ORIGINAL ARTICLE

Resistotyping and extended-spectrum beta-lactamase genes among *Escherichia coli* from wastewater treatment plants and recipient surface water for reuse in South Africa

B. Nzima[1], A. A. Adegoke[1,2], U. A. Ofon[2], H. O. M. Al-Dahmoshi[3], M. Saki[4,5], U. U. Ndubuisi-Nnaji[2] and C. U. Inyang[2]

1) *Institute for Water and Wastewater Technology, Durban University of Technology, Durban, South Africa,* 2) *Department of Microbiology, Faculty of Science, University of Uyo, Uyo, Akwa Ibom State, Nigeria,* 3) *Biology Department, College of Science, University of Babylon, Babylon Province-Hilla City, Iraq,* 4) *Department of Microbiology, Faculty of Medicine, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran and* 5) *Student Research Committee, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran*

Abstract

The spread of extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli* has increased in different environments. This study aimed to evaluate the patterns of antibiotic resistance and ESBL genes among *E. coli* isolates collected from wastewater and recipient surface water in South Africa. Fifteen samples containing nine wastewater and six river water samples were collected from a local wastewater treatment plant. The *E. coli* isolates were detected using standard microbiology methods. Antibiotic susceptibility testing was performed using disc diffusion agar. The occurrence of bla_{CTX-M}, bla_{SHV} and bla_{TEM} ESBL genes was investigated by PCR. Exactly 140 isolates were selected from the primary enumeration plates with a $\log_{10}$ CFU/mL count that ranged from 4.1 to 4.2 (influent), 4.2 to 4.5 (biofilter) and 2.5 to 3.3 (effluent). The wastewater effluent showed an impact on the receiving water environment, as the treatment efficiency was 92% and the downstream $\log_{10}$ CFU/mL count (range, 3.6–3.8 $\log_{10}$ CFU/mL) was higher than the upstream count (range, 3.3–3.6 $\log_{10}$ CFU/mL). Antibiotic testing results showed that 40% to 100% of *E. coli* isolates were resistant to ampicillin, penicillin, tetracycline and cefotaxime but susceptible to imipenem, meropenem and ciprofloxacin. A total of 40 studied isolates (28.6%) had both the bla_{TEM} and bla_{CTX-M} genes, while no bla_{SHV} was detected. The wastewater treatment plants contributed multidrug-resistant ESBL-producing *E. coli* isolates that can be potential environmental health risks. Regular monitoring policies are recommended to prevent the spread of antibiotic resistance in the region.

Keywords: ESBLs, *Escherichia coli*, South Africa, wastewater treatment plants, WWTP
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Corresponding author: A. A. Adegoke, Department of Microbiology, Faculty of Science, University of Uyo, PMB 1017 Uyo, Akwa Ibom State, Nigeria.
Corresponding author: M. Saki, Ahvaz Jundishapur University of Medical Sciences, Golestan Blvd, PO Box 159, Ahvaz, 61357-15794, Khuzestan, Iran.
E-mails: aayodegoke@gmail.com **(A.A. Adegoke),** morteza-saki1981@gmail.com **(M. Saki)**

Introduction

Water scarcity for irrigation has been one of the most important setbacks for agriculture in arid and semiarid regions of the earth. Agricultural reuse of treated wastewater has been acknowledged as an effective pathway to circumvent water scarcity. Although irrigation is recognized for its immense advantages, the benefits may be exacerbated if potentially antibiotic-resistant pathogens such as *Escherichia coli* are identified [1,2].

From a general perspective, antibiotic-resistant bacteria and antibiotic resistance genes, including extended-spectrum β-lactamases (ESBLs), are of special concern in wastewater as they can be conveyed into the food cycle [3,4]. Their effects on human health depend on the pathogenicity of the bacteria and their potential to resist conventional antibiotics. *Escherichia coli* as a commensal bacterium can be plentifully transmitted to the environment through the use of manure, animal faeces,

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improperly treated wastewater or sewage and sewage overflow caused by heavy rains [2]. Some of the commensal as well as pathogenic *E. coli* strains that are excreted into the environment may have the capacity to produce ESBL enzymes. The occurrence of ESBL-producing *E. coli* in surface waters has been reported in different parts of the world [5,6].

Bacteria with the potential to produce ESBL are on the rise globally, with hundreds of ESBL genes reported so far. Resistance associated with production of ESBL by *Enterobacteriaceae* in which *E. coli* has been identified led to high mortality and a huge cost of hospitalization [7,8]. There has been concomitant resistance to wide range of antibiotics among *E. coli* harbouring ESBL genes beyond β-lactam antibiotics. Human exposure to these bacteria may occur, for instance during recreation in contaminated surface water, or indirectly, when contaminated surface water is used for irrigation of fresh crops' produce, thus contributing to community-associated dissemination of ESBL-producing *E. coli* [9]. The most common classes of ESBLs include bla_{TEM}, bla_{SHV} and bla_{CTX-M}. Specific variants of these, such as bla_{SHV-5}, have ability to hydrolyze broad-spectrum cephalosporins and monobactams. These β-lactamase genes are usually harboured in mobile genetic elements like plasmids and can be transferred horizontally [7].

In order to prevent further spread of *E. coli* harbouring ESBLs in different environments, understanding the possible influence of wastewater treatment plant (WWTP) on the surface is essential to provide insight into the contribution of different possible environmental contamination sources and exposure routes. This necessitates regular surveillance of the wastewater being reused in agriculture because of its potential effects on the food cycle [10–13].

We aimed to determine the presence of ESBL-producing *E. coli* in surface water receiving effluent discharge from a WWTP, as well as the treatment efficiency and possible contribution of the WWTP to the distribution of ESBL-producing *E. coli* in surface water. Furthermore, the resistance profiles and presence of bla_{TEM}, bla_{SHV} and bla_{CTX-M} genes were also examined.

Materials and methods

Sample collection

This 3-month study was conducted on WWTP receiving domestic, industrial and hospital wastewater in Durban, South Africa, from May to July 2017. Influent and effluent wastewater (n = 9) and surface water samples (n = 6) from the final effluent were collected from four different sampling points including influent, biofilter, effluent, upstream and downstream at different sampling times and mixed with sodium thiosulphate (100 µL in 2 L of the bottle) immediately to decrease the level of and neutralize the activity of chlorine.

Isolation and identification of potential ESBL-producing *E. coli*

Microbial enumeration and isolation were carried out using *E. coli* CHROMagar ECC (bioMérieux, Marcy l'Étoile, France). Isolation procedures were based on standard isolation procedures for the selective isolation of *E. coli* using chromogenic media adapted to enable the selective growth of ESBL-producing variants. Specifically, isolation and recovery of bacteria was carried out using the membrane filtration method. Multiple volumes of samples (10 mL, 1 mL and 0.1 mL) were vacuum-filtered through 0.45 µm pore size filters. Filters were then placed onto CHROMagar ECC selective for the isolation of *E. coli* and incubated at 37°C for 24 hours. Blueish colonies were selected for further characterization by Gram staining and standard indole, methyl red/Vogues-Proskauer and Simmon citrate (IMViC) biochemical tests. Finally, the ESBL-production was assessed using ChromID ESBLagar (bioMérieux) [14]. The isolates identified as ESBL-producing *E. coli* were frozen in tryptic soy broth plus 20% glycerol at −80°C [15].

Molecular identification of *E. coli* isolates

Molecular identification was performed on the 140 presumptive ESBL-producing *E. coli* isolates by PCR using the specific primers for a conserved region situated within the *E. coli* alanine racemase (*Alr*) gene (Table 1) [13]. The primers were synthesized by Inqaba Biotechnical Industries (Pty) Ltd, South Africa. The boiling method was used for DNA extraction from isolates as previously described [16]. The PCR reaction consisted of initial denaturation at 95°C for 5 minutes, 35 cycles of 30 seconds' denaturation at 95°C, annealing at 58°C for 30 seconds per extension at 72°C for 30 seconds and a final extension for 5 minutes at 72°C. *E. coli* ATCC 25922 was used as a positive control. The standard reaction mixture contained 1.25 units of thermostable DNA polymerase, 1 × Ex Taq buffer, 2 mM $MgCl_2$, 10 pmol of each oligonucleotide primer, 10 nmol of dNTP and 2 µL of template DNA suspension in a final volume of 50 µL.

Antibiotic susceptibility testing

Antibiotic susceptibility testing was conducted following the 2017 guidelines of the Clinical Laboratory Standard Institute (CLSI) [17]. The bacterial suspensions were made in sterile phosphate-buffered saline (pH 7.4) to match a 0.5 McFarland standard in order to achieve an inoculum density of approximately 1×10^8 CFU/mL. Sterile swabs were used to inoculate the surface of Müller-Hinton agar (Merck, Darmstadt, Germany) plates from these suspensions. Antibiotic-impregnated

TABLE 1. Primer sequences and expected size of PCR-amplified genes

Gene target	Primer sequence 5′–3′	Amplicon size (bp)
Alr	F: CTGGAAGAGGCTAGCCTGGACGAG R: AAAATCGGCACCGGTGGAGCGATC	366
bla_{CTX-M}	F: CGGGAGGCAGACTGGGTGT R: TCGGCTCGGTACGGTCGA	381
bla_{TEM}	F: GTCGCCGCATACACTATTCTCA R: CGCTCGTCGTTTGGTATGG	258
bla_{SHV}	F: GCCTTGACCGCTGGGAAAC R: GGCGTATCCCGCAGATAAAT	319

discs were placed onto the Müller-Hinton agar surface with the use of sterile forceps. The plates were incubated for 18 to 24 hours at 37°C. The selected antibiotic discs used for this analysis included: ampicillin (10 µg), penicillin (10 U), ciprofloxacin (5 µg), tetracycline (30 µg), trimethoprim (10 µg), cefotaxime (30 µg), ceftazidime (30 µg), sulfamethoxazole (24 µg) and carbapenems (imipenem and meropenem) (10 µg). The interpretation criteria (sensitive/resistant) for the antibiotics were determined on the basis of the zone diameters provided in CLSI 2017 [17]. An isolate was designated multiple antibiotic resistant if it was resistant to at least three antibiotics classes [18–21]. The antibiotics used in this study were selected on the basis of their clinical and agricultural significance. Each of these antibiotics has either been found at potentially active concentrations in wastewater or has previously been associated with increased resistance in environmental *E. coli*.

Detection of ESBL genes among *E. coli* using multiplex PCR

Multiplex PCR (M-PCR) was performed by using the specific primers listed in Table 1 for screening for ESBL genes in *E. coli* isolates [13]. The screening of bla_{TEM}, bla_{SHV} and bla_{CTX-M} was done in a total volume of 25 µL containing 12.5 µL of master mix (DreamTag MM; Thermo Fisher Scientific, Waltham, MA, USA), 20 µM of each forward and reverse primers, distilled water and 5 µL of the DNA template. The M-PCR protocol was as follows: predenaturation at 95°C for 5 minutes, followed by 30 cycles of denaturation at 95°C for 30 seconds, annealing at 56°C for 40 seconds, extension at 72°C for 50 seconds and a final extension at 72°C for 10 minutes.

Statistical analyses

A statistically significant difference ($p \leq 0.05$) in *E. coli* concentrations between sampling points and sampling time was evaluated by two-way ANOVA. Correlation analysis was performed for inference on differences in average numbers of ESBL-producing *E. coli* between sampling locations and time. Analyses were performed by SPSS 22 software (IBM, Armonk, NY, USA).

Results

Density of ESBL-producing *E. coli* in WWTP and surface water

The PCR assay confirmed all 140 isolates to be *E. coli* strains. All four sampling points (influent, biofilter, effluent, upstream, downstream) had ESBL-producing *E. coli*, with an occurrence range of 44% (region B) to 100% (region D). The concentrations of ESBL-producing *E. coli* in WWTP ranged from 1.0 to 4.5 $\log_{10}$ CFU/mL, whereas it ranged from 3.3 to 4.0 $\log_{10}$ CFU/mL in surface water samples. The average concentrations of ESBL-producing *E. coli* in the influent water (in studied WWTPs) were in the same range or slightly higher than those in the biofilter, but were significantly reduced in the effluent samples discharged into surface waters (Tables 2 and 3). Concentrations of ESBL-producing *E. coli* at a distance from WWTP discharge points (upstream) were comparable to that in downstream and on average were 2- to 3-$\log_{10}$ units higher than that in the effluent (away from the discharged point). The best treatment efficiency was 92.5% at sampling time T_3, while the least was 91.9% at sampling time T_2 (Fig. 1).

Antibiotic resistance profiles of potential ESBL-producing *E. coli*

The majority of *E. coli* from influent samples were resistant to penicillin (70%) and 30% were intermediately resistant; biofilter isolates were resistant to penicillin (100%) and cefotaxime (100%); effluent isolates were resistant to penicillin (100%) and tetracycline (80%); surface water upstream isolates were resistant to penicillin (100%) and trimethoprim (50%); and samples from downstream surface water exhibited resistance to ciprofloxacin (60%), tetracycline (60%), trimethoprim (67%) and penicillin (80%). Resistance to penicillin, tetracycline and ampicillin was frequent, whereas resistance to cefotaxime, ceftazidime and trimethoprim were less frequently observed in other parts of WWTP except the biofilter. Resistance to the carbapenem antibiotics imipenem and meropenem was not seen in the studied isolates. Remarkably, in effluent samples, ESBL *E. coli* was 40% to 100% resistant to ampicillin, penicillin and tetracycline but susceptible to imipenem, meropenem and ciprofloxacin. Overall, 33.3% of isolates from biofilter, 44.4% from WWTP effluents, 55.6% from upstream surface water (under the influence of WWTP discharge points) and 44.4% from surface waters downstream (not under the direct influence of the investigated WWTPs) showed resistance to at least three antibiotic categories in addition to β-lactam antibiotics. It was thus designated as a multidrug-resistant pathogen.

Overall reduction of *E. coli* due to treatment was in the range 1.2 to 3.1 $\log_{10}$ CFU/mL (Table 4). The best treatment

TABLE 2. Presumptive *Escherichia coli* counts and pathogen log reduction from wastewater treatment plants

	Concentration of *E. coli* (log_{10} CFU/mL)											
	Influent				Biofilter				Effluent			
Sampling point	T_1	T_2	T_3	Mean	T_1	T_2	T_3	Mean	T_1	T_2	T_3	Mean
1	4.3	4.1	4.3	4.2	—	—	—	—	3.3	3.3	0	2.2
2	4.5	4.3	4.3	4.4	4.5	4.2	4.0	4.2	3.6	3.0	3.0	3.2
3	4.3	4.1	4.0	4.1	4.3	4.0	3.5	3.9	3.0	0	0	1.0

T_1, T_2 and T_3 refer to sampling time points.

efficiency was 92.5% and the least 91.9% (Fig. 1). Forty isolates (28.6%) had both the bla_{TEM} and $bla_{CTX\text{-}M}$ genes, while no bla_{SHV} was detected.

Discussion

Usually resistance of *E. coli* and other *Enterobacteriaceae* to antibiotics, particularly third-generation cephalosporins, is an expression of the $bla_{CTX\text{-}M}$ and bla_{TEM} genes, which code for ESBL [13]. These groups of organisms, which are of clinical significance, have recently been placed on the World Health Organization critical list, with a global spread from the clinical environment to the natural environment through WWTPs [13,22]. In this study, a significant decimation of *E. coli* cells was observed at all sampling times, suggesting that the WWTP was highly efficient. However, ESBL-producing *E. coli* were detected in all WWTP samples as well as upstream and downstream surface water samples situated nearby and/or under the influence of the WWTP under investigation. A significant percentage of these ESBL-producing *E. coli* isolates were multidrug resistant. Detection of ESBL-producing *E. coli* correlated strongly with the relatively high concentrations of total *E. coli* counts (r = 0.96 at p 0.05). Nonetheless, the proportion of ESBL-producing *E. coli* relative to total *E. coli* numbers varied among the sampling points, as well as between sampling times at the same site. This observation was in consonance with a previous study conducted by Blaak *et al.* [14].

The receiving surface water at the points of discharge (upstream) and WWTP effluents contained ESBL-producing *E. coli*. The occurrence of ESBL-producing *E. coli* in these sampling locations may be thought to directly reflect the strains load exist in the discharged effluents at the time of sampling, even though a small quantity of them may be obtained from upstream sites. The concentrations of ESBL-producing *E. coli* at effluent locations were on average 1- to 2-log_{10} units lower than those in upstream samples ($p < 0.05$), and were similar to concentrations downstream of the WWTP, suggesting a possible impact of the connecting water body receiving WWTP effluent. This study demonstrated the impact of WWTP in contributing to the load of ESBL-producing *E. coli* in surface water with possible risk of exposure to users of that water body. To this end, studies have identified recreational freshwater swimming in surface water as a significant risk factor for acquiring urinary tract infections caused by ESBL-producing *E. coli* [5,23]. The results of the present study provide substantial evidence to this epidemiologic and public health concern, as ingesting contaminated surface water may lead to intestinal colonization by extraintestinal ESBL *E. coli* and subsequent urinary tract infection [24]. Also, the frequent existence of ESBL-producing *E. coli* upstream of the WWTPs and in connecting water bodies was not influenced by the studied WWTPs, suggesting the presence of other sources of ESBL-producing *E. coli*. The current study focused on the possible impact of discharged effluents of WWTP as a possible source of ESBL-producing *E. coli* in nearby surface water, but it did not investigate the contribution of sewage overflows or more remote WWTPs. Because overflows contain untreated sewage, they serve as an important source of ESBL-producing *E. coli* in surface water during heavy rainfall. Although the locations of overflow exhausts in the area under investigation were not mapped in this study, both overflows and more remote WWTPs may have also contributed to the faecal contamination in the investigated surface water. Moreover, animal manure may again contribute, because ESBL-producing *E. coli* are ample in food animals, particularly in broilers, veal calves and pigs [14]. Further to this, faeces of wild animals such as birds may contribute ESBL-producing *E. coli* to surface water [25]. The

TABLE 3. Concentration of *Escherichia coli* in surface water samples

	Concentration of *E. coli* (log10 CFU/mL)							
	Upstream				Downstream			
Sampling point	T_1	T_2	T_3	Mean	T_1	T_2	T_3	Mean
1	3.5	3.0	3.3	3.3	3.8	3.4	3.5	3.33
2	3.3	3.5	3.5	3.3	4.0	4.0	3.6	3.9
3	3.8	3.3	3.6	3.6	3.7	3.7	3.9	3.8

T_1, T_2 and T_3 refer to sampling time points.

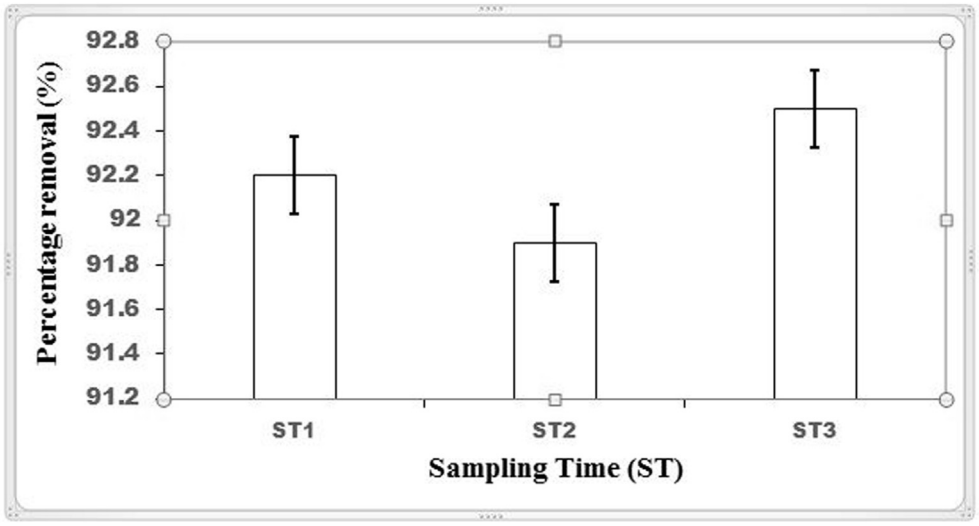

FIG. 1. *Escherichia coli* removal efficiency of treatment process at three different sampling times. High log reduction was observed at various stages of treatment. Treatment efficiencies was high on three sampling occasions. Error bars indicate standard deviation.

investigated river may be located within a suburban area, suggesting that ESBL-producing *E. coli* in the examined surface waters may be a mixture of human and animal origin. ESBL-producing *E. coli* recovered from recreational waters carried similar ESBL genes, partially in the same phylogenetic background, as ESBL-producing *E. coli* in effluents and/or upstream-located surface waters.

It is worth highlighting that the results this study obtained by antibiotic susceptibility profiling showed resistance to cefotaxime, a third-generation cephalosporin, in three out of the five sampling points. This is consistent with the findings of other studies that show an increasing emergence of resistance to third- and even fourth-generation cephalosporins [26,27]. Paterson and Bonomo [28] linked this resistance to hydrolysis by bla_{CTX-M} gene–coded β-lactamase enzyme. ESBL-producing *E. coli* recovered from surface waters have been revealed to carry similar ESBL genes or genes partially on the same phylogenetic background [5,14,24]. The epidemiology of ESBL genes, especially bla_{CTX-M} based, shows distinct variability around variation locations around the world [29] and are common among bacterial isolates from hospitals [30–32]. The current study showed some consistency with this observation, as all isolates showing resistance expressed at least one type of bla_{CTX-M} and bla_{TEM} genes. Several research reports from Nigeria also indicated the detection of bla_{CTX-M} [30–32], though in a clinical setting. Currently, $bla_{CTX-M-15}$ is the most dominant resistance gene in humans in the United States, which is accompanied by a broadly circulated strain of *E. coli* O:25b [33]. In tandem with our study, bla_{CTX-M} in *E. coli* from wastewater samples was reported by Čornejová *et al.* [34]. Unlike our study, where only bla_{CTX-M} and bla_{TEM} were

TABLE 4. **Antibiotic susceptibility results**

		Escherichia coli		
Sample points	**Antibiotics**	**S (%)**	**I (%)**	**R (%)**
Influent	Imipenem	45	55	0
	Meropenem	80	20	0
	Ciprofloxacin	100	0	0
	Tetracycline	100	0	0
	Penicillin	0	30	70
	Ceftazidime	100	0	0
	Ampicillin	100	0	0
	Cefotaxime	100	0	0
	Trimethoprim	100	0	0
Biofilter	Imipenem	100	0	0
	Meropenem	100	0	0
	Ciprofloxacin	100	0	0
	Tetracycline	90	10	0
	Penicillin	0	0	100
	Ceftazidime	100	0	0
	Ampicillin	50	20	30
	Cefotaxime	0	0	100
	Trimethoprim	100	0	0
Final effluent	Imipenem	60	40	0
	Meropenem	72	28	0
	Ciprofloxacin	72	28	0
	Tetracycline	0	20	80
	Penicillin	0	0	100
	Ceftazidime	100	0	0
	Ampicillin	60	0	40
	Cefotaxime	90	0	10
	Trimethoprim	60	0	40
Downstream	Imipenem	90	10	0
	Meropenem	70	30	0
	Ciprofloxacin	40	0	60
	Tetracycline	40	0	60
	Penicillin	0	20	80
	Ceftazidime	90	10	0
	Ampicillin	80	0	20
	Cefotaxime	100	0	0
	Trimethoprim	33	0	67
Upstream	Imipenem	100	0	0
	Meropenem	100	0	0
	Ciprofloxacin	100	0	0
	Tetracycline	90	10	0
	Penicillin	0	0	100
	Ceftazidime	80	0	20
	Ampicillin	55	0	45
	Cefotaxime	80	0	20
	Trimethoprim	50	0	50

I, intermediate; R, resistant; S, susceptible.

detected, a study in Bangladesh by Yesmin *et al.* [35] reported bla_{TEM} (50.5%), bla_{CTX-M} (46.7%) and bla_{SHV} (18.7%). The emergence of such resistant species in the environment limits the optimal treatment options for ESBL infections, thereby reducing the recovery rate of ESBL patients.

This study had several limitations. Firstly, we were unable to sequence the ESBL genes. Secondly, the source of ESBL-producing *E. coli* into the WWTPs and surface water was not traced.

Conclusions

This study revealed that WWTP and surface water are repositories of multidrug-resistant *E. coli* isolates harbouring ESBL genes. This finding highlights the serious health risk to humans upon exposure. In addition, the results showed the need for effective control of the release of bacterial contaminants into local surface waters and may form the basis of future research in adjoining surface waters.

Conflict of interest

None declared.

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