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Escherichia coli and *Salmonella* spp. Early Diagnosis and Seasonal Monitoring in the Sewage Treatment Process by EMA-qPCR Method

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Abstract

Numerous studies have recently shown that molecular biology tools can allow for early diagnosis of pathogens and can substitute existing cost and time-taking traditional methods. One of them, the qPCR, is successfully used in microbiology and its utility has been assessed for many different biological materials. The aim of this study was to: 1) determine, optimize and apply qPCR as a method to detect *Escherichia coli* and *Salmonella* spp. in primary influents and final effluents from municipal wastewater treatment plant 2) define if addition of ethidium bromide monoazide (EMA) before DNA extraction can allow to distinguish between alive and dead bacteria, 3) quantify *E. coli* and *Salmonella* spp. in wastewater during four seasons by qPCR and traditional spread plate method and determine the correlation between the indicator and pathogenic microorganisms. The obtained results has shown that qPCR can be used as a quantitative method in the diagnosis of investigated bacteria in wastewater with EMA pretreatment as a crucial step for a proper quantitative analysis of the presence of these bacteria in wastewater. Both *E. coli* and *Salmonella* spp. bacteria species were present in all samples of primary influents and final effluents. Our study shown that the quantity of investigated bacteria is strictly correlated with the season that they were obtained in.

Key words: *Escherichia coli*, *Salmonella* spp., ethidium bromide monoazide, qPCR, sewage treatment

Introduction

Municipal wastewater effluents may constitute a significant contribution to drinking and recreational water pollution. To assess the danger to the environment from the microbiological point of view, Total and Fecal Coliform Assays are used to indicate the hazard of the presence of pathogens associated with fecal material. Generally, it is expected that a reduction in their presence is equal to a reduction in quantity of all pathogens in the analyzed material (Hagendorf *et al.*, 2005). However this conventional method does not indicate the actual state of a microbiological pollution (Shannon *et al.*, 2007). Traditional methods are time consuming and are limited by proper media content and culture conditions. Some microorganisms may also divide faster on media causing the incorrect interpretation of obtained data (Gilbride *et al.*, 2006). In this case, the detection of indicator bacteria, like fecal coliforms or fecal streptococci is not sufficient, because inactivation of pathogens depends strongly on their nature and applied system for sludge treatment in the wastewater treatment plant (Sidhu and Toze, 2009).

Escherichia coli is one of facultative anaerobes of human colonic flora, and some strains, like *E. coli* O157:H7 may cause very dangerous enteric diseases, including hemorrhagic diarrhea, abdominal cramps and hemolytic uremic syndrome. *E. coli* is the main indicator organism used commonly in the evaluation of microbiological contamination. This enterohaemorrhagic *E. coli* may give rise to infections in the gastrointestinal tract of mammals and cross contaminate humans. In 1993 the first report on the potential transfer of bacterial pathogens from land applied biosolids to humans was published. Besser *et al.* (1993) described cases after the consumption of cider made from apples collected from field treated with cow manure. *Salmonella typhimurium* is a pathogen which causes foodborne infections and salmonellosis and in general is present in raw and treated biosolids (Sahlstorm *et al.*, 2004). According to the FDA (Food and Drug Administration), the infective dose of *Salmonella* can be lower than 20 cells depending on the age and health of the host organism (FDA, 2003).

Pathogens may be easily detected with molecular biology methods in water, food and in samples coming

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from wastewater treatment plants. Since 1990 the polymerase chain reaction (PCR) has been used as a method allowing for a completely culture-independent analysis of all microbial communities of bacteria (Giovannoni *et al.*, 1990). In qPCR technique, an increasing fluorescence signal is measured in real time, which enables the direct analysis of the results after PCR without additional detection steps. However, DNA is isolated not only from live, but also from dead bacteria, so a positive PCR result can be obtained irrespective of the viability of cells (Fijałkowski *et al.*, 2014). To avoid this problem ethidium bromide monoazide (EMA) may be used. This substance is a dye, which intercalates the DNA only of damaged bacteria after photoactivation (Nogva *et al.*, 2003). DNA with covalently bounded dye will not react in PCR reaction, thus completely eliminating the problem of live/dead cell differentiation.

The primary objective of the study discussed in this paper was to optimize the qPCR method for detection and identification of *Salmonella* spp. and *E. coli* in wastewater. We examined the SYBR® Green I dye, specific primers and EMA cross-linking to create an easy protocol for determining the quantity of investigated bacteria in samples derived from wastewater treatment plants.

Experimental

Materials and Methods

Wastewater samples. Wastewater (primary influents and final effluents) samples were collected from a municipal wastewater treatment plant with secondary treatment facilities based on an activated sludge process in southern Poland (unit per capita loading PE = 315 000, wastewater treatment plant capacity Q = 90 000 m^3/d) during four different time periods (December-February = winter, March – May = spring, June – August = summer, September-November = autumn). Samples were obtained in biological triplicates according to the Standard Methods for the Examination of Water and Wastewater (APHA, 1995). Samples were transported in 5 l sterile plastic bottles in 4°C and analyzed within 6 h. Due to the varying amount of solid biomass contained in each type of wastewater samples, different volumes of wastewater were used for biomass collection by centrifugation at 5000 × g for 10 minutes at 4°C, respectively: 10–100 ml for primary influents, 100–300 ml for final effluents.

Bacterial strains (for standard curve in real time PCR reaction). *Salmonella enterica* subsp. *enterica serovar Typhimurium str. LT2* (DSM 50912, DSMZ Germany) and *E. coli* (DSM 10235, DSMZ, Germany) bacterial strains were aseptically plated on Brain Heart Infusion broth (BHI) and incubated at 37°C with shaking at 350 rpm in thermomixer (Eppendorf, Germany) overnight. The actively growing cells were harvested by centrifugation 10000 × g for 5 minutes and used for genomic DNA extraction and bacteriological analysis. DNA was isolated using Qiagen DNeasy Blood & Tissue Kit (QIAGEN, Dusseldorf, Germany) according to the manufacturer's protocol. The quantity and purity of the isolated DNA was determined spectrophotometrically at an absorbance of 260 nm and A260/A280, respectively. DNA was stored at a temperature of minus 80°C.

Genomic DNA isolation from wastewater. Wastewater samples were first filtered on an cellulose acetate membrane filter 0.45 µm (Whatman, USA) with a vacuum filtration system (Merck KGaA, Germany). DNA was isolated from the filter using MO BIO PowerWater® DNA Isolation Kit (Mo Bio Laboratories Inc., Carlsbad, USA) according to the manufacturer's protocol. DNA purity was confirmed by spectrophotometry (BioPhotometer, Eppendorf, Hamburg, Germany) as a A260/A280 ratio, which was in range 1.7–2.0 for all analyzed samples. Isolated DNA was stored at a temperature of minus 80°C (Innova range U101, New Brunswick Scientific co., Inc., New Jersey, USA).

EMA cross-linking. EMA dye was dissolved in water to a stock concentration of 5 mg/ml and stored at minus 20°C in the dark. EMA cross-linking was carried out according to Nocker and Camper (2006) for all studied wastewater samples. Membrane filters were placed in tubes containing 1 ml of sterile water and EMA was added. Final concentration of EMA in samples was 100 µg/ml. After a 5-min incubation in the dark, samples were light exposed for 1 min using a 650-W halogen light source placed 20 cm from the samples. Through whole procedure samples were placed on ice to avoid excessive heating. After EMA cross-linking, DNA was isolated from the filters as described above.

Real time quantitative PCR. Purified genomic DNA and primers were added to PCR reaction tubes containing 2 × PCR mastermix (Power SYBR® Green PCR Master Mix, Life Technologies Corp., USA) to final volume 20 µl. The final PCR solution contained 900 nM of each foward (reverse) primer and 10 to 100 ng of DNA. PCR was carried out in a Mastercycler Ep Realplex^2 (Eppendorf, Germany). The PCR reaction with all reaction components using sterile Milli-Q water (Merck KGaA, Germany) in place of DNA template was always run as a negative control in order to rule out any carry over contamination. Primers were as follows: forward 5'-GGTCTGCTGTACTCCACCTTCAG-3' and reverse 5'-TTGGAGATCAGTACGCCGTTCT- 3' (Calvó *et al.*, 2008) for *Salmonella* spp. bipA gene and EcoF 5'-GTCCAAAGCGGCGATTTG-3' and EcoR 5'-GAGGCCAGAAGTTCTTTTTCCA-3' for uidA gene for *E. coli* (Lee *et al.*, 2006). A standard curve was obtained by analysing 10-fold serial dilutions of DNA isolated

from *S. enterica* subsp. *enterica* serovar *Typhimurium str. LT2*. Log-linear regression analysis showed a significant correlation ($R^2 = 0.995$) between the CT values and the copy numbers of *bipA* gene of *S. typhimurium*. A similar standard curve was prepared for *E. coli uidA* gene analysis. The quantity of target gene copy number in analyzed samples was calculated from those standard curves. The sample was considered negative if: the fluorescent signal did not increase within 40 cycles or its peak in melting curve was out of 80.7–82.0°C for *Salmonella* spp. and 76.1–77.6°C for *E. coli*.

The PCR program was as follows: initial denaturation at 95°C for 10 min and then 40 cycles at 95°C for 15 s, respectively: 64°C for 35 s for *Salmonella* spp. and 56°C for *E. coli*, followed by 72°C for 30 s. The melting curve program was: 95°C for 1 min, 60°C for 5 min then linear increase of temperature to 95°C in 30 min.

Traditional spread plate technique. The bacteriological analyses were conducted immediately after samples were transferred to the laboratory. Appropriate sample volumes in triplicate were diluted (10^{-2}–10^{-6} dilution) in a 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. Inoculated plates were incubated for: 48 h at 37°C on Bismuth sulfite agar acc. to WILSON-BLAIR (Merck KGaA, Germany) for *S. typhimurium* detection and for 24 h at 37°C on Chromocult® Coliform Agar (Merck KGaA, Germany) for *E. coli* detection.

Statistical analysis. All results concerning the target gene copy number and colony forming units were expressed as means ± standard errors. Differences between means were determined by the Tukey's test, with the level of significance established at $P < 0.05$. The Pearson's correlation was used to test the relationship between *E. coli* and *Salmonella* spp. concentrations in raw and treated waste water samples.

Results and Discussion

QPCR and EMA-qPCR analyses of *Salmonella* spp. and *E. coli*. Both *Salmonella* spp. and *E. coli* were present in primary influents samples during all analyzed time periods (Table I). EMA pretreatment resulted in statistically important decrease in detected target gene copies number for *E. coli* in all analyzed samples and for *Salmonella* spp. in samples taken in autumn and spring. The major differences among target gene copy numbers were observed between final effluents samples. This is caused by the influence of the wastewater treatment process, in which microbiological contamination is partly reduced by chemical and biological treatment. Similar observations were done by Soejima *et al.* (2008) who discriminated live and heat-treated *Listeria monocytogenes* cells by EMA-qPCR. EMA-qPCR was also used to study *E. coli O157*, *Salmonella* spp. and *L. monocytogenes* survival under decontamination and antibiotic treatments (Rudi *et al.*, 2005). Those authors suggested the usage of this method for complex samples with mixed food borne bacterial communities, especially present in food. In this study, authors confirmed that this method may be useful in the case of wastewater samples and may help to evaluate the effectiveness of the wastewater treatment process because of elimination of false positive results caused by detection of DNA from dead cells.

On the other hand, many authors have been critical of EMA treatment as a viability assay for qPCR-based

Table I
Enumeration of *Salmonella* spp. and *E. coli* in primary influents and final effluents by qPCR (target gene copy/100 ml) and traditional Petri dish method (C.F.U./100 ml). Results shown as means + SE n = 6

	Primary influents			Final effluents		
	Target gene copy/100 ml × 10^4		C.F.U/100 ml × 10^4	Target gene copy/100 ml × 10^4		C.F.U/100 ml × 10^4
	qPCR	EMA-qPCR		qPCR	EMA-qPCR	
Salmonella typhimurium						
Summer	110.22 ± 26.95 a	74.29 ± 27.71 ab	25.00 ± 7.07 b	27.98 ± 1.7 a	6.28 ± 0.28 b	2.00 ± 0.00 b
Autumn	5810.00 ± 438.41 a	9300 ± 989.95 b	66.50 ± 31.82 c	0 a	0 a	0 b
Winter	365.03 ± 41.26 a	254.95 ± 22.31 a	64.50 ± 6.36 b	280.06 ± 31.5 a	3.01 ± 0.33 b	0 c
Spring	3427.53 ± 7.88 a	1668.88 ± 34.33 b	143.50 ± 4.95 c	49.87 ± 3.22 a	2.95 ± 0.21 b	14.75 ± 1.06 c
Escherichia coli						
Summer	30.34 ± 2.96 a	14.22 ± 0.93 b	1160.00 ± 84.85 c	4.88 ± 2.07 ab	1.12 ± 0.14a	0 c
Autumn	1206.64 ± 135.26 a	491.35 ± 31.44 b	626.5 ± 2.12 a	2.75 ± 0.48 a	0 b	3.50 ± 0.71 b
Winter	85.81 ± 13.98 a	38.45 ± 4.33 b	105.00 ± 22.63 ab	46.22 ± 4.87 a	8.43 ± 0.6 b	0 c
Spring	391.35 ± 39.10 a	215.55 ± 40.88 b	140.50 ± 2.2 c	27.73 ± 2.92 a	5.06 ± 0.36 b	7.5 ± 0.71 c

a, b, c – shows statistically significant differences within experimental groups in rows ($p < 0.05$)

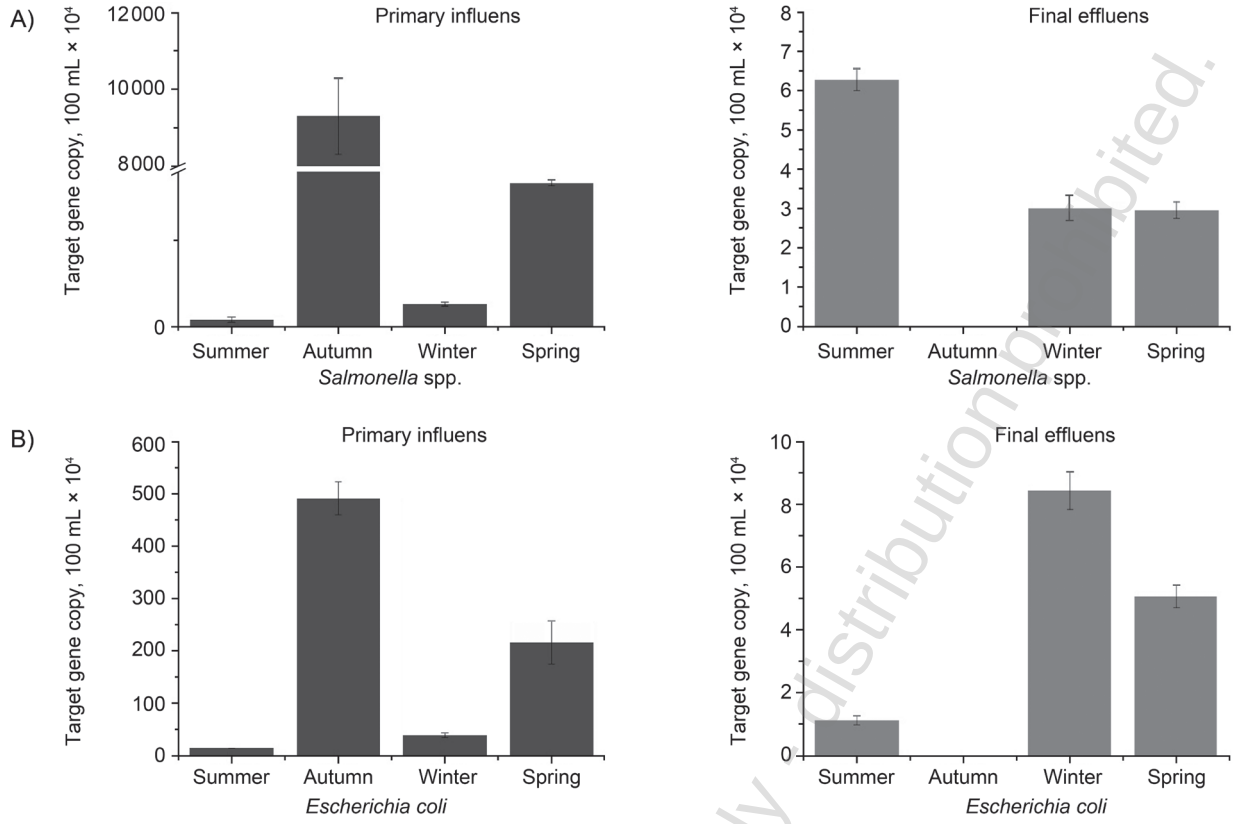

Fig 1. Evaluation of effectiveness of wastewater treatment process by EMA-qPCR method: A) *Escherichia coli uid*A target gene copies/100 ml × 10^4 analyzed in primary influents (left) and final effluents (right) through four seasons; B) *Salmonella* spp. *bip*A target gene copies/100 ml × 10^4 analyzed in primary influents (left) and final effluents (right) through four seasons

methods, because at high concentrations it is able to penetrate viable cells resulting in lower qPCR results and reduced total DNA recovery (Gedalanga and Olson, 2009). Moreover, authors have shown how many factors can influence EMA treatment regarding not only specific conditions of environmental samples (such as turbidity), but also specific cell membrane composition or cell physiology of target organisms that naturally occur in wastewater.

Viable bacteria quantification by the spread plate method. Results obtained by qPCR method were compared with the traditional spread plate method. For all analyzed samples, the target gene copy number was higher than the number of colony forming units per 100 ml of sample (Table I). Similar conclusions were stated by Morio *et al.* (2008) while comparing traditional and qPCR method for detection of *Legionella pneumophila* in environmental water samples. Lee *et al.* (2006) also explained that direct comparison between gene copy numbers and colony forming units is inappropriate because of DNA isolation from dead bacterial cells. Moreover, even though we assume that there is only single copy of a gene in one bacterial cell, it may appear in other bacterial strains in multiple copies.

Evaluation of the effectiveness of wastewater treatment process by EMA-qPCR method. *E. coli* and *Salmonella* spp. were present in primary influents during all four seasons (Fig. 1). According to Shannon (2007) the number of *Salmonella* spp. population ranges from 10^2–10^4 cells per 100 ml, which corresponds to the results obtained in this study. *Salmonella* spp. gene copies number was the highest in autumn and spring, medium in winter and the lowest in summer. *E. coli* was present mostly during spring and winter and was slightly detected during summer and autumn. Statistically important differences between all samples were observed. Similar results were obtained by Ulrich *et al.* (2005) who indicated that *E. coli O157:H7* is sporadically present or absent in primary influents in warm seasons.

Quantification of bacteria in final effluents has shown very good effectiveness of treatment of wastewater in the analyzed plant. Reduction in *Salmonella* spp. cells varied from 1.07 log units during summer, 1.92 log units in winter, 2.75 log units during spring, to more than 2.75 in autumn. Similar results were obtained for *E. coli* – 0.65 log units reduction in winter, 1.1 in summer, 1.63 in spring and 1.92 reduction of viable bacteria in autumn, prove that the process leads to reducing the risk of contamination from pathogens.

Correlation between microorganisms. Pearson's correlation analysis was used to identify whether any correlation existed between the concentration of indica-

Table II
Relationships between indicators concentrations measured as target gene copy number/100 ml by qPCR and EMA-qPCR method and colony forming units by traditional spread plate technique

Salmonella spp.	*E. coli*		
	qPCR	EMA-qPCR	CFU
Primary effluents	0.95*	0.98*	–0.72*
Final effluents	0.88*	0.09	0.85*

* Significant correlation for $P < 0.05$; qPCR – without ethidium ethidine monoazide; EMA-qPCR – with ethidium ethidine monoazide; CFU – colony forming units by traditional spread plate technique

tor and pathogenic microorganisms in the dependence on the used identification method. The significant correlations were obtained in the case of primary effluents (Table II) independently on the used method. However for final effluents the values were lower, moreover no significant correlations were observed between *E. coli* and *S. typhimurium* presence when EMA was used. In the literature contradictory data can be found on the relationship between microorganisms occurring in water and wastewater. Some authors suggest that fecal coliforms (FC) are good indicators of the presence in water of such microorganisms as *Salmonella, Shigella*, *Klebsiella*, *E. coli*, *Vibrio* or *Pseudomonas* (Patra *et al*., 2009). Others suggest no significant correlation between the concentration of *E. coli* and *Salmonellae* in wastewater (Song *et al*., 2010).

Conclusions. In this study, EMA-qPCR was successfully used for determining the degree of contamination of wastewater with *E. coli* and *Salmonella* spp. cells. The analysis made in four different time periods allows to assess the rate of occurrence of pathogens in the municipal wastewater treatment plant in Poland.

The qPCR reaction is a highly sensitive molecular tool which allows for the quantification of bacterial cells in wastewater. Application of EMA – pre-treatment eliminates the problem of false-positive results in standard PCR reaction and may become an effective tool to assess the effectiveness of a wastewater treatment process. Compared to the traditional spread plate method, it is more precise and can give information about the presence of specific microorganism, not only the indicator bacteria. The most important advantage of using qPCR for diagnosis of pathogens is the possibility of obtaining the result within 5 hours, which is sometimes crucial for a proper reaction.

The concentrations of *E. coli* and *Salmonella* spp. were higher when molecular methods (including EMA treatment) were used. In most cases significant correlations were observed between the concentration of the fecal indicator and pathogen. However taking into consideration negative results, *E. coli* could not be reliably used to predict the presence of pathogens in wastewater.

Moreover, in our opinion, further research is required to understand the persistency of the traditional fecal indicators in environmental water samples in relation to other pathogenic microorganisms. Hence, quantitative PCR data could be required to assess the survival of traditional fecal indicators along with the other pathogens depending on wastewater treatment and time.

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