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Short communication

An overlooked source of false positives in eDNA-based biodiversity assessment and management

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ABSTRACT

Biodiversity conservation and management in urban aquatic ecosystems is crucial to human welfare, and environmental DNA (eDNA)-based methods have become popular in biodiversity assessment. Here we report a highly overlooked source of significant false positives for eDNA-based biodiversity assessment in urban aquatic ecosystems supplied with treated wastewater - eDNA pollution originating from treated wastewater represents a noteworthy source of false positives. To investigate whether eDNA pollution is specific to a certain treatment or prevalent across methods employed by wastewater treatment plants, we conducted tests on effluent treated using three different secondary processes, both before and after upgrades to tertiary treatment. We metabarcoded eDNA collected from effluent immediately after full treatment and detected diverse native and non-native, commercial and ornamental fishes (48 taxa) across all treatment processes before and after upgrades. Thus, eDNA pollution occurred irrespective of the treatment processes applied. Release of eDNA pollution into natural aquatic ecosystems could translate into false positives for eDNA-based analysis. We discuss and propose technical solutions to minimize these false positives in environmental nucleic acid-based biodiversity assessments and conservation programs.

1. Introduction

Urbanization has accelerated globally in the past century. By 2050, 64% of the developing and 86% of the developed world will be urbanized, and >2/3 (~7 billion) of the global population are projected to live in urban areas (Gu, 2019). This rapid and large-scale shift to urbanization is causing significant ecological and environmental impairment of urban ecosystems, particularly aquatic ones such as rivers, lakes, and ponds, as most major cities are established around waterbodies (Grimm et al., 2008). Disturbances associated with anthropogenic activities collectively reduce water quality and/or quantity in megacities and result in biodiversity losses in urban ecosystems at global, regional, and local scales (Fanelli et al., 2019; Xiong et al., 2017, 2023). Thus, assessment and conservation of urban aquatic ecosystems and its biodiversity have become an increasingly important issue to human welfare.

The application of molecular approaches has revolutionized biodiversity assessment and conservation in aquatic ecosystems (Miyata et al., 2022), particularly for rare species such as endangered and newly introduced non-native species (Bohmann et al., 2014; Zhan and MacIsaac, 2015; Xiong et al., 2020). Specifically, environmental DNA (eDNA), which comprises genetic material shed by organisms in a habitat, has emerged as a direct and noninvasive method for rapidly assessing biodiversity. Consequently, the application of eDNA-based approaches has become highly efficient, requiring less expertise, and providing a cost-effective tool for characterizing biodiversity by utilizing species' eDNA at spatiotemporal scales (Zhang et al., 2022; Xia et al., 2024). However, eDNA-based biodiversity characterizations are susceptible to multiple factors that can result in both false positives and false negatives (Burian et al., 2021; Rishan et al., 2023). Fortunately,

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many of these factors are well known and can be technically managed (Burian et al., 2021; Diana et al., 2021). However, recent studies raised concerns about more potential sources of errors in eDNA-based biodiversity surveys, such as false positives derived from eDNA pollution originating from treated wastewater (Jo et al., 2017; Miyata et al., 2021; Inoue et al., 2023). Such eDNA pollution released into natural waterbodies can result in false positives in eDNA-based biodiversity assessments (Inoue et al., 2023), and these false positives may be a more severe problem for studies of urban aquatic ecosystems because unconventional water inputs - such as effluent discharges from wastewater treatment plants (WTPs) - are a common and sometimes major or even the only source (in some semi-arid and arid regions) to aquatic ecosystems in urban areas (Pernet-Coudrier et al., 2012).

There is no doubt that water derived from treated effluent can help address regional water scarcity and contribute to restoration of urban aquatic ecosystems, thereby improving urban sustainability (Schwabe et al., 2020). However, discharges of treated effluent from WTPs commonly cause negative ecological effects on receiving waterbodies (Thanigaivel et al., 2024; Xiong et al., 2023). In order to minimize ecological disturbances from treated effluent, many megacities worldwide are embracing advanced treatment processes in WTPs, such as integrating tertiary treatment with secondary treatment or implementing treatment processes tailored to specific water quality goals. When compared with conventional treatment such as the activated sludge system, advanced processes utilizing UV, NaClO, and O_3 significantly enhance water treatment efficiency and, more importantly, the quality of discharged effluent (Ma et al., 2021). Since eDNA is sensitive to the aforementioned oxidation treatments (McCartin et al., 2022; Snyder et al., 2023), these technical treatment upgrades have a high potential to degrade eDNA in treated effluent. Thus, we hypothesize that the effluent treated using advanced technical upgrades will be far less susceptible to generation of false positives in eDNA-based biodiversity assessments than traditional treatment such as the activated sludge system.

To test this hypothesis, we sampled effluent immediately after full treatment but before release into natural aquatic ecosystems in four major WTPs employing different treatment processes (Table 1) in Beijing, China to assess eDNA-based fish biodiversity. Specifically, we sought to test whether false positives derived from eDNA contaminations discharged by WTPs were common across various treatment processes, as well as before and after plant upgrades to advanced treatment using oxidating reagents. Subsequently, we compared the detected species list from this study with historically documented fish species in Beijing to evaluate the eDNA pollution originating from WTPs on biodiversity assessments. Following Darling et al. (2021), we define false positives as the positive detection of species not naturally present at tested sites but whose eDNA was introduced to recipient ecosystems with treated WTP effluent.

2. Materials and methods

2.1. Sampling

The four WTPs (Fig. 1) chosen in this study are large-scale municipal plants that treat a mixture of domestic, industrial, and runoff wastewater using conventional secondary treatment, including the conventional activated sludge system (CAS, WTP1), the anaerobic-anoxic-aerobic method (A/A/O, WTP2 and WTP3), and the membrane bioreactor (MBR, WTP4). All four WTPs were upgraded in April–May 2017 with the addition of tertiary treatment based on advanced oxidation. These four WTPs treat >67% of the total wastewater in Beijing, a megacity with >21 million residents (Table S1). After full treatment, effluents are released into rivers of the Haihe River Basin, the largest watershed in Northern China (Fig. 1).

We conducted sampling before and after the treatment upgrades over four consecutive years (Table S1). As both eDNA and eRNA sampling was completed *in situ* from treated effluents, no samples were taken from natural waters in recipient rivers. In all cases, 2 L of treated effluent was collected for eDNA and eRNA enrichment, while 2 L sterile pure water (Milli-Q®, MA, USA) served as a negative control. Both the sampled effluent and negative control for each sampling effort were simultaneously filtered through membranes (0.45 μm, mixed cellulose esters, Merck-Millipore) to collect nucleic acid. After filtration, each membrane was placed in an individually-labelled, sterile 50 mL tube and stored at −20°C until eDNA extraction. All membranes for eRNA were immediately preserved in RLT buffer (Qiagen) with 1% β-mercaptoethanol and stored in liquid nitrogen during the shipment to the laboratory. All eRNA samples were processed and PCR-amplified within 8h after sampling. We collected a total of 19 eDNA samples from these four WTPs across years (Table S1). Since eRNA-based methods were recognized for their effectiveness in biodiversity assessment only after technical upgrades in these wastewater treatment plants, we collected only nine eRNA samples after treatment upgrades in all four WTPs.

2.2. DNA/RNA extraction, PCR amplification, and sequencing

All processes of eDNA/eRNA metabarcoding, including nucleic acid extraction and PCR, were completed in our specific eDNA laboratory, with all equipment, laboratory consumables, and surfaces cleaned/sterilized before use for each sample. Total eDNA was extracted from preserved filters (including negative controls and water samples) using the DNeasy PowerWater Kit (Qiagen). Total RNA (including all negative controls) was extracted using the RNeasy Mini Kit (Qiagen) with minor modifications according to Littlefair et al. (2022). To prepare eRNA samples for PCR, the DNA-free DNA Removal Kit (ThermoFisher Scientific) and the PrimeScript II 1st Strand cDNA Synthesis Kit (TaKaRa) were used to avoid DNA contamination and implement cDNA synthesis, respectively.

For PCR amplification, the mitochondrial 12S rRNA gene fragment was amplified using the fish metabarcoding primer pair - fish_F1 (5′-TCGTGCCAGCCACCGCGGTTA-3′) and fish_R1.1 (5′-ATAGTGGGG-TATCTAATCCCAG-3'; Miya et al., 2015; Yang et al., 2023). This primer pair was modified to reduce nonspecific amplification and improve effectiveness of eDNA metabarcoding for fish (Yang et al., 2023). PCRs were performed in eight replicates to avoid possible biased amplifications for rare species (Zhan et al., 2013, 2014). For each sample, we used uniquely-tagged primers to distinguish individual samples. Each PCR

Table 1
The number of recovered fish taxa based on environmental DNA (eDNA)-metabarcoding for treated effluent before and after treatment upgrades in four wastewater treatment plants (WTPs). See Supplementary Table S1 for more information on secondary treatment and tertiary treatment after upgrade for each WTP.

	WTP1		WTP2		WTP3	WTP4		Total	
	before	after	before	after	after	before	after	before	after
Native freshwater commercial	13	15	15	13	14	12	14	17	17
Non-native freshwater commercial	5	6	5	3	5	5	7	5	8
Native freshwater ornamental	4	4	4	4	4	4	5	4	5
Non-native freshwater ornamental	2	2	0	1	2	1	2	2	4
Marine commercial	0	1	0	0	0	0	11	0	12
Total	24	28	24	21	25	22	40	28	48

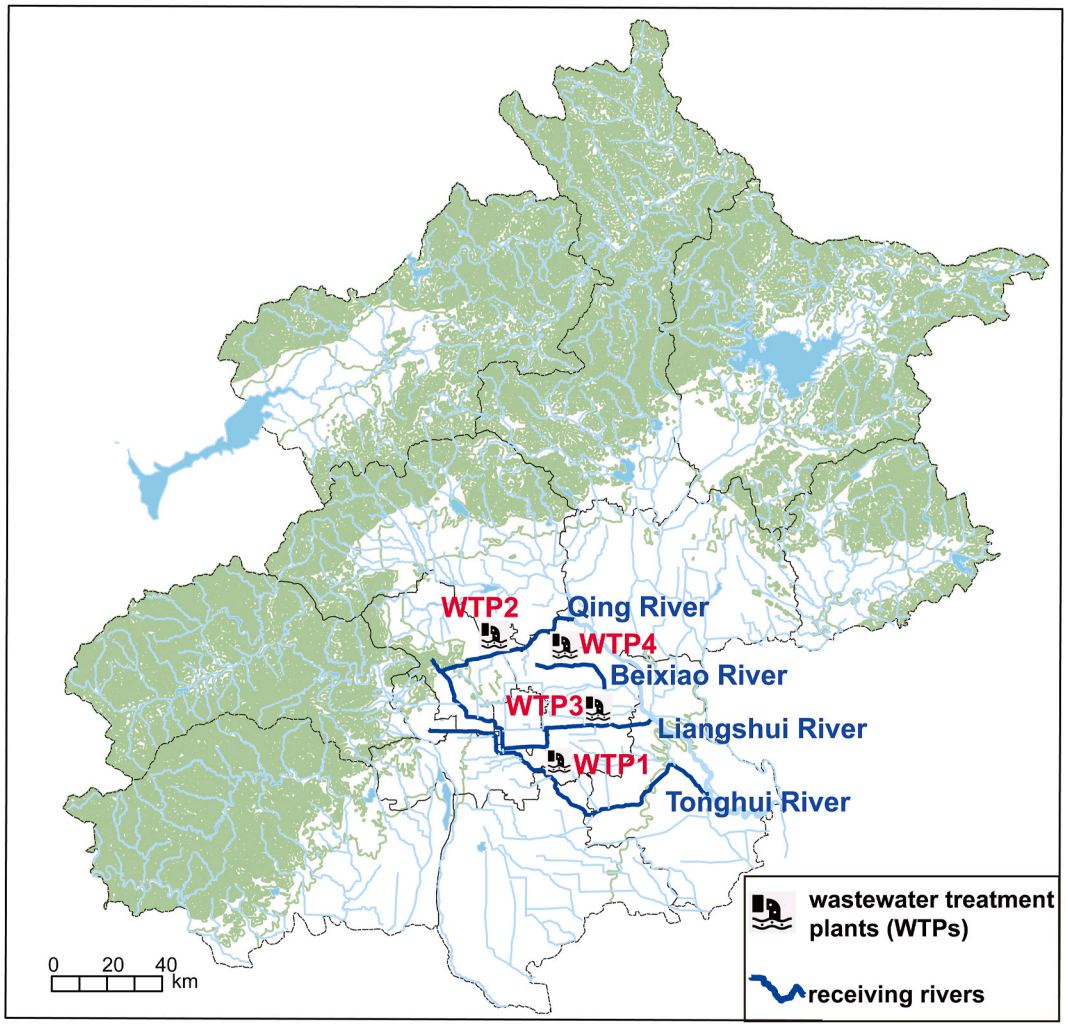

Fig. 1. The location of the four wastewater treatment plants (WTPs) chosen in Beijing, China. These four large-scale municipal plants treat >67% of the total wastewater (including domestic, industrial, runoff wastewater) in Beijing, a megacity with the population of >21 million. After full treatment, effluents are released into rivers of the Haihe River Basin (only waterbodies in Beijing are shown here).

was prepared with a total volume of 25 μL, consisting in 1 × PCR buffer, 0.2 mM dNTPs, 2U *Taq* DNA polymerase (Takara, Japan), 0.2 μM each primer, and 2 μL eDNA or cDNA. PCR conditions included an initial 10min at 95 °C, then 40 cycles of 30s at 95 °C, 30s at 58 °C, and 90s at 72 °C, and a final 10min elongation at 72 °C. PCR replicates of each sample were pooled and purified, and then one sequencing library was constructed by pooling equimolar purified PCR products derived from each sample. The library was sequenced *via* 2 × 150bp paired-end sequencing on a NovaSeq Illumina platform using NovaSeq Xp 4-Lane Kit (Illumina, San Diego, CA, USA).

2.3. Bioinformatic analysis

All sequence reads were demultiplexed, and the adaptor and primer sequences were trimmed from the paired-end sequences with one mismatch allowed, then merged by FLASH v1.2.5 with at least 50bp of overlap in the bioinformatic platform (http://mem.rcees.ac.cn:8080/; Feng et al., 2017). We used Btrim to remove any sequences with degenerate bases and lengths <150bp (Xiong and Zhan, 2018). All cleaned sequences were clustered into Zero-radius Operational Taxonomic Units (ZOTUs) using UNOISE3 with default parameters. The representative sequence of each ZOTU was annotated against GenBank nucleotide database using BLASTN program embedded in SEED 2 platform. For fish taxonomic annotation, we used Zhang et al.'s (2022) methods.

3. Results

All eDNA filters derived from negative controls and all eRNA samples returned no PCR amplification, and randomly chosen samples for sequencing did not detect any fish taxa after bioinformatic analyses. After sequencing and strict quality filtering, we obtained a total of 924,941 clean sequences from 19 effluent eDNA samples, with sequence number varying from 17,798 to 91,618 per sample. In total, we obtained 119 fish ZOTUs.

After taxonomic annotation, we detected a large number of diverse fish taxa in all 19 samples collected from four WTPs (Table 1). Taxa-level rarefaction curves indicated a sufficient capture of fish biodiversity from each sampling effort (Fig. S1). In total, we detected 48 taxa encompassing 10 freshwater and four marine orders (Table 1; Fig. 2), among which 35, nine, and four taxa were identified to the species, genus, and family levels, respectively (Fig. 2). Consistently across all tests, we only recovered freshwater and marine commercial fish (39 taxa) and freshwater ornamental pets (9 taxa; Table 1). For freshwater fishes, 12 taxa were non-native to Beijing, including four taxa (all commercial species) reported previously and eight (four commercial species and four ornamental pets) novel taxa (Fig. 2). All commercial taxa detected here are widely sold and consumed and all ornamental species are popular pets in

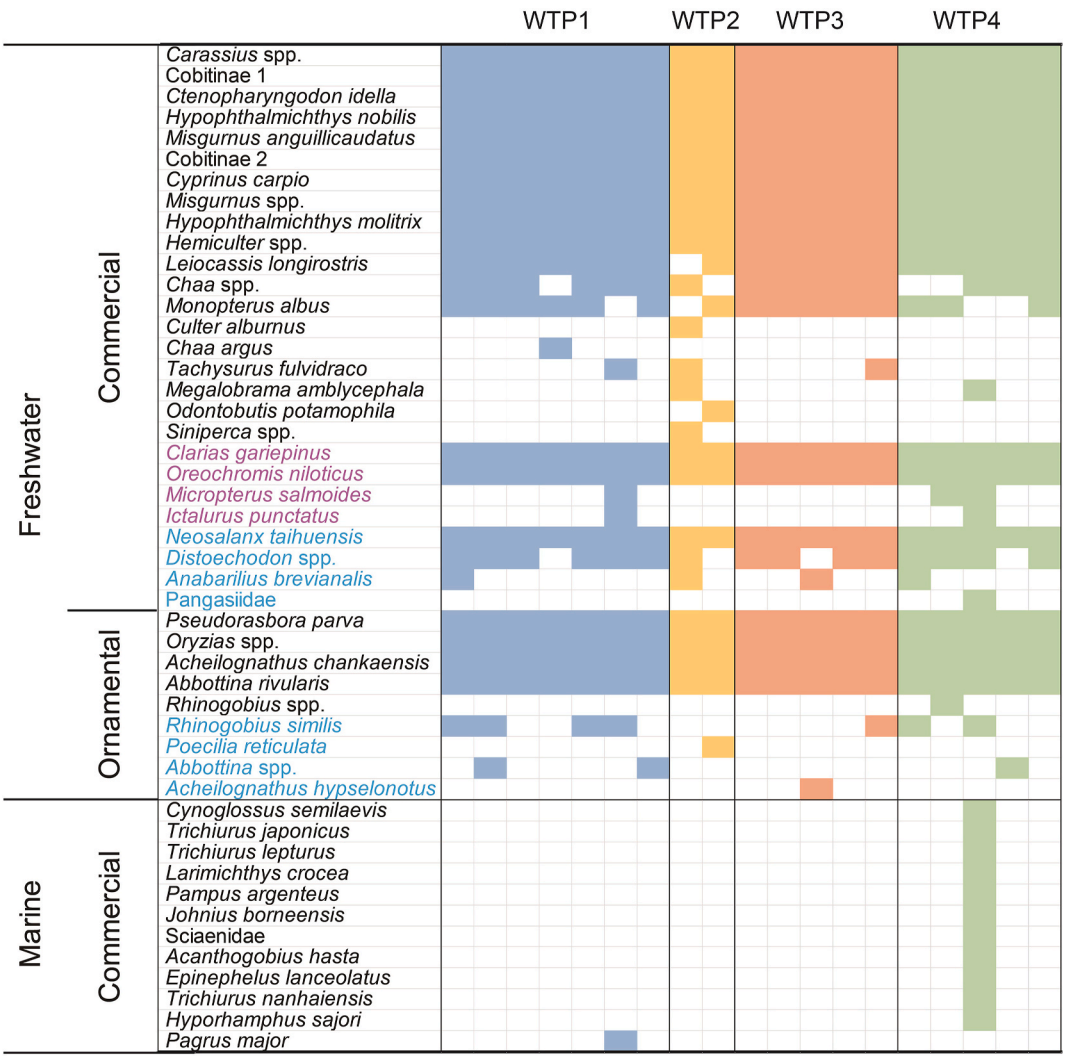

Fig. 2. Fish taxa detected by environmental DNA (eDNA)-metabarcoding in the four wastewater treatment plants (WTPs). We conducted 5, 7, 2, and 5 tests (color blocks) in different months (see details in Table S1) for WTP 1–4 located in Liangshui, Qing, Tonghui and Beixiao rivers, respectively. Positive detections of fish taxa in each WTP are color-coded, while blank spaces indicate negative detections. Taxa names of non-native freshwater fishes documented previously are highlighted in purple, while non-native taxa never previously recorded in the region are in blue.

Beijing.

Notably, eDNA contamination in effluent was not specific to a treatment process, as a variety of fish species were consistently detected across CAS, A/A/O, and MBR treatment processes, regardless of whether sampling occurred before or after treatment upgrades (Table 1). All marine species were detected only once, whereas eight freshwater species were unique to a single sampling effort (Fig. 2). Additionally, we observed temporal variation in the number of taxa identified within the same WTPs. For instance, marine commercial species yielded negative results in 17 of 19 tests in WTP4, but suddenly 11 taxa were identified in a single test (Fig. 2). Such large fluctuation is likely associated with temporary collection and treatment of wastewater from industry such as fish processing plants and/or the stochasticity associated with eDNA sampling of rare species.

4. Discussion

The input of eDNA pollution derived from treated wastewater, together with its relatively slow degradation in natural waterbodies (~several weeks; Harrison et al., 2019), can lead to a high level of false positives in eDNA-based biodiversity assessment (Inoue et al., 2023). Such false positives can be severe in semi-arid and arid regions such as Beijing in this study, as treated effluents are the major or only source of water supply to lotic and lentic ecosystems. In this study, we sampled eDNA directly from fully treated wastewater by WTPs and demonstrated that residual eDNA in treated effluent represents a significant source of false positives for eDNA-based biodiversity assessment. Since eDNA-based biodiversity assessments are now widely used, while the problem of eDNA pollution has less been well documented and systematically addressed, a pressing need exists to determine the frequency and consequences of false positive occurrences to biodiversity assessment and conservation programs.

Surprisingly, eDNA pollution was consistently detected across three advanced and commonly-used treatment processes (CAS, A/A/O, MBR; Table 1). This finding builds on a recent study on contamination limitations with the CAS process (Inoue et al., 2023) and provides clear evidence that the risk of false positives is not specific to a particular treatment process but rather is common across mainstream techniques employed in WTPs. More importantly, we found that the upgrades to the third stage of treatment based on advanced oxidation did not reduce risks of the eDNA contamination in treated effluent, as we still observed a wide range of diverse fish taxa after the upgrade (Table 1). While previous studies have demonstrated that oxidation can significantly accelerate eDNA degradation (Snyder et al., 2023), degradation rate

varies with the nature of the eDNA collected (e.g., intracellular, intra-organellar, dissolved, or particle-adsorbed eDNA) (Mauvisseau et al., 2022). Our results clearly show that the use of oxidation-based processes - such as NaClO and O_3 and UV light - in the third stage of treatment does not thoroughly degrade eDNA in the treated effluent, thus rejecting our proposed hypothesis in this study.

After treated wastewater is released into natural ecosystems - where biodiversity assessment is typically conducted - residual eDNA associated with anthropogenic activities can be detected as a false presence in recipient waterbodies. Unfortunately, false positives are not easy to detect in regular eDNA-based biodiversity assessments in natural ecosystems because it is impossible to differentiate true eDNA signals from those associated with anthropogenic activities, such as those released from WTPs. However, the presence of eDNA of marine species in effluent of a WTP far from the ocean, provides a strong indication of false positives in freshwater ecosystems (and vice versa). We consistently detected marine commercial and ornamental species in freshwater ecosystems including rivers in Beijing and beyond in our eDNA-based surveillance programs, highlighting the common occurrence of false positives in urban aquatic ecosystems caused by residual eDNA derived from anthropogenic activities. Failure to detect marine taxa does not mean contamination of eDNA derived from anthropogenic activities has not occurred, as marine taxa were detected in only two out of 19 sampling efforts in this study (Fig. 2). Marine taxa are easily ruled out as errors after they are detected from freshwater waterbodies. However, 28 (24 native and four non-native) freshwater taxa detected from WTP effluents were previously documented in Beijing rivers, and many of these species were identified across all 19 of our tests (Fig. 2). When compared with historically-documented species, eDNA detection of never-recorded, non-native species can result in further comprehensive surveillance and validation. For example, for non-native fishes never recorded in Beijing, several invasive taxa, such as the Yangtze icefish *Neosalanx taihuensis*, have extensive histories of invasions and economic damages in China and beyond (Kang et al., 2013). As eDNA-based methods can successfully detect presence of invaders at the earliest stage of invasions when their abundance is very low (Wang et al., 2023), false positive eDNA signals, particularly for highly invasive species, can drive researchers and managers to conduct deep, follow-up investigations. Indeed, the previously-recorded taxa in an area may also cause false positives for sites where they have never occurred in the wild, but rather represent eDNA pollution associated with anthropogenic activities. This may be severe for species with geographically restricted distributions in a given region. Thus, we call for caution in data interpretation for eDNA-based biodiversity assessment and conservation programs for ecosystems subject to intense anthropogenic disturbance.

We propose that false positive errors derived from unconventional water inputs may be common in all ecosystems influenced by anthropogenic activities, such as coastal zones. Urban and coastal systems may be most vulnerable to this problem since they are most prone to humans intentionally or unwittingly introducing eDNA. Technical solutions to this issue include well-designed sampling plans and the use of eRNA. As the residual eDNA from anthropogenic activities typically flows downstream, sampling upstream sites distant from WTPs or other point sources of pollution derived from anthropogenic activities may help rule out false positives. Meanwhile, the study of eDNA degradation with distance in a given waterbody with local hydrological features can help establish possible sampling sites downstream (Balasingham et al., 2017; Jo and Yamanaka, 2022). Also, the use of eDNA biodiversity data derived from WTPs samples may provide background information and assist in identifying false presence of species during biodiversity assessments. In addition to these measures, employing eRNA instead of eDNA could potentially reduce the occurrence of false positives, as eRNA is typically less stable than eDNA in environmental conditions. Indeed, Inoue et al. (2023) found less eRNA signals than eDNA in effluent treated by the conventional activated sludge system. Similarly, our tests yielded no eRNA-based PCR amplifications of valid taxa after treatment process upgrades. These results also highlight the potential utility of eRNA-based methods in reducing false positives in biodiversity surveys versus eDNA-based methods, which would be especially meaningful in anthropogenically-impacted ecosystems.

5. Conclusion

Our study affirms that the treatment methods employed for wastewater inadequately degrade eDNA in the treated effluent. Despite implementing technical enhancements to tertiary treatment, a substantial number of fish taxa were still detected in the treated effluent. Consequently, eDNA pollution persists regardless of the treatment processes employed by wastewater treatment plants. The residual eDNA discharged into natural water bodies can result in false positives in eDNA-based biodiversity assessments, highlighting an often overlooked yet significant source of errors. Such erroneous positives stemming from atypical water inputs may be prevalent in ecosystems influenced by human activities, such as urban aquatic ecosystems and coastal zones. Addressing this error issue requires well-designed and structured sampling protocols, along with the adoption of eRNA as a potential technical solution, and robust data interpretation practices in sustainable biodiversity management programs.

CRediT authorship contribution statement

Wei Xiong: Writing – review & editing, Visualization, Validation, Software, Resources, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Hugh J. MacIsaac:** Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Investigation, Funding acquisition. **Aibin Zhan:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

All sequencing data in this paper will be permanently archived in NCBI GenBank after the paper is accepted for publication.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jenvman.2024.120949.

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