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Occurrence changes of *Escherichia coli* (including O157:H7 serotype) in wastewater and sewage sludge by quantitation method of (EMA) real time—PCR

K.L. Fijalkowski*, M.J. Kacprzak, A. Rorat

Institute of Environmental Engineering, Czestochowa University of Technology, Brzeznicka 60a, Czestochowa 42-200, Poland
Tel. +48 34 372 13 03; Fax: +48 34 372 13 04; email: kfijalkowski@is.pcz.czest.pl

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ABSTRACT

There are hundreds of *Escherichia coli* serotypes and among them enterohaemorrhagic strain *E. coli* O157:H7 causing gastrointestinal disorders such as: bloody diarrhea, cramping and abdominal pain and the infectious "hemolytic uremic syndrome". Therefore, it's important to develop some rapid and reliable methods of detecting this pathogen in wastewater and sewage sludge. This will allow one to determine the potential risk of infection for humans and animals as far as wastewater and sewage management is concerned. *E. coli* non- and -O157:H7 gene copies were detected in primary influents and final effluents in winter from municipal wastewater treatment plant. The ethidium monoazide bromide (EMA) application revealed false-positive detection of this bacteria in final effluents. In spring and summer, *E. coli* gene was not found either in wastewater or in sludge. In autumn, *E. coli* genes were found in primary influents (20,000 copies of gene/100 mL) and final effluents (2,511 copies of gene/100 mL). High amounts of *E. coli* O157:H7 gene were detected in both kinds of sludge: waste activated sludge (3,890,451 copies of gene/100 mL) and final sewage sludge (1,819,700 copies of gene/100 mL). We detected the large amount of "free DNA" that is derived from dead cells, which can give false-positive results (overestimation). The use of EMA will make it possible to avoid this inconvenience and allow for effective and appropriate selection of EMA concentration, however, it needs further analysis.

Keywords: Wastewater; Sewage sludge; EMA; real time—PCR; *Escherichia coli*

1. Introduction

A wastewater stream contains a variety of human pathogens at different concentrations depending on the region with the highest levels in areas where a faecal-oral disease is widely endemic. Wastewater treatment processes usually significantly reduce the number of pathogenic organisms and the risk of their transmission to animal or human. However, some of them are able to survive in the environment and even to multiple, simultaneously increasing the danger of infection.

*Corresponding author.

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These phenomena are limited by many factors including: temperature, moisture, exposure to UV, presence of appropriate intermediate hosts, time, the type of crops as well some other factors highly specific to the region of interest. There are many indicators that have been used to assess the contamination of water/wastewater by pathogens (i.e. *Escherichia coli*, *Shigella* spp., *Salmonella* spp., *Clostridium perfringens*, selected bacteriophages and fungi) [1–4]. The most commonly monitored indicators related to faecal contamination is thermotolerant coliform/*E. coli* group of bacteria that have been used for 100 years. In 1914, the US Public Health Service adopted the enumeration of coliforms as a more convenient standard of sanitary significance.

1.1. Background

The natural habitat of *E. coli* is the intestinal tract. The bacteria produce vitamins (including B and K) that are absorbed by warm-blooded organisms. *E. coli* is an opportunistic pathogen and is well recognized in very high amount in wastewater. It has been established as the most reliable indicator of human faecal contamination and included in total and faecal coliform assays indicating strong sewage or animal waste contamination. There are several types of *E. coli* that are opportunistic pathogens and capable of causing diseases. These opportunistic pathogens, including *E. coli*—enteroinvasive, *E. coli*—enteropathogenic (ETEC), *E. coli*—enterotoxigenic and *E. coli*—enterohemorrhagic O157:H7 (EHEC) [5]. Traditionally *E. coli* is classified on the basis of biochemical test—lactose fermentation and confirmed on the highly selective medium. Shiga-like toxin producing (STEC) *E. coli* O157 is a Gram-negative rod, that does not usually ferment sorbitol and is β-glucuronidase-negative. Most of the serotype O157 are motile and possess the flagellar antigen H7. The strain is highly infective, and the infective dose is less than 50 organisms. The microorganism can cause a variety of clinical symptoms such as frequent severe bloody diarrhea and abdominal cramps and/or a complication called hemolytic uremic syndrome (HUS). According to WHO EHEC, it is the most important serotype in relation to public health. However, other serotypes have frequently been involved in sporadic cases and outbreaks as well. An outbreak attributed to *E. coli* O104: H4 occurred in 2011, in which more than 4,000 people fell ill in Germany, Switzerland, Poland, the Netherlands, Sweden, Denmark, the UK, Canada and the USA. More than 800 people out of all cases suffered from HUS, the severe kidney condition. This outbreak resulted in 54 deaths. Therefore, the usage of routine applications to demonstrate the presence of STEC *E. coli* O157 necessitates the development of more specific methods such as PCR-based tools. Recently, molecular techniques significantly have improved pathogen detection sensitivity and specificity, as well as reduced the time to result. One of them, quantitative-PCR or q-PCR (often referred to as real-time PCR) is now widely used to determine gene and/or transcript numbers present within environmental samples [6] including *E. coli* [7–11]. In spite of the fact that many PCR methods are proposed as supplement of conventional guidelines for monitoring microbial indicators [12,15], specific national regulations usually recommended using culture-based assays (i.e. Polish Law).

The goal of this research is to determine, optimise and apply real-time PCR methods to detect *E. coli* and *E. coli* O157 in samples taken from different stages of wastewater treatment process in specific Polish conditions. The results were compared with the ones obtained from standard plate methods of bacteria detection.

2. Materials and methods

2.1. The collection of samples

Wastewater and sewage sludge samples were collected from a municipal and industrial wastewater treatment plant (WWTP) in southern Poland based on the activated sludge process described as municipal WWTP (*PE* = 315,000, *Q* = 90,000 m^3/d) and industrial WWTP (*PE* = 9,133, Q = 550 m^3/d). The samples were obtained in triplicates according to the Standard Methods for the examination of water and wastewater [12]. The wastewater and sewage sludge samples were stored in sterile plastic containers having a capacity of five liters. Subsequently, samples were transferred in an ice box to the laboratory within 6 h of collection for bacteriological analyses.

Due to the varying amount of solid biomass contained in each type of samples (wastewater, sewage sludge), different volumes (or weight) of each were used for biomass collection. The biomass from wastewater samples were collected by centrifugation at 5,000×g for 10 min at 4°C. The biomass from sewage sludge samples was collected according to their hydration, respectively, by centrifugation at 5,000×g for 10 min at 4°C (hydration >5%) and by weighting (hydration <5%).

E. coli O157:H7 (DSM 19206, DSMZ, Germany) were aseptically plated on brain heart infusion broth with tryptone (10 g/L) and incubated at 37°C by shaking at 350 rpm in thermomixer (Eppendorf, Germany) overnight. The actively growing cells were harvested by centrifugation 2,600× g for 30 min and used for genomic DNA extraction and bacteriological

analyses (stored at −80˚C). In order to confirm the viability and identity of harvested cells, after appropriate dilutions the cells were placed on plates according to the method described below.

2.2. Bacteriological analyses

The bacteriological analyses were performed immediately after the samples had been transferred to laboratory. Appropriate sample volumes, in triplicates were diluted (10^{-2}–10^{-6} dilution) in phosphate saline buffer (140 mM NaCl, 10 mM phosphate buffer, and 3 mM KCl, pH 7.4) and varied according to sample source to ensure obtaining plates with 30–300 colonies. Plates were incubated for 24 h at 37˚C on highly specific medium Fluorocult *E. coli* O157:H7 agar (Merck, Germany).

According to manufacturer's protocol, on the Fluorocult *E. coli* O157:H7 agar the greenish colonies (no sorbitol-cleavage capacity within 48 h) and no fluorescence (MUG-negative) were counted. The presumptive colonies were aseptically transplanted on fresh medium for additional confirmation. PCR confirmation was used.

2.3. Ethidium monoazide bromide cross-linking

Ethidium monoazide bromide (EMA, Sigma-Aldrich Co., USA) is a photoactive stain which covalently binds to nucleic acids in solution and in cells with damaged membranes thus preventing replication in PCR (false positive results). EMA was dissolved in water to a stock concentration of 5 mg/mL and stored at −20˚C in the dark. EMA cross-linking was carried out according to [13] for all tested samples. EMA was added to samples before DNA extraction. Final concentration of EMA in samples was 100 µg/mL. After samples' centrifugation the resulting pellet was mixed in 1 mL of ultra clean water and EMA was added. Following a 5 min incubation in the dark with occasional flipping, the samples were exposed to light for 1 min using 650 W halogen light source placed approximately 20 cm from the samples. Through the whole procedure, samples were placed on ice to avoid excessive heating. After EMA

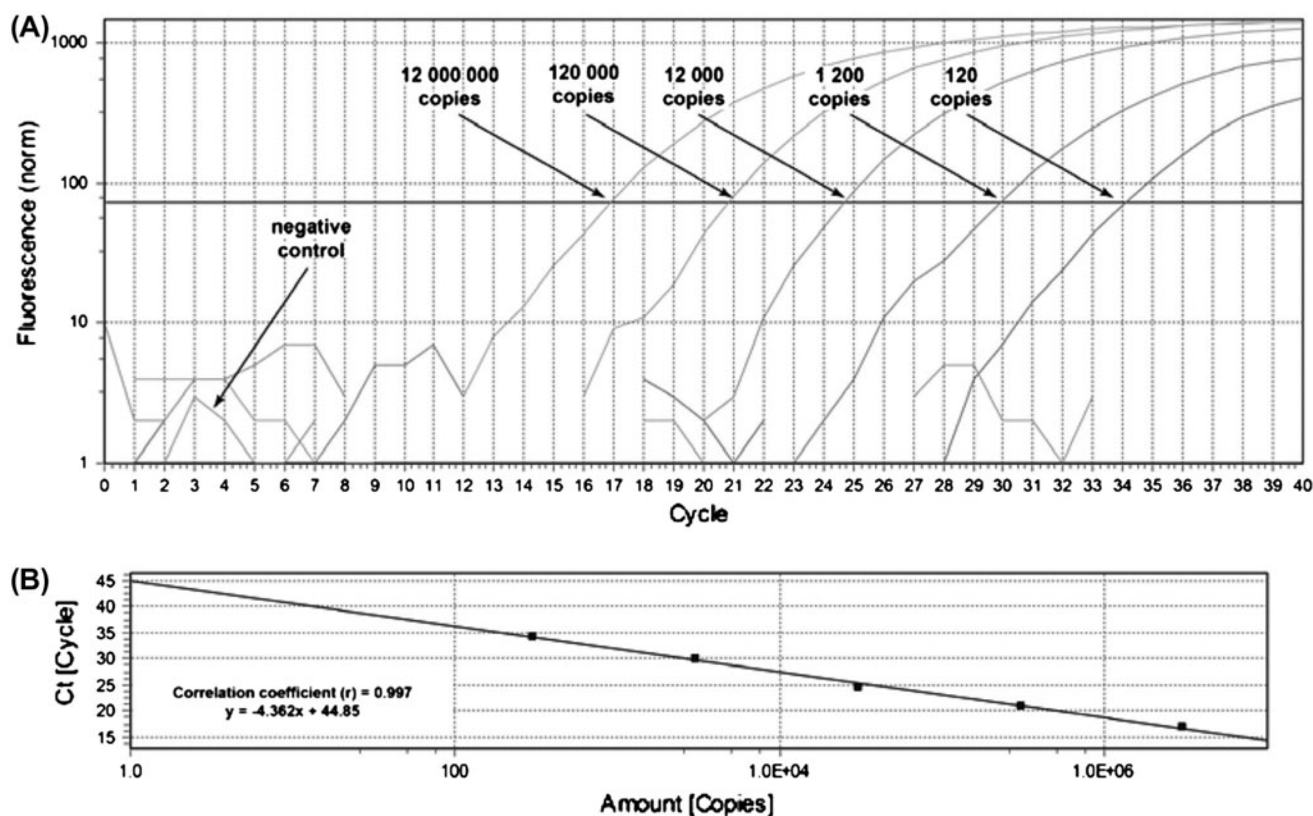

Fig. 1. Fluorescence curves (A) generated from 10-fold serially diluted genomic DNA of *E. coli* DSM 19206. (B) standard curve, Ct—cycle threshold, copies of investigated DNA.

cross-linking, the samples were pelleted at 5,000×g for 5 min prior to DNA extraction.

2.4. Genomic DNA extraction and purification

Genomic DNA was extracted from pure (for PCR standards) cultures with a QIAamp DNA Mini Kit (Qiagen Inc.) according to the manufacturer's protocol. From all samples genomic DNA was extracted by Ultra Clean Soil DNA Kit (MO BIO Laboratories Inc., Carlsbad, USA). Due to large amounts of impurities contained in the wastewater DNA extraction was performed according to the manufacturer's protocol with additional purification by total ethanol to further purify DNA. The quantity and purity of the DNA extract was determined spectrophotometrically at an absorbance of 260 nm and A260/A280, respectively.

2.5. PCR primers

The sequences of the primers that were used to detect *E. coli* and O157:H7 serotype according to [9] was: for *E. coli*—forward 5´-GTCCAAAGCGGCGATT TG-3´, reverse 5´-CAGGCCAGAAGTTCTTTTTCCA-3´, Gen-uidA, GenBank No. S69414; for *E. coli* O157:H7 forward 5´-TCGAGCGGACCATGATCA-3´, reverse 5´-GGCGGCGTCTGAGATAACA-3´, Gen-tir, GenBank No. AF125993 (Genomed, Poland).

2.6. Real time quantitative PCR

Purified genomic DNA and primers were added to PCR reaction tube containing 2× PCR mastermix (Power SYBR Green PCR Master Mix, Life Technologies Corp., USA) to final volume 20 μL. The final PCR solution contained 1.8 μL of each forward (reverse) primer (900 μM) and 2 μL DNA. PCR was carried out in a Mastercycler epgradient S (Eppendorf, Germany). The PCR reaction with all reaction components with sterile Milli-Q water (Millipore, USA) in the place of DNA template was always run as a negative control in order to rule out any carry-over contamination. These negative controls were included to ensure that the fluorescence signals observed were specific for PCR amplification of template DNA. After each PCR the melting curve was performed to check the presence of other constituents of the PCR assay mixture. The standard curve was automatically generated by the Mastercycler software system by plotting the cycle number, at which the threshold fluorescence was

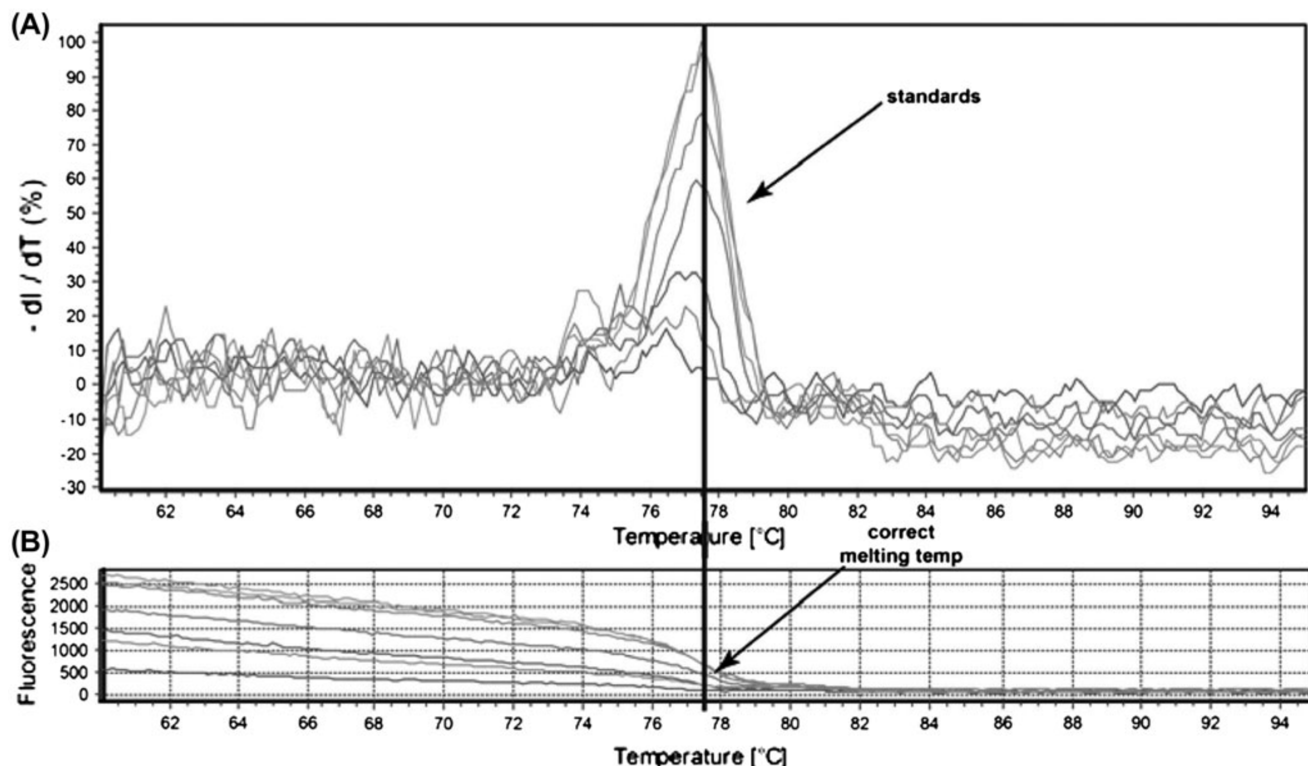

Fig. 2. Quantitative dissociation curves (melting curves) generated from 10-fold serially diluted genomic DNA of *E. coli* DSM 19206.

reached, compared to the logarithmic concentration of positive control DNA. Sample concentrations were calculated based on this standard curve. The sample was considered negative if the fluorescent signal did not increase within 45 cycles or its peak in melting curve was out of 78°C.

The PCR program was as follows: initial denaturation at 95°C for 10 min and then 45 cycles at 95°C for 15 s, 46.8°C for 35 s and 72°C for 30 s. The melting curve program was 95°C for 1 min and 60°C for 5 min then linear increase of temperature to 95°C in 30 min.

2.7. Generation of standard curve

The quantity and quality of extracted DNA from pure cultures of *E. coli* was measured at 260/280 nm with BioPhotometer spectrophotometer (Eppendorf, Germany). To determine the mass of gDNA template that corresponds to copy numbers of target nucleic acids sequence was calculated as follows: $m \times n$ (1.096×10^{-21} g/pb), where m is the mass and n is the number of base pairs according to [14], genome was 4,780 for non-O157:H7 *E. coli* [15] and 5,529 for O157:H7 *E. coli* (strain EDL933 from NCBI data base). The standard curve was made in the concentration range of $1.2-120 \times 10^7$ genomic DNA copies per PCR (Fig. 1).

3. Results and discussion

The observed *E. coli* (non- and O157:H7) specific real-time PCR fluorescence amplification curves are shown in Fig. 1. DNA isolated from harvested cells of *E. coli* (DSM 19206) was detected in all serial dilutions containing an equivalent of $1.2-120 \times 10^7$ uidA (O157:H7 serotype) and tir (*E. coli*) gene copies. Because of reagent contamination by low numbers of *E. coli* cells (Taq DNA polymerase is produced from recombinant *E. coli* cells) the PCR detection limit was 120 gene copies. Amplification of the correct PCR fragments was verified by analysing the melting curve after PCR reaction. Melting curves showed curves without any primer–dimer formation (Figs. 2 and 3). In contrast *E. coli* melting curves for serotype O157:H7 analysis revealed two melting temperatures (Fig. 3), lower for O157 but non-H7 and higher for O157:H7 serotype. This is due to sequence variation within the *E. coli* specific uidA gene fragment [8].

E. coli non- and -O157:H7 gene copies were detected in primary influents and final effluents in

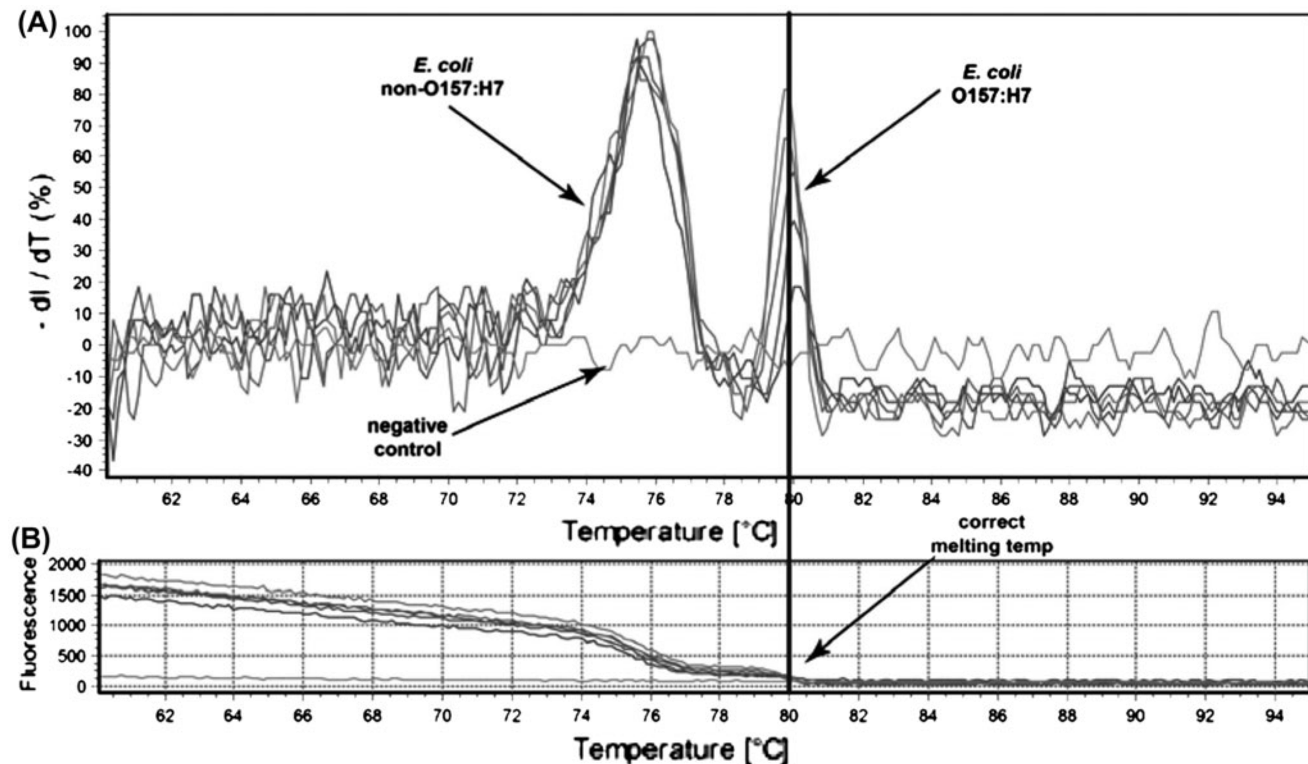

Fig. 3. Quantitative dissociation curves (melting curves) generated from 10-fold serially diluted genomic DNA of *E. coli*—O157:H7.

Table 1
Log_{10} target gene copies of *E. coli* and *E. coli*—O157:H7 obtained from municipal WWTP using wastewater and sludge DNA samples from: primary effluents, final effluents, final sewage sludge and waste activated sludge in four seasons

Season	Winter				Spring				Summer				Autumn			
	Log_{10} gene copies/100 mL				Log_{10} gene copies/100 mL				Log_{10} gene copies/100 mL				Log_{10} gene copies/100 mL			
Microorganism	*E. coli*		*E. coli* O157:H7		*E. coli*		*E. coli* O157:H7		*E. coli*		*E. coli* O157:H7		*E. coli*		*E. coli* O157:H7	
EMA—application	No	Yes	No	Yes	No	Yes	No	Yes	No	Yes	No	Yes	No	Yes	No	Yes
Primary influents	6.31	5.90	4.88	4.00	n.d.	n.d.	n.d.	n.d.	3.87	n.d.	n.d.	n.d.	6.86	5.10	5.99	3.90
Final effluents	4.83	n.d.	3.94	n.d.	n.d.	n.d.	3.70	3.23	3.38	3.58	n.d.	n.d.	4.13	n.d.	4.27	n.d.
Waste activated sludge	6.56	6.25	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Final sewage sludge	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

Note: n.d.—PCR target genes were below detection limits.

Table 2
Log_{10} target gene copies of *E. coli* and *E. coli*—O157:H7 obtained from industrial WWTP using wastewater and sludge DNA samples from: primary effluents, final effluents, final sewage sludge and waste activated sludge in four seasons

Season	Winter				Spring				Summer				Autumn			
	Log_{10} gene copies/100 mL				Log_{10} gene copies/100 mL				Log_{10} gene copies/100 mL				Log_{10} gene copies/100 mL			
Microorganism	*E. coli*		*E. coli* O157:H7		*E. coli*		*E. coli* O157:H7		*E. coli*		*E. coli* O157:H7		*E. coli*		*E. coli* O157:H7	
EMA—application	No	Yes	No	Yes	No	Yes	No	Yes	No	Yes	No	Yes	No	Yes	No	Yes
Primary influents	5.30	4.43	3.99	2.84	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	5.21	4.30	5.26	4.80
Final effluents	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	3.40	n.d.	4.83	n.d.
Waste activated sludge	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	7.87	6.59
Final sewage sludge	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	6.25	6.26

Note: n.d.—PCR target genes were below detection limits.

winter from municipal WWTP (Table 1). The EMA application revealed false-positive detection of the bacteria in final effluent by PCR. Thus, there was no *E. coli* O157:H7 in final effluents either. The *E. coli* was also detected in waste activated sludge but not in final sewage sludge. In the spring, 3.28 Log_{10} gene copies/100 mL of *E. coli* O157:H7 were detected only in the final effluents. Similarly, in the summer only in final effluents 3.38 Log_{10} gene copies/100 mL of *E. coli* non-O157:H7 were found and in autumn *E. coli* was detected only in primary influents (5.10 Log_{10} gene copies/100 mL) and final effluents (4.13 Log_{10} gene copies/100 mL).

In industrial WWTP the same pattern was observed (Table 2). Only in primary influents and final effluents the copies of *E. coli* gene were detected. In the spring and summer *E. coli* gene was not found in any of the analysed wastewater and sewage sludge samples. In the autumn, *E. coli* was found in primary influents (4.30 Log_{10} gene copies/100 mL) and final effluents (3.40 Log_{10} gene copies/100 mL). But a high amount of *E. coli* O157:H7 gene was detected in both the sludges: waste activated sludge (6.59 Log_{10} gene copies/100 mL) and final sewage sludge (6.26 Log_{10} gene copies/100 mL).

The collected results indicate that bacterial DNA remains relatively intact after wastewater treatment process giving false-positive PCR results. Therefore, EMA application could be successfully used as an appropriate tool to eliminate problems described by [16]. The EMA concentration needs to be calculated for each sample separately. Similar values of *E. coli*

Table 3
Log_{10} colony forming units of *E. coli*—O157:H7 obtained from municipal WWTP using wastewater and sludge samples from: primary effluents, final effluents, final sewage sludge and waste activated sludge in four seasons

Season	Winter Log_{10} CFU/100 mL	Spring Log_{10} CFU/100 mL	Summer Log_{10} CFU/100 mL	Autumn Log_{10} CFU/100 mL
Microorganism	*E. coli* O157:H7	*E. coli* O157:H7	*E. coli* O157:H7	*E. coli* O157:H7
Primary influents	6.98	n.d.	7.90	7.07
Final effluents	5.21	n.d.	5.74	5.74
Waste activated sludge	6.89	n.d.	7.66	8.39
Final sewage sludge	6.53	n.d.	6.57	9.33

Note: n.d.—no bacterial colonies were found on the plates in all dilutions.

Table 4
Log_{10} colony forming units of *E. coli*—O157:H7 obtained from industrial WWTP using wastewater and sludge samples from: primary effluents, final effluents, final sewage sludge and waste activated sludge in four seasons

Season	Winter Log_{10} CFU/100 mL	Spring Log_{10} CFU/100 mL	Summer Log_{10} CFU/100 mL	Autumn Log_{10} CFU/100 mL
Microorganism	*E. coli* O157:H7	*E. coli* O157:H7	*E. coli* O157:H7	*E. coli* O157:H7
Primary influents	6.11	n.d.	6.04	5.59
Final effluents	n.d.	n.d.	5.75	n.d.
Waste activated sludge	n.d.	n.d.	8.02	6.29
Final sewage sludge	n.d.	n.d.	7.67	6.35

Note: n.d.—no bacterial colonies were found on the plates in all dilutions.

gene were obtained by [17], and for primary influents was 6.55 and for final effluents 4.94 of Log_{10} gene copies/100 mL, respectively.

The obtained results of *E. coli* by PCR detection in wastewater and sewage samples for four seasons have shown that *E. coli* is mainly present in cold seasons (winter, autumn). This is confirmed by the results obtained by [18] which also indicated that *E. coli* O157:H7 is mainly present in primary influents in cold seasons.

The data received from microbiological analysis of wastewater and sewage samples from both WWTP's (Tables 3 and 4) have shown significant overestimation of *E. coli* O157:H7 cells compared to PCR results. This phenomenon can result from the fact that on Fluorocult media *E. coli* O157:H7 isolated from wastewater or sludge produced negative results in MMO-MUG (but possess the uidA gene) were not *E. coli* O157:H7 but related *Escherichia* species [8,19]. To overcome this problem, the serotype confirmation by PCR is essential.

4. Conclusions

- Real-time PCR can be used to monitor pathogens in wastewater treatment processes from different WWTPs.
- Lack of or sporadic presence of pathogenic *E. coli* O157:H7 in the warm seasons, but the presence only in primary influents in the cold ones.
- High amounts of *E. coli* O157:H7 gene were detected only in the final sewage sludge (in autumn)—this creates a risk of water (also groundwater) microbiological contamination if they are improperly stored or used.
- Uncertainty of the microbiological analysis of *E. coli* O157:H7 number in wastewater or sludge as compared to PCR data—probable overestimation because of false positive results
- EMA application is effective in eliminating false-positive results in the PCR analysis of environmental samples but appropriate selection of EMA concentration needs to be calculated for each sample separately.

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