-specific IS*900* sequence), and U00679 (16S rRNA genes of *H. pylori*). Primer specificity was determined with sequence alignments using BLAST and NCBI entries. In testing for cross-reactions with the respective sensitive strains, 7 to 10 strains from the Environmental Microbiology strain collection were applied for each resistance gene. Detailed information about the strains can be obtained upon request.

Quantification of antibiotic resistance genes and pathogenic bacteria in water samples by real-time qPCR. PCR amplifications for the quantification of *ampC*, *ermB*, *mecA*, *blaSHV-5*, *tetO*, and *vanA* genes and *Y. enterocolitica*, *M. avium* subsp. *paratuberculosis*, and *H. pylori* organisms in total DNA extracted from water samples were performed with an RG-6000-5 Plex real-time DNA cycler

TABLE 2. Primers and probes for real-time qPCR

Primer or probe	Target gene	Sequence (5′–3′)[a]	Reference or source
ampCF	*ampC*	GGGAATGCTGGATGCACAA	44
ampCR	*ampC*	CATGACCCAGTTCGCCATATC	44
ampCP	*ampC*	FAM–CCTATGGCGTGAAAACCAACGTGCA–BHQ-1	44
ermBF	*ermB*	GGATTCTACAAGCGTACCTTGGA	This study
ermBR	*ermB*	GCTGGCAGCTTAAGCAATTGCT	This study
ermBP	*ermB*	FAM–CACTAGGGTTGCTCTTGCACACTCAAGTC–BHQ-1	This study
mecAF	*mecA*	CATTGATCGCAACGTTCAATTTAAT	This study
mecAR	*mecA*	TGGTCTTTCTGCATTCCTGGA	17
mecAP	*mecA*	FAM–CTATGATCCCAATCTAACTTCCACATACC–BHQ-1	This study
SHVF	*blaSHV-5*	AACAGCTGGAGCGAAAGATCCA	This study
SHVR	*blaSHV-5*	TGTTTTTCGCTGACCGGCGAG	This study
SHVP	*blaSHV-5*	FAM–TCCACCAGATCCTGCTGGCGATAG–BHQ-1	This study
tetOF	*tetO*	AAGAAAACAGGAGATTCCAAAACG	40
tetOR	*tetO*	CGAGTCCCCAGATTGTTTTTAGC	40
tetOP	*tetO*	FAM–ACGTTATTTCCCGTTTATCACGGAAGCG–BHQ-1	This study
vanAF	*vanA*	CTGTGAGGTCGGTTGTGCG	44
vanAR	*vanA*	TTTGGTCCACCTCGCCA	44
vanAP	*vanA*	FAM–CAACTAACGCGGCACTGTTTCCCAAT–BHQ-1	44
YestF	16S rRNA gene from *Y. enterocolitica*	AGATTGAACGCTGGCGGCA	36
YestR	16S rRNA gene from *Y. enterocolitica*	CTACAAGACTCTAGCTTGCCA	36
YeP	16S rRNA gene from *Y. enterocolitica*	FAM–AAGGTCCCCCACTTTGGTCCGAAG–BHQ-1	36
MapF2	IS*900* sequence from *M. avium* subsp. *paratuberculosis*	AATGACGGTTACGGAGGTGGT	14
MapR2	IS*900* sequence from *M. avium* subsp. *paratuberculosis*	GCAGTAATGGTCGGCCTTACC	14
MapP	IS*900* sequence from *M. avium* subsp. *paratuberculosis*	FAM-TCCACGCCCGCCCAGACAGGTTG-BBQ	14
HpF	16S rRNA gene from *H. pylori*	TGCGAAGTGGAGCCAATCTT	47
HpR	16S rRNA gene from *H. pylori*	GGAACGTATTCACCGCAACA	47
HpP	16S rRNA gene from *H. pylori*	FAM-CCTCTCAGTTCGGATTGTAGGCTGCAAC-BBQ	47

[a] FAM, 6-carboxyfluorescein; BHQ-1, black hole quencher 1; BBQ, blackberry quencher.

(Rotor-Gene 6000; LTF Labortechnik, Wasserburg, Germany). TaqMan Universal PCR master mix (Applied Biosystems, Foster City, CA) was used, and the final reaction volume was 25 μl. All real-time qPCR amplifications were performed with the following three-step PCR program: (i) a uracil-DNA glycosylase step at 50°C for 2 min, (ii) an initial denaturation and *Taq* polymerase activation step at 95°C for 10 min, and (iii) 50 cycles of 95°C for 15 s and 60°C for 1 min. Tenfold dilution series (gene copy numbers of 10, 100, 1,000, 10,000, 100,000, and 1,000,000/reaction) of the standards for the respective genes were run along with the unknown samples. Each sample was tested in duplicate for each run. Quantification was performed using standard curves obtained from the amplification profiles of known concentrations of the respective standard. All reactions were performed at least in duplicate to confirm the results (9).

RESULTS

Water quality assessment of three artificial aquifer recharge systems. The fates of total bacteria (heterotrophic plate counts), total coliforms, *E. coli* cells, enterococci, and *Clostridium* spores were monitored in the aquifer recharge systems at Sabadell, Nardò, and Torreele in various sampling campaigns (3 to 16 samples). The monitoring period was approximately 1 year (January to December 2007). The data are given as minimum, maximum, and average values in Table 3. Five sampling points from the effluent of the WWTP to the reclaimed water in Nardò and Sabadell and six in Torreele were monitored for pH, electrical conductivity, and microbiological parameters. Except in samples from Torreele, the pH remained relatively stable over the reclamation process. In samples from Torreele, as reverse osmosis is part of the treatment scheme, the average pH decreased by 1 pH unit from the WWTP effluent to the infiltration water before transport (sample 3). Electrical conductivity was drastically reduced in the Torreele plant from 1,228 μS/cm at sampling point 1 to 51 μS/cm at sampling point 3 due to the reverse osmosis treatment and slightly increased again after passage through the infiltration pond and mixing with the water in the aquifer to 321 μS/cm in groundwater and 357 μS/cm in drinking water. Conductivity decreased in the Nardò system (from 1,653 μS/cm at sampling point 1 to 966 μS/cm at sampling point 4, reclaimed water at a 500-m distance from the sinkhole) and slightly increased in the Sabadell system (from 1,843 μS/cm at sampling point 1 to 2,021 μS/cm at sampling point 5). The latter can be explained by the fact that in Sabadell there is no dilution of the treated wastewater with the water from the aquifer, as the water exploited from the aquifer comes mostly from the river. The conductivity of the

TABLE 3. Assessment of water quality at different stages of the aquifer recharge process in Nardò, Sabadell, and Torreele

Case study	Sample no.	pH			n[a]	Conductivity (μS/cm)			n	Total bacteria (log CFU/100 ml) at 22°C			n	Total coliforms (log CFU/100 ml or log MPN/100 ml)[b]			n	*E. coli* cells (log CFU/100 ml)			n	Enterococci (log CFU/100 ml)			n	*Clostridium* spores (log CFU/100 ml)			n
		Min[c]	Avg	Max[d]		Min	Avg	Max		Min	Avg	Max		Min	Avg	Max		Min	Avg	Max		Min	Avg	Max		Min	Avg	Max	
Nardò	1	7.10	7.46	7.73	7	1,320	1,653	1,740	7	3.20	4.27	5.24	7	1.98	4.37	5.96	7	0.95	2.42	3.73	7	2.90	3.57	4.21	7	2.60	3.68	4.34	7
	2	7.10	7.85	8.27	7	950	1,289	1,500	7	3.93	4.51	5.54	7	2.96	4.08	5.21	7	0.70	1.59	2.96	7	1.54	2.67	4.20	7	2.30	2.69	3.52	7
	3	7.00	7.38	7.76	7	320	846	1,400	7	2.88	3.80	5.15	7	2.24	3.37	4.96	7	0.48	1.06	1.54	7	0.30	1.87	3.45	7	1.49	2.53	3.90	7
	4	7.40	7.58	7.82	7	850	966	1,230	7	3.08	3.94	4.57	7	2.96	3.71	4.21	7	0.48	1.26	2.54	7	0.70	2.55	3.42	7	1.40	1.94	2.78	7
	5	7.10	7.41	7.66	7	900	1,177	1,360	7	2.55	2.85	3.08	7	1.98	2.75	3.54	7	0.00	0.77	1.54	7	0.48	1.61	2.70	7	1.79	2.16	2.48	7
Sabadell	1	6.50	7.16	7.72	16	1,088	1,843	2,270	16	5.98	6.78	8.48	12	5.72	6.04	6.42	16	3.30	4.69	5.52	16	3.78	4.00	4.27	12	2.26	2.93	3.73	13
	2	7.53	7.95	8.37	16	1,224	2,204	2,850	16	4.67	5.80	8.48	12	4.76	5.27	6.03	16	2.00	3.60	4.79	16	2.43	3.01	3.53	12	0.90	1.65	2.99	13
	3	7.10	7.85	8.59	16	1,277	1,984	2,550	16	4.85	6.42	8.48	12	4.72	5.79	6.57	16	1.91	4.30	5.36	16	2.15	3.50	4.38	12	0.70	2.11	3.61	13
	4	6.79	7.17	7.57	16	1,770	2,009	2,440	16	1.62	2.75	4.51	12	1.19	2.32	3.79	16	0.00	1.02	2.26	16	0.10	0.74	1.70	12	0.00	0.10	0.47	13
	5	7.29	7.59	7.97	16	1,740	2,021	2,590	16	0.00	1.46	2.91	12	0.00	0.70	2.33	16	0.00	0.12	0.90	16	0.00	0.37	1.27	12	0.00	0.00	0.00	13
Torreele	1	7.63	7.71	7.85	3	1,131	1,228	1,292	3	3.86	4.41	4.92	3	4.51	4.84	5.38	3	4.02	4.33	4.94	3	3.51	4.11	4.83	3	3.28	3.60	4.26	3
	2	7.83	8.13	8.39	11	609	1,092	1,610	11	0.00	0.46	1.08	4	0.00	0.00	0.00	11	0.00	0.00	0.00	11	0.00	0.00	0.00	11	0.00	0.00	0.00	3
	3	6.49	6.80	7.09	11	24	51	68	11	0.00	0.03	0.30	11	0.00	0.00	0.00	11	0.00	0.00	0.00	11	0.00	0.00	0.00	11	0.00	0.00	0.00	3
	4	—[e]	—	—	—	—	—	—	—	2.51	2.88	3.11	3	1.40	2.27	3.41	3	0.00	1.08	2.55	3	0.00	0.59	1.78	3	0.00	0.76	1.51	3
	5	7.58	7.70	7.90	11	284	321	359	11	0.00	0.44	1.79	11	0.00	0.03	0.30	11	0.00	0.00	0.00	11	0.00	0.00	0.00	11	0.00	0.00	0.00	4
	6	6.94	7.79	8.14	11	305	357	398	11	0.00	0.55	1.91	11	0.00	0.00	0.00	11	0.00	0.00	0.00	11	0.00	0.04	0.48	11	0.00	0.00	0.00	4

[a] n, number of samples.
[b] Results from Nardò are given in log MPN/100 ml.
[c] Min, minimum.
[d] Max, maximum.
[e] —, not determined.

river itself is already high, as the river collects effluents from other WWTPs.

The bacteriological quality of the reclaimed water was assessed by measurement of the total bacterial counts at 22°C and by quantification of total coliforms, *E. coli* cells, enterococci, and *Clostridium* spores (Table 3). Total bacterial counts decreased in Nardò only slightly from sampling point 1 to reclaimed water (sampling point 4), in Torreele by 4 orders of magnitude, and in Sabadell by more than 5 orders of magnitude. Total coliforms decreased in Nardò by less than 1 log, in Sabadell by approximately 5.5 log, and in Torreele from 4.84 log CFU total coliforms/100 ml in the WWTP effluent to none in the infiltration water. In Torreele, all of the coliforms were already removed in the first barrier, the ultrafiltration. No coliforms were ever detected at sampling points 2 and 3. However, passage through the infiltration pond resulted in recontamination by coliforms, which are drastically reduced again by aquifer passage (sample 5) and completely eliminated in the drinking water due to UV treatment (sample 6). The picture for *E. coli* was quite similar. At the Nardò site, a decrease of more than 1 log was observed; in Sabadell, a decline of 4.5 log; and in Torreele, *E. coli* cells dropped from 4.33 log in the WWTP effluent to no detectable *E. coli* in the ultrafiltration filtrate. *E. coli* was never detected at Torreele sampling points 2 and 3. Recontamination in the infiltration pond (sample 4) was also detected with *E. coli*. Nevertheless, *E. coli* was completely absent at sampling points 5 and 6. The reduction trend for enterococci at the three sites is similar to the elimination efficiency of total coliforms and *E. coli*: in Nardò, a reduction of 1 log; in Sabadell, a reduction of 3.5 log; and in Torreele, an almost-complete elimination of enterococci (0.04 log CFU/100 ml at sampling point 6). *Clostridium* spores were reduced in Nardò by 1.5 log and in Sabadell and Torreele to no detectable *Clostridium* spores in the irrigation and infiltration water, respectively.

Accuracy of the real-time qPCR assays. Calibration curves were generated using the threshold values of the standard amplification plots. The threshold values of the assays were linear over a measurement range of 6 orders of magnitude. Only real-time qPCR runs without inhibition of the internal activity control were included in the analyses. The efficiencies of the real-time qPCR assays for the pathogens were 98% for *M. avium* subsp. *pseudotuberculosis*, 90% for *H. pylori*, and 85% for *Y. enterocolitica*. The average values over all real-time qPCR assays were applied for determination of pathogen concentration at the three sites. The efficiencies of the real-time qPCR assays for the antibiotic resistance genes were 96% for *tetO*, 95% for *blaSHV-5*, 94% for *ermB*, 91% for *mecA*, 85% for *vanA*, and 78% for *ampC*. The average values over all real-time qPCR assays were applied for determination of antibiotic resistance gene concentration at the three sites. The detection limit of all real-time qPCR assays was 10 gene copies per reaction. The application of less than 10 gene copies of the resistance genes and the pathogenic bacteria (dilutions down to 1 gene copy per reaction were tested) gave results that were not accurately reproducible (data not shown). Therefore, only runs resulting in ≥10 gene copies per reaction were applied for the calculation of pathogen and resistance gene concentrations.

Quantitative monitoring of *H. pylori*, *M. avium* subsp. *pseudotuberculosis*, and *Y. enterocolitica* in three artificial aquifer recharge systems. In the reclaimed water samples for potable use (Torreele) or irrigation purposes (Nardò and Sabadell), none of the three pathogens were detected. In February 2007 at Nardò sampling point 1, the effluent of the Galatone WWTP showed a signal positive for *Y. enterocolitica*. Nevertheless, the copy number was below the limit for accurate quantification (10 target genes/reaction) of the *Y. enterocolitica* 16S rRNA gene. At sampling point 2 (sinkhole), *Y. enterocolitica* was not detectable anymore. *H. pylori* and *M. avium*

TABLE 4. Antibiotic resistance genes detected at Nardò

Mo and sample no.	Mean copy no./100 ml (SD)[a]					
	tetO	*ampC*	*ermB*	*vanA*	*mecA*	*blaSHV-5*
February						
1	8.65×10^4 (9.19×10^3)	0 (0)	1.35×10^5 (2.69×10^4)	0 (0)	0 (0)	0 (0)
2	3.20×10^4 (1.81×10^4)	0 (0)	4.00×10^3 (3.82×10^2)	0 (0)	0 (0)	0 (0)
3	1.39×10^5 (1.41×10^4)	0 (0)	4.54×10^4 (1.17×10^4)	0 (0)	0 (0)	0 (0)
4	1.27×10^5 (3.25×10^4)	0 (0)	3.87×10^4 (1.91×10^3)	0 (0)	0 (0)	0 (0)
5	9.99×10^2 (4.69×10^2)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
May						
1	2.67×10^5 (1.68×10^5)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
2	1.01×10^4 (1.50×10^3)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
3	2.19×10^4 (1.33×10^4)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
4	8.74×10^3 (1.92×10^3)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
5	1.21×10^3 (4.04×10^2)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
September						
1	3.76×10^6 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
2	1.22×10^4 (8.38×10^3)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
3	1.07×10^4 (7.06×10^3)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
4	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
5	1.93×10^4 (3.54×10^2)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

[a] The given values are the means from two to four measurements, with standard deviations. Nardò was sampled three times, in February, May, and September 2007. The sampling points are described in detail in the text. Also see Fig. S1 in the supplemental material.

subsp. *pseudotuberculosis* were never detected in any of the samples, not even in the WWTP influents from Sabadell, Torreele, or Nardò (1-liter samples each).

Quantitative monitoring of six antibiotic resistance genes in three artificial aquifer recharge systems. From January 2007 to October 2007, the reclamation sites Sabadell, Nardò, and Torreele were investigated in three (Torreele and Nardò) or four (Sabadell) sampling campaigns for the presence of the antibiotic resistance genes *ampC*, *ermB*, *mecA*, *blaSHV-5*, *tetO*, and *vanA*. The real-time qPCR results for all of the resistance genes are summarized in Tables 4, 5, and 6.

The resistance gene *vanA* was never detected. It was also absent from the effluents of the WWTPs. *ampC* was found twice in Sabadell, at sampling point 3 (river after effluent recharge) in January 2007 and at sampling point 1 (WWTP effluent) in June 2007. *tetO* and *ermB* were detected in all three sites. In Torreele, *tetO* was found in low concentrations in the infiltration water before transport ($5.92 \times 10^3 \pm 1.39 \times 10^3$ gene copies) and in the groundwater after infiltration ($3.13 \times 10^3 \pm 1.52 \times 10^3$ gene copies) in October 2007 but never in the drinking water. *tetO* was present in the Sabadell irrigation water in March 2007 at a concentration of $6.23 \times 10^3 \pm 1.03 \times 10^3$ gene copies and in October 2007 at a concentration of $2.39 \times 10^4 \pm 1.41 \times 10^2$ gene copies/100 ml water and in the Nardò sampling campaigns from February and May 2007 up to $1.27 \times 10^5 \pm 3.25 \times 10^4$ gene copies/100 ml irrigation water. However, background values in a range from $9.99 \times 10^2 \pm 4.69 \times 10^2$ to $1.93 \times 10^4 \pm 3.54 \times 10^2$ *tetO* gene copies/100 ml reference groundwater were detected in Nardò. In Torreele, *ermB* was found only once in the WWTP effluent; in Sabadell, it was detected in three of the four sampling campaigns. In March 2007, *ermB* was found in the irrigation water, with copy numbers of $9.50 \times 10^2 \pm 7.07 \times 10^1$/100 ml water; this is a decline of 2 log from the WWTP effluent to the irrigation water. In Nardò, *ermB* was found only in the February 2007 sampling campaign until sampling point 4 (well with reclaimed water at a 500-m distance from the sinkhole), at a concentration of $3.87 \times 10^4 \pm 1.91 \times 10^3$ gene copies/100 ml water. The reference groundwater was free of *ermB* resistance genes. The *blaSHV-5* gene, conferring resistance to extended-spectrum β-lactamases, was detected in the January, March, and June sampling campaigns in Sabadell. However, it has to be pointed out that this gene was never found in the water used for park irrigation. *mecA* was found only in the Sabadell reclamation site, in the January sampling campaign in all sampling points. Only a slight decrease of the *mecA* concentration of 1 order of magnitude, from $5.65 \times 10^4 \pm 1.56 \times 10^3$ gene copies/100 ml water in the WWTP effluent to $3.04 \times 10^3 \pm 2.26 \times 10^2$ gene copies/100 ml water in the irrigation water, was observed.

DISCUSSION

In this work, three European artificial groundwater recharge systems were studied with respect to their capacities for generating water of drinking water quality (in Torreele) and water for irrigation purposes (in Sabadell and Nardò). Two different surveys were performed over a time period of 1 year: (i) a classical monitoring of microbiological parameters and (ii) a molecular analysis of three pathogens causing waterborne diseases and six different antibiotic resistance genes.

Fecal-contamination analysis as well as monitoring of pH and electrical conductivity showed that Torreele fulfilled the criteria of the Flemish drinking water ordinance, which is in accordance with European Directive 98/83/EC with respect to all tested parameters. In only 1 of the 11 sampling campaigns did we observe 2 CFU enterococci/100 ml reclaimed water for potable use, but this was not confirmed and could be due to sampling errors (E. Van Houtte, personal communication). For the case studies where the aquifer was recharged for irrigation purposes, we obtained the following results. Spanish

TABLE 5. Antibiotic resistance genes detected at Sabadell

Mo and sample no.	Mean copy no./100 ml (SD)[a]					
	tetO	*ampC*	*ermB*	*vanA*	*mecA*	*blaSHV-5*
January						
1	0 (0)	0 (0)	1.35×10^5 (2.69×10^4)	0 (0)	5.65×10^4 (1.56×10^3)	0 (0)
2	0 (0)	0 (0)	1.87×10^5 (3.04×10^4)	0 (0)	1.35×10^5 (1.06×10^4)	0 (0)
3	0 (0)	3.85×10^3 (1.34×10^3)	2.28×10^5 (4.95×10^3)	0 (0)	4.40×10^4 (3.61×10^3)	0 (0)
4	0 (0)	0 (0)	1.78×10^4 (1.41×10^3)	0 (0)	1.19×10^5 (9.90×10^3)	1.60×10^2 (2.83×10^1)
5	0 (0)	0 (0)	0 (0)	0 (0)	3.04×10^3 (2.26×10^2)	0 (0)
March						
1	7.23×10^5 (3.18×10^5)	0 (0)	9.51×10^4 (1.69×10^4)	0 (0)	0 (0)	4.72×10^3 (2.48×10^3)
2	9.18×10^6 (7.85×10^5)	0 (0)	2.64×10^6 (4.38×10^5)	0 (0)	0 (0)	2.19×10^3 (3.75×10^2)
3	2.62×10^6 (8.49×10^4)	0 (0)	2.03×10^5 (1.48×10^4)	0 (0)	0 (0)	1.07×10^3 (3.75×10^2)
4	6.67×10^5 (8.49×10^4)	0 (0)	1.10×10^5 (8.49×10^3)	0 (0)	0 (0)	1.45×10^3 (3.22×10^2)
5	6.23×10^3 (1.03×10^3)	0 (0)	9.50×10^2 (7.07×10^1)	0 (0)	0 (0)	0 (0)
June						
1	2.77×10^6 (1.07×10^6)	6.88×10^3 (5.69×10^3)	2.00×10^4 (8.49×10^2)	0 (0)	0 (0)	1.14×10^4 (0)
2	9.16×10^4 (9.12×10^3)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
3	2.83×10^4 (1.05×10^4)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
4	4.27×10^3 (7.02×10^2)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
5	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
October						
1	1.59×10^6 (3.25×10^5)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
2	1.67×10^6 (4.03×10^5)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
3	3.22×10^6 (3.32×10^5)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
4	2.11×10^4 (4.38×10^3)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
5	2.39×10^4 (1.41×10^2)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

[a] The given values are the means from two to four measurements, with standard deviations. Sabadell was sampled four times, in January, March, June, and October 2007. The sampling points are described in detail in the text. Also see Fig. S1 in the supplemental material.

royal decree 1620/2007 for reclaimed water for park irrigation contains a single microbiological parameter, namely, that *Escherichia coli* has to be monitored and that levels are not allowed to exceed 200 CFU/100 ml water (41, 42). The *E. coli* threshold value was never reached in the Sabadell reclaimed water; in all sampling campaigns, it was actually clearly below this maximum permissible value. For the Italian case study site in Nardò, the Italian law of the main quality parameters for agricultural reuse of municipal wastewater (D. Lgs n. 185/03 [summarized in reference 25]) applies. It contains strict thresholds for two microbiological parameters, namely, a maximum of 10 CFU *E. coli*/100 ml water and no detectable CFU of *Salmonella* spp./100 ml water. The *E. coli* threshold was exceeded in four of the seven sampling campaigns in Nardò sampling point 4 (well at a 500-m distance from the sinkhole).

No regulations for the presence of the human-pathogenic bacteria *H. pylori*, *M. avium* subsp. *pseudotuberculosis*, and *Y. enterocolitica* in drinking water or irrigation water exist. This is based on a general lack of data about particular pathogens, with the exception of *Salmonella* and *Legionella* spp., in drinking water, water for irrigation, and recreational water. However, our data demonstrate that there is no risk for contamination with *H. pylori*, *Y. enterocolitica*, and *M. avium* subsp. *pseudotuberculosis* when the reclaimed water from the three reclamation sites is used, as none of the pathogens were ever detected in the purified water. The presence of *Y. enterocolitica*, *H. pylori*, and *M. avium* subsp. *pseudotuberculosis* was also investigated in a WWTP in Salisbury (South Africa) and in samples from the world's largest sewage field in Mezquital Valley (Mexico). One-liter water samples were processed from each site. Influent samples from the Mexican site showed the presence of *M. avium* subsp. *pseudotuberculosis* at concentrations between 1.6×10^2 and 2.6×10^3 gene copies/100 ml (U. Böckelmann and S. Hergett, unpublished data). *Y. enterocolitica* was also present in some of the samples from both sites. However, the concentration was below the limit for accurate quantification (10 gene copies/reaction). *H. pylori* was not detected in any of the samples.

For the antibiotic resistance genes, the situation is the same. No regulations with respect to the presence of resistance genes in drinking water or water for irrigation or recreational purposes exist. Our study aimed to show the potential impact of reclaimed wastewater on the antibiotic resistance gene pool in the environment. To this end, we applied a potent rapid molecular technique, real-time qPCR, to measure the concentrations of antibiotic resistance genes in the reclaimed water. The method proved to be very efficient, reproducible, and sensitive. A minimum of 10 gene copies of the respective antibiotic resistance gene could be detected reproducibly per real-time qPCR reaction.

Only two resistance genes, *tetO* and *ermB*, were detected in all of the three reclamation sites. Chen and coworkers and Cauwerts and colleagues found that the levels of abundance of the *erm* resistance genes often appeared to be positively correlated with those of the *tet* resistance genes (11, 13). Martins da Costa and coworkers investigated antimicrobial resistance in *Enterococcus* spp. isolated in inflow, effluent, and sludge from municipal WWTPs in Portugal (26). They found that

TABLE 6. Antibiotic resistance genes detected at Torreele

Mo and sample no.	Mean copy no./100 ml (SD)[a]					
	tetO	*ampC*	*ermB*	*vanA*	*mecA*	*blaSHV-5*
January						
1	1.05×10^7 (3.54×10^6)	0 (0)	1.92×10^5 (1.06×10^4)	0 (0)	0 (0)	0 (0)
2	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
3	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
4	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
5	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
6	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
July						
1	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
2	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
3	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
4	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
5	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
6	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
October						
1	4.35×10^6 (5.59×10^5)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
2	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
3	5.92×10^3 (1.39×10^3)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
4	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
5	3.13×10^3 (1.52×10^3)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
6	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

[a] The given values are the means from two to four measurements, with standard deviations. Torreele was sampled three times, in January, July, and October 2007. The sampling points are described in detail in the text. Also see Fig. S1 in the supplemental material.

34.6% of the isolates were resistant to tetracycline and 24.8% to erythromycin. Wastewater treatment in the Portuguese plants showed an enterococcal decrease up to 4 log. Nevertheless, more than 4.4×10^5 CFU/100 ml were present in the outflow of the plants (26). The reclamation sites investigated in this study were more efficient in the elimination of enterococcal contamination, with a maximum of 1.27 log CFU/100 ml for Sabadell and a maximum of 3.42 log CFU/100 ml for Nardò. We could also detect some correlation between the abundance of enterococci in the reclaimed water and the concentration of tetracycline and erythromycin resistance genes, respectively. In Nardò, in the February and May sampling campaign, *tetO* resistance genes were found in the reclaimed water at concentrations of 1.27×10^5 and 8.74×10^3, respectively. In these sampling campaigns, we noticed a twofold increase in the enterococcal contamination in comparison with that of the September sampling campaign, where neither *tetO* nor *ermB* was detected. The same scenario was observed in Sabadell: the highest *tetO* concentration in the irrigation water was measured in October 2007 (2.39×10^4 gene copies/100 ml). The October sampling campaign was the only one where enterococcal contamination was detected in the sprinkler water (1.27 log CFU/100 ml water). In Nardò, we additionally noticed a correlation between the concentration of total culturable bacteria at 22°C, the number of *E. coli* cells, and the abundance of *tetO* resistance genes. Sample 4 from the Nardò May sampling campaign (8.74×10^3 *tetO* copies/100 ml) showed the highest contamination by total bacteria and *E. coli* cells of all of the sampling campaigns (data not shown). In Sabadell, the highest *tetO* concentration was measured in October 2007. This is in agreement with the occurrence of contamination by coliforms and *E. coli* in the sample, in comparison with the absence of these indicators in the January and June samples, where no *tetO* and *ermB* genes were detected. There was no correlation between the presence of the oxacillin resistance gene, *mecA*, in the Sabadell samples from the January sampling campaign and elevated levels of total bacteria or enterococci (M. N. Ayuso-Gabella, personal communication).

Our data on the human intracellular pathogens are consistent with the data of others, as in the case of *Y. enterocolitica* with Alexandrino and coworkers. They could not detect any *Y. enterocolitica* in wastewater samples or purified water from two different constructed wetlands in Germany by multiplex PCR (3). For *H. pylori*, the results vary with different countries and different wastewater purification systems: Shannon and coworkers could not detect *H. pylori* in any stage of municipal wastewater treatment in Canada in a real-time qPCR study (38), whereas a nested-PCR study of water systems for human use in Mexico City demonstrated the absence of *H. pylori* in water from dams used as water sources but found that 17% of treated wastewater samples contained the pathogen (27). For *M. avium*, no molecular studies of WWTPs are available.

It is well known that effluent released by WWTP can be a source for antibiotic resistance genes in the environment (16, 20). The best-investigated antibiotic resistance genes with respect to presence in the environment are the tetracycline resistance genes, including *tetO*. Ferreira da Silva and coworkers (16) showed that tetracycline-resistant enterococci were not eliminated during wastewater treatment consisting of primary and secondary activated sludge processes. The data are consistent with our Sabadell and Nardò results. At both case study sites, secondary WWTP effluent is processed by soil passage and dilution with surface waters. Both artificial aquifer recharge sites resulted in a release of enterococci and in a temporary release of *tetO* and *ermB* genes in the environment via the irrigation water. Volkmann et al. performed real-time

qPCR analysis for the presence of the resistance genes *ampC*, *vanA*, and *mecA* in municipal wastewater in Germany. *vanA* was detected in 21% of the wastewater samples and *ampC* in 78%. *mecA* was not found in municipal wastewater but was found in two clinical wastewater samples (44). Ferreira da Silva and coworkers also looked for vancomycin-resistant enterococci in raw and treated wastewater, but no resistance among the enterococcal isolates was observed (16). The detection of *ampC* in wastewater is in agreement with our data from the Sabadell site, where *ampC* was found in two of the four sampling campaigns. *vanA* was never detected in our survey, not even in the raw wastewater (data not shown). The presence of *mecA* in all samples from the Sabadell January 2007 sampling campaign could be explained by the fact that the Sabadell WWTP receives clinical wastewater from the local hospital. For the resistance gene *blaSHV-5*, no comparable analyses have been conducted.

No clear trend in the extent of contamination by antibiotic resistance genes over the course of the year was observed for the three sites. Consistent for all three sites is that the lowest resistance gene contamination was found in the summer sampling campaign. No resistance genes were detected in Torreele in the July samples, in Sabadell in the irrigation water in June, or in Nardò in the irrigation water from September 2007.

The three investigated artificial aquifer recharge systems demonstrated different capacities of removal of fecal contaminators and antibiotic resistance genes. Our data demonstrated that *tetO*, *ermB*, and *mecA* can occur in groundwater derived from artificial recharge, suggesting that groundwater may be a potential source of antibiotic resistance in the food chain.

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