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Research Article
Distribution and Potential Effects of 17β-Estradiol (E2) on *Aeromonas* Diversity in Wastewater and Fish Samples

[1]Murendeni Manavhela, [2]Alfred Sichilima and [1]Amidou Samie

[1]School of Mathematical and Natural Science, Department of Microbiology, University of Venda, Private Bag X5050, 0950 Thohoyandou, South Africa
[2]School of Mathematics and Natural Sciences, Department of Biological Sciences, Copperbelt University, P.O. Box 21692, Kitwe, Zambia

Abstract

Background and Objective: Recently, there has been evidence for the accumulation of steroid hormones in the water environment with negative consequences on fish and humans. However, there is paucity of information on how the steroid hormones influence the microbial community in environmental waters. The objective of this study was to determine the occurrence of 17β-estradiol (E2) and its potential influence on the diversity of *Aeromonas* spp. **Materials and Methods:** Wastewater samples were obtained from sewage treatment plants in northern South Africa and fish samples were collected from the Nandoni dam. *Aeromonas* spp. were isolated using microbiological methods and PCR protocols were used for their identification. A commercial Elisa kit was used for measuring the concentration of 17β-estradiol (E2) from the wastewater samples as well as the fish samples. **Results:** 17β-estradiol (E2) was found in high concentration in sewage samples varying from 0.32-348.6 pg mL^{-1} while in fish samples, it ranged from 1.1-73.6 pg mL^{-1}. There was a tendency of samples with high E2 concentrations to have higher diversity of *Aeromonas* spp., implying that steroid hormones may serve as nutrient for *Aeromonas* spp. *Aeromonas hydrophila* was the most prevalent species (71%), followed by *A. sobria* with (68%). **Conclusion:** The presence of *Aeromonas* spp. in environmental waters and fish that is consumed by the local community poses a serious health concern. The high content of E2 in treated wastewater is of serious concern as well. For the first time, the present study showed a positive impact of E2 on *Aeromonas* growth.

Key words: *Aeromonas* spp., environment, fish, 17β-estradiol (E2), steroid hormones, ELISA, wastewater

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Corresponding Author: Amidou Samie, School of Mathematical and Natural Science, Department of Microbiology, University of Venda, Private Bag X5050, 0950 Thohoyandou, South Africa Tel: +27159628186 Fax: +27159628468

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

For over a decade now, there has been a rising concern about the effects of endocrine disrupting compounds in human and animal health[1,2]. Estrogens are among those compounds that are classified as endocrine disruptors. Natural and synthetic steroid hormones are known to interfere with growth, reproduction and development in fish and humans[3]. Natural Estrogens include estrone (E1), 17β-estradiol (E2) and estriol (E3), while 17α-ethinylestradiol (EE2) is a synthetic estrogen among others[4]. Steroid hormones have been reported to cause adverse effects on fish organisms in concentration[5] as low as 1 ng L^{-1}. Some of the effects of hormones in fish include feminization of male fish, induction of vitellogenin, intersex and interference with growth[6]. These chemicals find their way to environmental waters through sewage discharge and animal wastes, also industries that synthesize these steroid hormones play a role in contaminating the environmental waters through the disposal of their industrial waste materials that includes active forms of these endocrine disruptors[7]. However, the distribution of these substances in the environment in South Africa is not well researched.

In the aquatic environment, there is a variety of microorganisms, which form part of the normal microflora in water as well as in fish. Such are *Aeromonas* spp. which are also known to cause a wide spectrum of diseases in man and animals[8]. The genus *Aeromonas* comprises of Gram-negative rod-shaped bacilli which are autochthonous in the aquatic environment, i.e., fresh and brackish water, sewage and wastewater, untreated and treated drinking water[9]. *Aeromonas* spp. are known as contaminants in fish and a variety of raw meat, milk and milk products and other raw foods[10,11]. The genus *Aeromonas* is very diverse with 31 species and 12 subspecies and a few of those are known to be primary pathogens of fish and warm-blooded animals[12]. These include *Aeromonas hydrophila*, *Aeromonas sobria*, *A. caviae*, *A. veronii*, *A. jandei*, *A. trota*, *A. salmonicida*, *A. media*, *A. popoffi* and *A. schubertii*[13].

Some of the *Aeromonas* spp., such as *Aeromonas hydrophila* cause gastroenteritis, septicemia, chronic diarrhea, wound infections, urinary tract infections, peritonitis and respiratory tract infections in humans[14]. Some are isolated from environmental samples such as fresh water and wastewater as well as from fish tissues and in fish, *A. hydrophila* causes motile *Aeromonas* septicaemia[15-17]. These motile *Aeromonas* cause such infections in numerous species of cultured and wild freshwater fish such as carp, rainbow trout, brown trout, salmon, eel, carp, channel catfish, tilapia and goldfish[18,19]. *Aeromonas* tends to cause infections in fish when their normal environment is compromised, for example, due to poor water quality, nutritional deficiencies and a general poor environment in the aquarium[20]. Therefore, contamination of aquatic environments by steroid hormones is thought to compromise water quality which may lead to microorganisms causing infections to aquatic organisms. However, there is very limited data on the distribution of steroid hormones in environmental water and wastewater in most African regions including South Africa. Although the impact of steroid hormones on human and fish has been studied, there is no data on the impact of these steroid hormones on bacterial organisms including *Aeromonas* spp. Therefore, the aim of this study was to determine the distribution of 17β-estradiol (E2) and *Aeromonas* spp. in wastewater and fish samples and to evaluate the effects of this hormone on *Aeromonas* diversity.

MATERIALS AND METHODS

Study area: The study was conducted in the northern part of the Limpopo Province, South Africa from February to November, 2015. Nandoni dam is situated about 20 km from Thohoyandou town along the Luvuvhu River and is surrounded by Ha-Mutoti and Ha-Budeli villages in the Vhembe district. The dam serves primarily for water supply while local populations also catch fishes from the dam as a mean to provide a supplemental protein ration to their meals. Wastewater treatment plants (WWTP), where samples were collected include Muledane, Louis Trichardt, Malamulele, Waterval as well as Elim . The treated wastewater from these plants is sent directly to surrounding rivers.

Sample collection: A total of 30 fishes which weighed between 41.8 and 183.4 g were collected from Nandoni dam at different occasions. About 2 L of water samples was collected from the dam and from the sewage treatment plants at 3 different sections of the plant including the influent, the sedimentation tank and the effluent. All the samples were put in a cooler box with ice to maintain temperature of 4°C and were transported immediately to the Parasitology Laboratory at the University of Venda.

Bacterial isolation

From fish: *Aeromonas* selective agar from Oxoid (England), was used for the primary isolation of *Aeromonas* from the samples after a preliminary enrichment in peptone water. Briefly, different parts of the fish were aseptically dissected and inoculated into 9 mL peptone water in sterile bottles. The

bottles were closed and shaken thoroughly and were allowed to stand for 30 min, after which a 3-fold serial dilution was carried out in duplicates. About 50 µL Aliquots of each sample was inoculated onto *Aeromonas* selective agar and incubated at 37°C. After incubation, colony forming units were counted from each plate and recorded and one colony from each plate was taken and inoculated in a 2 mL nutrient broth and incubated for 24 h. After incubation, DNA was extracted from each sample.

From water: Following collection of water samples from different WWTP, samples were transported to the University of Venda, Parasitology Lab. Within 3 h of arrival at the laboratory *Aeromonas* was isolated using *Aeromonas* selective agar enriched with ampicillin as follows: 50 µL of each sample was transferred to agar plates and the sample was spread using the glass rod spreader.

Detection of *Aeromonas* spp. by PCR: Three different PCR protocols were used as previously described using the primers indicated[19] in Table 1. All PCR protocols were performed in a total volume of 25 µL. The reaction mixtures contained 12.5 µL dream tag, 0.6 µL of each primer (Aer.hyF, Aer.verF and reverse), 0.25 µL BSA and 5.45 µL PCR water and 5 µL DNA. PCR was performed using the following programs:

- For genus specific detection, genus specific primers A16SF and A16SR were used with an initial denaturation at 95°C for 5 min, 50 cycles of denaturation at 95°C for 30 sec, annealing at 59°C for 30 sec and extension at 72°C for 30 sec and 72°C for 7 min
- For *Aeromonas hydrophila* and *Aeromonas veronii*, initial denaturation at 98°C for 150 sec, 5 cycles of denaturation at 93°C for 30 sec, annealing 64°C for 30 sec, extension at 72°C for 30 sec and 28 cycles of denaturation at 93°C for 30 sec, annealing at 60°C for 30 sec, extension at 72°C for 45 sec and final extension at 72°C for 5 min
- For *Aeromonas trota* and *Aeromonas caviae*, the cycling conditions were as follows: initial denaturation at 95°C for 2 min, 35 cycles of denaturation at 94°C for 1 min, annealing at 68°C for 1 min, extension at 72°C for 1 min and final extension at 72°C for 3 min

The detection of *Aeromonas sobria* and *Aeromonas salmonicida* was performed as per a protocol previously described by Wang *et al.*[21]. The primers ASA1F and ASA1R were used for the detection of *A. sobria* with the following conditions: initial denaturation at 95°C for 5 min, 50 cycles of denaturation at 95°C for 30 sec, annealing at 59°C for 30 sec and extension at 72°C for 30 sec and 72°C for 7 min. The amplification parameters for *Aeromonas salmonicida* included an initial denaturation at 95°C for 30 sec, followed by 30 cycles of denaturation at 94°C for 2 min, primer annealing at 57°C for 30 sec, extension at 72°C for 1 min 30 sec. A final extension was performed at 72°C for 3 min. Five microliters of each reaction mixture were then analyzed by gel electrophoresis in 1% agarose at 100 V and the reaction products were visualized with a Bio-Rad gel doc machine after staining with 3% ethidium bromide.

RESULTS

Characteristics of samples used in the study: A total of 103 samples were collected and used in the study. Of these 90 (87.3%) were fish samples, 12 (11.7%) were wastewater samples and 1 was dam sample. All fish samples were collected from Nandoni dam while wastewater and treated wastewater were collected from sewage treatment plants including Malamulele, Muledane, Louis Trichardt, Elim and

Table 1: List of primers that were used in detecting *Aeromonas* spp. in fish samples

Primers	Sequences	Base pairs	References
A16SF	5′-GGG AGT GCC TTC GGG AAT CAG A-3′	356	Wang *et al.*[21]
A16SR	5′-TCA CCG CAA CAT TCT GAT TTG-3′		
ASA1F	5′-TAAAGGGAAATAATGACGGCG-3′	249	Khan and Cerniglia[22]
ASA1R	5'-GGCTGTAGGTATCGGTTTTCG-3′		
Aer.hyF	5'-GAAAGGTTGATG CCTAATACGTA-3'	685	Dorsch *et al.*[19]
Aer.verF	5'-GAGGAAAGGTTGGTAGCTAATAA-3'	658	
Reverse	5'-CGTGCTGGCAACAAAGGACAG-3'		
AER8	5′-CTGCTGGCTGTGACGTTACTCGCAG-3′	260	Khan and Cerniglia[22]
AER9	5′-TTCGCCACCGGTATTCCTCCAGATC-3′		
PAAS1	5′-CGTTGGATATGGCTCTTCCT-3′	243	Byers *et al.*[23]
PAAS2	5′-CTCAAAACGGCTGCGTACCA-3′		
AER1	5′-AGTTGGAAACGACTGCTAATA-3′	316	Khan and Cerniglia[22]
AER2	5′-ACGCAGCAGATATTAGCTTCAG-3′		

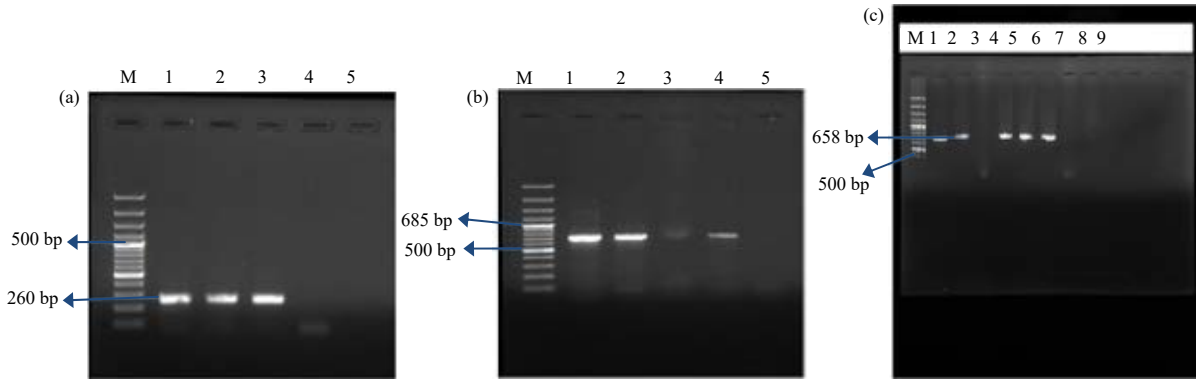

Fig. 1(a-c): Representative agarose gels of PCR amplification products for (a) *A. caviae* (260 bp) lane 1-5 are flesh, intestine, gills and intestine from fish and negative control respectively, (b) *A. hydrophila* (685 bp) lane 1-5 are flesh, intestine, gills, gills from fish and negative control respectively and (c) *A. veronii* (658 bp) lane 1-9 are influent, influent, effluent, effluent, sediment, effluent and sediment and negative control respectively from wastewater samples

Table 2: Characteristics of the samples used in the study

Variables	Frequency	Percentage
Parts		
Dam	1	1.0
Final	5	4.9
Gills	30	29.1
Intestines	30	29.1
Meat	30	29.1
Raw	5	4.9
Sediment	2	1.9
Total	103	100.0
Type of samples		
Fish	90	87.3
Wastewater	12	11.7
Water	1	1.0
Total	103	100.0
Location		
Elim hospital	2	1.9
Louis Trichardt	2	1.9
Malamulele	3	2.9
Muledane	3	2.9
Nandoni dam	91	88.3
Waterval	2	1.9
Total	103	100.0
Hormones		
Negative	13	29.5
Positive	31	70.5
Total	44	100.0

Waterval. The samples were grouped according to the section of the plant from which the original samples were collected. Of the 103 samples 44 samples were tested for the presence of estradiol (E2) and 13 (29.5%) were negative and 31(70.5%) were positive. The results are shown in Table 2.

Prevalence of *Aeromonas* spp. according to type of samples: Following PCR amplification, the correct bands were obtained for the different *Aeromonas* spp. as shown in Fig. 1 from fish samples and wastewater samples. *Aeromonas sobria* occurred in all the wastewater samples while *A. hydrophila* occurred in 75.0% of the wastewater samples. However, *A. caviae* was most prevalent in fish samples occurring in 67.8% of the samples, but the difference was not statistically significant $p = 0.358$ ($p>0.05$). *Aeromonas salmonicida* and *Aeromonas trota* were not detected in any of the samples. The results are shown in Table 3.

Prevalence of *Aeromonas* spp. in the presence of estradiol (E2): Table 4 shows the prevalence of *Aeromonas* spp. in relation to the presence of estradiol (E2). The prevalence of *A. sobria* was much higher in samples that had estradiol (E2) 90.3% compared to the samples that did not (53.8%). The difference was statistically significant with $p = 0.006$ ($p<0.006$). *Aeromonas caviae* had the prevalence of 71.0% in samples that had estradiol (E2), however the difference with samples that did not have estradiol (E2) was not significant. On the contrary, *A. hydrophila* was more common in samples that did not have the hormone although the difference was not significant.

Variation of estradiol (E2) concentration from wastewater and fish samples: Estradiol (E2) concentrations varied from 0.32-348.64 pg mL^{-1}. E2 concentrations were determined by ELISA which had been proven to be a very sensitive method. In the present study, raw samples had high concentration with the highest being 348.64 pg mL^{-1} although one effluent sample had high concentration of 279.31 pg mL^{-1} suggesting

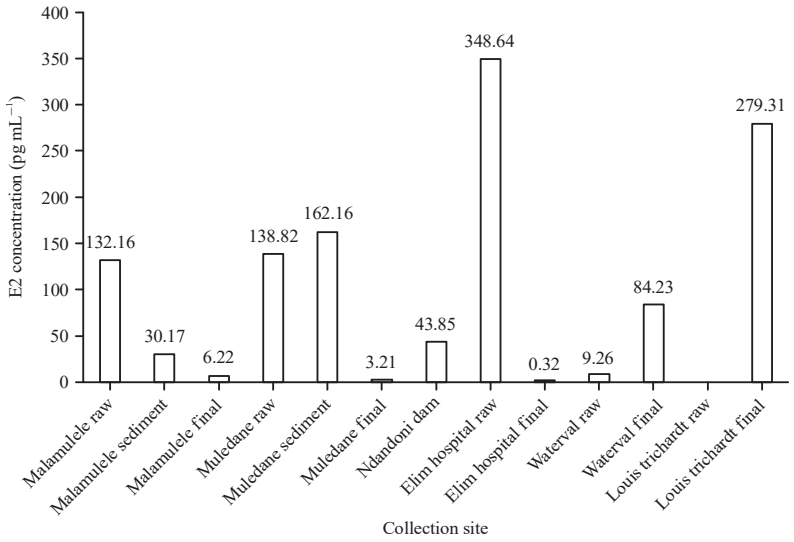

Fig. 2: A graph showing variation of concentration of estradiol (E2) from wastewater samples

Table 3: Prevalence of *Aeromonas* spp./type of samples

	Negative		*A. sobria*		*A. hydrophila*		*A. veronii*		*A. caviae*	
Variables	Number	Percentage	Number	Percentage	Number	Percentage	Number	Percentage	Number	Percentage
Fish	33	36.7	57	63.3	64	71.1	8	8.9	61	67.8
Wastewater	0	0.0	12	100.0	9	75.0	0	0.0	8	66.7
Water	0	0.0	1	100.0	0	0.0	0	0.0	0	0.0
Total	33	32.0	70	68.0	73	70.9	8	7.8	69	67.0

Table 4: Prevalence of *Aeromonas* spp. in the presence of estradiol (E2)

	Estradiol negative		Estradiol positive		Total		
Species	Number	Percentage	Number	Percentage	Number	Percentage	Statistics
A. sobria negative	6	46.2	3	9.7	9	20.5	$X^2 = 7.490$, $p = 0.006$
A. sobria positive	7	53.8	28	90.3	35	79.5	
A. hydrophila negative	2	15.4	9	29.0	11	25.0	$X^2 = 0.910$, $p = 0.340$
A. hydrophila positive	11	84.6	22	71.0	33	75.0	
A. veronii negative	12	92.3	31	100.0	43	97.7	$X^2 = 2.440$, $p = 0.118$
A. veronii positive	1	7.7	0	0.0	1	2.3	
A. caviae negative	4	30.8	9	29.0	13	29.5	$X^2 = 0.013$, $p = 0.908$
A. caviae positive	9	69.2	22	71.0	31	70.5	

that, that wastewater treatment plant was not working well. Having such large amount of hormones in the effluent may lead to their accumulation in environmental waters (streams, rivers as well as dams); which also results in aquatic organisms being affected by these compounds (Fig. 2).

The concentration of estradiol (E2) varied also according to the part of the fishes tested and ranged from 0.001-27.82 pg mL^{-1} with flesh samples having the highest concentration (Fig. 3). Further analysis showed that there is a correlation between hormone concentration and multiple infections. It was observed that the higher the hormone concentration, the higher the number of multiple infections by *Aeromonas* spp. (Fig. 4). Regression analysis also revealed that there is correlation between estradiol (E2) and multiple infection with $p = 0.0048$ ($p<0.05$) and R^2 with a value of 0.1822. This suggested that hormones do influence the members of *Aeromonas* spp. and particularly their occurrence in fish.

DISCUSSION

The present study found high concentrations of 17β-estradiol in wastewater as well as treated waste water with potential influence on the distribution of *Aeromonas* spp. in these samples. *Aeromonas hydrophila* was the most prevalent species isolated from fish and water

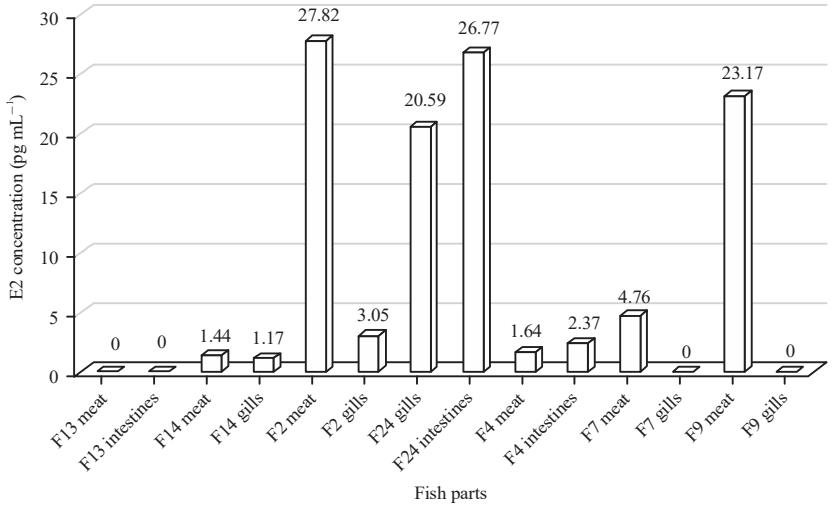

Fig. 3: A graph showing varying concentrations of estradiol (E2) from fish samples

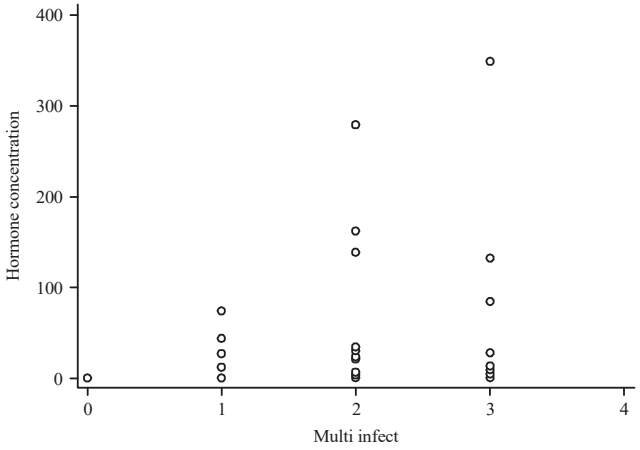

Fig. 4: Scatter graph shows correlation between hormone concentration and multiple infection
White dots on the graph represent the number of *Aeromonas* species

samples. In fact, *A. hydrophila* is the most common *Aeromonas* spp. that is also involved in human disease. Similar findings have been described by other authors such as Janda and Abbot, who also found that *A. hydrophila* was the most prevalent in fish samples and they suggested that it may be because it is known to be the most common pathogen linked to fish kills all over the world, which results in economic losses[13]. In the current study, the prevalence of *A. hydrophila* was followed by that of *A. sobria*, this is close to the results obtained by Beaz-Hidalgo *et al.*[24] and Austin and Austin[25] who showed that *A. sobria* was the commonly isolated species in diseased fish in Spain. These differences may be due to geographic difference. This study also shares the results with a study done by Hu[26] in 2012 who found *A. hydrophila* to be the most prevalent species isolated from diseased fish.

In the presence of estradiol (E2), our results showed that *A. sobria* was the most common species with (90.3%) and p-value of 0.006. This is an indication that *A. sobria* might be capable of metabolizing estradiol much better compared to the other *Aeromonas* spp. To our knowledge no studies have investigated the metabolism of this hormone by microorganism, this do not match with the occurrence of *Aeromonas* spp. in general, because the most prevalent was *A. hydrophila.* But now in the presence of estradiol (E2) the most common species is *A. sobria*. This may be due to that not all samples were tested for estradiol (E2) and many of the tested samples were positive for *A. sobria*, or it may be that *A. sobria* strives most in the presence of estradiol (E2). This was followed by *A. hydrophila*, but the difference was not significant. Most of the flesh samples from fish showed high concentration of Estradiol, followed by gills samples and then intestines samples; this suggests the possibility that hormones accumulate in the flesh of aquatic organisms. A previous study showed that *Aeromonas hydrophila* was not able to degrade estrone in a monoculture[27]. The increased growth of *Aeromonas* spp. in the present study is a proof that these organisms could be able to degrade estradiol. Further studies should investigate the metabolism of this hormone by *Aeromonas* spp.

From the different sewage treatment plants, samples from the influent (raw sewage) had higher concentration of estradiol which is not surprising considering that the influent contains lot of chemical pollutants from different sewage

drainage such as from households and institutions. The concentration of Estradiol in effluents is what is of concern because it is not expected to be high, yet from some treatment plants the effluents showed rather high concentration of Estradiol, which means that the plants were not effective in removing this kind of pollutants which might end up accumulating in environmental waters where the effluents are released. The accumulation of the hormones in the rivers will also lead to their accumulation in the bodies of aquatic organisms; affecting their health[28]. The concentration of estradiol (E2) from Nandoni dam in the current study was high with a value of 43.9 pg mL^{-1}. This was in comparison to the concentration of estradiol (E2) found in dams built on ephemeral rivers in a study done by Faul and colleagues who found the value of estradiol[29] (E2) to be 7.2 pg mL^{-1}. This may be because Nandoni dam receives large amounts of inflow from rivers that are catchments of effluents from different wastewater treatment plants and the estrogenic pollutants that are in the water accumulates in the dam over time.

Estradiol (E2) seemed to be an important factor on the diversity of *Aeromonas* spp., because the higher the estradiol (E2) concentration, the higher the number of *Aeromonas* spp. These results are in agreement with a study done by Zhang and colleagues who found that, the presence of Estrone and estradiol (E2) in soil increased soil microbial community, suggesting that microorganisms may be using hormones as nutrients for their growth[30]. This could also imply that the same could happen to microorganisms in the aquatic environment, which could lead to overflow of pathogenic microorganisms that may end up infecting aquatic organisms causing diseases.

Aeromonas salmonicida and *Aeromonas trota* were absent from the samples tested. The failure of the primer set to detect *A. salmonicida* may be related to the primer target site or that they did not grow at 37°C[21,31]. The failure to detect *Aeromonas trota* may also be related to the primer used. Or it could be that these 2 species were not available in all the samples. Of the samples that were sequenced the sequence information obtained covered approximately 600 nucleotides and allowed for identification of members of the genus *Aeromonas*. Based on the constructed phylogenetic tree using the KT371350 reference sequence, isolates showed clustering with the reference strains of respective species and were identified as *Aeromonas caviae/veronii* species and a cluster of *Aeromonas sobria*. Joseph *et al*.[32] also found that different strains of *Aeromonas* spp. that are closely related cluster together when a phylogenetic tree is constructed. *Aeromonas veronii* was found to be more prevalent in environmental samples. This is in alignment with a study done by Khor who also found that *Aeromonas veronii* was more common in environmental samples (freshwater)[6]. *Aeromonas* spp. as well as other bacterial species living in natural water sources are able to cause a wide range of diseases to both humans and animals. Therefore, it is essential to keep their levels down by implementing measures that discourage their growth. The presence of hormones and other endocrine disrupting chemicals (EDCs) in environmental niches may affect not only on the organisms living in these spaces, but also on microorganisms by enhancing their overgrowth in these environments. The development of methods for the improved elimination of these substances in the environment is of paramount importance. Further studies should include more hormones including the artificial 17 alpha ethinyl estradiol which is part of most birth control pills used by women throughout the world. Communities should be educated on the disposal of such material that may contain human body fluids to avoid the contamination of natural water sources.

CONCLUSION

In conclusion, this study demonstrated that *Aeromonas* spp. are prevalent in wastewater and in fish from the Nandoni Dam. There was a high concentration of estradiol (E2) in the sewage samples. Two STPs were able to reduce the concentration of estradiol in the wastewater while 2 others did not. This could lead to high concentrations of estradiol (E2) in environmental waters that come in contact with the partially treated wastewater. Although there was no straight association between estradiol (E2) and *Aeromonas* diversity there was a tendency of samples with high estradiol (E2) concentrations to have high numbers of *Aeromonas* spp. as well. However, further studies are needed to confirm this hypothesis.

SIGNIFICANCE STATEMENT

This study discovered the possible potential effect of estradiol (E2) on *Aeromonas* spp. which will be beneficial in serving as preliminary data for more research in order to explore such findings. These findings will help researchers to uncover the critical areas of the interaction between steroid hormones and bacterial organisms. Thus, a new theory on this type of interaction may be arrived at and further research is needed to explore these possibilities.

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REFERENCES

1. Filby, A., T. Neuparth, K.L. Thorpe, R. Owen, S. Galloway and C.R. Tyler, 2007. Health impacts of estrogens in the environment, considering complex mixture effects. Environ. Health Persp., 115: 1704-1710.
2. Naldi, A.C., P.B. Fayad, M. Prevost and S. Sauve, 2016. Analysis of steroid hormones and their conjugated forms in water and urine by on-line solid-phase extraction coupled to liquid chromatography tandem mass spectrometry. Chem. Cent. J., Vol. 10. 10.1186/s13065-016-0174-z.
3. Mohagheghian, A., R. Nabizadeh, A. Mesdghinia, N. Rastkari and A.H. Mahvi *et al*., 2014. Distribution of estrogenic steroids in municipal wastewater treatment plants in Tehran, Iran. J. Environ. Health Sci. Eng., Vol. 12. 10.1186/2052-336X-12-97.
4. Vymazal, J., T. Brezinova and M. Kozeluh, 2015. Occurrence and removal of estrogens, progesterone and testosterone in three constructed wetlands treating municipal sewage in the Czech Republic. Sci. Total Environ., 536: 625-631.
5. Huang, B., W. Sun, X. Li, J. Liu, Q. Li, R. Wang and X. Pan, 2015. Effects and bioaccumulation of 17β-estradiol and 17α-ethynylestradiol following long-term exposure in crucian carp. Ecotoxicol. Environ. Saf., 112: 169-176.
6. Khor, W.C., S.M. Puah, J.A.M.A. Tan, S.D. Puthucheary and K.H. Chua, 2015. Phenotypic and genetic diversity of *Aeromonas* species isolated from fresh water lakes in Malaysia. PLoS ONE, Vol. 10. 10.1371/journal.pone.0145933.
7. Meijide, F.J., G.R. Vazquez, Y.G. Piazza, P.A. Babay, R.F. Itria and F.L.L. Nostro, 2016. Effects of waterborne exposure to 17β-estradiol and 4-*tert*-octylphenol on early life stages of the South American cichlid fish *Cichlasoma dimerus*. Ecotoxicol. Environ. Saf., 124: 82-90.
8. Vincent, A.T., M.V. Trudel, L. Freschi, V. Nagar, C. Gagne-Thivierge, R.C. Levesque and S.J. Charette, 2016. Increasing genomic diversity and evidence of constrained lifestyle evolution due to insertion sequences in *Aeromonas salmonicida*. BMC Genom., Vol. 17. 10.1186/s12864-016-2381-3.
9. Khan, I.U., A. Loughborough and T.A. Edge, 2009. DNA-based real-time detection and quantification of aeromonads from fresh water beaches on Lake Ontario. J. Water Health, 7: 312-323.
10. Subashkumar, R., T. Thayumanavan, C. Thilagavathy, G. Vivekanandhan, K. Savithamani and P. Lakshmanaperumalsamy, 2006. Typing of haemolytic and antibiotic resistant *Aeromonas hydrophila* isolated from raw milk of coimbatore, South India. Int. J. Dairy Sci., 1: 70-83.
11. Porteen, K., R.K. Agarwal and K.N. Bhilegaonkar, 2007. Detection of *Aeromonas* sp. from chicken and fish samples by polymerase chain reaction. Am. J. Food Technol., 2: 30-37.
12. Martin-Carnahan, A. and S.W. Joseph, 2005. *Aeromonadalesord*. nov. In: Bergey's Manual® of Systematic Bacteriology, Volume 2: The Proteobacteria, Part B: The *Gammaproteobacteria*, Garrity, G., D.J. Brenner, N.R. Krieg and J.R. Staley (Eds.). 2nd Edn., Springer, Boston, MA., USA., ISBN-13: 9780387280226, pp: 556-587.
13. Janda, J.M. and S.L. Abbott, 2010. The genus *Aeromonas*: Taxonomy, pathogenicity and infection. Clin. Microbiol. Rev., 23: 35-73.
14. Kamble, S.R., S.U. Meshram and A.S. Shanware, 2013. Characterisation of *Aeromonas* species isolated from diseased fish using ERICRAPD markers. Asia-Pac. J. Mol. Biol. Biotechnol., 20: 99-106.
15. Igbinosa, I.H., E.U. Igumbor, F. Aghdasi, M. Tom and A.I. Okoh, 2012. Emerging *Aeromonas* species infections and their significance in public health. Scient. World J., Vol. 2012. 10.1100/2012/625023.
16. Harikrishnan, R. and C. Balasundaram, 2005. Modern trends in *Aeromonas hydrophila* disease management with fish. Rev. Fisher. Sci., 13: 281-320.
17. Saraceni, P.R., A. Romero, A. Figueras and B. Novoa, 2016. Establishment of infection models in zebrafish larvae (*Danio rerio*) to study the pathogenesis of *Aeromonas hydrophila*. Front. Microbiol., Vol. 7. 10.3389/fmicb.2016.01219.
18. Janda, J.M. and S.L. Abbott, 1996. Human Pathogens. In: The Genus *Aeromonas*, Austin, B., M. Altwegg, P.J. Gosling and S. Joseph (Eds.). Chapter 6, John Wiley and Sons, Chichester, UK., ISBN-13: 9780471967415, pp: 151-173.
19. Dorsch, M., N.J. Ashbolt, P.T. Cox and A.E. Goodman, 1994. Rapid identification of Aeromonas species using 16S rDNA targeted oligonucleotide primers: A molecular approach based on screening of environmental isolates. J. Applied Bacteriol., 77: 722-726.
20. Smith, K.F., V. Schmidt, G.E. Rosen and L. Amaral-Zettler, 2012. Microbial diversity and potential pathogens in ornamental fish aquarium water. PLoS ONE, Vol. 7. 10.1371/journal.pone.0039971.
21. Wang, G., C.G. Clark, C. Liu, C. Pucknell and C.K. Munro *et al*., 2003. Detection and characterization of the hemolysin genes in *Aeromonas hydrophila* and *Aeromonas sobria* by multiplex PCR. J. Clin. Microbiol., 41: 1048-1054.
22. Khan, A.A. and Cerniglia, 1997. Rapid and sensitive method for the detection of *Aeromonas caviae* and *Aeromonas trota* by polymerase chain reaction. Lett. Applied Microbiol., 24: 233-239.

23. Byers, H.K., N. Gudkovs and M.S.J. Crane, 2002. PCR-based assays for the fish pathogen *Aeromonas salmonicida*. I. Evaluation of three PCR primer sets for detection and identification. Dis. Aquat. Organisms, 49: 129-138.
24. Beaz-Hidalgo, R., A. Alperi, N. Bujan, J.L. Romalde and M.J. Figueras, 2010. Comparison of phenotypical and genetic identification of *Aeromonas* strains isolated from diseased fish. Syst. Applied Microbiol., 33: 149-153.
25. Austin, B. and D.A. Austin, 2016. Aeromonadaceae Representatives (Motile Aeromonads). In: Bacterial Fish Pathogens: Disease of Farmed and Wild Fish, Austin, B. and D.A. Austin (Eds.). Springer, Cham, Switzerland, ISBN: 978-3-319-32674-0, pp: 161-214.
26. Hu, M., N. Wang, Z.H. Pan, C.P. Lu and Y.J. Liu, 2012. Identity and virulence properties of *Aeromonas* isolates from diseased fish, healthy controls and water environment in China. Lett. Applied Microbiol., 55: 224-233.
27. Giese, C., N. Miethe and G. Schlenker, 2007. [Biodegradation of estrogens in stream water]. Berl. Munch. Tierarztl. Wochenschr., 120: 141-147, (In German).
28. Ting, Y.F., S.M. Praveena, A.Z. Aris, S.N.S. Ismail and I. Rasdi, 2017. Mathematical modeling for estrogenic activity prediction of 17β-estradiol and 17α-ethynylestradiol mixtures in wastewater treatment plants effluent. Ecotoxicology, 26: 1327-1335.
29. Faul, A.K., E. Julies and E.J. Pool, 2014. Steroid hormone concentrations and physiological toxicity of water from selected dams in Namibia. Afr. J. Aquat. Sci., 39: 189-198.
30. Zhang, X., Y. Li, B. Liu, J. Wang and C. Feng, 2014. The effects of estrone and 17β-estradiol on microbial activity and bacterial diversity in an agricultural soil: Sulfamethoxazole as a co-pollutant. Ecotoxicol. Environ. Saf., 107: 313-320.
31. Mooney, J., E. Powell, C. Clabby and R. Powell, 1995. Detection of *Aeromonas salmonicida* in wild Atlantic salmon using a specific DNA probe test. Dis. Aquat. Organisms, 21: 131-135.
32. Joseph, A.V., R.S. Sasidharan, H.P. Nair and S.G. Bhat, 2013. Occurrence of potential pathogenic *Aeromonas* species in tropical seafood, aquafarms and mangroves off Cochin coast in South India. Vet. World, 6: 300-306.