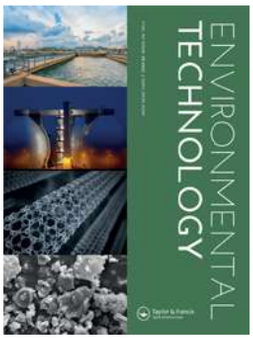

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Simultaneous nitrification-denitrification (SND) using a thermoplastic gel as support: pollutants removal and microbial community in a pilot-scale biofilm membrane bioreactor

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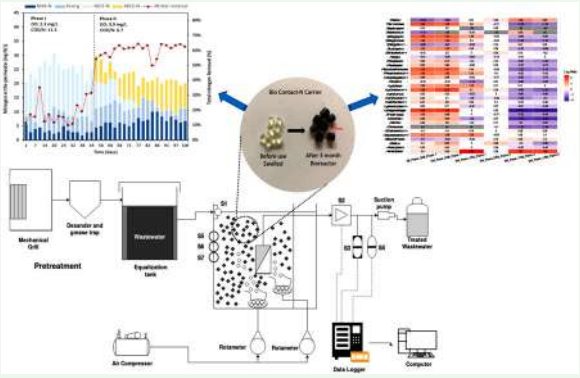
ABSTRACT

In this study, experiments were carried out to treat sanitary wastewater in a biofilm membrane bioreactor using a thermoplastic gel as a support to assist the nitrification-denitrification process. For this purpose, the system was operated in two different dissolved oxygen concentrations (2.3 ± 0.2 and 0.9 ± 0.3 mg O_2/L for Phases I and II, respectively) and the removal of organic compounds and nitrogen, as well as the microbial community in suspended biomass and biofilm were evaluated. The MB-MBR system was able to withstand raw wastewater variations and maintaining a low permeate COD concentration (18 mg/L) even at low DO concentrations. On the other hand, it was found that oxygen concentration significantly influenced the process of nitrogen conversion. In Phase I the average removal of total nitrogen was 18 ± 8%, while in Phase II it increased to 66 ± 11%. The denitrification rate was two times higher (7.8 mg NO_3^-–N/h) at low dissolved oxygen, with a significant contribution of the biofilm (41%). Additionally, the high-throughput 16S rDNA sequencing showed that the oxygen concentration was determinant for arrangement patterns of the samples and not the sampling site (suspended biomass and support material). *Thiothrix*, *Comamonas*, *Rhodobacter*, *Mycobacterium*, *Thermomonas*, *Sphingobium*, *Sphigopyxis*, *Pseudoxanthomonas*, *Nitrospira* and, *Novosphingobium* were the main genera regarding the nitrogen cycle.



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KEYWORDS
Biofilm carriers; sanitary wastewater treatment; simultaneous nitrification and denitrification; 16S rDNA sequencing

HIGHLIGHTS

- SND was achieved at low dissolved oxygen;
- The formation of biofilm improved nitrogen conversion process under anoxic condition;
- AOB, NOB and denitrifying bacteria was similar in the suspended biomass and biofilm;
- Different genera/species of bacteria were found in oxic and anoxic environments.

1. Introduction

The excessive discharge of nutrients, mainly nitrogen, is still one of the most critical problems considering its elimination in wastewater treatment. Due to environmental impacts, a number of regulatory agencies have established more restrictive standards for water quality and sewage discharge and have therefore included them as essential criteria for wastewater treatment projects.

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In the last years, several studies have shown that these two processes of total nitrogen removal may occur simultaneously in the same reactor, where biological flocs may include both aerobic and anoxic zones, such that nitrification and denitrification take place within the same tank [1–5]. The concentration gradient of dissolved oxygen (DO) within flocs or microbial biofilms due to diffusion limitations is the key hypothesis for the occurrence of SND [6]. In addition, other pathways, such as heterotrophic nitrification [7], aerobic denitrification [8], and short-circuit denitrification [9,10] will also lead to the removal of nitrogen by SND processes.

Membrane bioreactors (MBRs), which refer to the combination of biological and membrane separation processes, have been established as a potential technology for nitrogen removal by SND. The reduction in oxygen transfer as a result of high mixed liquor suspended solids (MLSS) makes it possible to create optimal concentrations of DO in the reactor for the occurrence of the SND process [11]. Therefore, the configuration of SND in MBR has been proposed and evaluated in recent years [5,12,13]. However, the high aeration requirements for fouling control in MBR lead to the development of a very small floc, which is easily penetrated by dissolved oxygen. This will result in less nitrogen removal efficiency due to inhibition of the denitrification process at higher concentrations of dissolved oxygen and restricting the application of SND [14].

The use of support material for biofilm development has been shown to be an important strategy for improving the removal of nitrogen through SND in biological reactors, including MBRs [15–19]. It is known that the process of adhesion and the consequent formation of biofilm is determined primarily by the interaction between microorganisms and the supporting material surface [20]. Therefore, in order to establish suitable conditions for the growth of microbial communities, it is very important to select an appropriate material that contributes to the increase in the richness of bacterial communities engaged in wastewater treatment, which might be one of the ways for the better performance of the biofilm in the removal of different pollutants. Furthermore, in the case of the MBR system, the use of a carrier for the development of biofilm must be chosen carefully due to certain properties that could damage the membrane material [21]. For this reason, various materials, including plastics, textiles and wood, have been used to facilitate the development of biofilm in bioreactors [20,22,23]. The use of carriers with a high porosity structure is an important feature of biofilm formation in order to achieve SND, as it enables the creation of a thicker biofilm with better redox stratification and the establishment of concentration gradients from both electron donors and acceptors [14]. However, the concentration of dissolved oxygen is still a critical factor to achieve a higher degree of nitrogen elimination, and even the establishment of a thicker biofilm may not be sufficient to achieve high levels through the SND process.

DO plays an important role in the conversion of nitrogen and could significantly regulate the activity and distribution of nitrobacteria and denitrobacteria in the biofilm. Zhuang et al. [24] demonstrated that in low oxygen concentration some toxic compounds can be converted into readily biodegradable intermediates, and the toxicity of refractory compounds reduced, creating even more sustainable conditions for the dominant growth of nitrifying and denitrifying bacteria in the SND process. As a result, the levels of DO in the bioreactor have a significant impact on both the physiological structure and the structure of the microbial community, improving the removal rate of some specific pollutants.

Since the use of biocarriers and DO are key factors that influence the quantity and distribution of the attached biomass and therefore affect the nitrification and denitrification processes in moving bed membrane bioreactor (MB-MBR), research into new materials that can be used in MBR for the occurrence of SND under different environmental conditions is still an important subject. This is even more important considering their application in realistic condition and moderate scale, since the real compounds present in the effluent, represent an additional challenge and may completely differ from the environments with model solutions and synthetic effluents used in the preliminary assessments.

To date, however, the use of an expansive thermoplastic gel as carrier in MBRs having an effect on nitrification and denitrification rates, as well as on the microbial community, has not yet been examined. In this context, in the present study, experiments were carried out using a novel thermoplastic gel as a support for the simultaneous nitrification and denitrification process in a pilot-scale MB-MBR. Over a period of more than 100 days, the pilot plant was fed with sanitary wastewater and monitored for its organic compounds and nitrogen removal performance under two different dissolved oxygen conditions. In order to investigate the effect of dissolved oxygen, an ammonium oxidation rate (AOR) and denitrification rate (DNR) were performed, as well as a robust description of the microbiome in the MB-MBR system using a high-throughput 16S rDNA sequencing. The results of this study will provide an important orientation for the application of a biofilm membrane bioreactor process, especially for those who need

improved nitrogen removal without altering the layout of the treatment plant.

2. Materials and methods

2.1. Experimental set-up and operating conditions

A pilot plant of MB-MBR (Figure 1) was operating at the International Reference Center on Water Reuse (IRCWR) of the University of São Paulo, CIRRA-USP. The reactor was constructed in acrylic with a working volume of 156 L, equipped with an automatic data acquisition system and the necessary instruments for measuring and registering, at an interval of 20 s, permeate flow, temperature, transmembrane pressure (TMP), Oxidation Reduction Potential (ORP), Dissolved Oxygen (DO) and pH. Flat sheet ultrafiltration membrane modules (FS-UF), fabricated in Polyvinylidene fluoride (PVDF) with a mean pore size of 0.1 microns from SINAP® were used for solid separation. The total membrane area was 1.8 m^2 (18 plates). The permeate was continuously withdrawn by a peristaltic pump operating with a cycle of 9 min of filtration and 2 min of relaxation. The physical structure of the membrane cassette was made of stainless steel with two diffusers in the base for the cleaning of the membranes [25].

The pilot plant was maintained under ambient temperature during the study period (104 days) and was continuously fed with domestic wastewater arising from the student housing and restaurant of the University of São Paulo (Table 1). Prior to the MB-MBR system, wastewater passed through a mechanical screening (step-screen) and a grit chamber (Figure 1). After the preliminary treatment, the sewage was stored in an equalization tank and transferred to the MB-MBR system by gravity. During the experiment, sludge was periodically removed from the reactor to control the MLSS concentration, resulting in an average Solids Retention Time (SRT) of 28 days.

In order to provide the specific process conditions, the MB-MBR system was operated with one fine bubble diffuser for the mixed liquor (intermittent aeration – 10 L air/min) and one coarse bubble diffuser for the membranes system (continuous aeration – 15 L air/min). The DO concentration was monitored using a luminescent dissolved oxygen probe connected to a controller (DS-NET, Policontrol). This controller, based on the concentration of DO, operated an electrically actuated valve installed in the mixed liquor aeration line.

To achieve the SND conditions the experiment was carried out in two different conditions of DO concentration: Phase I – DO was ≥ 2.0 mg O_2/L; and Phase II – DO ≥ 0.8 mg O_2/L. Although each phase operates at different concentrations of dissolved oxygen, the resulting velocity gradient when both aeration systems were activated was the same, with 111.3 s^{-1} for the membrane system and 90.8 s^{-1} for intermittent aeration, resulting in a total velocity gradient (G) of 202.1 s^{-1}. The velocity gradient was calculated according to Equation (1). The main characteristics of Phase I and II are shown in Table S2 of the supplementary material.

$$G = \sqrt{QH\gamma_w/V\mu} \quad (1)$$

where Q is the air flow rate (m^3/s), H is the depth of water column in m, V is the reactor volume in m^3, μ is the viscosity of water in Pa s, and γ_w is the specific weight of water in N/m^3.

The carrier material used for biofilm formation and supporting the NDS process was Bio Contact-N (BCN) (Nisshinbo Chemical Inc.) composed of polyurethane (thermoplastic gel) with a high-water absorption capacity. The surface of the material had a rough structure with a pore size of 5–10 µm in diameter and Specific surface area less than 0.01 m^2/g [20]. The size of the expanded media is 4 mm in diameter and 4 mm in length (Figure 2).

2.2. Analytical methods

Influent and effluent samples were analysed according to the Standard Methods [26] for Chemical Oxygen Demand – COD (5220 – D), Total Kjeldahl Nitrogen (TKN – 4500 – B), ammonium nitrogen (4500 – B/C), nitrate and nitrite nitrogen (4110 – B – Ion chromatography) and alkalinity (2320 – B). Five-day Biochemical Oxygen Demand ($BOD_{5,20}$) was determined using manometric systems from Aqualitic (BOD System, OxiDirect). Mixed Liquor Suspended Solids (MLSS) and Mixed Liquor Volatile Suspended Solids (MLVSS) samples were analysed according to Standard Methods number 2540 [26].

2.3. Calculation of nitrogen removal through different pathways and specific nitrification and denitrification rate

The amount of nitrogen removed via SND was estimated by a mass balance of the nitrogenous material according to Equations (2), (3) and (4). The total nitrogen content from biomass was estimated by the methodology described in Melcer [27]; this resulted in 7.0% ± 0.5 by

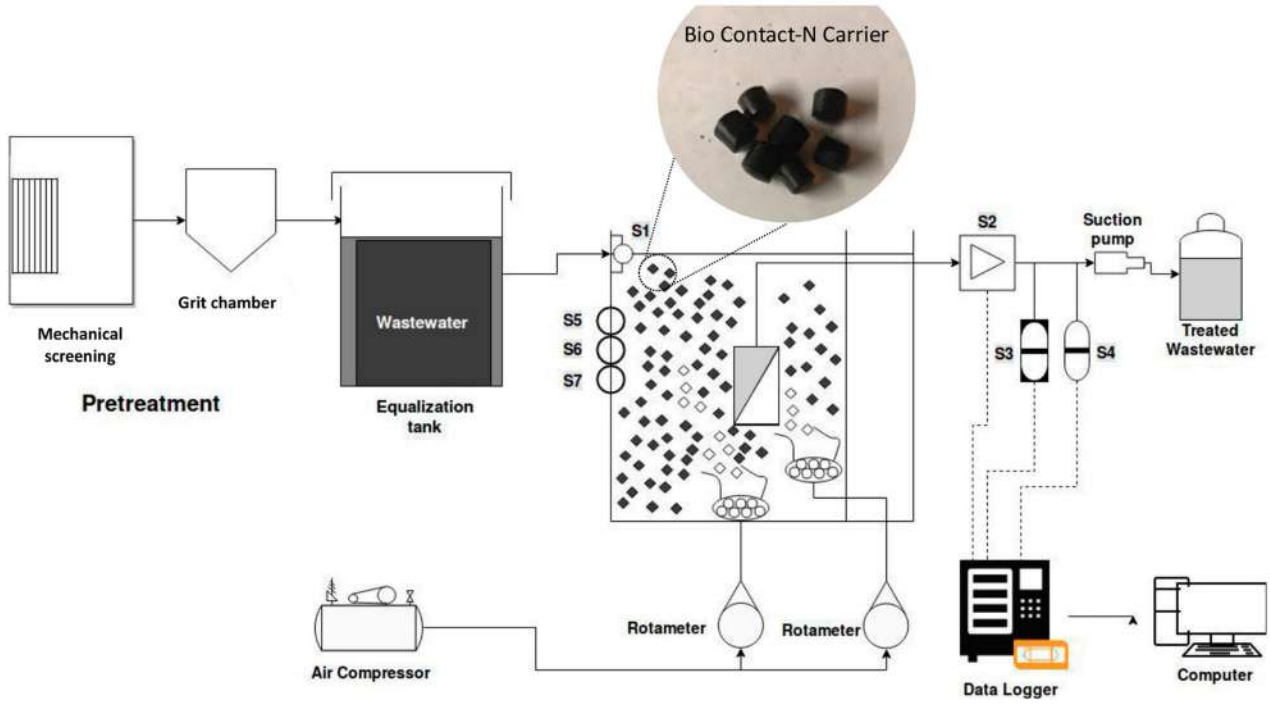

Figure 1. MB-MBR pilot plant configuration, where: S1 – level sensor, S2 – flow meter, S3 – pressure transmitter, S4 – temperature sensor, S5 – dissolved oxygen probe, S6 – ORP probe and, S7 – pH sensor.

weight of MLVSS.

$$\eta_{global} = \left(\frac{NT_{influent} - NT_{effluent}}{NT_{influent}}\right) \cdot 100\% \quad (2)$$

$$\eta_{assimilation} = \left(\frac{Y_{obs} \cdot \Delta COD \cdot g}{NT_{influent} - NT_{effluent}}\right).100\% \quad (3)$$

$$\eta_{NDS} = \left(\frac{NT_{influent} - NT_{effluent} - Y_{obs} \cdot \Delta COD \cdot g}{NT_{influent} - NT_{effluent}}\right) \cdot 100\% \quad (4)$$

where g = mass of nitrogen per biomass (mg N/mg/VSS), Yobs = observed yield (0.23 ± 0.10 mg-VSS/mg COD for Phase I and 0.32 ± 0.07 mg-VSS/mg COD for Phase II) ΔCOD = COD removed (mg/L), $NT_{influent}$ and $NT_{effluent}$ = influent and effluent total nitrogen concentrations (mg/L).

Determination of the ammonium oxidation rate (AOR) and the denitrification rate (DNR) were performed at the end of each phase in a batch test with a working volume of 3 L at ambient temperature [28]. In order to determine the contribution of each type of biomass to the nitrogen conversion process, experiments were carried out on suspended biomass and biofilm, which represents the total rate (AOR and DNR), and also on suspended biomass only (see Figures S1 and S2 of the supplementary material). Therefore, the biofilm contribution was calculated as the difference between total and suspended rates. In steady state condition the absorption of nitrogen was neglected and only the biological process associated with the biofilm was considered as the main mechanism for nitrogen removal.

$NaNO_3$ and CH_3COONa were used to provide nitrate and carbon source, respectively, for the DNR estimate. The solution contained NO_3^-–N of 16.5 mg/L. For the AOR tests NH_4Cl and $NaHCO_3$ were used to provide ammonium and alkalinity source, respectively. This resulted in a solution with 39 mg/L NH_4^+–N.

Table 1. Wastewater characteristics during experimental period.

Parameters	Phase I	Phase II
pH	6.7 ± 0.3	7.1 ± 0.4
COD, mg/L	464 ± 200	579 ± 70
TKN, mg/L	41 ± 4	87 ± 7
NH_4-N, mg/L	29 ± 3	65 ± 3
Alkalinity $CaCO_3$, mg/L	150 ± 13	284 ± 32
P_{total}	4.3 ± 0.9	4.1 ± 0.3
COD/TN ratio	11.3	6.7

Figure 2. Carrier material Bio Contact – N (BCN).

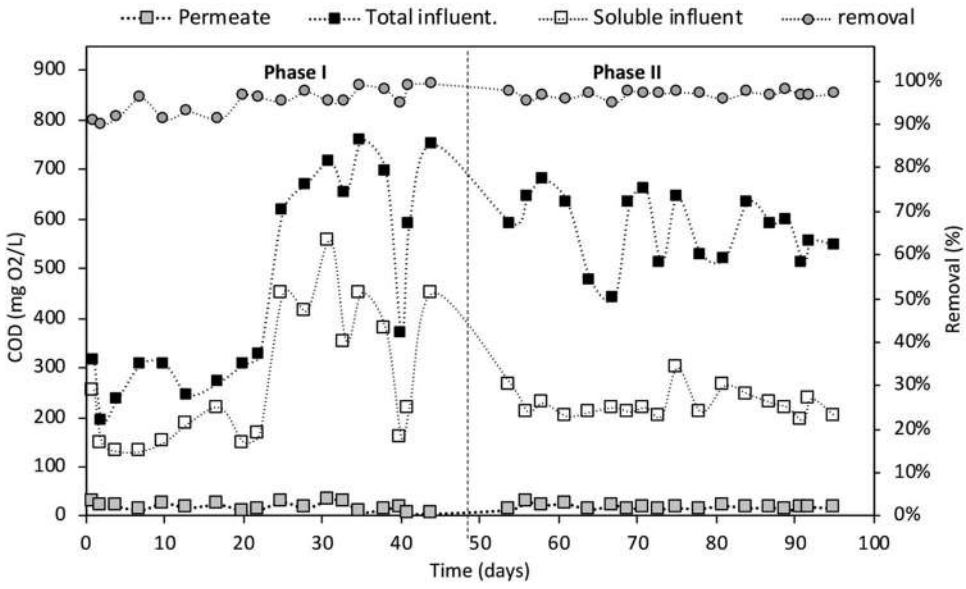

Figure 3. Variation in COD concentration during steady state operation.

2.4. Sampling, DNA extraction, Illumina sequencing and bioinformatic

For the in-depth microbiome characterization, the strategy adopted was 16S rDNA gene amplicons sequencing. For this approach, two technical replicates were sequenced from suspended biomass (SB) and biomass attached in the support material (SM) at the end of Phases I and II, yielding a total of eight sequencing datasets (SB_Phase_1; SM_Phase_1; SB_Phase_2 and SM_Phase_2). For the extraction of SB, 10 mL of sludge were collected, on the other hand, in order to collect biomass from SM, approximately, 1.0 L of support material (BCN) was collected and washed with distilled water to withdraw the excess of suspended sludge. After that, SM was put in an agitator table for 1 h to facilitate the detachment of biomass; following the centrifugation at 3000 rpm for three minutes to concentrate the biomass. The biomass from SB and SM were washed with phosphate buffered saline (PBS) and frozen at −20° C, without PBS.

Table 2. Nitrogen concentrations in permeate, removal efficiencies.

Parameters	Phase I	Phase II
Total nitrogen (mg N/L)	33.6 ± 3.9	34.7 ± 3.5
TKN (mg N/L)	8.5 ± 2.2	11.8 ± 1.6
Ammonia nitrogen (mg NH_4^+–N/L)	3.3 ± 1.1	6.7 ± 1.6
Nitrite (mg NO_2^-–N/L)	–	8.2 ± 2.4
Nitrate (mg NO_3^-–N/L)	16.6 ± 2.5	3.6 ± 2.8
TN removal (%)	18 ± 8	66 ± 11
TKN removal (%)	79 ± 5	86 ± 2
Ammonia removal (%)	89 ± 4	90 ± 3

The samples were sequenced using the MiSeq single-end 1 × 300 bp by Neoprospecta Microbiome Technologies, Florianópolis, SC, Brazil. The DNA was extracted using the PowerSoil ® DNA Isolation kit (MoBio Laboratories, Inc., Carlsbad, CA, USA) according to manufacture procedure. The 16S rDNA genes were amplified using a primer set that flanked the V3 and V4 hypervariable regions. The primers used were 341F (5′ – CCT ACG GGR SGC AGC AG – 3′) and 806R (5′ – GGA CTA CHV GGG TWT CTA AT – 3′) as described by Caporaso et al. [29].

The raw sequences were processed using QIIME software [30]. Initially, a quality check was performed by the FASTQC software [31]. Sequences with size less than 100 bp and sequences identified as chimera by VSearche 2.7.1 software were removed [32]. Operational taxonomic units (OTUs) were defined using 97% sequence similarity and taxonomic classification was performed using green genes as a database. The results were exported to R software for statistical analysis, alpha and beta diversity and graphs using mainly the phyloseq [33] and ggplot packages.

The statistical analysis was divided into reproducibility check of each technical replicate, replicate combination and, finally, data analysis. Beta diversity (Principal Coordinate Analysis – PCoA) by Bray Curtis and Jaccard metrics was used to compare the similarity of the samples, while the alpha diversity was obtained from parameters such as Shannon, Chao1 and observed richness. The sequences were submitted to the European Nucleotide Archive (https://www.ebi.ac.uk) under the project accession number PRJEB31623.

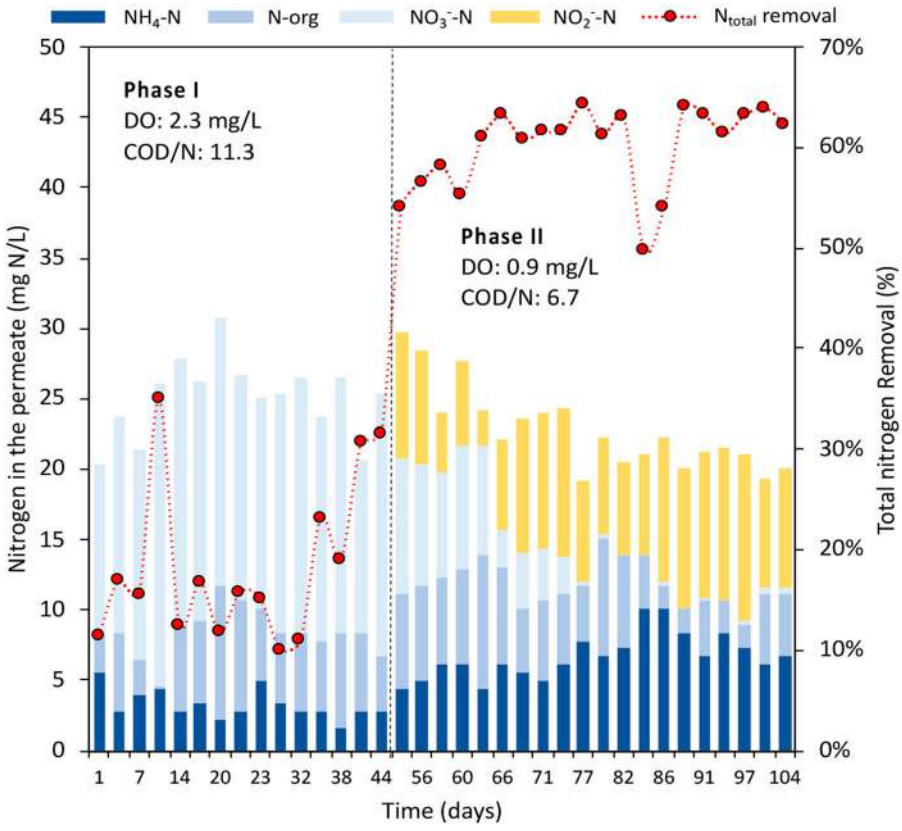

Figure 4. Nitrogen composition of the permeate and total nitrogen removal during steady state condition.

3. Results and discussion

3.1. Treatment performance of the MB-MBR

Figure 3 present the variation of COD concentration in the permeate and its removal efficiencies obtained in the two operational phases. During the experimental period the COD concentration in effluent was low. In Phase I (DO of 2.3 ± 0.2 mg O_2/L), the MB-MBR produced an effluent with only 18 ± 9 mg/L (95 ± 3% of removal). The same concentration was observed in the Phase II permeate when the system was operated at 0.9 ± 0.3 mg O_2/L and the COD concentration was 18 ± 5 mg/L. In addition, despite the variations in the influent COD (200–700 mg O_2/L), its concentration remained quite stable in the effluent, regardless the DO levels in the mixed liquor.

Indeed, several studies have demonstrated that the operation of the MBR system in SND conditions has low influence on the removal of organic compounds. Subtil et al. [5], which worked the same pilot plant without a biofilm, reported similar behaviour. The MBR system was able to remove more than 95% of COD even when the DO was maintained at 0.4 mg O_2/L. Rodríguez-Sánchez et al. [34], as well as in this study, also observed that there was no significant variation in the COD removal under different DO concentrations (0.4 to 2.4 mg/L).

The ability of the MB-MBR system to withstand raw wastewater variations, even at low DO concentrations, and to maintain low permeate COD levels can be explained, firstly, by the high membrane efficiency. Its high separating capacity prevents the release of suspended solids into the effluent (particulate COD), even under unfavourable floc formation conditions, which is expected for biological aerobic system under low levels of dissolved oxygen. Secondly, by the oxygen half-saturation coefficient for mixed cultures of heterotrophs ($K_{O,H}$), which depends on the DO concentration, and must be very low (0.01 to 0.15 mg/L) to affect the efficiency in the elimination of organic compounds due to grow of floc-forming and filament bacteria [35,36]. Therefore, the results of this study and other studies which also investigated the effect of DO suggest that the removal of organic compounds in MBR/MB-MBR with SND process, when operating with typical ranges of HRT (3–8 h), SRT (15–30 d) and F/M ratio (0.08–0.2 Kg BOD_5/Kg MLSSV.d), depended only on the non-biodegradable fraction of COD in wastewater.

In order to obtain a more in-depth understanding of the findings shown here, the efficiency in the conversion

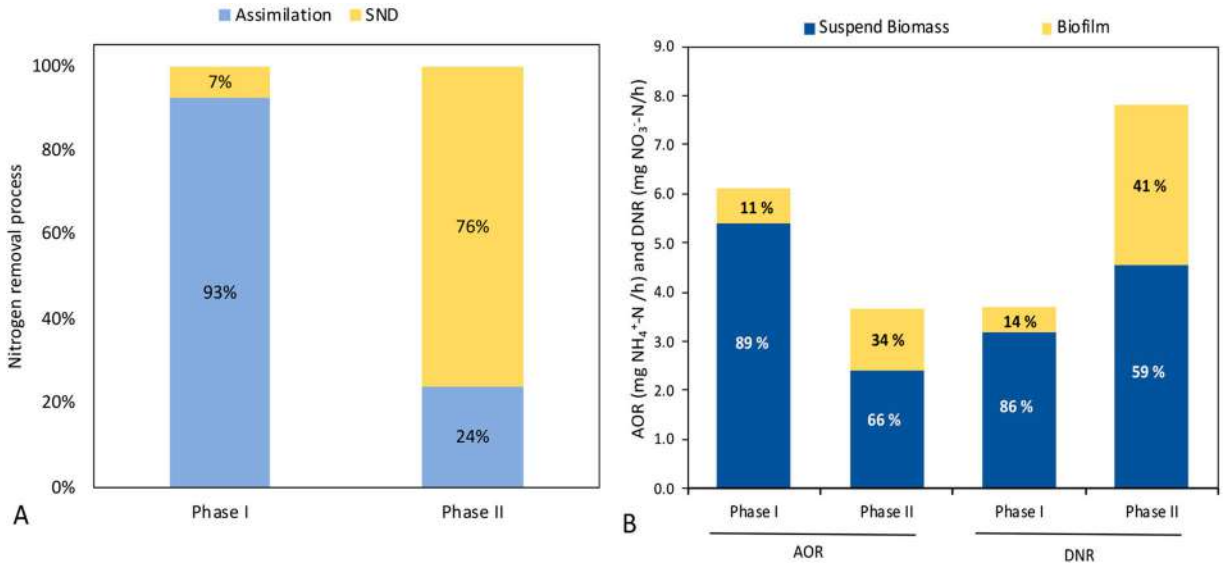

Figure 5. (A) Relative amounts of nitrogen removed by assimilation (Equation (3)) or SND (Equation (4)) and (B) AOR, DNR and the relative contribution of biofilm and suspended biomass.

of Total Nitrogen (TN) and the mean values in the treated wastewater of nitrogenous content were studied (Table 2). In Phase I, when DO was approximately 2.3 mg/L and COD/TN was approximately 11.3, it can be shown that NH_4^+–N and TN removal efficiencies averaged 89% and 18%, respectively. Similar NH_4^+–N removal efficiencies were observed in Phase II (90%), with no significant difference. On the other hand, TN removal increased significantly when the DO decreased to about 0.9 mg O_2/L, achieving an average TN removal of 66%. Throughout this time, the removal of TN consistently showed higher efficiency than that achieved in Phase I and did not show any distinct differences (Figure 4). As a result, approximately 76% of the removed total nitrogen (66%) in Phase II was via SND, compared to just 7% in Phase I (Figure 5A). In Phase I, therefore, the key process responsible for nitrogen removal was assimilation, which accounted for nearly 93% of the 18% of total nitrogen removed.

In both phases, the AOR and DNR were calculated to investigate the effect of dissolved oxygen and the contribution of each biomass in the processes of nitrogen conversion (nitrification and denitrification). As can be seen in Figure 5B, the AOR was higher in Phase I (6.11 mg NH_4^+–N/Lh) than in Phase II (3.66 mg NH_4^+–N/Lh) corresponding to a reduction of about 40%. Although in optimal conditions (only ammonia as substrate) a significant reduction in AOR is expected at DO concentrations of less than 0.5 mg/L [37], in the presence of organic substrates such as sanitary wastewater, ammonia oxidizing bacteria (AOBs) compete with fast-growing heterotrophic oxygen bacteria, limiting AOR. This was observed by Satoh et al. [38], who reported a decrease in the AOBs population compared to heterotrophs when biodegradable organic compounds were introduced, suggesting that heterotrophs can overcome competition with AOBs for oxygen. Similar result was found by Hanaki et al. [39], which suggested that organic load at low DO levels could inhibit the nitrification in suspended and attached growth reactor. This fact explains the 40% reduction in AOR observed in Phase II of this study.

However, despite the reduction in AOR at low DO concentrations, the nitrification capacity of the biomass was still able to promote high rates of ammonia removal (90%). In low DO concentration an efficient nitrification has been justified by the increase in oxygen affinity by AOBs, mainly of the groups *Nitrosomonas* sp., *Pseudomonas* sp., *Xanthomonadaceae*, *Rhodococcus* sp. and *Sphingomonas* sp. If high sludge age and low organic loads are maintained, near-complete nitrification can be achieved with low DO concentrations (<0.5 mg O_2/L) [40,41].

The decrease in DO in Phase II resulted in a substantial increase in DNR from 3.70 mg NO_3^-–N/L h in Phase I to 7.80 mg NO_3^-–N/L h in Phase II. Furthermore, the contribution of the biofilm to the nitrification and denitrification rates was higher when the concentration of DO was reduced. In the case of DNR, biofilm accounts for almost 41% at low DO levels, while in Phase I it was only 14%. The depth of the aerobic zone in the biofilm depends on the level of oxygen and the rate of reduction of the microorganisms. Since nitrification occurs on the carrier surface and denitrification occurs in the deepest

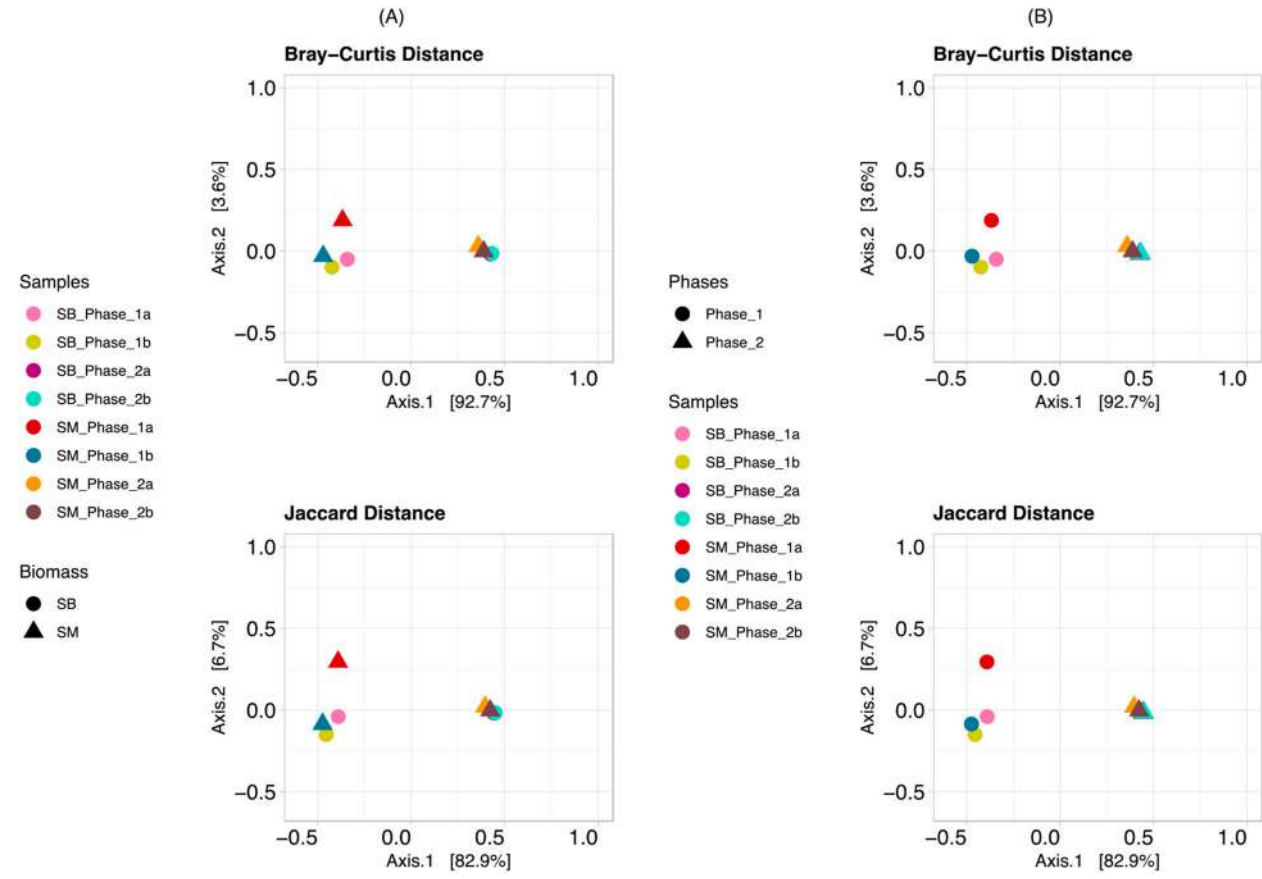

Figure 6. Principal coordinate analysis (PCoA) plot using Bray-Curtis and Jaccard distances from filtered data. (A) Samples divided according to the place of sampling. SB – suspended biomass and SM – support material. (B) Samples divided according to the Phase of operation.

layer of the biofilm, these results support the hypothesis that SND was caused by an oxygen diffusion limitation on the biofilm, thereby generating an anoxic condition within the biofilm [42,43].

The reduction of DO and the COD/N ratio led to a shift in oxidized nitrogen forms in the permeate (Figure 4). In Phase I, NO_2^-–N was not detected and NO_3^-–N was the main nitrogen compound (16.6 ± 2.5 mg N–NO_3^-/L), while nitrite became the major NOx compound in the permeate of Phase II, with an average concentration of 8.2 ± 2.4 mg NO_2^-–N/L. Although nitrite accumulation can occur due to incomplete nitrification at low DO concentrations, its presence in the effluent of Phase II may be mainly linked to a lack of adequate/sufficient carbon to allow for full denitrification, as well as a low COD/N ratio in the DO limiting environment. Therefore, the low COD/N ratio (6.7) in Phase II and the strong nitrification and denitrification efficiency shown by the results of AOR and DNR suggest that the key explanation for nitrite production and accumulation was predominantly incomplete denitrification. Ge et al. [44] observed that denitrification in a high COD/N ratio result in smaller total N_2O emission, which is related at the same time to the inhibition in the production of nitrite. On the other hand, using batch cultures of denitrifying bacteria, Sobieszuk and Szewczyk [45] showed the critical value for microbial denitrification of COD/N ratio (7.6) being similar to the value obtained in phase II of the present study. Furthermore, the lower AOR observed in Phase II may have favoured organic compound consumption by heterotrophs using O_2 as electron acceptor over denitrification.

3.2. Molecular biology analysis

3.2.1. Diversity and richness of microbial communities

Four samples with two technical replicates each were sequenced, yielding a total of eight datasets. In the total, 2,146,848 reads were obtained with a sequence length ranging from 205 to 305 bp. In the filtering step, 49.7% of reads were removed due to low-quality, remaining 1,068,110 reads. After that, in the clustering step, 455 different OTUs were obtained; the minimum and maximum number of reads per sample was 33,000

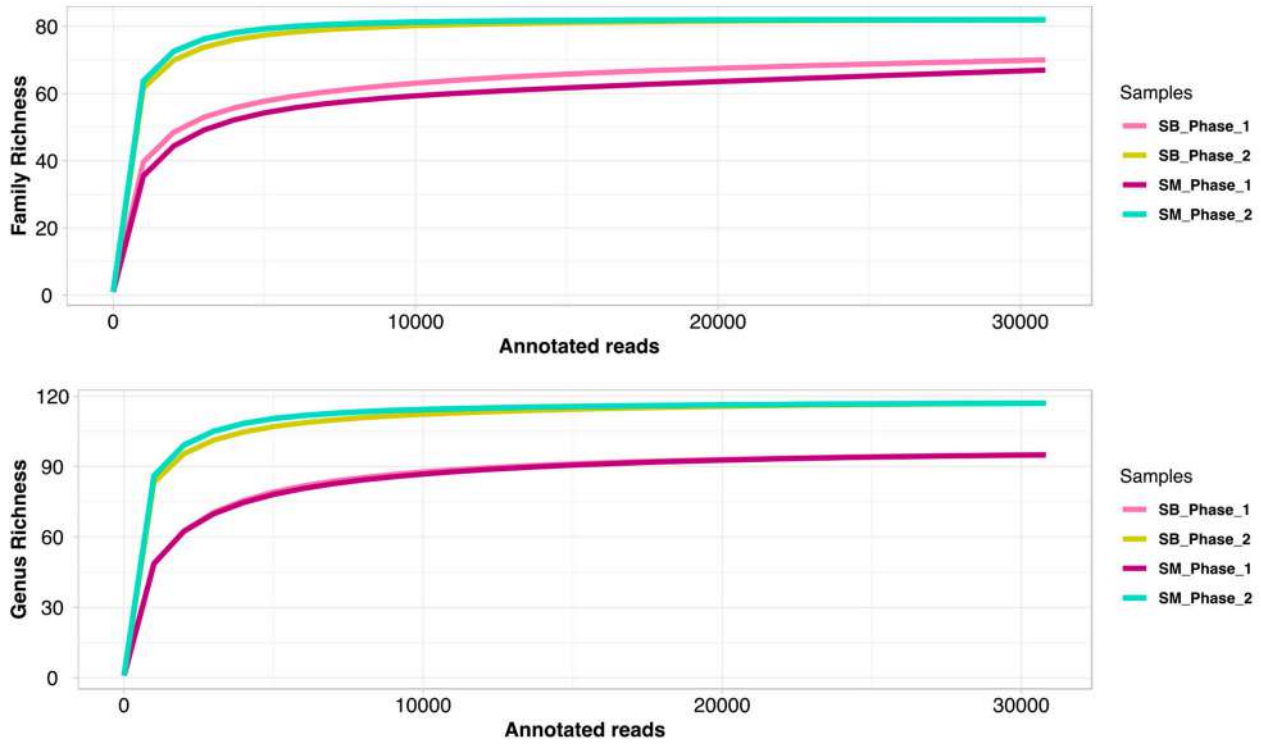

Figure 7. Rarefaction curve at family and genus level.

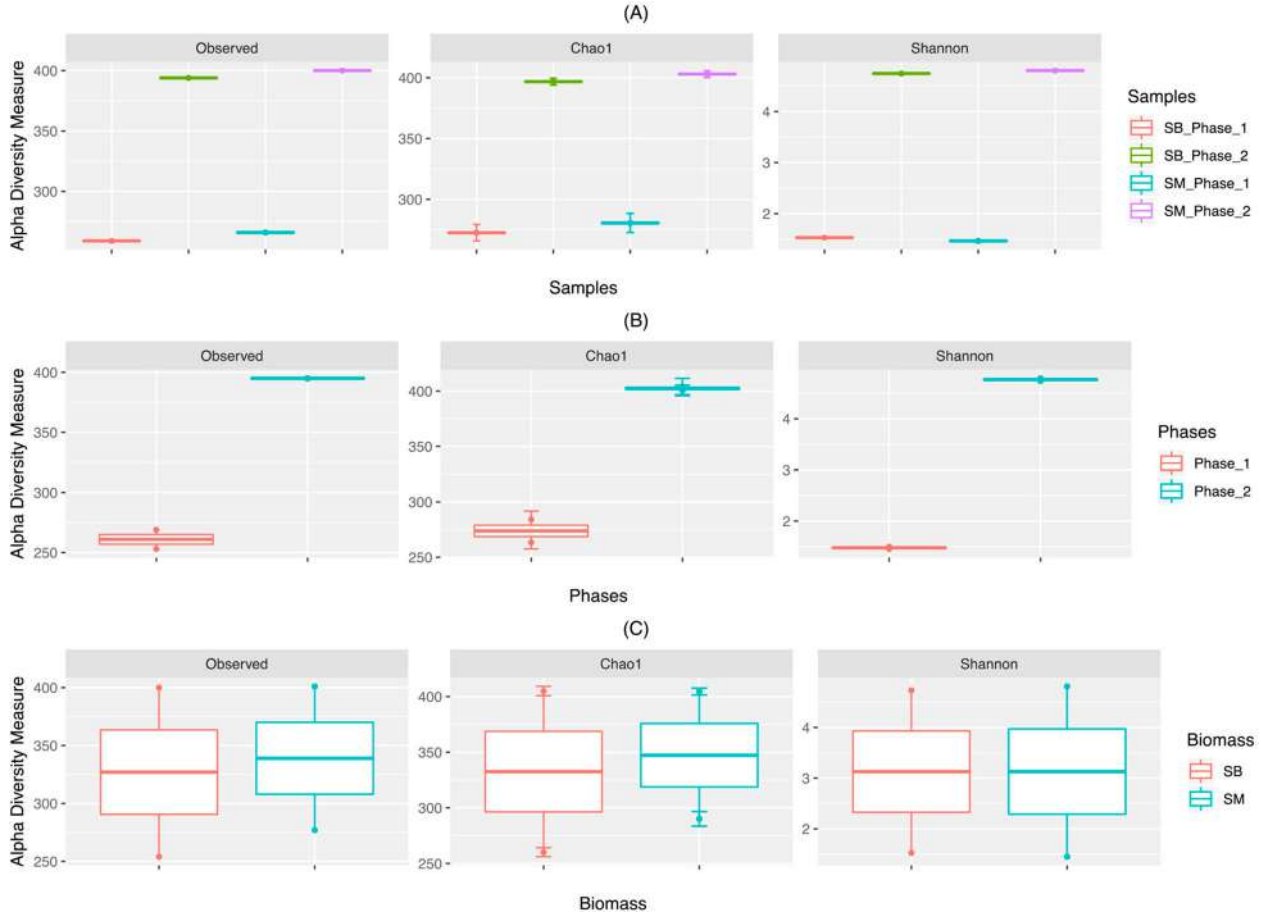

Figure 8. Alpha diversity measure. (A) Values according to each sample. (B) Values according to operation Phases. (C) Values according to place of sampling.

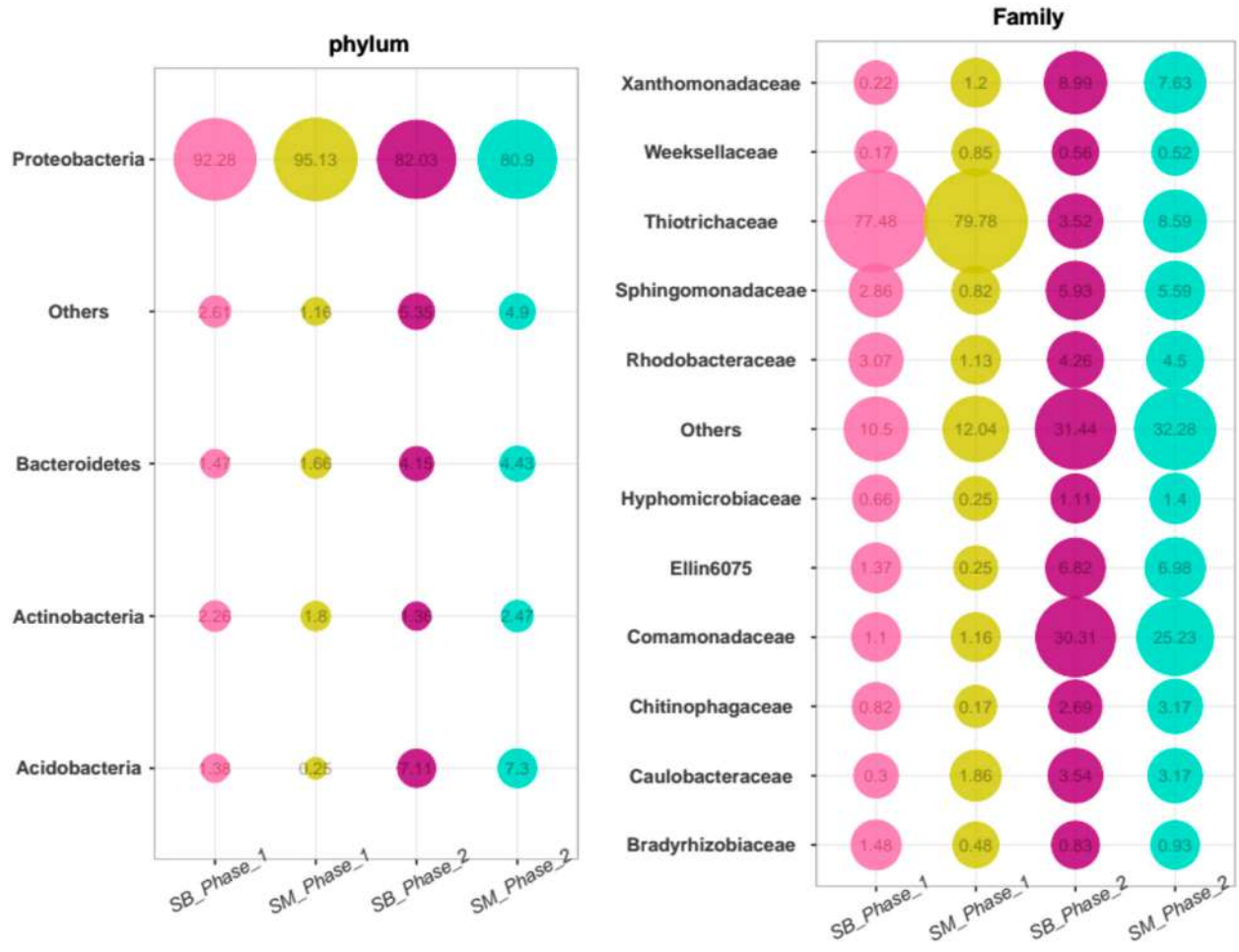

Figure 9. Relative abundance at Phylum and Family level.

and 157,000, respectively. Before the analysis, the samples were normalized by the smallest library.

Principal coordinate analysis (PCoA) method was applied in order to verify the similarities and dissimilarities of samples and the technical replicates (Figure 6). Regardless of distance metric (Bray–Curtis or Jaccard), the samples showed spatial arrangement based on the Phase of operation and not based on the sampling location (suspended biomass and support material). Moreover, the technical replicate was very close, indicating a high correlation between them. Rarefaction analysis revealed that 10,000 reads were sufficient to access the whole richness in the samples at family and genus level (Figure 7). These results were corroborated by values of richness observed and richness estimators (Chao 1; Figure 8) in each sample, in which both values were similar.

As well as observed in the results of PCoA, in the comparison alpha diversity values among the samples, the differences occurred on the basis of the operating phase and not between the sampling site (Figure 8B, C). SB_Phase_1 and SM_Phase_1 showed Shannon value ranging from 1.53 to 1.45, whereas, SB_Phase_2 and SM_Phase_2 showed ranging from 4.75 to 4.80, respectively. Probably, the DO concentration (Phase I: 2.3 ± 0.2 mg O_2/L and Phase II 0.9 ± 0.3 mg O_2/L) may have been responsible for the variation in microbial diversity between Phases. Additionally, as a consequence of the DO variation, it was observed variation of relative abundance of Comamonadaceae, Sphingomonadaceae and Xanthomonadaceae that shows nitrifying and denitrifying species. Yan et al. [45] reported that the diversity and relative abundance of denitrifying species increased under conditions of low oxygen concentration, with Comamonadaceae being the family with the highest abundance. Similar values of Shannon observed were presented by Yan et al. [46] and Matar et al. [47].

3.2.2. Overall taxonomic microbial population

The taxonomic profiles of datasets were determined from the annotation of reads against the GreenGenes database. Predominantly, the sequences were assigned to the bacterial domain. In the phylum level,

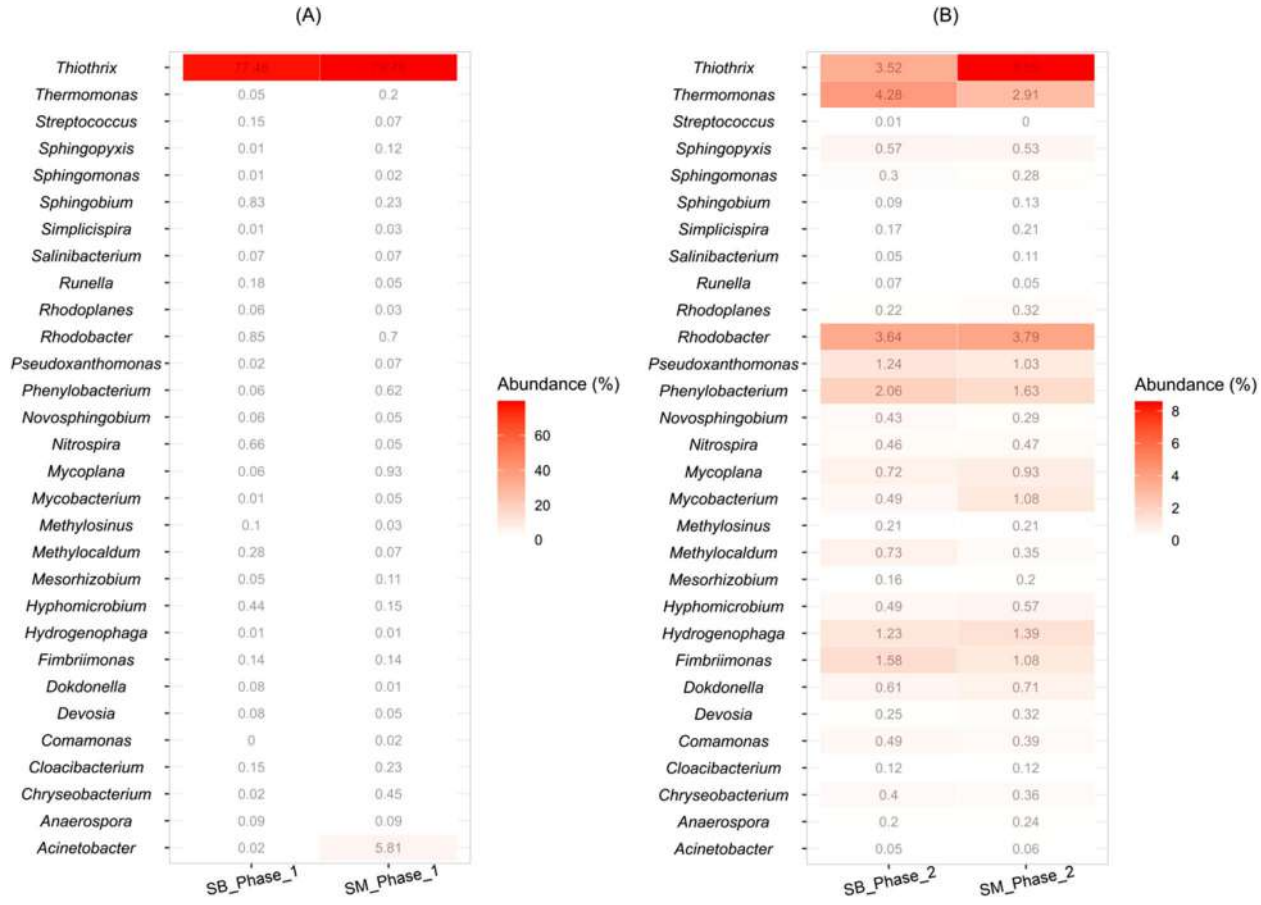

Figure 10. Heatmap of relative abundance of samples at genus levels. (a) Phase 1 and (b) Phase 2.

Proteobacteria, followed by Acidobacteria, Bacteroidetes and Actinobacteria were the most abundant (Figure 9). It can be seen that the percentage of Proteobacteria decreased from Phase I (92.3% and 95.1%) to Phase II (82% and 81%), while Bacteroidetes and Acidobacteria increased from 1.5–1.7% per and 1.4–0.3% in Phase I to 4.2–4.4% and 7.1–7.3% in Phase II, respectively.

Interestingly, at the family level, Thiotrichaceae was the most abundant in the samples SB Phase 1 and SM Phase 1, whereas Comamonadaceae and Xanthomonadaceae were the most abundant in the samples SB Phase 2 and SM Phase 2.

At the genus level (Figure 10), *Thiothrix* genus was the most abundant in the samples SB_Phase_1 and SM_Phase_1 with Log_{10} values of relative abundance ~1.9 and relative abundance ranging from 77% to 79% (Figure 9). On the other hand, in the samples SB_Phase_2 and SM_Phase_2, there was not a dominant genus. This contrasts with Phase I samples, where *Thiothrix* appears as the genus with the greatest relative abundance, not exceeding 8.5% though. It is important to emphasize that, at the genus level, the percentage of unclassified in these samples reached >70%, against 8–17% in the samples from Phase I. The majority of the unclassified in the Phase II families belong to Comamonadaceae, Sphingomonadaceae, Xanthomonadaceae, Chitinophagaceae, Rhodobacteraceae and Hyphomonadaceae. Others important genera identified in the Phase 2 were *Rhodobacter* (~0.57) and *Thermomonas* (~0.46–0.63).

The main genera able to utilize nitrite or nitrate as final receptor of electrons and to actuate in the nitrifying and denitrifying processes were: *Thiothrix, Comamonas, Rhodobacter, Mycobacterium, Thermomonas, Sphingobium, Sphigopyxis, Pseudoxanthomonas, Nitrospira and, Novosphingobium* [48–54]. With regard to the observed family Comamonadaceae of the genus *Comamomas*, which was the most abundant in the samples from Phase II (SB_Phase_2 and SM_Phase_2), Chen and Ni [49] demonstrated that they are able to nitrify heterotrophically and denitrify aerobically, presented in a maximum specific growth rate (μ_{max}) of 0.38 h^{-1}, about ten times higher than the nitrifying autotrophic microorganisms (*Nitrosomonaeuropea* presents an μ_{max}, the equivalent of 0.03 to 0.05 h^{-1}). The speed of *Comamonas* N-total removal obtained by the authors was 0.381 mmol L^{-1} h^{-1}. The experiment was developed by measuring the quantity of oxidized ammonia minus

the nitrite and nitrate quantity in the end, divided by the time. The authors concluded that, under great conditions of carbon source (quantity and quality), it is possible that the heterotrophic nitrifying process, beside the aerobic denitrifying, undertaken by this genus of microorganisms, may have a significant role in the process of nitrogen removal in the reactor.

Besides, another relevant genus in denitrifying process noticed in this study belong to Xanthomonadaceae; the *Pseudoxanthomonas* was regarded as the responsible for reducing nitrite to N_2O, since it is optional anaerobic [55–57]. The genus *Nitrospira*, observed in this study, was the main reported genus influencing the NOB process, being reported by Yao and Peng [57], Brito [48] and Daims et al. [53], as quimioautotrophic microorganisms that are able to oxidize nitrite and nitrate.

One relevant fact, described by Brito [48] and found in this present study, encompassing the wider relative abundance, was the presence of optional aerobic microorganisms in both operation Phases. In elevated DO concentrations (Phase I), it seems probable that these microorganisms utilized oxygen as a final receptor of electrons, but, in lower DO concentrations (Phase II), they utilized nitrite or nitrate, even both together, as final receptor of electrons. Probably, there might have been a bigger adaptation of theses microorganisms in the reactor, when the DO concentration variation existed, thereby oxidizing organic compounds aerobically in the Phase I and denitrifying heterotrophically in the Phase II.

Conclusion

The results show that it is feasible to eliminate organic contaminants with high efficiency at different DO concentrations, allowing SND process and energy savings. High organic compounds removal values were obtained in the two Phases. Another important outcome was the accumulation of nitrite in Phase II, mainly as a by-product of incomplete SND process. This showed that TN removal was impacted by DO concentration and COD/N ratio.

The biofilm plays an important role on nitrification and denitrification processes in low dissolved oxygen condition, which could be in fact an interesting approach to improve nitrogen removal in SND processes. In view of the high denitrification potential of the developed biofilm, increasing the carrier filling ratio is an important strategy for improving the removal of total nitrogen at lower DO concentrations. This is a relevant aspect for existing WWTPs where the addition of the carrier could increase the removal of total nitrogen, but without the need to build additional treatment units.

The molecular biology analysis allowed us to identify important bacteria in the processes of SND and organic compounds removal. No significant differences were identified in the bacterial communities depending on the type of biomass (suspended biomass and support material). However, the beta diversity analysis showed clustering of the samples in relation to the Phases of operation, indicating that the differences may have occurred due to the concentration of DO. *Thiothrix*, *Comamonas*, *Rhodobacter*, *Mycobacterium*, *Thermomonas*, *Sphingobium*, *Sphigopyxis*, *Pseudoxanthomonas*, *Nitrospira* and, *Novosphingobium* were the main genera regarding the nitrogen cycle.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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Data availability statement

The authors confirm that the data supporting the findings of this study are available at https://www.ebi.ac.uk under the project accession number PRJEB31623 and its supplementary material.

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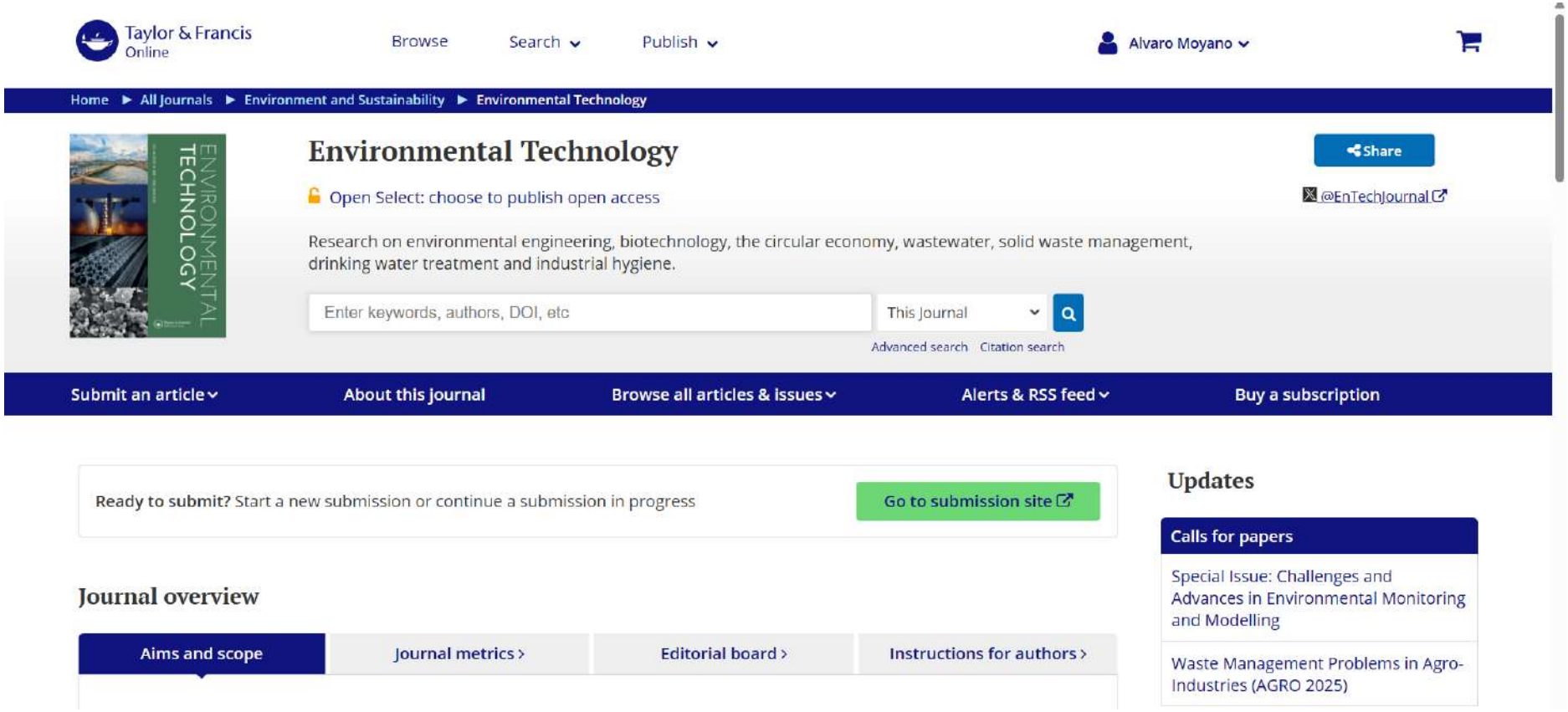

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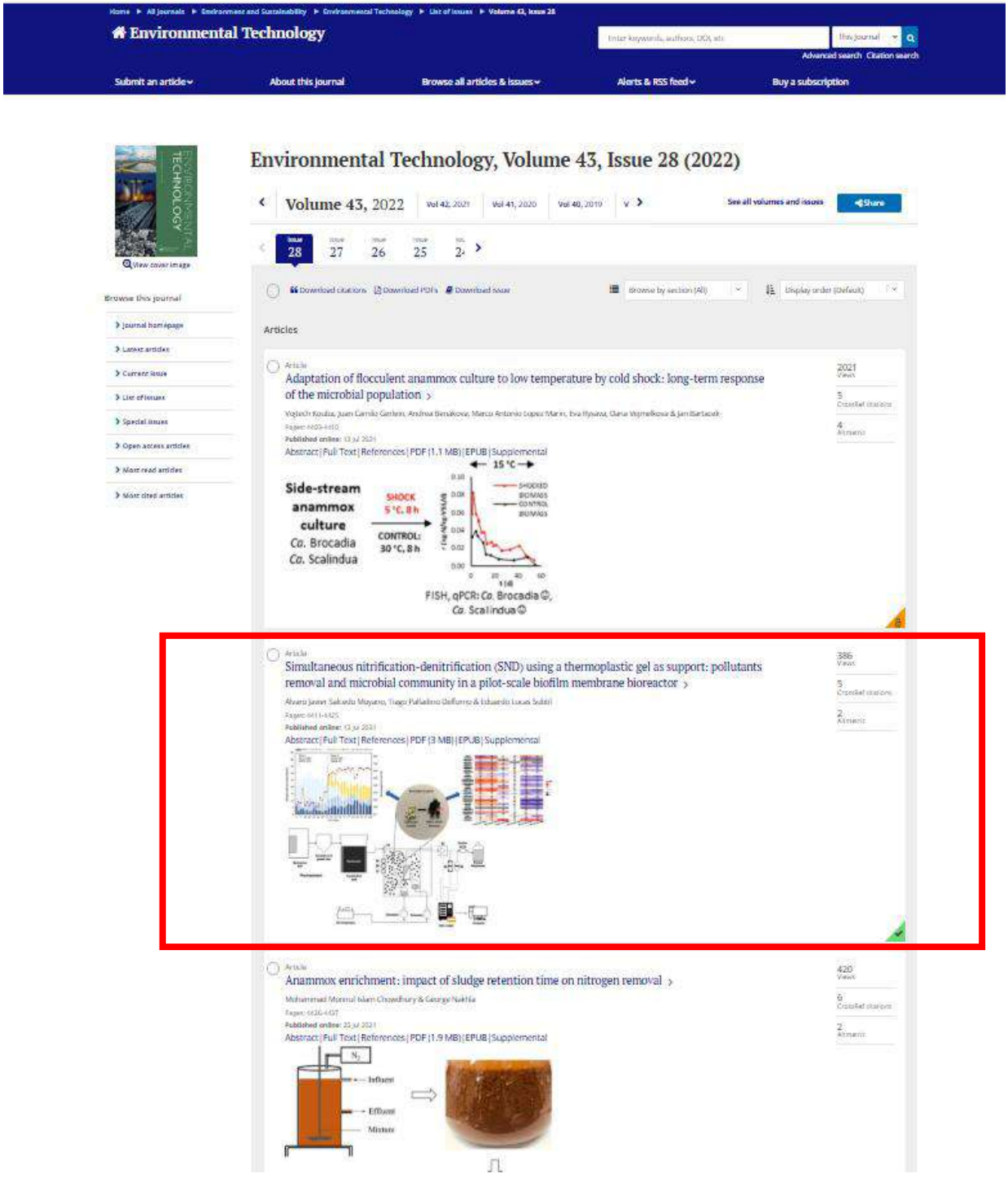

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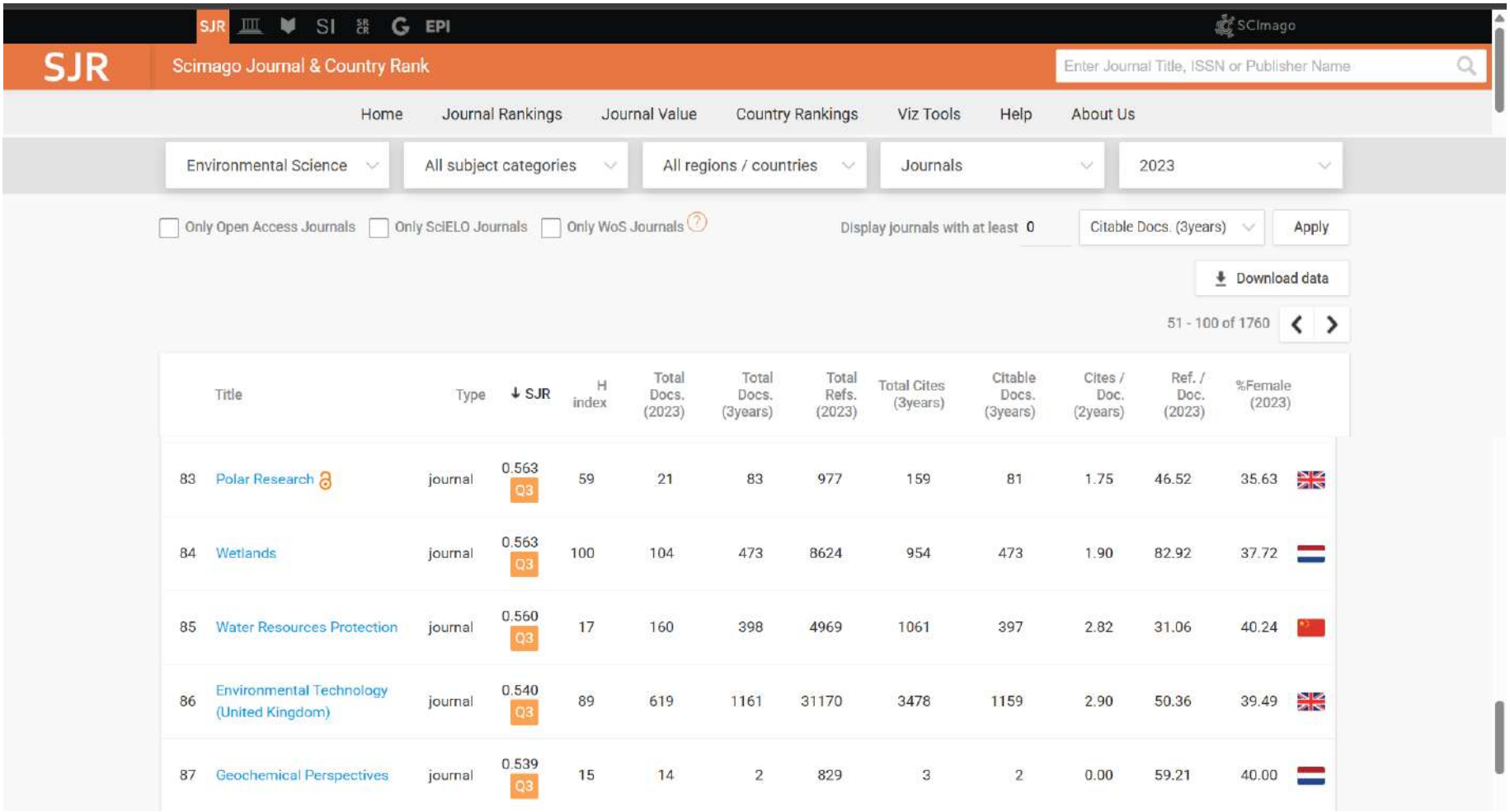

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83	Polar Research	journal	0.563 Q3	59	21	83	977	159	81	1.75	46.52	35.63
84	Wetlands	journal	0.563 Q3	100	104	473	8624	954	473	1.90	82.92	37.72
85	Water Resources Protection	journal	0.560 Q3	17	160	398	4969	1061	397	2.82	31.06	40.24
86	Environmental Technology (United Kingdom)	journal	0.540 Q3	89	619	1161	31170	3478	1159	2.90	50.36	39.49
87	Geochemical Perspectives	journal	0.539 Q3	15	14	2	829	3	2	0.00	59.21	40.00