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Faecal indicator bacteria and antibiotic-resistant β-lactamase producing *Escherichia coli* in blackwater: a pilot study

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The aim of this study was to identify and quantify faecal indicator bacteria in blackwater collected from a source separation unit and determine the amount of *E. coli* isolates resistant to antimicrobials and their potential to produce extended spectrum β-lactamases (ESβLs) and metallo-β-lactamases (MβLs), which hydrolyse the most important antibiotics used in clinical practice. Most of the isolates were resistant to amoxicillin with clavulanic acid (36.4 %), followed by ticarcillin with clavulanic acid (22.7 %) and tetracycline (18.2 %). ESβL-producing genes $bla_{CTX\text{-}M}$ and bla_{TEM} were found in three (13.6 %) and four (18.2 %) *E. coli* strains, respectively, while MβL genes were found in two (9.1 %). By separating at source, this pilot study clearly shows that gastrointestinal bacteria of healthy people can be an important source of antibiotic resistance released into the environment through wastewaters. One way to prevent that is to treat wastewater with a combination of TiO_2, UV light, or ozone, as successful methods to remove resistant bacteria and prevent their spread in the environment.

KEY WORDS: antimicrobial resistance; extended spectrum β-lactamases; metallo-β-lactamases; public health; wastewater treatment

In source separation sanitation systems wastewater is separated and collected on site as blackwater (composed of human faeces, urine, flushing water, and toilet paper), as yellow water (composed of urine), and as greywater (composed of handwashing and/or shower wastewater) (1, 2). Although faeces and urine account for less than 1 % of municipal wastewater volume, they contribute the majority of microorganisms (3). Blackwater also has a higher organic load than municipal wastewater (4).

Microorganisms in the municipal wastewater mostly originate from human excreta, since more than 300 phylogenetic bacterial groups make up the typical microbiota of the human gastrointestinal (GI) tract (5). Some percentage of the bacteria can be of environmental origin and not only from excreta (6). According to the Slovenian Decree on the Discharge and Treatment of Urban Wastewater (7), the efficiency of wastewater treatment is determined by decrease in the count of indicator bacteria of faecal contamination, i.e. coliforms [total (TC) and faecal (FC)], *Escherichia coli* (*E. coli*), enterococci (ENT), and sulphite-reducing clostridia (SRC) below the safety limit for release into watercourses. The limits for ENT are <4 CFU/mL and for *E. coli* <10 CFU/mL, while there are none for TC, FC, and SRC.

Wastewater treatment plants (WWTP) reduce bacterial counts, but some bacteria still remain in the effluent, depending on initial bacterial concentration, and bacterial physical and chemical features, as well as the type of treatment technology (8, 9). Blackwater treatment with anaerobic digestion technology showed a 99.6 % reduction of *E. coli* and TC, and 96.9 % reduction of ENT (8). In contrast, blackwater treatment efficiency with organic and bio-filters is no higher than 46.8 % for heterotrophic bacteria, 60.1 % for *E. coli*, and 81.5 % for coliforms (4). The remaining bacteria in the effluent from WWTP not only have a pathogenic potential but can also carry over antimicrobial resistance. Human GI tract as the primary source of microorganisms in blackwater provides an ideal combination of factors for antibiotic-resistant genes to emerge and spread through bacterial populations. These factors include high cell density, antibiotic exposure during therapy and subsequent selection, and the innate bacterial

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ability to transfer genes through a variety of mechanisms (10).

Bacteria have developed many defence mechanisms against antibiotics, one of which is the production of specific enzymes that metabolise antibiotics. These enzymes, β-lactamases, inactivate a β-lactam ring of antibiotic groups of penicillins, cephalosporins, clavams, cephamycins and, in some cases, even carbapenems. Extended-spectrum β-lactamases (ESβLs) (CTX-M, TEM, and SHV-type enzymes) are capable of hydrolysing penicillins, cephalosporins, and monobactams. Class B metallo-β-lactamases (MβLs) have a broad substrate spectrum and can catalyse the hydrolysis of virtually all β-lactam antibiotics including carbapenems, with the exception of monobactams. They belong to five different families with multiple variants of the VIM and IMP families and single members of the SPM, GIM, and SIM families (11). The presence of β-lactamases in *Enterobacteriaceae* was determined mostly in clinical samples (patient faeces), samples of river water, hospital wastewater, sewage effluent (9, 12, 13) but rarely in blackwater.

Therefore, the purpose of this study was to isolate and quantify surviving faecal indicator bacteria from the human GI tract in blackwater obtained from a pilot source separation unit. We wanted to determine isolates resistant to selected antimicrobials and investigate the presence of ESβL- and MβL-producing strains that can serve as input data for appropriate blackwater treatment to lower human health risk.

METHODS

Sampling and characterisation of blackwater

For the purposes of this study a pilot source separation unit was constructed in Ljubljana, Slovenia in 2014. It consisted of an unheated container (6×2.9×2.8 m), a vacuum toilet (model 59 M, JETS®, Hareid, Norway) for collecting blackwater, waterless urinal for yellow water (Enswico, Egg bei Zürich, Switzerland), and a washbasin (Kovinoplastika Lož, Stari trg pri Ložu, Slovenia) for greywater. The unit was placed in an animal waste-processing company and used by 15–20 employees every day. They were presumed healthy because they regularly came to work.

Blackwater was collected in a 100 L closed stainless steel tank (to prevent outside contamination) with two stirrers, which were running automatically for 15 minutes twice per day. Following the ISO 19458 (14) procedure, all thirteen 800 mL samples of blackwater were taken from the tank in the mornings (9–10 a.m.) over eleven months (January to November 2015) at regular intervals. Before each sampling, stirrers in the blackwater tank were manually switched on to stir blackwater for 5 minutes.

After each sampling, the temperature of blackwater in the tank was measured with a Checktemp 1 pocket thermometer (Hanna Instruments, Woonsocket, RI, USA). The samples were transported in a cooler to the microbiological laboratory and analysed within an hour of collection. One part was separated for microbiological analysis and another for pH (inoLab® pH 730, WTW GmbH, Weilheim in Oberbayern, Germany) and conductivity (EC/TDS meter 98311, Hanna Instruments) measurements.

Determination and enumeration of individual bacterial groups

We analysed the samples for the presence and number of total coliforms (TC) and faecal coliforms (FC), including *E. coli*, enterococci (ENT), staphylococci (ST), and sulphite-reducing clostridia (SRC) spores following a modified ISO 8199 method (15). Instead of membrane filtration, the samples were serially diluted in saline (0.9 % NaCl) and the number of colony-forming units determined with a pour plate method (15). The number of colonies was expressed as CFU/mL and converted to log units.

TC, FC, and *E. coli* were counted in colonies grown on Chromocult® Coliform agar (Merck KGaA, Darmstadt, Germany) after aerobic incubation at 36±3 °C for TC or 44.0±0.5 °C for FC and *E. coli* for 21±3 h (16, 17). The presence and number of ENT colonies were determined on enterococcus selective agar (Merck) after aerobic incubation at 36±2 °C for 44±4 h following the ISO 7899 procedure (18). Colonies of the genus *Staphylococcus* were determined on Mannitol Salt Phenol-red agar (Merck) after aerobic incubation at 30–35 °C for 72 h according to the manufacturer's instructions (19). To activate SRC spores, samples were heated at 80 °C for 10 min and cooled down before analysis. Typical colonies were counted on TSC agar supplemented with D-cycloserine (Biolife Italiana, Milan, Italy) after incubation in anaerobic conditions at 44±1 °C for 24 h (20).

Identification of isolated Gram-negative bacteria

At least one colony grown on Chromocult® Coliform agar (Merck, Germany) was identified on each positive sample with the API 10S biochemical test (bioMerieux, Marcy l'Etoile, France) and determined for oxidase and catalase production (17). Additionally, 22 confirmed *E. coli* isolates were tested for the presence of the O157 serogroup with a latex agglutination *E. coli* O157 kit (Oxoid, Basingstoke, UK) to identify strains producing the Shiga toxin (21).

E. coli *antimicrobial susceptibility testing*

Twenty-two *E. coli* isolates were tested for susceptibility to amoxicillin/clavulanic acid (CA) (20/10 μg), ampicillin/sulbactam (S) (10/10 μg), ciprofloxacin (5 μg), gentamicin (10 μg), kanamycin (30 μg), nalidixic acid (30 μg), tetracycline (30 μg), ticarcillin/CA (75/10 μg), and tobramycin (10 μg) with BD BBL™ Sensi-Disc™ Antimicrobial Susceptibility Test Discs (Becton Dickinson,

Wayne, PA, USA) using the Kirby-Bauer disc diffusion method (22). The tests were validated with control strains *E. coli* ATCC 25922 and *K. pneumoniae* ATCC 700603.

Phenotypic detection of β-lactamases production

To identify production of ESβL, the *E. coli* strains were inoculated on a HiChrome ESβL chromogenic screening medium (HiMedia Laboratories, Mumbai, India). ESβL producing *E. coli* colonies were dyed in pink or purple.

Detection of nucleotide sequences encoding β-lactamases

DNA was isolated from the twenty-two *E. coli* strains by boiling the cells in a water bath at 100 °C for 10 minutes (23). Its multiplication was determined with polymerase chain reaction (PCR) using a Thermo Scientific DreamTaq Green PCR Master Mix (2X) solution (Thermo Fisher Scientific, Waltham, MA, USA). For ESβL gene detection we used universal primers OT-3 and OT-4 (24), and MA-1/MA-2 (25, 12) targeting bla_{TEM} and bla_{CTX-M}, respectively. Strains positive to CTX-M β-lactamases were further tested with multiplex PCR with specific primers for bla_{CTX-M} groups 1, 2, 8, 9, and 25. The encoding MβLs $bla_{M\beta L}$ sequences (bla_{VIM} and bla_{IMP} class) as well as the bla_{GIM}, bla_{SPM-1}, and bla_{SIM-1} were detected with multiplex $bla_{M\beta L}$ primers VIM, IMP, GIM, SPM, and SIM (26, 27). The primers and the cycling conditions are shown in Table 1.

PCR products were visualised with agarose gel electrophoresis after staining with Sybr-safe (Thermo Fischer Scientific). Reference strains producing CTX-M1, CTX-M2, and CTX-M9 were provided by the National Institute of Biology, Ljubljana, Slovenia, while CTX-M15, TEM-1, VIM-2, and IMP-1-lactamases were provided by the Laboratory for Clinical and Molecular Microbiology, Clinical Hospital Centre Zagreb, Zagreb, Croatia.

Data analysis

Correlations between the groups of faecal indicator bacteria in blackwater were determined with Pearson correlation, with 11 degrees of freedom (df). For statistical analysis we used the IBM SPSS Statistics version 23.0 software (IBM, Armonk, NY, USA).

RESULTS

Concentration of faecal indicator bacteria in blackwater

Colonies of indicator bacteria were differentiated and counted based on their reaction with the growing medium according to the manufacturer's instructions. *E. coli* produced blue to purple colonies, while all other coliforms grew as pink colonies. ENT produced red colonies, ST colonies were colourless with or without a yellow zone, and SRC colonies were grey to black.

In all 13 samples of blackwater TC and ENT had the highest average counts (both 5.1 log CFU/mL), regardless of the season, and SRC had the lowest (2.2 log CFU/mL) (Figure 1). On average, *E. coli* accounted for 89 % of TC and all of FC.

Statistical correlations between the groups of indicator bacteria were as follows: r=0.90 (P<0.001) between TC and FC; r=0.98 (P<0.001) between FC and *E. coli*, r=0.94 (P<0.001) between TC and *E. coli*; r=0.60 (P=0.032) between TC and ENT; r=0.03 (P=0.025) between ST and ENT; r=-0.61 (P=0.027) between ST and SRC; and r=-0.64 (P=0.018) between SRC and ENT. Negative correlation was also observed between ST count and temperature in the blackwater container (r=-0.717, P=0.006).

Figure 2 shows that TC counts did not vary significantly between summer and winter, save for a slight increase in standard deviation (SD) in the summer. FC and *E. coli* had the highest counts in the summer and winter, when the average blackwater temperature was 22.1±1.8 °C and 12.8±3.7 °C, respectively. ENT showed the highest count in the winter, when blackwater temperature was 17.5±4.3 °C. The highest ST count was recorded in spring and winter, and that of SRC in the summer.

The pH values of blackwater kept in the range from 7.02 to 8.84 and did not significantly vary between seasons (P<0.001) (Figure 2).

Gram-negative bacteria findings

Twenty-two of the 39 typical purple colonies growing on chromogenic medium were biochemically confirmed as *E. coli* (56.3 %), and none belonged to the O157 serotype. Other isolated colonies belonged to different species of the genera *Enterobacter* spp. (6 or 15.3 %), *Citrobacter* spp. (3 or 7.7 %), *Plesiomonas* sp. (1 or 2.6 %), *Pantoea* sp. (1 or 2.6 %), *Pseudomonas* sp. (1 or 2.6 %), and *Hafnia* sp. (1 or 2.6 %). Four bacterial strains (10.3 %) were not identified successfully.

E. coli *susceptibility to antimicrobials and ESβL production*

Most *E. coli* isolates showed resistance to amoxicillin/CA (36.4 %), followed by those resistant to ticarcillin/CA (22.7 %) and tetracycline (18.2 %).

Nineteen of the 22 (86.4 %) were found to produce ESβLs (Figure 3).

E. coli *encoding ESβLs and MβL*

Genes encoding ESβLs and MβL were detected in eight (36.4 %) of the 22 *E. coli* isolates. The class bla_{CTX-M} sequences were detected in seven strains (31.8 %) and the bla_{TEM} genes in four strains (18.2 %). These isolates also carried the bla_{CTX-M} genes, more specifically bla_{CTX-M2} (four strains, 57.1 %), $bla_{CTX-M25}$ (two strains, 28.6 %), and bla_{CTX-M9} (one strain, 14.3 %), while the bla_{CTX-M1} gene was not detected.

Table 1 Oligonucleotide primers used for the detection of β-lactamase genes

Target sequence	Nucleotide sequence (5'→3')	Orientation	Designation	Amplicon's expected size (bp)	PCR conditions	Reference
bla$_{TEM}$	ATG AGT ATT CAA CAT TTC CG CCA ATG CTT AAT CAG TGA GG	F R	OT-3 OT-4	850	94 °C/3 min; 35 cycles 94 °C/30 s, 55 °C/30 s, 72 °C/45 s; 72 °C/5 min	24, 28
bla$_{CTX-M}$ consensus	SCS ATG TGC AGY ACC AGT AA CCG CRA TAT GRT TGG TGG TG	F R	MA-1 MA-2	554		25, 12, 27
bla$_{CTX-M1}$	AAA AAT CAC TGC GCC AGT TC AGC TTA TTC ATC GCC ACG TT	F R		415	94 °C/5 min; 30 cycles 94 °C/25 s, 52 °C/40 s, 72 °C/50 s; 72 °C/6 min	27
bla$_{CTX-M2}$	CGA CGC TAC CCC TGC TAT T CCA GCG TCA GAT TTT TCA GG	F R		552		
bla$_{CTX-M8}$	TCG CGT TAA GCG GAT GAT GC AAC CCA CGA TGT GGG TAG C	F R		666		
bla$_{CTX-M9}$	CAA AGA GAG TGC AAC GGA TG ATT GGA AAG CGT TCA TCA CC	F R		205		
bla$_{CTX-M25}$	GCA CGA TGA CAT TCG GG AAC CCA CGA TGT GGG TAG C	F R		327		
bla$_{IMP}$	GGA ATA GAG TGG CTT AAT TCT C CCA AAC CAC TAC GTT ATC T	F R		188		26, 27
bla$_{VIM}$	GAT GGT GTT TGG TCG CAT A CGA ATG CGC AGC ACC AG	F R		390		
bla$_{GIM}$	TCG ACA CAC CTT GGT CTG AA AAC TTC CAA CTT TGC CAT GC	F R		477		
bla$_{SPM}$	AAA ATC TGG GTA CGC AAA CG ACA TTA TCC GCT GGA ACA GG	F R		271		
bla$_{SIM}$	TAC AAG GGA TTC GGC ARC G TAA TGG CCT GTT CCC ATG TG	F R		570		

A, adenine; C, cytosine; G, guanine; T, thymine; S - G or C; Y - C or T; R - A or G; F, forward; R, reverse

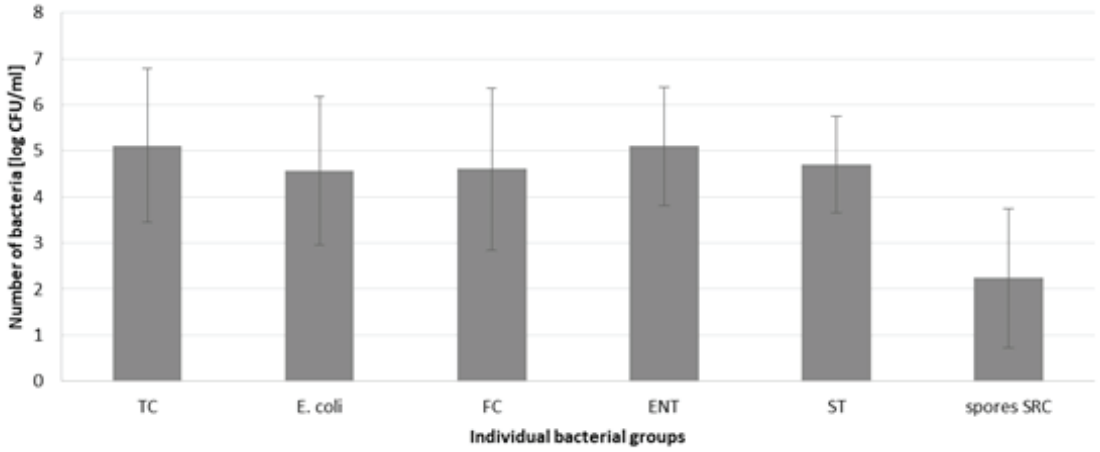

Figure 1 Average counts of total coliforms (TC), *E. coli*, faecal coliforms (FC), enterococci (ENT), staphylococci (ST), and sulphite-reducing clostridia spores (SRC) in blackwater samples collected over a year (n=13)

MβL genes were detected in two strains (9.1 %) (bla_{IMP} in one and bla_{VIM} in another), whereas bla_{GIM}, bla_{SIM}, and bla_{SPM} were not detected.

DISCUSSION

The average ENT and TC count (5.1 log CFU/mL) in our study is similar to the one reported in blackwater by Wendland (7). The same is true for the blackwater *E. coli* and TC counts reported by Oarga et al. (4). According to the Decree on the Discharge and Treatment of Urban Wastewater (6), effluents treated by disinfection at WWTPs containing less than 4 CFU/mL of ENT and less than 10 CFU/mL of *E. coli* can be released in watercourses. To comply with this regulation, treatment technology for blackwater from this study should be able to reduce the ENT and *E. coli* counts for 4 log and 4.1 log, respectively.

As expected, the number of TC, FC, and SRC positively correlated with ENT ($P<0.05$), since they are all part of the normal human gut microbiota (5). Positive correlation between ENT and ST ($P<0.05$) and ST and SRC ($P<0.05$) is in line with studies commonly reporting colonisation of the human GI tract with the usual representatives of STs, namely *S. aureus* and *S. epidermidis* (29, 30).

Nearly none of the indicator bacteria in our study showed statistically significant correlations between their count and blackwater temperatures in the tank, which confirms that they can survive in environmental temperatures (31) (in our case ranging from 15 °C to 25 °C) and are not affected by season. The exception is ST, which showed a negative correlation ($P=0.006$).

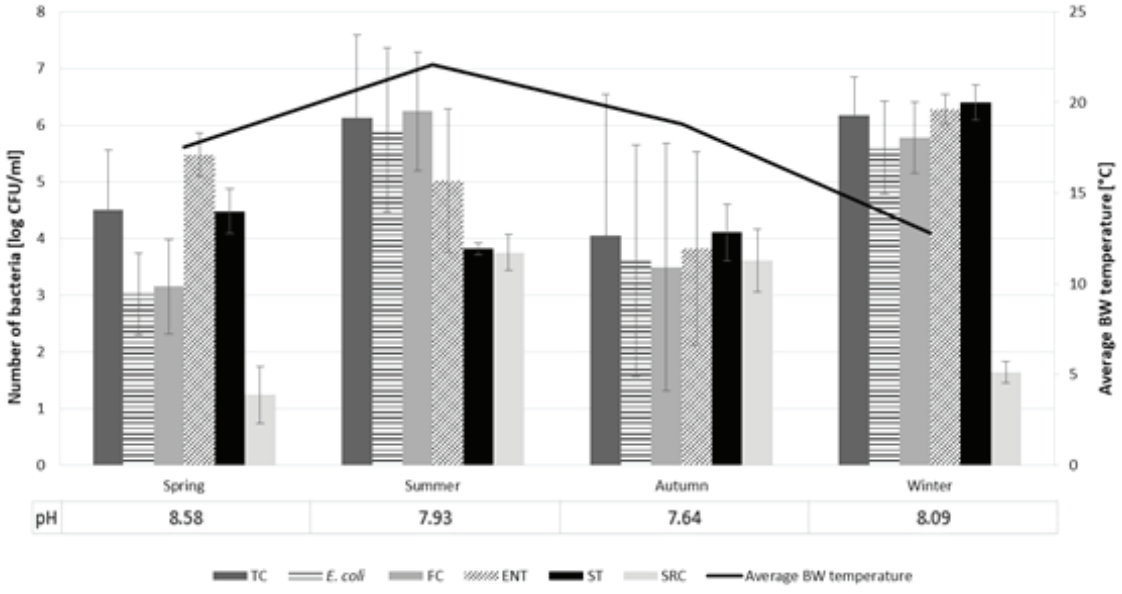

Figure 2 Average seasonal blackwater (BW) temperature, pH, and counts of total coliforms (TC), *E. coli*, faecal coliforms (FC), enterococci (ENT), sulphite-reducing clostridia spores (SRC), and staphylococci (ST) (n=13)

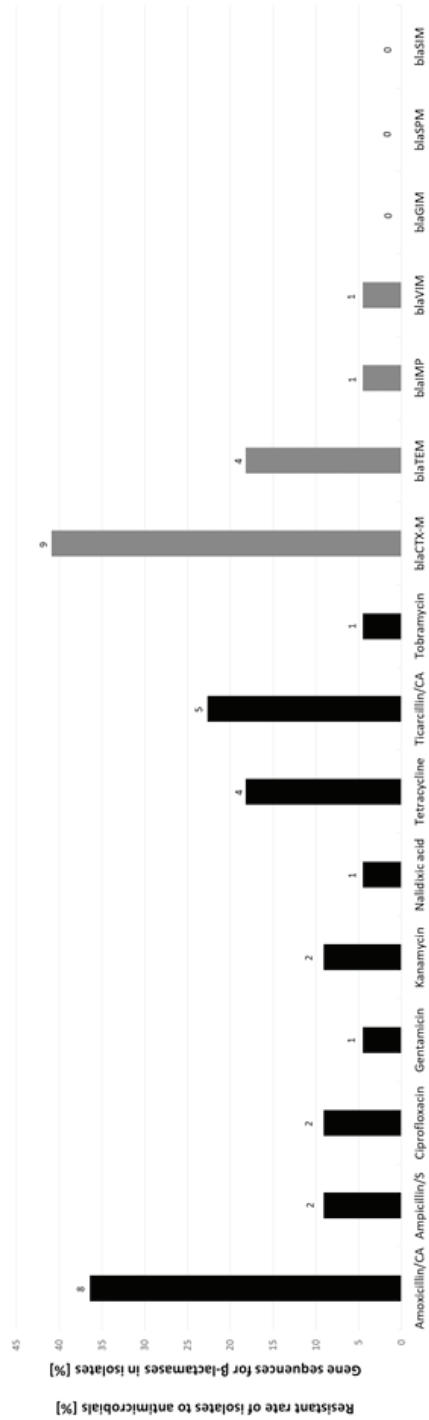

Figure 3 Antimicrobial resistance rates and the percentage of blackwater *E. coli* isolates containing β-lactamase-encoding genes (n=22)

Our study also revealed the presence of strains belonging to *Pseudomonas* spp., *Hafnia* spp. and *Pantoea* spp., which are only occasionally present in human faeces and are usually ingested with contaminated food and drinking water (32–35). As all the animal-waste processing facility employees were presumed healthy, they probably contracted these microorganisms from the contact with animal waste (skin, fleece, and hair) and ingested them with food or drinking water if they did not wash their hands properly before a meal. However, none of the *E. coli* strains tested positive to O157, which suggests that none of the employees had pathogenic *E. coli* strains.

So far, there are but a few reports about resistant *E. coli* isolates in blackwater (36, 37), as ESβL-producing *E. coli* strains have mostly been studied in hospital and municipal wastewater or directly in patient faeces (9, 13, 38, 39). Our *E. coli* isolates showed resistance to amoxicillin/CA (36.4 %), ticarcillin/CA (22.7 %), and tetracycline (18.2 %), which clearly suggests that antibiotic-resistant bacteria are also present in normal human GI tract. Similar conclusions were drawn by Vinué et al. (36). Bacterial strains in the GI tract can acquire antibiotic resistance from present resistant bacterial strains through horizontal transfer of resistance plasmids or through ingestion of antibiotic-resistant bacteria with food (13).

Phenotyping in our study showed that 86 % of the *E. coli* isolates were producing ESβL, while PCR confirmed that 31.8 % of these were positive to $bla_{\text{CTX-MA}}$ and 18.2 % to bla_{TEM}. $Bla_{\text{CTX-MA}}$ was also reported in 6.7 % of 85.7 % ESβL-positive *E. coli* isolates from faecal samples of healthy people, mostly from the group CTX-M9 and CTX-M1 (36). *E. coli* strains can carry more than one different *bla* sequence (9). The sequences of CTX-M and TEM can be transferred with the same plasmid (40), as confirmed in our study, in which all four isolates had the bla_{TEM} and $bla_{\text{CTX-M}}$ genes. A similar phenomenon was reported by Reinthaler et al. (13).

As for MβL resistance, we isolated two (9 %) MβL-positive strains of *E. coli*, one of the IMP and one of the VIM type.

CONCLUSION

Source separation sanitation systems provide a new sustainable approach to nutrient recovery from wastewater as well as reduction in water and energy consumption (4). By separating at source, this pilot study clearly shows that blackwater is a fraction with high concentration and diversity of microorganisms. We can conclude that the blackwater may be an important reservoir of MβL- and ESβL-producing enterobacteria as part of normal GI microbiota in healthy people. This should be taken into account while deciding which wastewater treatment process to use to prevent the dissemination of resistance genes into the aquatic environment. One of the options that has proved

successful is the oxidation processes. Öncü et al. (41) reported significant and dose-dependent oxidative damage to the plasmid DNA of multi-resistant *E. coli* HB101 treated with ozone and photocatalysis. Photocatalytic degradation with titanium oxide (TiO_2) nanoparticles and exposure to ultraviolet light was also reported very successful in removing antibiotic-resistant genes and bacteria (42, 43).

There are several limitations to this pilot study, one of which is the small number of blackwater samples due to relatively few sanitation unit users. To confirm our findings of the ESβL producing phenotypes, the next step will be to run PCR multiplication of the sequences encoding other common ESβLs like SHV, OXA, and CMY (36). In addition, more isolates of *E. coli* should be examined in the future for better comparison with other reports.

Conflicts of interest

None to declare.

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Ugotavljanje indikatorskih bakterij fekalnega onesnaženja in prisotnosti vrste *Escherichia coli*, ki tvori encime β-lactamase v črni vodi

V vzorcih črne vode, ki je ena od frakcij odpadne vode, smo ugotavljali prisotnost in število fekalnih indikatorskih bakterij, vključno z bakterijo *Escherichia coli* (*E. coli*). Pri osamljenih sevih *E. coli* smo ugotavljali njihovo odpornost proti izbranim antibiotikom in njihov potencial za tvorbo nekaterih β-laktamaz razširjenega spektra in metalo-β-laktamaz. Preizkušeni sevi so bili najpogosteje odporni proti amoksicilinu s klavulansko kislino (36,4 %), tikarcilinu s klavulansko kislino (22,7 %) in tetraciklinu (18,2 %). Nukleotidne sekvence za bla_{CTX-M} in bla_{TEM} smo našli pri treh (13,6 %) in štirih (18,2 %) sevih, medtem ko smo gene za izbrane metalo-β-laktamaze ugotovili pri dveh (9,1 %) sevih *E. coli*. Pilotna študija, z ločevanjem odpadne vode na viru nastanka, kaže, da so bakterije v prebavnem traktu zdravih ljudi lahko pomemben vir prenosa odpornosti proti antibiotikom v okolju preko odpadne vode. Eden izmed načinov za preprečevanje širjenja odpornosti proti antibiotikom je čiščenje odpadne vode z uporabo kombinacije TiO_2, UV svetlobe in ozona, ki so se pokazale kot uspešne metode za odstranjevanje bakterij, odpornih proti antibiotikom.

KLJUČNE BESEDE: β-lactamase s širokim spektrom delovanja; čiščenje odpadne vode; javno zdravje; metalo-β-lactamase; odpornost proti antibiotikom