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16S metabarcoding of the bacterial community of a poultry wastewater treatment plant in the Philippines

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Introduction

Wastewaters are loaded with many types of microorganisms but are predominantly composed of bacteria with different roles in the water treatment system (Kumar et al., 2022). Some belong to functional genera, which are mainly responsible for the removal of pollutants in wastewater while others are potentially pathogenic bacteria, which can pose threats to public health and the environment (Fan et al., 2018). The functional bacterial groups reported to be found in wastewater treatment plants (WWTPs) include ammonia-oxidizing bacteria (AOB), nitrite-oxidizing bacteria (NOB), polyphosphate-accumulating organisms (PAOs), glycogen-accumulating organisms (GAOs), acetogens, methanogens, and sulfur-reducing bacteria (Gallert and Winter, 2005; López-Vazquez et al., 2007; Dueholm et al., 2022). These types of bacteria are very favorable for plant operations because of their high pollutant removal rates, hence, ensuring their maintenance and sustainability in the systems is critical for the continuous efficacy of wastewater treatment operations (Gude, 2015). WWTPs are also known reservoirs of pathogenic microorganisms (Azli et al., 2022). Current microbial monitoring for contamination is limited to a few indicators such as coliforms, *Salmonella* sp., *Shigella* sp., *Vibrio* sp., *Legionella* sp., and fecal streptococci and enterococci (Naidoo and Olaniran, 2014; Garrido-Cardenas et al., 2017). However, the microbial indicators may mask the concentration of pathogens due to their lower concentration than the microbial indicators, hence a more sensitive approach is needed for pathogen detection (Zhang et al., 2021). Understanding the composition of bacterial communities is thus crucial in providing the basis for optimizing treatment conditions favoring the functional bacteria and detecting the presence of pathogenic bacteria. With the advent of new sequencing technologies, 16S metabarcoding may be utilized to explore and characterize wastewater microbiomes without the need for conventional culture methods. For these reasons, we sought to profile the bacterial communities in a poultry WWTP to serve as reference and baseline information on potentially functional and pathogenic bacteria present in wastewater, and to infer possible implications to wastewater treatment and management.

Here, we report data on the taxonomic composition of four treatment stages, i.e., Influent Tank, two aeration tanks where activated sludges were used (Aeration Tank 3 and Aeration Tank 1), and the Effluent flow, in a poultry wastewater treatment plant. The metabarcoding data offer insights into the bacterial diversity through ecological

indices and may be used for further analyses relevant to the role of bacteria in wastewater treatment.

Methods

Sample collection, processing, and sequencing

The samples were collected from a poultry dressing plant with a wastewater treatment facility in Cavite, Region IV-A, Philippines on 1 August 2022. Short interviews were conducted with the plant engineers to determine the operation protocols of the plant. The following consequent sampling points were chosen: (1) Influent Tank, where raw wastewater from the dressing plant was kept before entering the treatment system, (2) Aeration Tank 3, where the raw wastewater entered the system and where activated sludge was used, (3) Aeration Tank 1, where activated sludge was also used after treatment in the anaerobic digester, and the (4) Effluent, where it was free-flowing towards the nearest creek.

Triplicate 1L samples were collected using properly labeled sterile bottles at four sampling areas: Influent Tank, Aeration Tank 3, Aeration Tank 1, and Effluent flow. For the Influent Tank, only the top portion of the tank was collected due to the limitations of the sampler and the small opening of the tank. For the Aeration Tanks, the sampling protocol of the Global Water Microbiome Consortium (GWMC) was followed with modifications (Wu et al., 2019), wherein the sampling points depended only on the accessible areas of the tanks instead of having three different positions per zone. Hence, only one sampling point at a time was accessed with a time interval of 10 min per sampling. For the Effluent, since this was free-flowing, the samples were collected directly from the effluent water.

The twelve samples collected were immediately processed for sub-sampling. Approximately 300 mg of sludge/precipitate was collected from each sampling container and transferred into 1.5 mL cryovials. Triplicate samples from each treatment stage were processed. DNA was extracted following the manufacturer's protocol using Qiagen DNeasy PowerSoil Pro (QIAGEN, United States). DNA concentration was measured using a Denovix DS-11 fluorometer (Denovix, United States) and samples that met sequencing standards were sent to Macrogen, South Korea for 16S V1-V3 metabarcoding employing 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 534R (5'-ATTACCGCGGCTGCTGG-3') primers (Dueholm et al., 2022). The sequencing platform used was an Illumina MiSeq platform, 2 × 300 bp, 100,000 reads per sample, using Nextera XT-prepared libraries. Raw reads were deposited in the National Center for Biotechnology Information (NCBI) Short Read Archive (SRA) (Supplementary Table S1).

Bioinformatics analysis

An end-to-end workflow for 16S metabarcoding was employed using QIIME2-2022.8 (Hall and Beiko, 2018). Raw reads were quality-checked, primer trimmed at 0% error rate, and merged using 70 as the optimal $-p$ -minovlen parameter. All merged

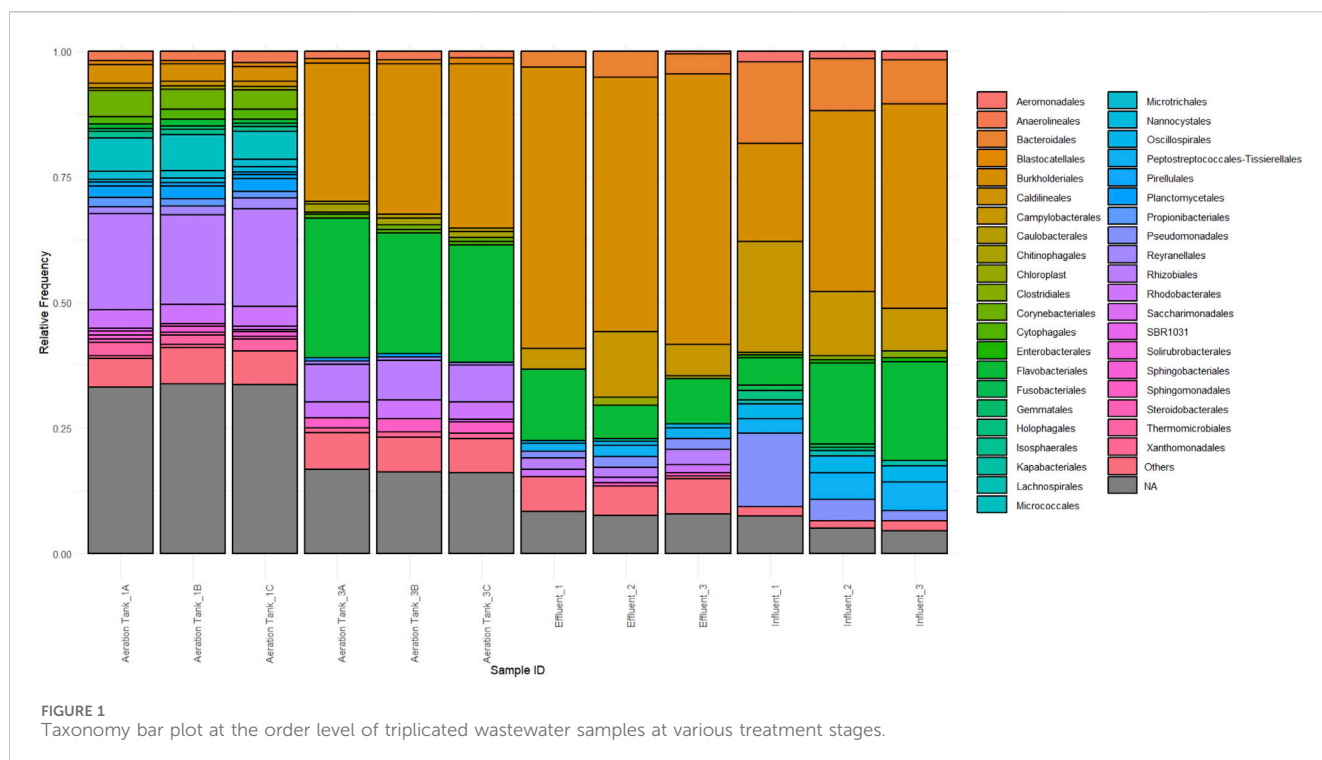
reads were quality-filtered at Phred 30, dereplicated, and clustered at 97% using an open reference OTU picking technique through VSEARCH (Rognes et al., 2016). Subsequently, singletons and chimeras were removed before taxonomy assignment using a Naive-Bayes trained classifier from SILVA 138-99 (Quast et al., 2012). The resulting taxonomy assignments were filtered to retain only bacterial sequences. Samples were rarefied to a depth of 8,900 features and subsequently, ecological indices along with statistical comparisons were done through the QIIME2 --diversity plugin. The taxonomy bar plot showing relative frequency was generated in R (R Core Team, 2021) using the package ggplot2 (Wickham, 2016) while other figures were directly lifted from QIIME2-generated tools.

Results and discussion

Resulting raw reads for all samples yielded a total of 1029761 sequences which was reduced to 806085 after primer trimming. Merging at a minimum overlap length of 70bp yielded 513586 merged reads and subsequent quality trimming at Q30, dereplication, and open-reference-based OTU clustering at 97% using SILVA 138-99 produced 306092 features. Filtering of features to include only bacterial sequences yielded 180324 features. Figure 1 shows the taxonomy bar plots of bacterial sequences present in each sample and treatment stage.

Effluent samples are dominated by *Delftia* sp. which comprises approximately 40% of each replicate. This genus has displayed remarkable biotechnological potential in wastewater applications. *Delftia tsuruhatensis* SDU2 showed excellent ammonium removal in high-strength nitrogen wastewater (Chen et al., 2023). Other studies demonstrated the ability of *Delftia* to metabolize organic pollutants (Custodio et al., 2022), inhibit biofilm formation in sludge through quorum quenching (Xu et al., 2023), and assimilate terephthalate from sludge (Shigematsu et al., 2003). *Flavobacterium* sp. is the next dominant member, with a relative frequency of 4.093%–7.283%. Other bacterial community members with a relative frequency of more than 1% are as follows: unclassified and uncultured bacteria (3.224%–4.497%), a bacterium under family *Comamonadaceae* (2.735%–5.153%), a bacterium under family *Rhodocyclaceae* (1.186%–1.938%), a bacterium under order Burkholderiales (1.832%–2.655%), a bacterium under order Rhizobiales (1.195%–1.389%), a bacterium under order Campylobacterales (2.126%–9.594%), a bacterium under family *Arcobacteraceae* (1.730%–3.234%), and an uncultured *Tepidibacter* sp. (1.616%–2.050%).

Influent samples show a high frequency of family *Comamonadaceae*, family *Arcobacteriaceae* members, and *Aquaspirillum* uncultured bacterium with relative frequency ranges of 7.247%–19.041%, 6.547%–7.799%, and 4.777%–10.197%, respectively. Family *Comamonadaceae* members were previously reported in influent raw wastewater samples collected from Canadian Arctic ponds (Huang et al., 2021). However, their role in other stages such as in activated sludge is more documented (Sadaie et al., 2007; Ge et al., 2015). Ge et al. (2015) have reported a novel PAO clade *Comamonadaceae* as integral in the removal of biological phosphorus from abattoir wastewater while its domination in sludge microbiome was seen as a result of sludge reduction due to reduced oxygen supply in a food-processing



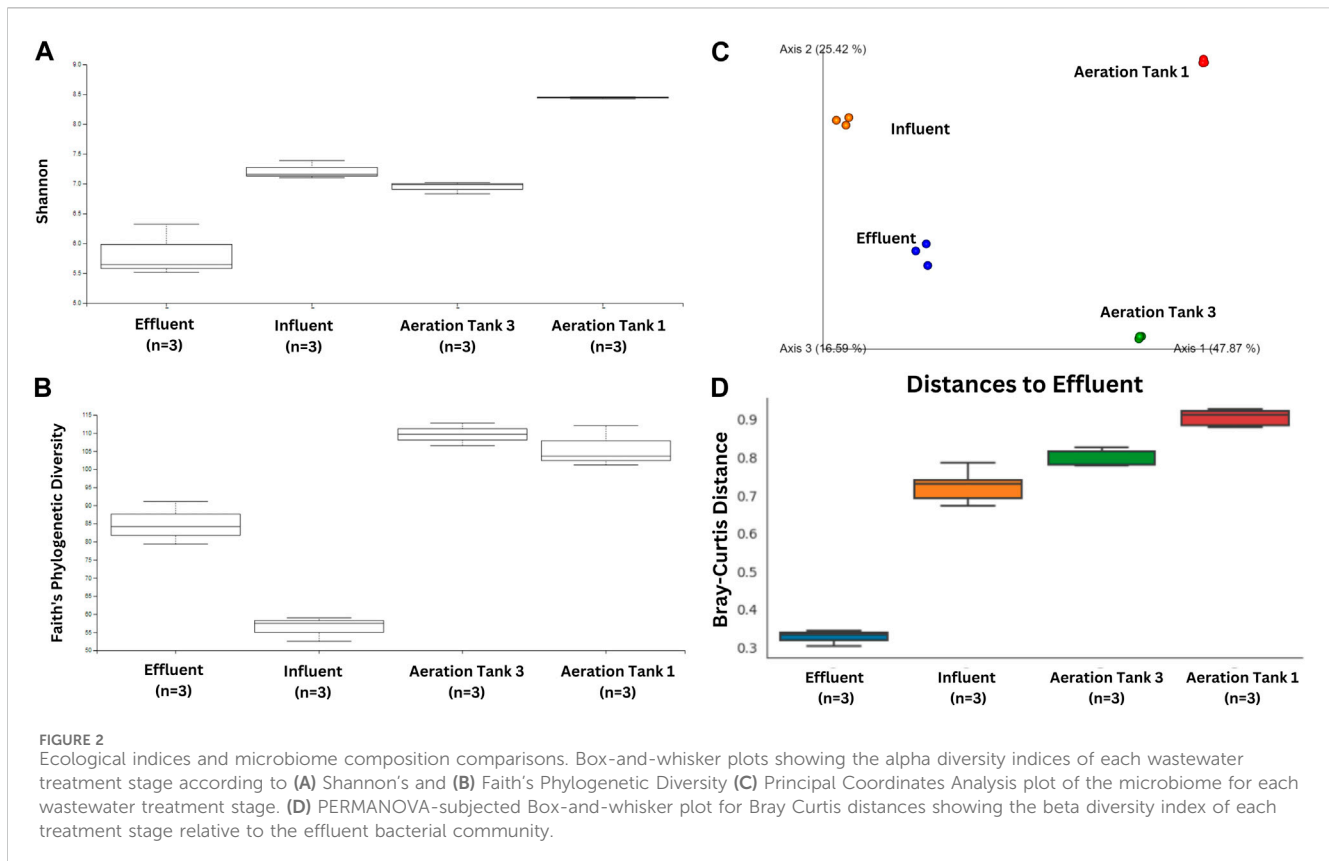
wastewater treatment plant (Sadaie et al., 2007). Family *Arcobacteriaceae* was found to be the dominant influent and “clean” effluent microbiome member in 14 Danish wastewater treatment plants (Kristensen et al., 2020). In addition, *Arcobacter* species have been isolated and characterized to harbor various multi-antibiotic resistance genes and phenotypes from poultry in Ghana and Tunisia (Jribi et al., 2020; Zautner et al., 2023). Thus, it is not surprising that this was also dominant in our Influent samples. Lastly, *Aquaspirillum* has been consistently found to be a major denitrifier in various reactors in WWTPs in Korea (Lee et al., 2008).

Some highly abundant members in the Effluent were also found in the Influent, namely, *Flavobacterium* sp. (1.956%–4.881%), a family *Rhodocyclaceae* member (1.179%–1.392%), an order Burkholderiales member (2.553%–3.306%), and an order Campylobacteriales member (1.498%–14.929%). Other detected bacteria with high relative frequency are a class Bacteroidia member at 1.784%–3.634%, a family *Prevotellaceae* member at 2.200%–2.668%, a family *Weeksellaceae* member at 1.328%–2.842%, *Bacteroides luti* at 1.324%–1.616%, and *Aeromonas* E1-54 at 1.403%–1.997%.

Aeration Tank 3 samples are dominated by *Flavobacterium* sp. at 18.578%–19.575%, followed by the family *Rhodocyclaceae* at 12.960%–19.813%, and order Burkholderiales members at 7.266%–10.564%. *Flavobacterium* has been previously reported as an abundant member in wastewater effluent in springtime from a site in Korea (Chen et al., 2019); *Flavobacterium* has also been extensively documented in wastewater (Ryu et al., 2007; Fujii et al., 2014; Liu et al., 2017). *Rhodocyclaceae* is also a dominant member of wastewater microbiomes such as in poultry, cattle, and pigs WWTPs (Boukerb et al., 2021). In addition, they were pointed out as important denitrifiers in treatment plants as indicated by metagenomic and phylogenomic analyses (Wang et al., 2020). Order Burkholderiales, another dominant Aeration Tank 3 taxon,

has been well documented in wastewater samples such as in the fermentation tank of a piggery WWTP (Shi et al., 2021), in a microbial fuel cell-treated wastewater input with high antibiotic load (Chen et al., 2022), and effluent input into riverine systems (Ruprecht et al., 2021). The following frequency ranges are detected in this treatment stage with their relative frequency ranges: the same unclassified and uncultured bacteria detected on the Effluent at 8.883%–9.320%, a family *Comamonadaceae* member at 2.961%–3.319%, a class Actinobacteria member at 1.459%–1.788%, an order Rhizobiales member at 4.394%–4.606%, a family *Rhizobiaceae* member at 1.235%–1.569%, *Flavobacterium cucumis* at 3.295%–7.102%, a family *Rhodobacteraceae* member at 1.981%–2.442%, a class Gammaproteobacteria member at 1.412%–1.774%, a class Alphaproteobacteria member at 1.119%–1.409%, and a family *Sphingomonadaceae* member at 1.995%–2.635%.

Lastly, Aeration Tank 1 samples show a high frequency of class Actinobacteria (12.824%–14.199%), the same uncultured bacteria seen on the Effluent and Aeration Tank 3 (8.835%–10.582%), an order Rhizobiales member (8.285%–8.680%), a family *Rhizobiaceae* member (5.472%–6.126%), and *Lapilicoccus* sp. (5.260%–6.918%). A family *Comamonadaceae* member (1.384%–1.892%), a class Bacteroidia member (1.286%–1.663%), a family *Rhodobacteraceae* member (1.648%–1.857%), a class Gammaproteobacteria member (2.081%–2.140%), a class Planctomycetes member (3.068%–3.358%), a class Alphaproteobacteria member (1.491%–1.589%), *Mycobacterium* sp. (2.215%–3.132%), *Gemmobacter* sp. (1.157%–1.715%), *Reyranelia* sp. (1.350%–2.197%), an order Thermomicrobiales member (1.851%–2.691%), a family Rhizobiales *incertae sedis* member (1.468%–1.857%), and an order Corynebacteriales member (1.301%–1.791%) were also present in this treatment stage. A class Actinobacteria member showed high predominance in Aeration Tank 1 microbiomes. Such



has been reported of high predominance in effluent microbiomes in a full-scale plant (Kaevska et al., 2016). Other studies show the potential of Actinobacterial members as biofloculants that aid the treatment process (Agunbiade et al., 2016). Meanwhile, order Rhizobiales and class Rhizobiaceae members have increased in frequency in Aeration Tank 1 which is recapitulative of what is reported by Bia et al. (2021) where a switch of anoxic to oxic conditions permitted its sudden increase. Another group has also reported increased counts of Rhizobiales in activated sludge (Fredriksson et al., 2013). *Lapilicoccus* also showed high counts in this treatment stage. Interestingly, to our knowledge, there is no report of this genus in other wastewater microbiomes. To date, only two published studies are available: one which discusses its isolation from a botanical garden in Indonesia (Ratnakomala et al., 2016) and another that showed its presence in zinc-contaminated soil microbiome (Zaborowska et al., 2022).

In the case of the functional bacteria in WWTPs, their frequency in the wastewater treatment plant is quite low. *Nirosomonas* sp., a common NOB, is only present in Aeration Tank 3, with an average of 0.001%. Another bacterium, *Thauera* sp., which was reported to have denitrification and phosphorus removal capabilities (Ren et al., 2021), is more abundant in Aeration Tank 3, with an average relative frequency of 0.468%, than Aeration Tank 1 with an average relative frequency of 0.086%. A sulfate-reducing bacterium, *Ochrobactrum* sp. (Yang et al., 2021) was also detected in both Aeration Tanks 3 and 1 with mean relative frequencies of 0.260% and 0.215%, respectively. Two *Nitrospira* spp., which are AOB, were also detected on both Aeration Tanks with mean relative frequencies of 0.238% and

0.064% in Aeration Tank 3 and 0.051% and 0.115% in Aeration Tank 1. These may not reflect the true frequency of functional bacteria as there are many unidentified bacteria from the samples.

The use of metabarcoding for microbial monitoring revealed some pathogenic bacteria present in the poultry wastewater. Here, we report some of the potential pathogens found in the poultry wastewater treatment plant. *Mycobacterium* sp., a possible pathogen, has been detected in all treatment stages, with averages of 0.020%, 0.676%, 2.640%, and 0.157% in Influent, Aeration Tank 3, Aeration Tank 1, and Effluent, respectively. Another pathogen, *Aeromonas* sp., was also detected on the influent, with a relative frequency range of 1.403%–1.997%, in Aeration Tank 3 at 0.014%–0.034%, and in the Effluent at 0.151%–0.520%. *Bacteroides* spp. are clinically important pathogens where three different species, aside from *B. luti*, were detected in the Influent and Effluent with a total mean relative frequency of approximately 1.00% per sample. *Pseudomonas* sp., a ubiquitous and opportunistic pathogen, was present in all sites with the highest frequency in the Influent and the lowest at Aeration Tank 1. *Leptospira* sp. Was also detected with mean relative frequency of 0.040% in the Effluent and 0.094% in Aeration Tank 3.

The water flow in the treatment plant was as follows: Influent → Aeration Tank 3 → Aeration Tank 1 → Effluent (Supplementary Figure S1). Aeration Tank 1 had the highest Shannon entropy values ranging from 8.42 to 8.45, which suggests the highest species diversity among all samples, while effluent had the lowest (Figure 2A). Also, Faith's Phylogenetic Diversity of bacterial composition increased along the treatment stage except for the Effluent where there was a decline in bacterial diversity relative to Aeration Tank 1 (Figure 2B).

As for bacterial composition, distinct bacterial communities across treatment stages suggested significant alterations in communities as they passed through different tanks (Figure 2C). This observation was corroborated by Bray-Curtis distances relative to Effluent's bacterial composition where a significant difference in beta diversity was observed. Shifts in each treatment stage's bacterial community were observed (Figure 2D). In a microbial ecosystem, stochastic and deterministic processes determine community assembly (Dottorini et al., 2021). In the case of WWTPs, bacterial communities are affected by the plant's capacity, which are composed of equipment capacity, volume of reactors, characteristics of the influent, sludge retention time, and hydraulic retention time (Kim et al., 2019). Hence, these processes may have contributed to the bacterial community shifts in the poultry WWTP system.

Conclusion

The bacterial community profiles of a poultry farm wastewater treatment plant in the Philippines showed that different bacteria dominated the different phases. In the Influent, the most prominent are members of the family *Comamonadaceae* and of family *Arcobacteriaceae*, and an uncultured *Aquaspirillum* bacterium while *Delftia* sp. and *Flavobacterium* sp. were dominant in the Effluent. Despite both having activated sludges, the bacterial composition of Aeration Tanks 3 and 1 were also different. For Aeration Tank 3, members of the family *Rhodocyclaceae* and order Burkholderiales, and *Flavobacterium* sp. had the highest frequencies while members of class Actinobacteria, order Rhizobiales, family *Rhizobiaceae*, and *Lapilicoccus* sp. were the highest for Aeration Tank 1. Common nitrifiers such as *Nitrosomonas* and *Nitrospira* were found at a low frequency. *Mycobacterium* sp., *Aeromonas* sp., *Bacteroides* spp., *Pseudomonas* sp., and *Leptospira* sp. were the potential pathogens detected in the system that may be disseminated in the environment. This will serve as baseline information on the microbial dimensions in poultry wastewater treatment plants and will allow other researchers to use it as a reference. The bacterial taxonomic information can guide the development of wastewater-related policies and guidelines for improved treatment processes and biotechnological explorations in the future.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

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Author contributions

MR-S: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Writing–original draft, Writing–review and editing. PG: Data curation, Formal Analysis, Investigation, Methodology, Validation, Visualization, Writing–original draft, Writing–review and editing. MO: Conceptualization, Funding acquisition, Methodology, Resources, Supervision, Writing–review and editing.

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Conflict of interest

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fenvs.2024.1390323/full#supplementary-material>

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