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Abstract:	The aim of this study was the evaluation of the occurrence of pathogenic Campylobacter, E. coli O157:H7, E. coli virulence genes and Salmonella spp. in different wastewater treatment plants (WWTPs) using a method based on an enrichment step and PCR. This method was sensitive enough to detect low levels (2 CFU/100 ml of raw sewage) of all the investigated pathogens. In the WWTPs samples, E. coli O157:H7 DNA and the eae gene were never found, but 33% of influents and effluents exhibited amplicons corresponding to Shiga-like toxin I. 25% of the influent and 8% of the effluent exhibited the presence of Shiga-like toxin II. Campylobacter jejuni and C. coli DNA were identified in 50% and 25% of the influents and in 8% and 25% of the effluents, respectively. Salmonella spp. DNA was present in all the samples. Considering the results obtained the method tested here offer a reliable and expeditious tool for evaluating the efficiency of the effluents treatment in order to mitigate contamination risk. Influent contamination by Salmonella spp. and Campylobacter spp. provides indirect information about their circulation, moreover their presence in effluents underlines the role of WWTPs in the contamination of the receiving surface waters, which affects public health directly or indirectly.
Response to Reviewers:	Reviewer 2 We agree that the “best practise” procedure in the study design is the use of a positive control as confirmed by the approach utilized in this study, but it was not possible to buy the toxigenic strain (not commercialized in Europe by ATCC). Despite this, we think that the positive results obtained in the wastewater samples don’t represent an

	artefact. In fact during the sampling, amplicons with the expected sizes of these genes (Hu et al., 1999, JAM, vol 87) were obtained. Reviewer 3 Major point Binary logistic regression was performed to study the association between the concentration of indicator bacteria and the occurrence of pathogens in wastewater samples. Differences in occurrence of pathogenic bacteria between different wastewater treatment plant (Chieri, Lanzo and Castiglione) and sampling time (Spring; Summer; Autumn; Winter) were studied, but the results obtained did not show a relationship. This information was added in the text. Minor points 1 The volume of spiking sample was added in the text 2. The description of the wastewater treatment methods were clarified. 3. The approach used to evaluate the sensitivity of the protocol was the approach of the “worst case”. The sample used to verify the sensitivity of the protocol was the raw sewage of Castiglione Torinese WWTP, that collects domestic and industrial sewage of the Turin city. Considering the information available at the start of this study, the characteristics of this sample (e.g. turbidity, kind of input) were the worst also respect to the effluent samples. 4. The text was modified as required.
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TITLE PAGE

Detection of pathogenic *Campylobacter*, *E. coli* O157:H7 and *Salmonella* spp. in wastewater by PCR assay

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ABSTRACT

The aim of this study was the evaluation of the occurrence of pathogenic *Campylobacter*, *E. coli* O157:H7, *E. coli* virulence genes and *Salmonella* spp. in different wastewater treatment plants (WWTPs) using a method based on an enrichment step and PCR. This method was sensitive enough to detect low levels (~2 CFU/100 ml of raw sewage) of all the investigated pathogens. In the WWTPs samples, *E. coli* O157:H7 DNA and the *eae* gene were never found, but 33% of influents and effluents exhibited amplicons corresponding to Shiga-like toxin I. 25% of the influent and 8% of the effluent exhibited the presence of Shiga-like toxin II. *Campylobacter jejuni* and *C. coli* DNA were identified in 50% and 25% of the influents and in 8% and 25% of the effluents, respectively. *Salmonella* spp. DNA was present in all the samples. Considering the results obtained the method tested here offer a reliable and expeditious tool for evaluating the efficiency of the effluents treatment in order to mitigate contamination risk. Influent contamination by *Salmonella* spp. and *Campylobacter* spp. provides indirect information about their circulation, moreover their presence in effluents underlines the role of WWTPs in the contamination of the receiving surface waters, which affects public health directly or indirectly.

Keywords: *Campylobacter* spp., *E. coli* O157:H7, virulence genes, *Salmonella* spp., PCR, wastewater

INTRODUCTION

Fecal contamination is a source of environmental bacterial contamination and it is responsible for the presence of pathogens in natural environment (Bertrand and Roig 2007). Surface water can generally be contaminated by human pathogens from enteric origin as a consequence of soil contamination from animal manures or the release of untreated slurry or effluents from wastewater treatment plants. Although wastewater is treated to eliminate pathogenic microorganisms and prevent waterborne transmission, numerous studies indicate that conventional wastewater treatment does not guarantee their complete elimination (Espigares et al. 2006). The microbiological quality of wastewater can also pose a number of potential risks in terms of public health and environment contamination when considering the possible reuse of wastewater effluents (Levantesi et al. 2010). Different pathogenic and opportunistic bacteria have been found in treated effluent, and these bacteria include Shiga toxin (Stx) *Escherichia coli* (STEC or VTEC), *Salmonella* spp., *Campylobacter jejuni*, *Bacillus cereus*, *Clostridium difficile*, and *Listeria monocytogenes* (Varela and Manaia 2013).

Campylobacter spp. and Shiga toxin-producing *E. coli* are important causes of bacterial gastroenteritis in the United States and around the world (Park et al. 2011). In 2013, *Campylobacter* spp. was the most commonly reported gastrointestinal bacterial pathogen in humans in the European Union (EU), and has been since 2005. Moreover, 6,043 confirmed cases of VTEC infections were reported in the EU (EFSA 2015). Nevertheless, the reported cases of *Campylobacter* spp. and VTEC enteritis in Italy are below average relative to reported cases in other European and non-European countries with analogous characteristics (e.g., environmental contamination, routes of transmission). This condition could be ascribed to many factors such as a very low diffusion of these pathogens in the environment and in the Italian human population, or case underestimation (Kaakoush et al. 2015; Pennington 2010).

The water cycle plays an important role in the *Campylobacter* spp. and VTEC enteritis transmission. *C. jejuni*, *C. coli* and VTEC are often present in aquatic environments (e.g. wastewater), which may provide a reservoir, or even a direct source, of their infection (Saxena et al. 2015; Ugarte-Ruiz et al. 2015). *Campylobacter* spp. and VTEC are most frequently associated with waterborne disease outbreaks worldwide (CDC 2013, EFSA 2015; Guzman-Herrador et al. 2015).

The detection of pathogenic microorganisms in wastewater samples is labour-intensive, time-consuming and difficult. Indicator microorganisms (e.g., *E. coli*, faecal enterococci) and *Salmonella* spp. are

generally used to assess the microbiological quality of effluents produced by wastewater treatment plants. However, the use of traditional indicators is known to have limitations because of their inability to predict the presence of all pathogen types in environmental water (Teklehaimanot et al. 2014). The introduction of molecular methods, such as the polymerase chain reaction (PCR), has resolved some problems in pathogen detection research. PCR has high specificity, speed and sensitivity (Shannon et al. 2007). These characteristics are required to evaluate public health risks accurately, quickly identify contaminated wastewater and minimize human exposure (Bertrand and Roig 2007).

The aim of this study was to evaluate the occurrence of pathogenic *Campylobacter*, *E. coli* O157:H7, *E. coli* virulence genes and *Salmonella* spp. in influents and effluents of different wastewater treatment plants (WWTPs). Data about the presence of these pathogenic bacteria in untreated sewage can reflect clinical and subclinical infections that are prevalent in human populations; moreover, the results obtained in treated effluents can provide useful information about the role of WWTPs as a possible source of environmental contamination. To evaluate the occurrence of *Campylobacter* spp., *E. coli* O157:H7, *E. coli* virulence genes and *Salmonella* spp. in wastewater samples, a method based on an enrichment step and PCR was tested.

MATERIALS AND METHODS

Bacterial strains and culture media

C. jejuni (ATCC 33291), *E. coli* O157:H7 (NCTC 129, non-toxigenic strain, encoding eae gene) and *S. typhimurium* (ATCC 14028) were used as quality control strains throughout this study. *C. jejuni* strain was cultivated on blood-free Campylobacter Medium base (Karmali; Biolife, Milan, Italy) or Bolton broth (Oxoid, Cambridge, UK) at 42°C under a microaerobic atmosphere (Campygen; Oxoid), and *E. coli* O157:H7 and *S. typhimurium* were grown on Tryptic Soy Agar (TSA; Applichem, Darmstadt, Germany) or in Tryptic Soy Broth (TSB; Applichem,) at 37°C.

Sampling

Influent and final effluent samples were collected from three Italian Wastewater Treatment Plants located in the Piedmont region (Italy) as follows: Castiglione Torinese (untreated sewage corresponding to a population equivalent of 2,500,000), Chieri (untreated sewage corresponding to a population equivalent

60,000) and Lanzo Torinese (untreated sewage corresponding to a population equivalent 8,000). The WWTPs located in Castiglione Torinese, Chieri and Lanzo employ screening, aerated grit removal, biological treatment in a denitrification-nitrification process based on activated sludge, and secondary settling. Finally, the effluents are discharged into the receiving water bodies (Po, Tepice-Banna, and Stura Rivers, respectively). The plant at Castiglione Torinese uses primary settling before the biological treatment, then dephosphatization and a filtration step after the secondary settling.

Wastewater samples were collected during four sampling periods (spring-May 2014, summer-July 2014, autumn October 2014 and winter-February 2015) at the three WWTPs.

Wastewater composite samples (24 h) were collected in sterile plastic bottles, transported on ice to the laboratory and tested within 24 h.

Sample processing

Samples of raw sewage (100 ml) and treated effluent (1 l) were used for pathogen detection with the PCR protocol. During each sampling, a raw sewage sample spiked with a high concentration of pathogens (10^6 CFU) was also prepared (positive control). The main steps of the protocol for pathogen detection in wastewater samples are summarized in Fig. 1. In brief, wastewater sample was concentrated by filtration through 0.45 μm pore size nitrocellulose filters (Merck Millipore, Vimodrone, Italy). The filters were then vortexed in peptone water (Oxoid) for *E. coli* O157:H7 and *Salmonella* spp. detection and in Bolton broth containing an antibiotic supplement (Oxoid) for *Campylobacter* spp. detection. These broths were cultivated (enrichment step) at 37°C for 18 h for *E. coli* O157:H7 and *Salmonella* spp. detection and at 42°C for 48 h under a microaerobic atmosphere for *Campylobacter* spp. detection. Following incubation, 2 ml of each broth were centrifuged at 4,500 g for 20 min to recover the bacteria. The DNA was extracted and purified with a PowerSoil® DNA Isolation Kit (Mo Bio Laboratories Inc., Carlsbad, USA) according to the manufacturer's protocol. The resulting DNA was used for PCR amplification.

PCR analysis

A triplex PCR amplification assay was performed to target a genus-specific 16S rRNA for *Campylobacter* spp. and species-specific mapA and ceuE genes for the detection of *C. jejuni* and *C. coli* species. The PCR amplification reactions were performed with previously documented primer pairs and

PCR protocol (Khan et al. 2009). Each 50 µl reaction mixture contained 15 µl of template DNA, 1X PCR buffer (20 mM Tris-HCl [pH 8.4], 50 mM KCl), 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.2 µM of each primer and 1 U of Taq Gold Polymerase (Applied Biosystems, Milan, Italy). The amplification reaction was performed with an initial template denaturation step at 95°C for 10 min, followed by 35 cycles of amplification consisting of denaturation at 95°C for 30 s, annealing at 59°C for 1.5 min, and extension at 72°C for 1 min, followed by a final extension at 72°C for 5 min.

Details about the PCR protocol for *E. coli* O157:H7, *E. coli* virulence gene and *Salmonella* spp. were previously reported in Bonetta et al. (2011).

Detection sensitivity

In order to verify the sensitivity of the protocol, raw sewages (100 ml) were collected and artificially inoculated with different concentrations of *C. jejuni, E. coli* O157:H7 and *S. typhimurium* bacterial cells (10-fold dilution series, 2 10^7-2 CFU). The exponential phase cultures were serially diluted and the number of CFU was determined by plating the dilutions on agar media.

In all the experiments, the same non-inoculated wastewater sample was analysed to verify the absence of the pathogens (negative control).

Microbiological analyses

E. coli, enterococci, *Clostridium perfringens* spores, coliforms and *Salmonella* spp. were analysed in all samples. In brief, the membrane filtration method was used to process wastewater samples for *C. perfringens* enumeration as reported by the ISO 14189:2013. Wastewater samples were assayed for *E. coli*, coliforms and enterococci with a commercial Quanti-Tray™ 2000 (IDEXX Laboratories, Milan, Italy) (method EN ISO 9308-2:2014 and ISS F 003B rev.009). For *Salmonella* spp. detection, 100 ml of influent sample and 1 l of effluent sample were filtered through 0.45 µm-pore (47 mm diameter) nitrocellulose membranes (Merck Millipore) followed by a pre-enrichment step (Peptone Water, Oxoid), a selective enrichment step (Rappaport Vassililadis Broth, Oxoid) and a selection in XLD Agar (Oxoid) (method ISS F002C rev00). Bacterial colonies with typical *Salmonella* morphology were subcultured onto TSA for 18-24 h and then tested for oxidase, and with API® 20E identification kit (BioMerieux, Marcy L'Etoile, France).

Statistical analyses

Statistical analyses were performed with SPSS Package version 22.0 for Windows. Qualitative data for the pathogen variables and a Log 10 conversion of the indicator bacteria variables were used in the statistical analysis. Binary logistic regression was performed to study the association between the concentration of indicator bacteria and the occurrence of pathogens in wastewater samples. Differences in occurrence of pathogenic bacteria between different types of wastewater treatment plant and sampling time were studied with binary logistic regression.

RESULTS AND DISCUSSION

Development of PCR assays for pathogenic *Campylobacter*, *E. coli* O157:H7, *E. coli* virulence genes and *Salmonella* spp.

For successful detection in wastewater samples, we have proposed a method for *Campylobacter* (spp., *jejuni*, *coli*), *E. coli* O157:H7, *E. coli* virulence genes and *Salmonella* spp. based on an enrichment step and PCR.

In evaluating the experiments on wastewater-spiked samples, an initial DNA purification protocol using Chelex and proteinase K was performed on raw wastewater samples that had been inoculated with a high concentration (2 10^7 CFU) of each pathogen (*C. jejuni*, *E. coli* O157:H7 and *S. typhimurium*), but no amplicons were recovered. Considering the difficulties experienced with this protocol, the DNA was extracted and purified with a commercial method that was usually used for DNA extraction from soil (Power Soil® DNA Isolation Kit). Typical PCR amplicons from *Campylobacter* spp. and *C. jejuni* were obtained by analysing inoculated raw wastewater (Fig. 2a). A multiplex PCR analysis of a wastewater sample inoculated with a non-toxigenic *E. coli* O157:H7 strain revealed three distinct bands of the expected sizes that corresponded to H7, intimin and O157 (Fig. 2b). As expected, no PCR amplicons were observed for Shiga-like toxin I and II genes. Additionally, the PCR primers used for the detection of *Salmonella* spp. generated a specific PCR product (Fig. 2c).

The results obtained for artificially contaminated wastewater samples (Range 2 10^7-2 CFU) to verify the sensitivity of the protocol are shown in Table 1. The tested method showed a high sensitivity for all microorganisms, with levels as low as ~2 CFU in the spiked wastewater samples (100 ml of raw sewage).

For all the pathogens, the presence of PCR products was observed in all duplicate wastewater samples and at the lowest inoculum concentration; no PCR amplicons were observed in the unspiked samples (negative control). The tested method has the following advantages: i) the method involve the use of an enrichment step. Broth enrichment is often used to promote the recovery when the number of pathogens cells is low and can also dilute the inhibitory compounds produced by competing bacteria in the sample as well as aid the recovery of injured, stressed or lag-phase bacterial cells (Pitkanen 2013; Touron et al. 2005) ii) the enrichment broth (peptone water) can also be used for *Salmonella* spp. detection in wastewater with the cultural method; iii) the multiplex PCR protocol for VTEC detection uses primer pairs for *E. coli* O157:H7 DNA that can simultaneously reveal serotype O157:H7 and its virulence traits, as previously reported in surface water samples (Bonetta et al. 2011); and iv) the presence of *Campylobacter* spp., *C. jejuni* and *C. coli* DNA can be monitored with a single multiplex PCR.

Occurrence of pathogenic *Campylobacter, E. coli* O157:H7, *E. coli* virulence genes and *Salmonella* spp. in wastewater samples

The results of analyses performed on wastewater samples with the developed protocol are reported in Table 2. *Salmonella* spp. DNA was present in all raw sewage analysed by molecular methods. Moreover all the samples were positive for *Salmonella* spp. detection by cultural method. These data are consistent with the results obtained by Cataldo et al. (2001) that verify the occurrence of *Salmonella* spp. in raw sewage from a wastewater treatment plant located near Rome (Italy). A high *Salmonella* spp. concentration was detected in municipal Finnish wastewaters (Koivunen et al. 2003), and in municipal wastewater treatment plants located in France (Wery et al. 2008) and South Africa (Teklehaimanot et al. 2015). Minor contamination (33% of influent samples) was revealed in a similar study conducted in different wastewaters from the province of Venice (Ostoich et al. 2007). The results obtained in our work suggest that the presence of *Salmonella* spp. in the influents could be a consequence of the local epidemiological situation as proposed in other studies (Berge et al. 2006; Ostoich et al. 2007). In our study, the *Salmonella* spp. contamination was also monitored in all of the effluents of WWTPs, highlighting that this bacteria survived the treatment processes and was discharged to the natural receiving waters. This behaviour indicated that conventional municipal wastewater treatment without efficient tertiary treatment may constitute a risk to public health (Koivunen et al. 2003).

The results obtained by PCR highlighted that 83% (20/24) of the wastewater samples were positive for H7 DNA, but *E. coli* O157:H7 DNA was never found. The prevalence of Shiga toxin-producing *E. coli* in sewage has previously been evaluated in different studies, showing a wide range of positive samples for the isolated bacteria depending on the volume of analysed samples and the given method (Muniesa et al. 2006). The molecular analysis conducted in our study on the major virulence factor genes of pathogenic Shiga toxin-producing *E. coli* strains highlighted the presence of amplicons that correspond to *stx1* and *stx2* according to the results obtained in other studies (Dumke et al. 2006; Martinez-Castillo et al. 2012). A total of 4 influent (4/12 or 33%) and 4 effluent (4/12 or 33%) samples revealed the presence of amplicons corresponding to Shiga-like toxin I. These amplicons were found during the spring and summer seasons. The stx_2 gene (Shiga like toxin II) was detected only in 3 wastewater influent samples (3/12 or 25%) during the summer and winter and in a single effluent sample during the summer (1/12 or 8%). These results highlighted the possible presence of other pathogenic bacteria. In fact, the *stx*1/*stx*2 genes are widely distributed among *E. coli* (Shiga-toxin-producing *E. coli* or verotoxin-producing *E. coli*) and *Shigella* strains, as well as other waterborne bacteria, because of their dissemination via bacteriophages (James et al. 2001; Strauch et al. 2008). It is important to highlight that although *Stx* is the primary factor that defines the virulence of Shiga toxin-producing *E. coli*, its presence is essential but not sufficient to cause infection. Other major virulence factors such as the *eae* gene, which encodes the intimin protein, could play an important role (Loukiadis et al. 2006; Martinez-Castillo et al. 2012; Yang et al. 2014). In our study, the *eae* gene related to intimin expression was never observed.

During sampling, the presence of the *Campylobacter* genus DNA was observed in 83% (10/12) of wastewater influent samples. This result highlights widespread contamination by thermotolerant *Campylobacter* species in the inlet of monitored wastewater samples. Different studies showed that *Campylobacter* spp. are ubiquitous in sewage (Rechenburg and Kistemann 2009; Whiley et al. 2013). In almost all the samples (67%, 8/12), the presence of this genus was related to *C. jejuni* and/or *C. coli* contamination. 50% (6/12) and 25% (3/12) of the influent samples were positive for *C. jejuni* and *C. coli* DNA, respectively. These data allowed us to suggest the real circulation of these microorganisms in the population, underlining the need to investigate this issue further. A lower frequency of *C. jejuni* contamination was revealed in raw sewage samples (36.4%) in British Columbia (Canada) (Jokinen et al.

2010), and a higher frequency (98%) was observed in untreated urban effluents in Spain (Ugarte-Ruiz et al. 2015).

Considering the results obtained in our study, 42% (5/12) of wastewater effluents were contaminated by *Campylobacter* spp. DNA; in particular the frequency of positive wastewater samples was 8% (1/12) and 25% (3/12) for *C. jejuni* and *C. coli* DNA, respectively. These results indicated that the treatment of raw sewage may be insufficient to eliminate these pathogenic microorganisms. Previous studies have reported that the majority of *Campylobacter* are already eliminated during primary sedimentation, even if the effectiveness of a sewage treatment plant in reducing *Campylobacter* contamination also depends on the complexity of the plant as well as on the characteristics of the raw sewage (Whiley et al. 2013). Moreover, even if a lower frequency of these bacteria was observed with respect to influent samples, it is important to highlight that the samples were collected simultaneously, and thus a real relation between the influent and effluent is not possible.

Seasonal frequency variation was not observed in all the monitored pathogens ($p>0.05$), and their detection in wastewater did not seem to be affected by the capacity of the wastewater treatment plant ($p>0.05$).

To verify the correlation with emerging pathogens, some classic faecal indicators such as *E. coli*, coliforms, enterococci and *C. perfringens* spores were also analysed. *Salmonella* spp. was monitored with the culture method because it is generally used as a pathogen indicator in wastewater samples; moreover, this parameter is used to evaluate the possible reuse of wastewater effluent as fertilizer. The results for the faecal indicators and the mean abatement of the three WWTPs are reported in Figure 3. The coliform, *E. coli* and enterococci loads had values ranging between 7.3-5.5, 6.8-4.4 and 5.8-3.7 log MPN 100 ml^{-1}, respectively. The concentration of *C. perfringens* spores ranged from 5.6-3.6 log CFU 100 ml^{-1}. Generally, the lowest values for all the parameters were observed in the influent of the Lanzo wastewater treatment plant. As observed for the molecular method, *Salmonella* spp. was present in all the analysed samples. The greatest reduction in faecal indicators occurred in the WWTP of Castiglione (range 2.0-2.5 log) followed by Chieri, with a lower overall average (range 1.3-2.0 log), and Lanzo (range 0.7- 0.8 log).

No significant association between the occurrence of bands corresponding to H7, O157, intimin (*eae* gene), Shiga-like toxin I (stx_1 gene), Shiga-like toxin II (stx_2 gene) and microbiological indicators (*E. coli*, enterococci, *C. perfringens* spores, and coliforms) were obtained by binary logistic regression ($p>0.05$).

Moreover the results of the binary logistic regression did not show a relationship between the bacterial indicator load and the occurrence of *Campylobacter* spp., *C. jejuni* and *C. coli* DNA in wastewater samples ($p>0.05$), except the coliform count for *Campylobacter* spp. and *C. coli* DNA. No relation was observed between *Salmonella* spp. contamination and faecal indicators or other pathogens ($p>0.05$). These results suggest that the indicator bacteria were not good indicators of different pathogenic bacteria in municipal wastewater samples, according to different studies that showed that the presence of indicator bacteria does not clearly correlate with the presence of pathogenic bacteria including *Salmonella* spp. and *Campylobacter* spp. (Li et al. 2013; Wery et al. 2008).

In conclusion, the method tested here offer a reliable and expeditious tool for evaluating the efficiency of the effluents treatment in order to mitigate contamination risk and could be used as a routine analysis for the evaluation of microbiological quality in wastewater samples. Moreover, the data obtained from the samples showed the presence of pathogenic bacteria, especially *Salmonella* spp. and thermotolerant *Campylobacter* spp., in the untreated sewage of wastewater treatment plants by providing useful information about those circulating in the population. The presence of these microorganisms in the treated effluents, even at a lower frequency with respect to the wastewater influent, underlines the possible role of wastewater treatment plants in environmental contamination. This is true when the contaminated effluents are discharged into the receiving surface waters and consequently affect public health directly or indirectly to a severe degree.

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Fig 1. Primary steps of the protocol for detecting *Campylobacter* (spp., *coli* and *jejuni*), *Salmonella* spp., *E. coli* O157:H7 and *E. coli* virulence genes in wastewater samples.

Fig 2. (a) Agarose gel electrophoresis of PCR products amplified from raw wastewater inoculated with *C. jejuni* (2 10^7 CFU). Lane L: 100 bp DNA ladder (size marker); Lane 1: negative control (no template); Lane 2-3: *C. jejuni* with the expected amplicon sizes of this genus (857 bp) and *jejuni* species (589 bp). (b) Agarose gel electrophoresis of PCR products amplified from raw wastewater inoculated with non-toxigenic *E. coli* O157:H7 (2 10^7 CFU). Lane L: 100 bp DNA ladder (size marker); Lane 1: negative control (no template); Lane 2: *E. coli* strain using H7 (625 bp), intimin (368 bp) and O157 PCR (292 bp) primers. (b) Agarose gel electrophoresis of PCR products amplified from raw wastewater inoculated with *S. typhimurium* (2 10^7 CFU). Lane L: 100 bp DNA ladder (size marker); Lane 1: negative control (no template); and Lane 2: *S. typhimurium* strain (284 bp).

Fig 3. Faecal indicator contamination and abatement in the three WWTPs (mean and SD). I: Influent; E: Effluent; ABAT: abatment; 1: Castiglione plant; 2: Chieri plant; 3: Lanzo plant.

Table 1. Sensitivity of the PCR assay for detecting *Campylobacter* (spp., *jejuni*), *E. coli* O157:H7 and *S. typhimurium* in spiked wastewater samples (100 ml of raw sewage) (each experiment was performed in duplicate).

Pathogens	Number of cells[a] detected by PCR								
	$2\ 10^7$	$2\ 10^6$	$2\ 10^5$	$2\ 10^4$	$2\ 10^3$	$2\ 10^2$	2 10	2	C-
Campylobacter spp.	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	-/-
C. jejuni	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	-/-
S. typhimurium	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	-/-
E. coli O157:H7eae+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	-/-

[a] The number of bacterial cells was determined by plating in triplicate on TSA (Oxoid) and on Karmali Agar (Biolife).
C-: not spiked wastewater
+: positive PCR
-: negative PCR

Table 2. Detection of *Campylobacter* (spp., *coli*, and *jejuni*), *E. coli* O157:H7, *E. coli* virulence genes and *Salmonella* spp. by PCR method in wastewater samples

Sample	Season	*Salmonella*	*E. coli* O157:H7					*Campylobacter*		
		invA	O157	H7	Intimin	SLT-I	SLT-II	Genus	*C. jejuni*	*C. coli*
I1	S	+	-	+	-	+	-	+	+	-
E1	S	+	-	+	-	+	-	+	-	-
I2	S	+	-	+	-	+	-	+	+	+
E2	S	+	-	+	-	+	-	+	+	-
I3	S	+	-	+	-	+	-	+	-	-
E3	S	+	-	-	-	-	-	-	-	-
I1	Su	+	-	+	-	-	-	+	+	-
E1	Su	+	-	+	-	+	-	-	-	-
I2	Su	+	-	+	-	+	+	+	+	-
E2	Su	+	-	+	-	+	+	-	-	-
I3	Su	+	-	+	-	-	-	+	+	-
E3	Su	+	-	-	-	-	-	-	-	-
I1	A	+	-	+	-	-	-	+	+	-
E1	A	+	-	+	-	-	-	-	-	-
I2	A	+	-	+	-	-	-	+	-	-
E2	A	+	-	+	-	-	-	-	-	-
I3	A	+	-	+	-	-	-	-	-	-
E3	A	+	-	+	-	-	-	-	-	-
I1	W	+	-	+	-	-	+	+	-	+
E1	W	+	-	+	-	-	-	+	-	+
I2	W	+	-	-	-	-	+	+	-	+
E2	W	+	-	+	-	-	-	+	-	+
I3	W	+	-	+	-	-	-	-	-	-
E3	W	+	-	-	-	-	-	+	-	+

I: Influent; E: Effluent; S: Spring; Su: Summer; A: Autumn; W: Winter; 1: Castiglione plant; 2: Chieri plant; 3: Lanzo plant.
+: positive PCR
-: negative PCR

Figure1

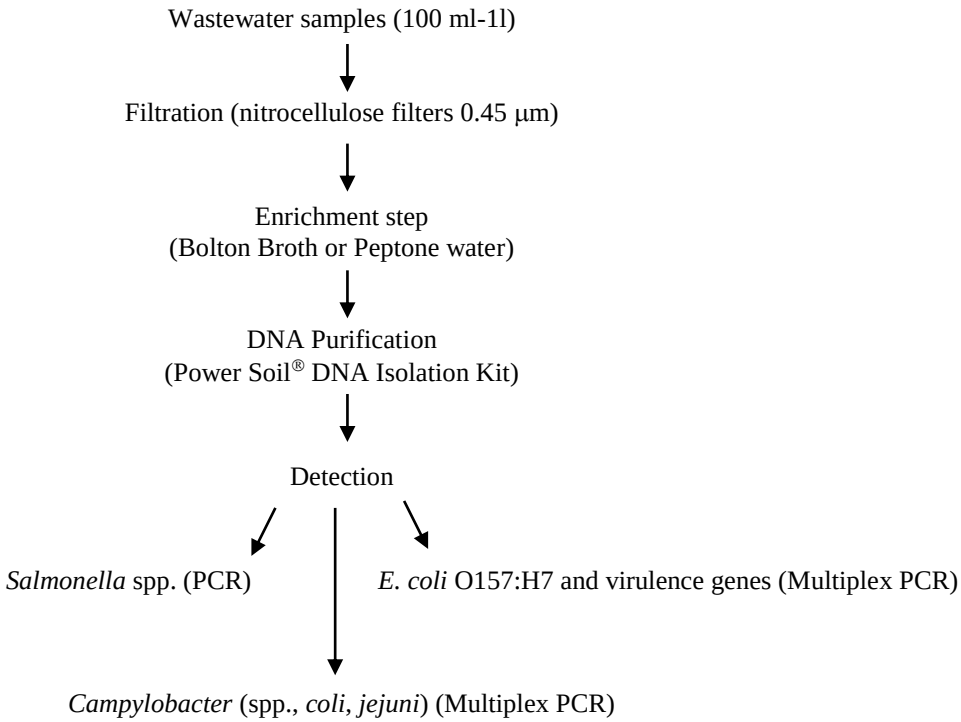

Wastewater samples (100 ml-1l)
Filtration (nitrocellulose filters 0.45 μm)
Enrichment step
(Bolton Broth or Peptone water)
DNA Purification
(Power Soil® DNA Isolation Kit)
Detection
Salmonella spp. (PCR)
E. coli O157:H7 and virulence genes (Multiplex PCR)
Campylobacter (spp., *coli*, *jejuni*) (Multiplex PCR)

Figure2

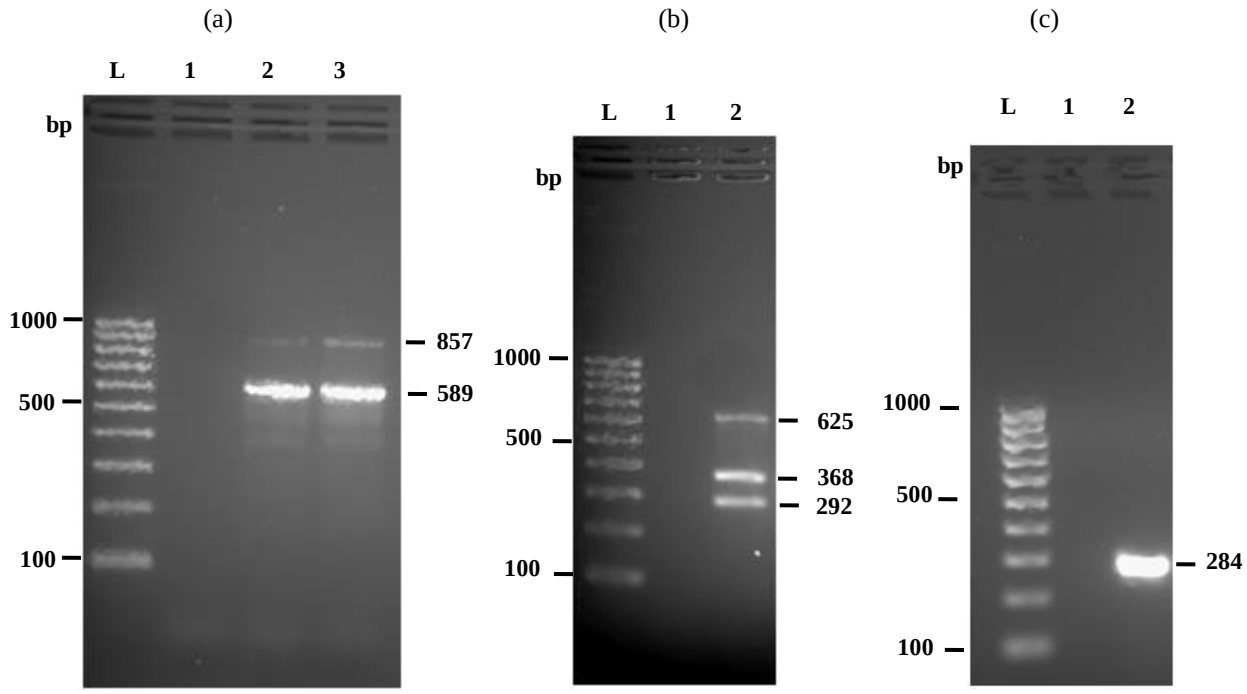
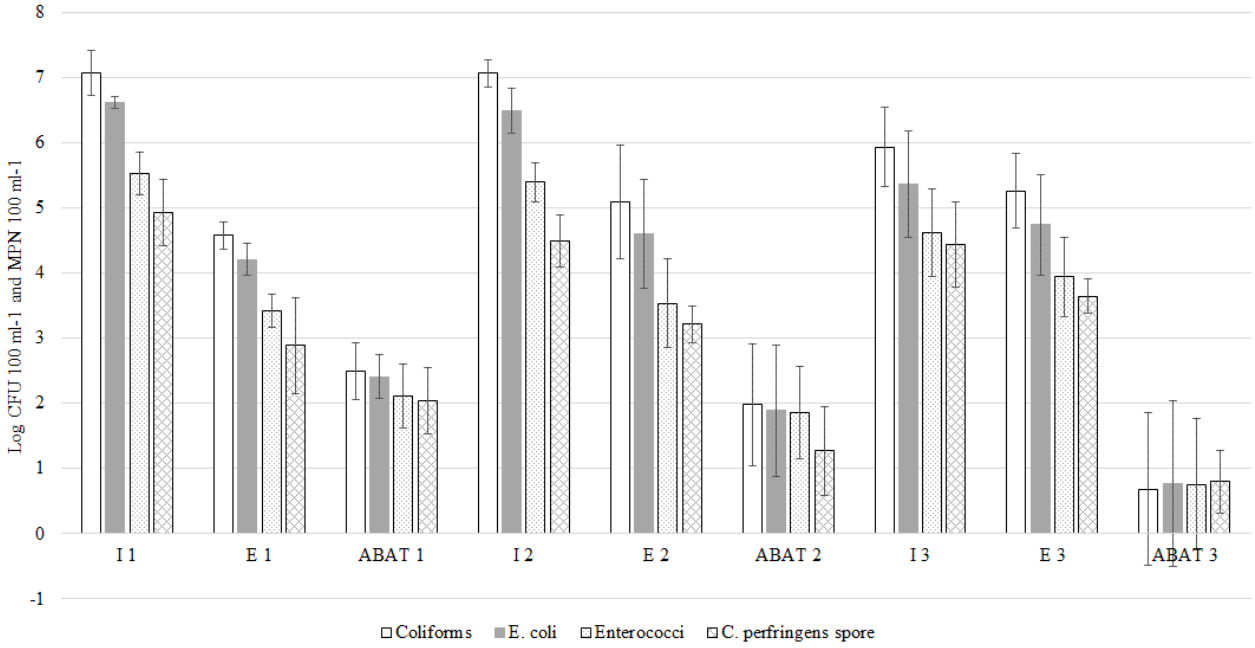

(a)
L
1
2
3
bp
1000
500
100
857
589
(b)
L
1
2
bp
1000
500
100
625
368
292
(c)
L
1
2
bp
1000
500
100
284

Figure3


8
7
6
5
4
3
2
1
0
-1
Log CFU 100 ml-1 and MPN 100 ml-1
I 1
E 1
ABAT 1
I 2
E 2
ABAT 2
I 3
E 3
ABAT 3
Coliforms
E. coli
Enterococci
C. perfringens spore