



Quantification of pathogenic microorganisms and microbial indicators in three wastewater reclamation and managed aquifer recharge facilities in Europe

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ABSTRACT

Managed Aquifer Recharge (MAR) is becoming an attractive option for water storage in water reuse processes as it provides an additional treatment barrier to improve recharged water quality and buffers seasonal variations of water supply and demand. To achieve a better understanding about the level of pathogenic microorganisms and their relation with microbial indicators in these systems, five waterborne pathogens and four microbial indicators were monitored over one year in three European MAR sites operated with reclaimed wastewater. *Giardia* and *Cryptosporidium* (oo)cysts were found in 63.2 and 36.7% of the samples respectively. *Salmonella* spp. and helminth eggs were more rarely detected (16.3% and 12.5% of the samples respectively) and *Campylobacter* cells were only found in 2% of samples. At the Belgian site advanced tertiary treatment technology prior to soil aquifer treatment (SAT) produced effluent of drinking water quality, with no presence of the analysed pathogens. At the Spanish and Italian sites amelioration of microbiological water quality was observed between the MAR injectant and the recovered water. In particular *Giardia* levels decreased from 0.24–6.14 cysts/L to 0–0.01 cysts/L and from 0.4–6.2 cysts/L to 0–0.07 cysts/L in the Spanish and Italian sites respectively. *Salmonella* gene copies and *Giardia* cysts were however found in the water for final use and/or the recovered groundwater water at the two sites. Significant positive Spearman correlations ($p < 0.05$, r_s range: 0.45–0.95) were obtained, in all the three sites, between *Giardia* cysts and the most resistant microbial markers, *Clostridium* spores and bacteriophages.

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1. Introduction

Increasing demand for water to meet agricultural, industrial, environmental and municipal needs over the last few decades has extended the need for reclaimed water reuse. The number of water reclamation and reuse schemes in Europe, and around the world (i.e. USA, Australia), has increased considerably this century (Angelakis and Bontoux, 2001; Asano et al., 2007; Dillon et al., 2008). One of the main concerns for reclaimed water reuse is the microbiological quality of water and thereby the possibility of spreading diseases as it has

been found that pathogenic microorganisms are not completely eliminated by conventional secondary wastewater treatment (Rose et al., 1996; Castro-Hermida et al., 2008; Wéry et al., 2008) and are also detected in tertiary and final effluents of water reclamation plants for non potable purposes (Rose et al., 1996; Gennaccaro et al., 2003; Harwood et al., 2005; Costán-Longares et al., 2008).

To reduce the health risk associated with reuse of reclaimed water to an acceptable level, it is very important to design multiple barrier systems and to carefully examine, from a microbiological point of view, any kind of process directed to reclaim water (Asano and Cotruvo, 2004). In MAR systems operated with pre-treated wastewater a further improvement of microbiological water quality is achieved by natural attenuation processes during soil filtration and/or transport in the aquifer (Dillon et al., 2008; John and Rose, 2005; Asano et al., 2007).

A great variety of pathogenic microorganisms, characterised by different survival capacities in the environment and differing resistance to water treatments can be transmitted through water

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contact and ingestion to humans (WHO, 2006). The type and abundance of waterborne pathogens highly varies among countries and it is therefore fundamental to identify the microorganisms that can be of health concern in water reuse cases in the specific region of interest (Jiménez, 2003). Many papers dealing with all aspects of water recycling in various water treatment processes are available; however recent publications on the occurrence and removal of pathogenic and indicator microorganisms in wastewater reclamation processes (Bonadonna et al., 2002; Gennaccaro et al., 2003; Harwood et al., 2005; Costán-Longares et al., 2008) focused mainly on enteroviruses, *Cryptosporidium* oocysts and *Giardia* cysts and their attenuation in tertiary treatments. Furthermore field studies data on the occurrence and fate of microorganisms of concern to human health in aquifer recharge with reclaimed water are still scarce in spite of the relevance of these natural attenuation processes in water reuse schemes. Evidence of pathogens and indicators *in situ* decay in the aquifer were however shown (Bitton et al., 1983; Toze et al., 2010; Dillon and Toze, 2005). In particular *in situ* pathogen decay during MAR was determined measuring the reduction over time of microorganisms in diffusion chamber seeded with the pathogen of interest and located in the aquifer (Toze et al., 2010; Dillon and Toze, 2005). Two recent studies (Toze et al., 2010; Page et al., 2010) used the quantitative microbial risk assessment approach to determine pathogen risk in MAR systems. The risk assessment calculations were developed with pathogen decay rates obtained from *in-situ* decay studies using the diffusion chambers and direct data on pathogens concentration in the aquifer were not provided.

Due to the expected low occurrence and the difficulties of pathogens quantification in environmental samples available guidelines (USEPA, 2004; WHO, 2006) and specific national regulations (i.e. Italian Law DM185, 2003; Spanish Official Bulletin, RD, 1620/2007) for reclaimed water quality and reuse are usually based on the levels of bacterial indicators, namely faecal coliforms or *E. coli*. Costán-Longares et al. (2008) and Harwood et al. (2005) described the failure entailed in using single microbial indicators to predict the presence and removal of protozoan and viral pathogens in water reclamation processes indicating that further data on the correlation between marker microorganisms and pathogens in water reuse processes are required to define more reliable indicators of water quality in these systems. New molecular methods, such as quantitative PCR (qPCR), which allows the specific and rapid detection and quantification of target microorganisms in environmental samples, represent a promising approach as an alternative to and/or as a support to microbial indicators monitoring for water quality assessment and compliance with regulations.

The aims of the study were to describe the occurrence of different types of waterborne pathogens and microbial indicators at three European sites of aquifer recharge with treated municipal wastewater, including:

1. an extensive low-technology system where a mixture of secondary effluent and river water is recharged in the aquifer by river bank filtration for production of non potable water (Sabadell, Spain);
2. a technically advanced process of MAR with extensively treated wastewater for drinking water production (Torreale, Belgium);
3. a case of direct injection of secondary treated wastewater in a karstic fractured aquifer as source of non potable water (Nardò, Italy).

The frequency and concentration of five pathogens (Helminth eggs, *Giardia* and *Cryptosporidium* (oo)cysts, *Salmonella* spp. and *Campylobacter* spp.) and four microbial indicators of water contamination (*E. coli*, enterococci, *Clostridium* spores, and somatic coliphages) characterised by different resistance to water treatment and survival capacities were investigated during one year at the three sites and the correlation between presence of indicators and pathogens was assessed.

2. Material and methods

2.1. Sites description and sampling campaigns

The study was performed in three European MAR sites with treated municipal wastewater. The main characteristics of the sites, including the level of wastewater treatment, the aquifer recharge scheme, aquifer characteristics, the final use of water and the sampling points, are described in Table 1. Pathogenic microorganisms diverse in their sizes, infection doses, and capacity of survival in the environment, which are known to be pathogens of major importance with regard to waterborne diseases and are reference microorganisms for national/international legislation and guidelines for water reuse, were selected.

The presence of *Giardia* and *Cryptosporidium* (oo)cysts, helminth eggs, *Salmonella* spp., *Campylobacter* spp. and microbial indicators was monitored in three sampling campaigns at the Sabadell (March 2007, June 2007 and October 2007) and Torreale (February 2007, July 2007 and October 2007) sites. The presence and attenuation of *Salmonella* spp., helminth eggs and microbial indicators in the fractured karstic subsoil of the Nardò aquifer recharge site was investigated during four sampling campaigns (November 2006, February 2007, May 2007 and September 2007). The presence and abundance of *Giardia* cysts and *Cryptosporidium* oocysts in the Nardò site, partially described in La Mantia et al. (2008), are also reported for comparison.

2.2. Enumeration of *Giardia* and *Cryptosporidium* (oo)cysts

For *Giardia* cysts and *Cryptosporidium* oocysts detection, sample volumes varied depending upon the treatment stage and the amount of water that could be filtered. For example, approximately 1 L of wastewater, 10–30 L of secondary effluent, 30–60 L of surface water, and 50–115 L of ultra-filtration (UF) filtrate, groundwater and water for final use were filtered. Samples were concentrated and analyzed as previously described (La Mantia et al., 2008). The protozoan detection limits varied with the total volume of sample filtered and analysed and ranged between 0.0087 cysts/L and 1 cyst/L.

2.3. Helminth eggs analysis

The protocol used for the determination of helminth eggs is based on the Baileger technique adapted by Gracenea and de Montoliu (2003). Different volumes were sampled: 20–25 L for the influent of the wastewater treatment plant (WWTP) and 20–100 L for the effluent, surface water, groundwater and finished water. Helminth eggs were concentrated as previously described by Gracenea and de Montoliu (2003), identified according to Thienpont et al. (1986) and counted by microscopic observation in phase contrast at 100× magnification (Laborlux K microscope Leitz).

2.4. Pathogenic bacteria detection

Real time qPCR was applied for the quantitative detection of *Salmonella* in environmental water samples, using primers and probe targeting within the *trrSBCA* locus (Malorny et al., 2004). *Salmonella enterica* strain LT2 (DSM 10506) and *S. enterica* strain 51 K61 (kindly provided by Dr. Burkhard Malorny) were used as reference strains. *Salmonella* LT2 and 51 K61 were cultivated overnight on TGA and LB medium respectively for DNA extraction and PCR standard production. Different volumes of water samples, from 7 to 40 L, were collected according to water pollution and particulate concentration. Samples were concentrated and DNA was extracted and quantified as described in Böckelmann et al. (2009). Serial dilutions of LT2 and 51 K61 DNA, in Tris–EDTA buffer (Tris–HCl 10 mM, EDTA 0.5 mM) containing 10 µg/mL herring sperm, were applied as standards in the

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