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Wastewater treatment effectively eliminates exogenous eDNA, preventing downstream false-positive detections

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Abstract – Environmental DNA (eDNA) is widely applied to monitor aquatic biodiversity, but false-positive detections may arise when DNA from consumed or processed organisms is discharged to waterways through domestic wastewater. We experimentally tested whether such wastewater-derived eDNA persists through municipal treatment and contributes to downstream false-positive detections. Controlled spikes of homogenized tissue from six target taxa (two marine fishes and four freshwater fish/amphibians) were introduced into the influent of four wastewater treatment plants (WWTPs) in Flanders, Belgium. Water samples were collected at three points: influent, treated effluent, and the receiving river. Sampling was performed before and up to 24 h after spiking, and eDNA concentrations of each of the six target taxa were quantified using droplet digital PCR. All species showed post-spike increases in influent eDNA concentrations, although individual taxa were not detected in every influent sample, particularly at the largest facilities where hydraulic dilution reduced short-term detectability. In contrast, effluent and downstream concentrations remained zero across all WWTPs, indicating that no spiked eDNA persisted beyond the treatment process. One WWTP located near a marine harbor showed background detection of marine fish species even before spiking, likely originating from seafood processing or domestic discharge. However, these signals were also fully removed during treatment. Overall, modern secondary treatment effectively eliminates exogenous eDNA, preventing its release into receiving waters. While systems with incomplete sewer connectivity or combined sewer overflows may still discharge untreated domestic wastewater, under standard operating conditions riverine eDNA detections can be interpreted as reflecting local biota rather than wastewater contamination.

Keywords: Droplet digital PCR (ddPCR) / DNA removal efficiency / combined sewer overflow / environmental monitoring reliability / eDNA transport dynamics

1 Introduction

Environmental DNA (eDNA)-based species detection has revolutionized aquatic biodiversity monitoring by enabling the detection of organisms through genetic material emitted into the environment (*e.g.*, released via mucus, scales, feces, gametes, or decaying tissue; Sassoubre *et al.*, 2016) (Thomsen and Willerslev, 2015; Deiner *et al.*, 2017). This approach offers a non-invasive, highly sensitive alternative to traditional monitoring techniques, particularly for assessing fish and amphibian communities across diverse aquatic ecosystems (Hånfling *et al.*, 2016; Valentini *et al.*, 2016). Specifically in lowland rivers and ponds, recent applications have demonstrated the robustness of eDNA for detecting both native and invasive fish and amphibian species under a range of environmental conditions (Brys *et al.*, 2021; Everts *et al.*, 2021; Van Driessche *et al.*, 2022, 2024a).

In lotic environments, eDNA at a given downstream location can originate from multiple upstream inputs, posing challenges for spatially attributing species presence and distinguishing local from transported signals (Jane *et al.*, 2015; Shogren *et al.*, 2017; Van Driessche *et al.*, 2022, 2023). Beyond presence of upstream populations contributing to the eDNA particle pool, eDNA signals may also be supplemented or distorted by a variety of non-local anthropogenic or biological vectors, including trophic transfer, faunal-mediated dispersal, fisheries activities, shipping or ballast-water

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movement, and broader human-mediated transport processes (Ragot and Villemur, 2022). While the majority of locally detected eDNA originates from resident specimens in the upstream vicinity, hydrological transport and retention processes can carry genetic material over variable distances depending on flow dynamics, substrate type, and degradation rate (Deiner and Altermatt, 2014; Van Driessche *et al.*, 2024a). In addition to these processes, domestic and industrial wastewater constitute consistent and high-volume inputs of exogenous eDNA entering river systems, introducing DNA from consumed species or processed biological materials and thereby creating potential false-positive detections unrelated to the local biota (Inoue *et al.*, 2023; Clare *et al.*, 2022). Recent studies have identified such consumption-derived DNA in wastewater as a credible and underrecognized source of non-local eDNA, capable of persisting through treatment processes and entering receiving water bodies under certain conditions (Xiong *et al.*, 2024b; Inoue *et al.*, 2023). This form of DNA, originating from ingested and excreted tissue, has been shown to contribute to spurious detections in both marine and freshwater systems, especially when using highly sensitive analytical techniques such as qPCR or droplet digital PCR (ddPCR) (Clare *et al.*, 2022; Mauvisseau *et al.*, 2021). These approaches, and particularly multi-marker assays, can further increase detection rates and lower quantification thresholds (Brys *et al.*, 2023), making the distinction between true ecological signals and trace anthropogenic inputs even more critical. This challenge is compounded by the fact that eDNA in wastewater exists in multiple physical states (*e.g.*, dissolved, particle-bound), each with distinct degradation rates influenced by temperature, microbial activity, and water chemistry (Mauvisseau *et al.*, 2021; Barnes *et al.*, 2014). Consequently, interpreting eDNA patterns in urbanized catchments remains complex, as the variable persistence of these different states makes it difficult to distinguish whether detections of common species reflect resident populations or "consumption noise" from domestic discharge. To address this uncertainty, experimental designs must utilize non-local or marine taxa as proxies to explicitly isolate and track the fate of exogenous genetic material across the wastewater-river interface.

Across much of Western Europe, wastewater is treated in wastewater treatment plants (WWTPs) prior to discharge. However, the efficiency of these facilities in removing non-local, perhaps consumption-derived eDNA before it enters natural water bodies remains poorly understood. These facilities receive domestic and industrial wastewater that may carry eDNA from dietary sources or imported species, which may remain detectable if not fully removed during treatment. Modern WWTPs in industrialized regions typically employ a combination of mechanical, biological, and chemical filtration steps designed to remove organic pollutants and microbial loads (Qasim, 2017; Karia and Christian, 2003). Despite these advanced treatments, the persistence of intra- and extracellular eDNA through WWTPs and its potential to confound eDNA-based assessments remains poorly quantified (Nielsen *et al.*, 2007; Caldwell *et al.*, 2021). Although the limited number of studies assessing eDNA in treated effluents has reported variable outcomes, most detections to date appear to be low-level and context-dependent (Inoue *et al.*, 2023; Xiong *et al.*, 2024a, 2024b). Overall, the scarce available evidence suggests that while wastewater-derived eDNA is readily detected in influents, its persistence into effluents is inconsistent and likely influenced by differences in treatment configuration, operational conditions, and analytical sensitivity. Moreover, as wastewater treatment is increasingly regulated to meet standardized performance thresholds in many regions, understanding how effectively such systems prevent the release of non-local eDNA becomes essential, thereby motivating controlled empirical assessments using WWTPs that comply with these standards.

In this study, we aimed to experimentally determine whether consumption-derived eDNA introduced via domestic wastewater can persist through standard WWTP processes and remain detectable in treated downstream water that is discharged in rivers. We conducted a controlled spike experiment at four WWTPs in Flanders, Belgium, encompassing large (Ghent, Ostend) and small (Nevele, Zomergem) facilities with varying treatment capacities but using the same procedure to remove organic pollutants and microbial loads. A predefined mixture of species, comprising commonly consumed flatfish (*Solea solea* and *Pleuronectes platessa*), a native but rare freshwater fish species (*Misgurnus fossilis*), and three non-native species even more unlikely to occur in the environment (*i.e.*, *Misgurnus bipartitus, Xenopus laevis, and Lithobates catesbeianus*), was introduced into the influent stream of each facility. Water samples for eDNA analyses were subsequently taken before and after spiking at three points: the influent, the treated effluent, and the receiving river 100 meters downstream of the WWTP effluent. We then used ddPCR, a highly sensitive molecular technique suitable for quantifying species-specific trace DNA levels (Doi *et al.*, 2015; Hindson *et al.*, 2011). We hypothesized the WWTP treatment chain effectively removes consumption-derived eDNA from wastewater. By explicitly evaluating the detectability and spatial persistence of experimentally introduced and highly concentrated target DNA, this work offers new empirical insights into the accuracy and reliability of eDNA-based monitoring in riverine and lowland aquatic ecosystems. It also offers practical insights for interpreting eDNA data in human-impacted catchments, with potential relevance for eDNA surveillance protocols, water quality assessments, and environmental regulation.

2 Materials and methods

2.1 Experimental set-up

This study was performed on the 9th and 10th of September, 2024, at four WWTPs located in Flanders, Belgium: Ostend, Ghent, Nevele, and Zomergem (Fig. 1). Although the filtering procedure steps are the same, the selected WWTPs differ markedly in terms of capacity and population equivalents served. Ostend and Ghent represent large facilities, treating 50 099 m^3/day and 78 720 m^3/day during the experiment, respectively, corresponding to 198 000 and 207 000 population equivalents. The two smaller facilities at Nevele and Zomergem, on the other hand, treat 4351 m^3/day and 1877 m^3/day, with respective population equivalents of 9000 and 5400. At each WWTP, eDNA water samples were collected from three site-specific locations: (i) the influent (*i.e.*, incoming untreated wastewater), (ii) the effluent (*i.e.*, treated water discharged by the facility), and (iii) a site in the river 100 m downstream of the WWTP effluent (Fig. 1).

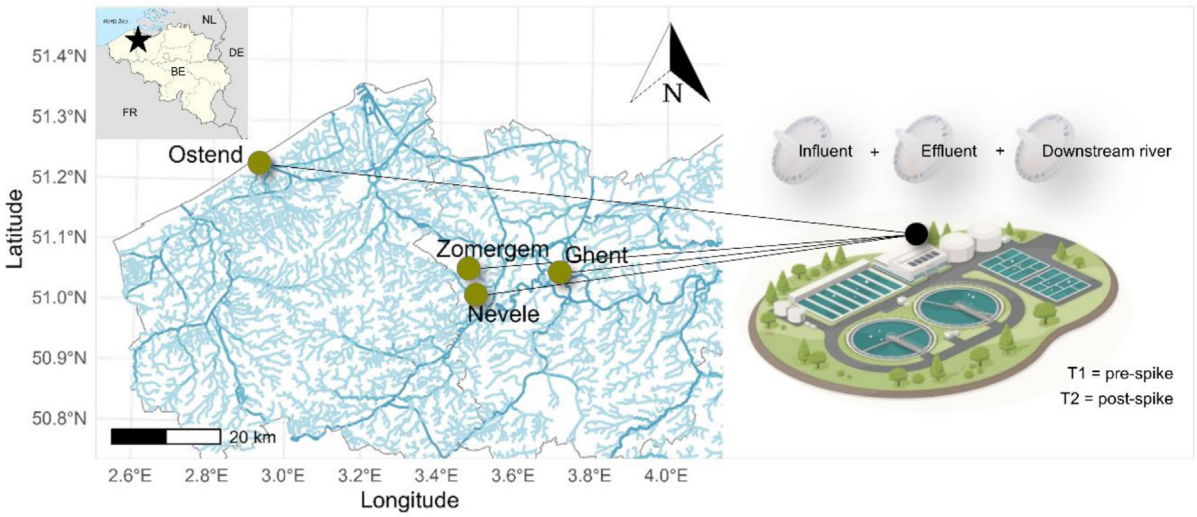

Fig. 1. Overview of the wastewater treatment plant (WWTP) experiment. eDNA samples were collected at four WWTPs in Belgium (Ostend, Ghent, Zomergem, and Nevele) at three sampling locations per WWTP: the influent (untreated wastewater), the effluent (treated water leaving the WWTP), and the receiving river 100 m downstream. A predefined DNA spike was introduced at the influent of each WWTP, with samples collected before (T1) and after (T2) the spiking event to assess the persistence and detectability of consumption-derived fish DNA following wastewater treatment. The map shows the location of the four WWTPs within Flanders, with major waterways indicated in blue. The schematic on the right illustrates the sampling strategy within the WWTP system. Base illustration of the wastewater treatment plant adapted from NoBrokerHood (2021).

At the onset of the experiment, eDNA samples were taken at each of these three site-specific locations at every WWTP. For this eDNA-sampling, water was collected from the influent and the effluent using the facilities' automated collectors, which provide a standardized 24-h composite sample representing the full daily wastewater cycle. For the sampling in the river downstream of the site, water was collected cross-sectionally to the river using a 0.5-L sterile sampling bag attached to a telescopic sampling pole. To capture the expected passage of the spiked material through the treatment system, 15–20 subsamples were collected in the river downstream of the site at 12 h and again at 24 h post-spiking, corresponding to the typical hydraulic retention time (HRT) of medium-scale activated-sludge WWTPs, which generally ranges from ~8 to 24 h depending on plant size and flow conditions (Qasim, 2017; Aquafin, pers. comm.). All subsamples were combined and homogenized to form a pooled water sample for each location. From each sample, 2 L of water was then filtered through an enclosed disk filter containing an integrated 5 μm glass fiber prefilter and a 0.8 μm PES membrane (Nature-Metrics, Surrey, England). At the end of each filtration, the remaining water inside the capsule was expelled by pushing air through the filter capsule until it dried completely. The filters were then capped at both ends and stored at −21°C until further laboratory processing.

Next, a controlled eDNA "spike" was introduced into the influent stream of each WWTP. This spike (Fig. 2) consisted of a homogenized tissue mixture comprising six species: two commonly consumed flatfish species (*Solea solea* and *Pleuronectes platessa*) and four non-consumed species, including the native *Misgurnus fossilis* and the non-native invasive *Misgurnus bipartitus, Xenopus laevis*, and *Lithobates catesbeianus*. Those non-native taxa were deliberately chosen because they do not occur naturally in the system, thereby enabling clear discrimination between experimentally introduced DNA and any background eDNA originating from genuine wastewater inputs. In contrast, the two flatfish species were included because they represent realistic, consumption-derived taxa that may occur in domestic wastewater, thereby reflecting plausible real-world sources of exogenous eDNA entering WWTPs. The flatfish specimens were sourced from a local fish market, while *M. fossilis* was obtained from the conservation breeding facility at the INBO Research Center for Aquatic Fauna (Linkebeek). Specifically, these *M. fossilis* specimens consisted of naturally deceased individuals that originated from routine cultivation and harvesting operations in the breeding facility's rearing ponds (Van Wichelen *et al.*, 2023). The invasive *M. bipartitus*, *X. laevis*, and *L. catesbeianus* were captured specimens retrieved through an invasive species management program. No animals were killed for the specific purpose of this experiment but obtained as already deceased individuals. All specimens were blended to produce a uniform mixture, which was then equally divided into four equal portions, one for each WWTP. Because this spike consisted of raw homogenized tissue rather than digested material, it contained far higher DNA quantities than would typically be present in domestic wastewater, where consumption-derived eDNA is strongly degraded and diluted. This intentional use of a high-yield source material was designed to represent a conservative, worst-case scenario for testing removal efficiency. Once introduced into the influent stream, however, the spike was immediately dispersed into very high flow volumes, meaning that the effective concentration entering the treatment system was strongly reduced. Given the high flow rates and turbulent mixing at influent inlets, particularly in large facilities, the spike likely behaved as a

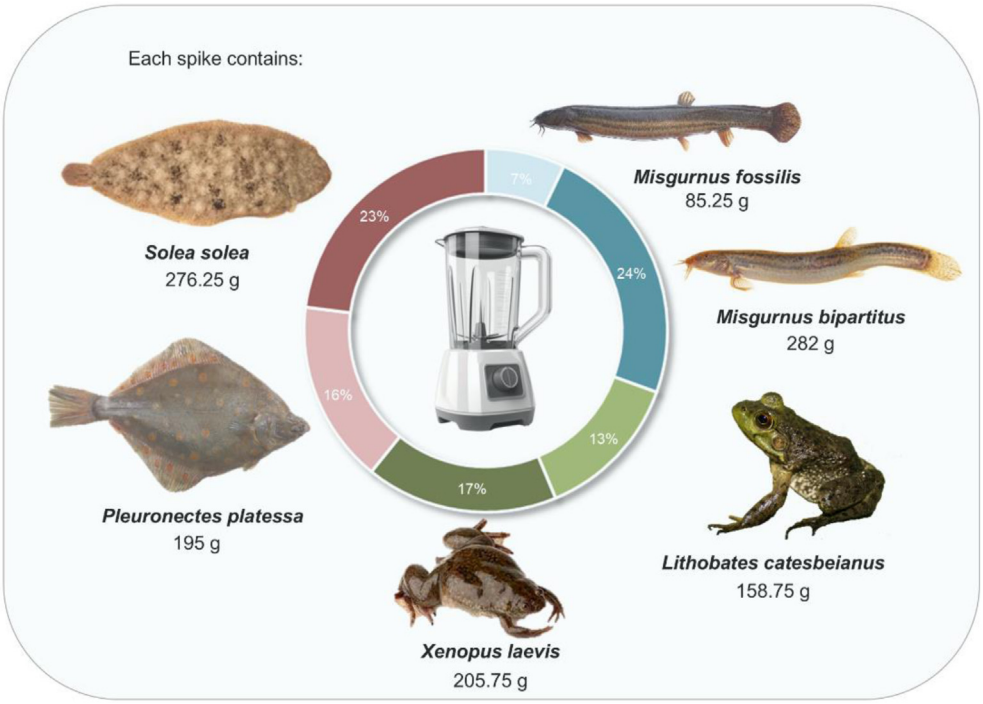

Fig. 2. Composition of the eDNA spike introduced at the influent of each WWTP. Each spike contained a predefined mixture of six species and their biomasses, including two commonly consumed flatfish species (*Solea solea* and *Pleuronectes platessa*) as well as the rare *Misgurnus fossilis* and three non-native species (*i.e.*, *Misgurnus bipartitus, Xenopus laevis*, and *Lithobates catesbeianus*) that are unlikely to occur naturally in the river or to be present in human diets.

short-lived pulse rather than an instantaneously homogenized solution, further diluting the introduced material within the 24−h composite influent samples.

Twenty-four hours after this spiking event (T2, Fig. 1), water samples were again collected at the same site-specific locations per WWTP and filtered for collection of eDNA following the same procedure as described for the pre-spiking samples. In total, 24 eDNA samples were collected.

2.2 Laboratory procedures

DNA extraction from the filters was conducted according to Brys *et al.* (2021), using the $SX_{CAPSULE}$ method with the DNeasy Blood & Tissue Kit (Qiagen), following the manufacturer's guidelines. This extraction process was performed in a dedicated PCR-free room for low-copy-number template extractions, with particulate air-filtered compartments, avoiding contamination of eDNA samples. The resulting eDNA extract was quantified prior to amplification with a Quantus Fluorometer according to the manufacturer's instructions.

Species-specific eDNA detection and quantification of each of the six target species were obtained using ddPCR on a QX200 ddPCR system (Bio-Rad, Temse, Belgium) in 20 µL reaction volumes. The species-specific primer/probe assays used in this study were previously developed and validated, and are further detailed in Table 1. Final eDNA concentrations per assay were obtained as described in (Brys *et al.*, 2021; Everts *et al.*, 2021; Van Driessche *et al.*, 2022). For each of the six target species assays, all 24 water samples were analyzed in triplicate, resulting in three technical replicates. Each ddPCR plate included extraction blanks, no-template controls, and an assay-specific positive control consisting of diluted tissue-derived reference DNA. eDNA concentrations (copies μL^{-1}) were calculated using Poisson correction of positive droplet counts (QuantaSoft).

To provide broader context on the background fish community and validate ddPCR results, parallel eDNA metabarcoding was conducted on all samples using 12S rRNA primers (see Supplementary Information S1 for details).

2.3 Statistical analyses

All statistical analyses were conducted in R version 4.5.1 (R Core Team, 2025) using the packages *lme4* v1.1.37 (Bates *et al.*, 2015), *lmerTest* v3.1.3 (Kuznetsova *et al.*, 2017), *emmeans* v1.11.2.8 (Lenth, 2023), and *dplyr* v1.1.4 (Wickham, 2023). Analyses focused on evaluating whether eDNA introduced during the spike event was effectively removed by wastewater treatment and whether any residual DNA could be detected in downstream waters. Figures were created using the additional package *ggplot2* v3.5.2 (Wickham, 2016).

Because effluent and downstream samples produced no detections, the presence–absence analysis was restricted to the influent. Pre- and post-spike detection frequencies were compared using a Fisher's exact test. Secondly, the quantitative analysis used ddPCR concentration data from all locations, as zero concentrations in effluent and downstream samples

Table 1. Characteristics of the primer/probe assays used for the detection of *Solea solea, Pleuronectes platessa, Misgurnus fossilis, Misgurnus bipartitus, Xenopus laevis*, and *Lithobates catesbeianus*, respectively.

Species	Gene	Amplicon size	Temp	Length (bp)
Solea solea	COI	100 bp	56 °C	
Forward: Ss COI F	5′-CCCCTGCTTTCCTGCTACTT-3′			20
Reverse: Ss COI R	5′-GCATGGGCGAGGTTACTTGA-3′			20
Probe: Ss COI P	5′-ACCTCATCCGTTGTTGAAGC-3′			20
Maes *et al.* (2024)				
Pleuronectes platessa	CytB	90 bp	56 °C	
Forward: Plepla CytB F	5′-TAGGCTTCGCAGTCCTCCTC-3′			20
Reverse: Plepla CytB R	5′-TTGCAGGCGTGAAGTTGTCT-3′			22
Probe: Plepla CytB P	5′-CTAAAAGATTTGGGGAAAATAGGGCGAGT-3′			29
Knudsen *et al.* (2019), Maes *et al.* (2024)				
Misgurnus fossilis	COI	119 bp	56 °C	
Forward: Mf COI F	5′-CCCCCGACATAGCATTTCCG-3′			20
Reverse: Mf COI R	5′-AACTGTTCAGCCTGTCCCAG-3′			20
Probe: Mf COI P	5′-CTCGTTCCTCCTTCTGCTGG-3′			20
Brys *et al.* (2021)				
Misgurnus bipartitus	COI	154 bp	56 °C	
Forward: Mb COI F	5′-TTTAGCCGGGGTTTCGTCT-3′			19
Reverse: Mb COI R	5′-TAGGACCGGCAAGGAGAGT-3′			19
Probe: Mb COI P	5′-ACCAGACCCCCTTGTTTGT-3′			19
Brys *et al.*, (2021)				
Xenopus laevis	12S	83 bp	53 °C	
Forward: SPYGEN XenLea F	5′-AGGCTTAATGATTTTGCATC-3′			20
Reverse: SPYGEN XenLea R	5′-AGGGTATAGAAAATGTAGCC-3′			20
Probe: SPYGEN XenLea P	5′-ACGTCAGGTCAAGGTGTAGCA-3′			21
Secondi *et al.* (2016)				
Lithobates catesbeianus	16S	120 bp	55 °C	
Forward: qLC16S F	5′-GCAGAGATAACCTCTCGT-3′			18
Reverse: qLC16S R	5′-GTCCCATAGGACTGTTCT-3′			18
Probe: qLC16S P	5′-TGCCCTCCCGAAACTAAGTGAGC-3′			23
Lin *et al.* (2019)				
Lithobates catesbeianus	CytB	84 bp	60 °C	
Forward: Bullfrog F	5′-TTTTCACTTCATCCTCCCGTTT-3′			22
Reverse: Bullfrog R	5′-GGGTTGGATGAGCCAGTTTG-3′			20
Probe: Bullfrog BHQ P	5′-TTATCGCAGCAGCAAGTATGATCCACC-3′			27
Everts *et al.* (2021), Strickler *et al.* (2015), Brys *et al.* (2023)				

represent meaningful values for evaluating treatment performance. To evaluate quantitative variation in eDNA concentration, a linear mixed-effects model (LMM) was fitted to the log-transformed ddPCR concentrations. eDNA concentrations (copies μL^{-1}) were transformed using $\log_{10}(x+1)$ to improve normality and reduce the influence of zero concentrations. The fixed-effects structure included sampling location (influent, effluent, and downstream), sampling period (pre- or post-spiking), and their interaction, whereas WWTP identity was included as a random intercept to account for repeated measurements within facilities:

$$log_{10}(\text{eDNA concentration} + 1) \sim \text{Site location} \times \text{Sampling period} + (1|\text{WWTP}). \quad (1)$$

Model residuals were visually inspected to verify approximate normality and homoscedasticity. Estimated marginal means (EMMs) and Tukey-adjusted pairwise contrasts were obtained using the *emmeans* package to compare differences in mean eDNA concentration among locations and sampling periods.

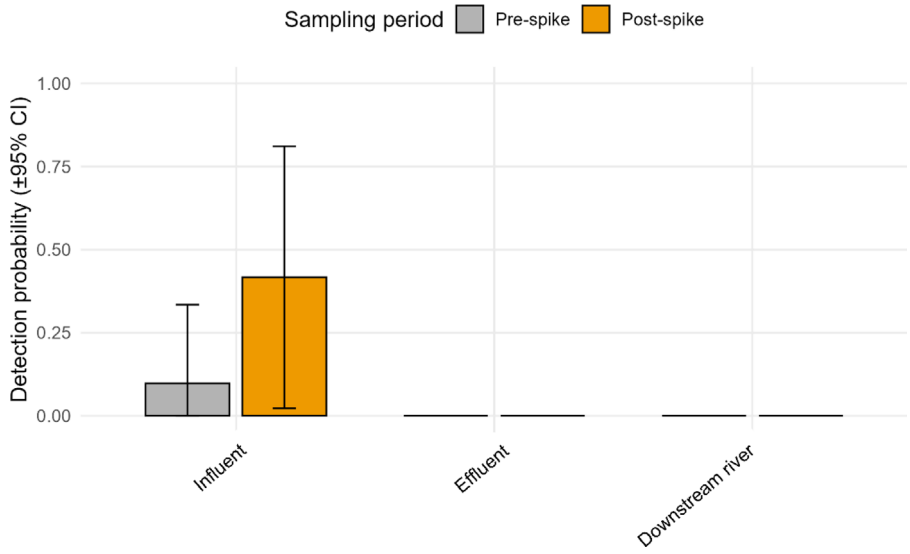

Fig. 3. Overall treatment barrier effect: Mean eDNA detection probability (±95% CI) of the six spiked species across WWTPs, based on the proportion of positive ddPCR detections before (pre-spike) and after (post-spike) the controlled introduction of the homogenized tissue mixture at the influent. Bars show mean detection probabilities aggregated across all taxa and facilities. The *X*-axis represents the sampling locations per WWTP, *i.e.*, the influent (incoming untreated wastewater), the effluent (treated discharge water), and the downstream river site (100 m from the effluent outflow).

3 Results

3.1 Data quality

No amplification was observed in extraction blanks or PCR negative controls for any of the spiked target taxa, indicating the absence of detectable contamination during field sampling, DNA extraction, and amplification procedures.

3.2 Detection patterns and eDNA removal

Across all four WWTPs, background eDNA levels of the spiked taxa were largely absent prior to the experiment (pre-spiking), confirming minimal existing contamination prior to the onset of the experiment. An exception occurred at the Ostend WWTP, where low eDNA concentrations of *Solea solea* and *Pleuronectes platessa* were detected in the pre-spike influent samples (Figs. 3 and 4). These detections likely originated from local seafood processing or domestic discharge, consistent with the city's proximity to a major fishing harbor (Ostend Fish Market, Port of Ostend). Such background detections illustrate the relevance of the research question and the realistic presence of consumption-derived marine eDNA in untreated wastewater.

Across all facilities, influent samples showed more detections after the spike than before (Fig. 3). A Fisher's exact test confirmed a significant increase in influent detection probability after the spike (odds ratio = 6.54, 95% CI = 2.52−19.31, $P < 0.0001$). Effluent and downstream samples showed no detections at any facility or sampling time.

At the two smaller facilities (Nevele and Zomergem), all taxa were consistently detected, whereas the larger WWTPs (Ghent and Ostend) showed occasional non-detections (Fig. 4). Log-transformed eDNA concentrations ($\log_{10}$[copies $\mu^{-1} + 1$]) showed a clear and location-specific response to the spike event. The linear mixed-effects model showed significant effects of sampling location ($F = 40.56$, $P < 0.001$), spike period ($F = 12.49$, $P < 0.001$), and their interaction ($F = 12.49$, $P < 0.001$). Estimated marginal means indicated that influent concentrations increased after the spike (pre: 0.094 ± 0.034; post: 0.329 ± 0.034), while effluent and downstream concentrations remained near zero at both sampling periods (Fig. 4). Tukey-adjusted pairwise contrasts showed that the spike significantly increased eDNA concentrations only in the influent ($P < 0.0001$). Concentrations did not differ between effluent and downstream sites (all $P > 0.05$).

Metabarcoding results corroborated these ddPCR findings (Supplementary Fig. S1). While resident fish communities were successfully characterized across all sites, the experimentally introduced target species were only detectable via eDNA metabarcoding in the Ostend site pre-spiking and in the influent samples across all WWTP locations post-spiking.

4 Discussion

This study provides clear experimental evidence that consumption-derived environmental DNA (eDNA), introduced at relatively high concentrations via domestic or household wastewater, can effectively be removed during standard secondary wastewater treatment. Across all four WWTPs, eDNA from the spiked species showed increased concentrations in the post-spike influent samples, although detections were not uniformly present for every species at every facility. In both effluent and downstream samples, however, all species were consistently undetectable. Detection frequencies increased significantly in the influent after the spike, as confirmed by the Fisher's exact test, while no detections were observed in either effluent or downstream

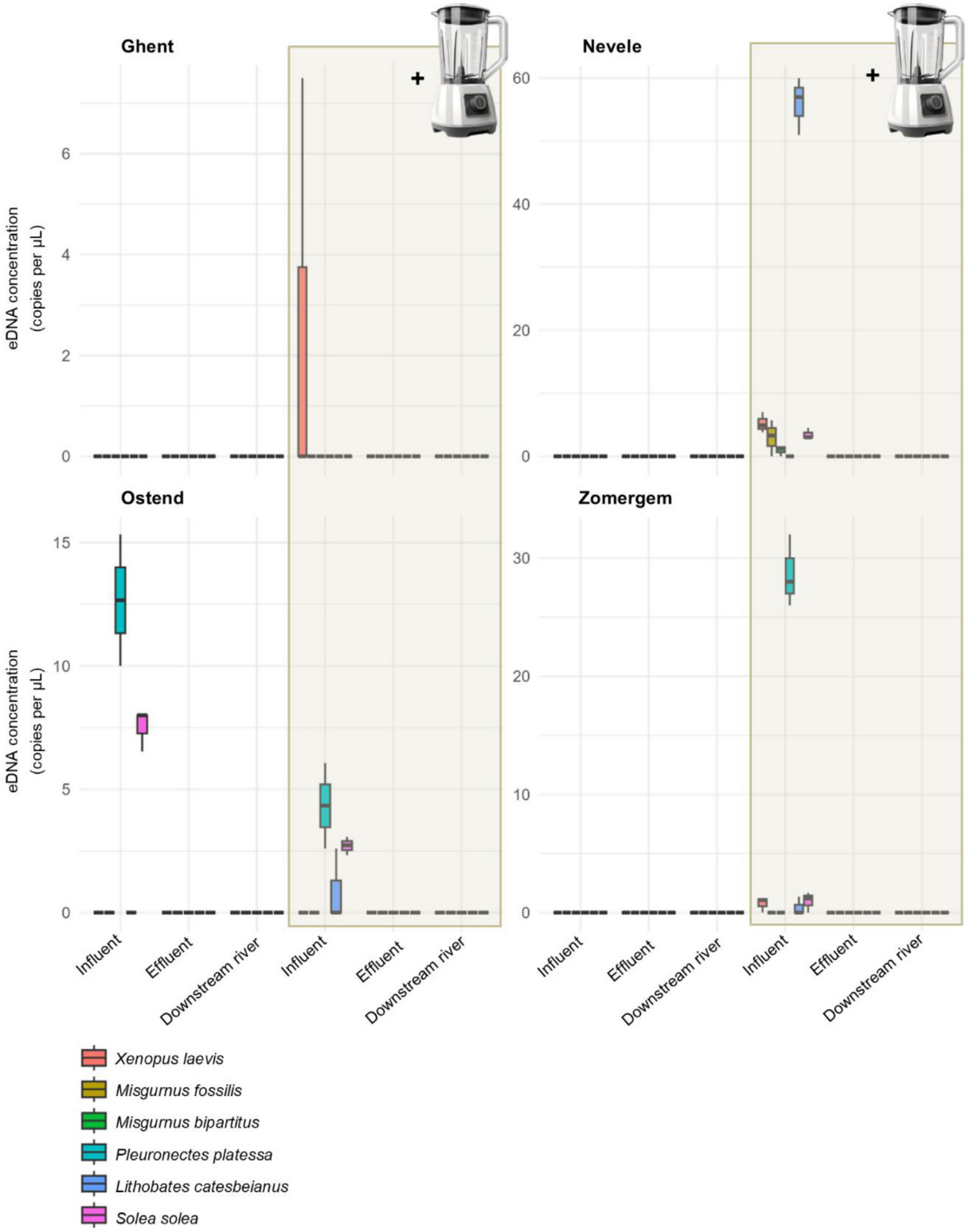

Fig. 4. Measured eDNA concentrations (copies per µL) of the six spiked species (*i.e., Xenopus laevis, Misgurnus fossilis, Misgurnus bipartitus, Pleuronectes platessa, Lithobates catesbeianus*, and *Solea solea*) across four WWTPs (Ghent, Ostend, Nevele, and Zomergem) at three sampling locations per WWTP: influent (incoming wastewater), effluent (treated water released from the WWTP), and downstream river (100 m from effluent discharge point). The shaded areas with blender icons indicate the introduction of the DNA spike containing six species. Box plots show the distribution of eDNA concentrations for each species across filter replicates. Note that the *Y*-axis range differs for each panel.

samples. Quantitatively, influent eDNA concentrations were significantly higher than those measured at subsequent treatment stages, as shown by the linear mixed-effects model and Tukey contrasts. Together, these findings demonstrate that modern WWTP processes are highly efficient at removing exogenous DNA, thereby minimizing the potential for false-positive detections of non-local fish or amphibian species in downstream waters.

Interestingly, background detections at the Ostend facility, where marine fish species such as *Solea solea* and *Pleuronectes platessa* were identified in pre-spike influent samples, highlight the realistic presence of consumption-derived or industrially sourced eDNA in urban wastewater streams. These detections likely originate from seafood processing or domestic discharge associated with Ostend's coastal economy. Similar observations have been reported in other urban and coastal settings, where DNA from market fish, aquaculture products, or industrial effluents was detected in influent or sewage samples (Inoue *et al.*, 2023; Clare *et al.*, 2022). Comparable coastal cases have also been described by Van Driessche *et al.* (2024b), where eDNA from marine and estuarine species reflected local economic and hydrological linkages rather than ecological presence. Importantly, no corresponding signals were observed in effluent or receiving waters, confirming that the treatment process acts as an effective barrier against discharge of exogenous DNA. This aligns with findings from Clare *et al.* (2022) and Zhang *et al.* (2025).

Parallel metabarcoding (Supplementary Fig. S1) detected the target spike species in post-spike influent samples, though relative read abundance remained low compared to dominant local species, and there was no targeted detection in effluent or downstream river samples. This illustrates that while metabarcoding is effective for broad biodiversity assessment, targeted ddPCR is more robust for quantification of trace-level DNA in complex matrices. As noted by Wood *et al.* (2019), targeted assays are less influenced by primer competition or taxonomic bias in high-biomass environments. Together, these results highlight the robustness of ddPCR and the reliability of eDNA-based monitoring in anthropogenically influenced systems. Furthermore, even if trace eDNA particles bypassed treatment, its ecological impact would likely remain negligible. In lotic systems, locally emitted and less fragmented resident biota typically dominate eDNA patterns. These local signals tend to overwhelm distant or degraded anthropogenic inputs in relative read abundance data (Van Driessche *et al.*, 2023). This reinforces the reliability of eDNA monitoring, as well-functioning infrastructure prevents exogenous DNA from distorting the local ecological signal.

In our experiment, post-dilution influent concentrations ranged from approximately 3 to 60 copies μL^{-1} (Fig. 4), equivalent to 3×10^6 to 6×10^7 copies L^{-1}. These values fall within, or even exceed, the upper ranges reported for fish mitochondrial eDNA in wastewater-associated environments (Inoue *et al.*, 2023). Because our spike consisted of undigested homogenized tissue, it represents a conservative, high-load scenario compared to genuine consumption-derived eDNA, which undergoes significant degradation during digestion and sewer transport. The complete absence of detectable eDNA in effluent and receiving waters despite these high influent loads indicates that standard secondary treatment effectively retains and degrades exogenous DNA before discharge. This high removal efficiency is driven by the microbial purification inherent to the activated sludge process. Our results align with Xiong *et al.* (2024a), who demonstrated that the majority of extracellular DNA is removed through sorption to extracellular polymeric substances (EPS) and subsequent microbial degradation within the biological treatment stage. Mechanistically, this process acts as a critical filter where DNA is removed via a combination of physical retention in primary sedimentation, sorption to activated sludge flocs, and enzymatic cleavage during aeration (Nielsen *et al.*, 2007; Caldwell *et al.*, 2021; Xiong *et al.*, 2024a). These findings mirror eDNA decay mechanisms observed in natural sedimentary environments (Jo *et al.*, 2023). While the homogenized tissue used in this study primarily contained DNA protected within cellular or organellar membranes, the complete removal observed suggests that dissolved DNA, which lacks such protection and is more susceptible to enzymatic cleavage, would be eliminated even more rapidly. Our experiment thus represents a conservative assessment, as intracellular DNA is typically more stable than the dissolved fragments often associated with highly degraded anthropogenic inputs (Xiong *et al.*, 2024a; Nielsen *et al.*, 2007). Recent synthesis work identifies anthropogenic eDNA inputs, including wastewater and urban runoff, as an important challenge for obtaining accurate and ecologically interpretable molecular biodiversity data (Çevik and Çevik, 2025). Our findings add nuance to this ongoing discussion about wastewater as a potential source of non-local DNA that could confound eDNA monitoring surveys in adjacent rivers. By demonstrating that even elevated influent concentrations are fully removed, our results show that well-functioning secondary treatment largely mitigates this source of uncertainty.

Regional and infrastructural differences are likely to influence the extent to which wastewater contributes detectable eDNA to the aquatic environment. In our study, these infrastructural effects manifested clearly at the influent stage of the larger facilities, where the spike was diluted beyond full species detectability. This reduced detectability at large facilities is consistent with the hydraulic behavior of influent streams, where very high flow rates and turbulence across upstream inlet structures (*e.g.*, screw pumps, grit chambers, and inlet channels) prevent instantaneous homogenization of the added material (Metcalf and Eddy, 2014; Tchobanoglous Leverenz, 2013). Under such conditions, a spike behaves as a short-lived pulse rather than a uniformly mixed signal, a dynamic well documented for shock or slug loads in large facilities (Metcalf and Eddy, 2014). Consequently, the introduced spike can be temporarily unevenly dispersed or strongly diluted within the 24-h composite samples, which are known to dampen short-term concentration peaks (Ort *et al.*, 2010). While this 'averaging' effect dampens the measured concentration of a short-lived pulse, it provides a quantitatively robust measure of the total DNA mass load handled by the facility. By capturing the integrated mass of the spike in the influent, which remained significantly elevated despite dilution, and observing its total absence in the effluent, we can confirm a complete removal efficiency. This mass-balance approach ensures that our conclusions reflect the plant's overall processing capacity rather than transient peak dynamics. Such mixing-related variability is therefore an

important consideration when interpreting influent eDNA concentrations but does not compromise assessments of downstream treatment performance. In regions with lower treatment efficiency, incomplete sewer connectivity, or frequent combined sewer overflows, influent concentrations of consumption-derived DNA may be higher, and a greater proportion of untreated material may enter natural waterways (Inoue *et al.*, 2023; Xiong *et al.*, 2024b). Such overflows occur when heavy rainfall or high hydraulic loads exceed treatment capacity, causing temporary bypasses that discharge untreated domestic and stormwater directly into rivers (Mailhot *et al.*, 2015). These episodic events can release dietary or industrially derived eDNA into receiving waters, potentially leading to short-lived, spatially restricted false-positive detections. Similarly, households not connected to centralized WWTP networks, which is still common in rural or developing areas, may discharge greywater directly into streams, potentially introducing dietary or domestic eDNA (Khajvand *et al.*, 2022; Sha *et al.*, 2024). Such variability underscores the importance of local infrastructure and hydrological context when interpreting eDNA detections near populated areas. For ecological surveys, sampling designs should therefore account for proximity to wastewater outfalls and hydrological connectivity. Collecting samples either upstream or sufficiently downstream from effluent discharge zones, or verifying flow separation from urban drainage, can help ensure that eDNA signals are representative of local biota rather than anthropogenic inputs. Comparative studies across regions differing in treatment technologies, discharge regulation, and catchment type will be essential to generalize these findings and refine eDNA-based monitoring frameworks under varying environmental conditions.

Overall, our results show that secondary wastewater treatment effectively eliminates consumption-derived DNA, preventing its release into receiving waters. Consequently, effluents from modern WWTPs are unlikely to generate false-positive eDNA detections downstream, reinforcing the reliability of eDNA-based biodiversity monitoring in urbanized landscapes. These conclusions apply to well-functioning treatment systems in lowland temperate regions. Areas with higher wastewater loads, intensive seafood processing, or incomplete sewer networks may exhibit different dynamics and warrant further study. By demonstrating complete removal of exogenous DNA under realistic operational conditions, this work strengthens confidence in interpreting riverine eDNA signals as reflections of local biota rather than wastewater contamination. Future efforts should integrate molecular and hydrological approaches across facilities of varying design to refine predictions of eDNA persistence and transport under diverse environmental conditions.

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Conflicts of interest

The authors declare that they have no conflicts of interest in relation to this article.

Data availability statement

All data are available from the Zenodo Digital Repository (https://doi.org/10.5281/zenodo.17952416).

Author contribution statement

Charlotte Van Driessche: Conceptualization, Methodology, Software, Formal analysis, Investigation, Writing – Original Draft, Visualization, Supervision, Project administration, Funding acquisition. Teun Everts: Investigation, Writing – Review & Editing, Funding Acquisition. Berdien De Beer: Investigation, Writing – Review & Editing. Sabrina Neyrinck: Investigation, Writing – Review & Editing. Johan Auwerx: Investigation, Writing – Review & Editing. Dries Bonte: Validation, Supervision, Writing – Review & Editing. Rein Brys: Conceptualization, Investigation, Supervision, Writing – Review & Editing.

Supplementary material

Figure S1. Spatiotemporal fish community composition across four wastewater treatment plants (WWTPs) based on eDNA metabarcoding. Relative read abundance is shown for three sampling points per WWTP (influent, effluent, and downstream river), both before and after a controlled spike of six target species. Red stars indicated the detection of at least one of the six target species (*Misgurnus fossilis, Solea solea, Pleuronectes platessa, Xenopus laevis*, and *Lithobates catesbeianus*). Note that target species were detected across all influent post-spike samples but were absent from all effluent and downstream river samples.

The Supplementary Material is available at https://www.kmae-journal.org/10.1051/kmae/2026007/olm.

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