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Effect of 4-nonylphenol on the performance and microbial community of a sequencing batch reactor

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ABSTRACT

4-nonylphenol (4-NP) is one of the most relevant endocrine-disrupting compounds that can be found in wastewaters. In this work, the effect of dosing 1 $mg{\cdot}L^{-1}$ of 4-NP to simulated wastewater on the activated sludge process was assessed. For it, two laboratory sequencing batch reactors (SBR) were operated for 94 days, adding 1 $mg{\cdot}L^{-1}$ of 4-NP to the wastewater entering one of them (SBR-NP), while the other one (SBR-B) worked as a control reactor. Holistic study of 4-NP influence on activated sludge treatment was carried out, which included both the evolution of the biomass characteristics and the effect of this substance on reactor performance. Although the COD removal efficiency in SBR-NP was lower than in the reactor without 4-NP addition (SBR-B), COD removal efficiency of SBR-NP was always higher than 90%. From day 50, nitrification bacteria were inhibited in SBR-NP and cellular viability decreased from 85.7 ± 11.0% in the first 50 days to 63.0 ± 10.2% in the last 44 days. Concerning the microbial community analysis, both *Nitrosomonas* and *Nitrospira* abundances decreased in SBR-NP (from 0.62% to 0.45%, and from 2.39% to 1.01%, respectively). *Proteobacteria* abundance was considerably higher in SBR-NP at the end of the experiment (44.28% in SBR-NP and 25.88% in SBR-B), which was due to increase of *Aquabacterium* genus (13.00% and 0.00% in SBR-NP and SBR-B, respectively), playing an important role in 4-NP degradation. Thus, 4-NP presence, in the concentrations studied, affected heterotrophic and autotrophic bacteria differently, having a negative effect in the second group.

1. Introduction

Occurrence of endocrine-disrupting compounds (EDCs) in wastewater has been investigated in the last two decades. These compounds are harmful for the endocrine system and are capable of altering the development and the reproductive functions of animals and humans [1]. 4-nonylphenol (4-NP) is one of the most relevant EDC, whose effect on aquatic species has been widely studied [2,3].

Nonylphenol (NP) is a degradation product of nonylphenol ethoxylate (NpEO), as a result of long-chain break of NpOE in shorter-chain metabolic intermediates, due to biotransformations in sewer systems and activated sludge processes [4,5]. NpEO is included in alkylphenol ethoxylates (APEO) family [6], which are non-ionic surfactants widely used in the industry, and other products like NP phosphites (chemicals used in the rubber and plastic industries), detergents, emulsifiers and aminocarb insecticide spray [7,8]. Approximately 80% of produced APEO are NpEO, 60% of which reach the environment according to estimations of some researchers [9,10].

The use of NP and NpEO has been regulated by Directive nº 2003/53/EC of European Commission. These substances cannot be placed on the market, or used as constituents of preparations, in concentrations equal or higher than 0.1% (mass). For example, it has to be accomplished in cleaning, textile, leather and cosmetic products. In spite of it, many authors have reported about NP and NpEO occurrence in natural waters. Grund et al. [11] found NP and other EDCs in sediments of Danube River (Germany) in samples collected in 2006. More recently (2019), Spataro et al. [12] detected 4-nonylphenol and nonylphenol mono- and di-ethoxylate in all the wastewater treatment plants (WWTPs) treating Rome wastewaters. Occurrence of these substances was also reported in other countries. Lee et al. [13] measured 4-NP concentration in 16 rivers in Taiwan, detecting it in all the samples analyzed. Jiang et al. [14] reported that NPs were the predominant endocrine-disrupting chemicals in the influents and effluents of the 38 studied WWTPs in China. In view of the data described above, the

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presence of NP in wastewaters is a global problem, which must be studied in depth to solve it.

NP structure depends on the location of nonyl group in the phenol ring, being the 4- position the most usual [15]. Focusing on 4-NP biodegradation, Liwarska-Bizukojc et al. [16] studied the removal of three persistent pollutants, 4-NP among them, at laboratory scale by activated sludge process. These authors worked at concentrations of 10 $\mu g \cdot L^{-1}$ and reported a 71% of removal efficiency. In another study [17], performed for 485 $\mu g \cdot L^{-1}$ of 4-NP, authors reported a biodegradation of 67%, which was enhanced to 91% after acclimation of activated sludge. In that work, authors concluded that biodegradation was the main removal mechanism of 4-NP, playing adsorption an almost negligible role. On the contrary, Bouki et al. [18] performed adsorption tests using 4-NP as adsorbate and active and inactive biomass as adsorbent. Results indicated that more than 90% of 4-NP was adsorbed in 1 h, which means that 4-NP accumulates on the sludge due to its hydrophobicity.

Other studies about NP degradation reported in the bibliography have been carried out by means of some isolated species such as *Aspergillus* strains [19,20] or fungus [21], among others. In these works, the initial 4-NP concentrations ranged between 20 and 100 $mg \cdot L^{-1}$, achieving removal percentages higher than 80%. NP biodegradation by microbial consortia has been also studied. Bai et al. [22] reported biodegradation of 75.61% and 89.75% of 1000 $mg \cdot L^{-1}$ NP by facultative microbial consortium named NP-M2, after 48 h and 8 days, respectively.

The contradictory results and the lack of detailed information about what happens with 4-NP in the biological treatment make necessary more research on this field. In addition, the changes on diversity in the microbial community of an activated sludge reactor treating wastewater with 4-NP has hardly been studied.

Toxicity of 4-NP on activated sludge biomass was reported by Stasinakis et al. [23]. These authors performed batch respirometric tests concluding that concentrations between 100 and 1000 $mg \cdot L^{-1}$ inhibited the heterotrophic biomass, meanwhile considerably lower values inhibited autotrophic bacteria.

In this work, two SBRs were operated in parallel for 94 days. Both reactors were fed with the same simulated wastewater, adding 1 $mg \cdot L^{-1}$ of 4-NP in the influent of one of these reactors (SBR-NP). The other reactor operated as a control system without 4-NP. To evaluate the effect of 4-NP, a deep study of mixed liquor and effluent of both reactors was performed to assess the changes in the physico-chemical and biological characteristics, including microbial community.

2. Materials and methods

2.1. Sequencing batch reactors

Two identically sequencing batch reactors (SBRs) worked for 94 days at room temperature under the same operating conditions. Fig. 1 shows a scheme of a SBR. The main parts of this laboratory plant are the following: a cylindrical tank (30×20 cm of height and diameter), a mechanical stirrer (200 rpm), an air compressor (400 $L \cdot h^{-1}$) with two air diffusers (located at the bottom of tank) and two peristaltic pumps.

In Fig. 1, some operating parameters and the steps followed in each SBR cycle are also included. In the filling and aerobic reaction (first step), both stirrer and compressor were running for 6 h to maintain homogeneous conditions and dissolved oxygen concentration above 1.5 $mg \cdot L^{-1}$. Filling (the feed volume in each cycle was 2 L) was carried out in the first 25 min of this step. In the second step, stirrer and compressor stopped for 1.5 h to allow the activated sludge sedimentation. Both devices also remained off in the third step, in which draw pump was connected (25 min) to perform the effluent drawing (2 L). Finally, to complete the 8 h of each cycle, an idle time of 5 min was necessary. The number of cycles per day was 3.

Both reactors were seeded with activated sludge from a WWTP located in Comunitat Valenciana (Spain). To maintain the mixed liquor suspended solids (MLSS) around 2.5 $g \cdot L^{-1}$ throughout the experimental period, suitable sludge withdrawal was performed in each reactor. In addition, to achieve a food to microorganisms (F/M) ratio of 0.2 kg $COD \cdot kg\ MLSS^{-1} \cdot d^{-1}$, influent (simulated wastewater; SWW) with a COD of 500 $mg \cdot L^{-1}$ was prepared. For it, 225 $mg \cdot L^{-1}$ of peptone and meat extract (as nitrogen and organic matter sources) and 28 $mg \cdot L^{-1}$ of K_2HPO_4 (as phosphorus source) were mixed with tap water. This formulation ensures a COD:N:P ratio of 100:11:1. In Table 1, the influent quality is presented.

The difference between both reactors was the presence of 4-nonylphenol (Sigma-Aldrich; CAS 104-40-5) in influent composition of one of these reactors. Both reactors worked with SWW in the first 15 days, to ensure the biomass adaptation to the new influent. From this day on, 1 $mg \cdot L^{-1}$ of 4-NP was added to the influent of SBR-NP (SWW-NP), while SBR-B worked as a control reactor without 4-NP addition.

2.2. Effluent analysis

The following physicochemical parameters were measured in the effluents of both SBRs: pH, conductivity, soluble COD and ammonia nitrogen (NH_4-N), which were analysed three times a week, and total

Table 1
Influent characteristics.

Parameters	Influent values
pH	7.6 ± 0.1
Conductivity ($mS \cdot cm^{-1}$)	0.98 ± 0.05
COD ($mg \cdot L^{-1}$)	616 ± 27
Total nitrogen; N_T ($mg \cdot L^{-1}$)	65 ± 2
Total phosphorus; N_T ($mg \cdot L^{-1}$)	7.2 ± 0.4

Operating parameters		
V_R	6	L
HRT	24	h
F/M ratio	0.2	kg COD·kg MLSS^{-1}·d^{-1}
MLSS	≈2.5	g·L^{-1}
Number of cycles	3	cycles/d
Cycle characteristics		
Filling + Aerobic reaction	6	h
Sedimentation	1.5	h
Draw	25	min
Idle	5	min

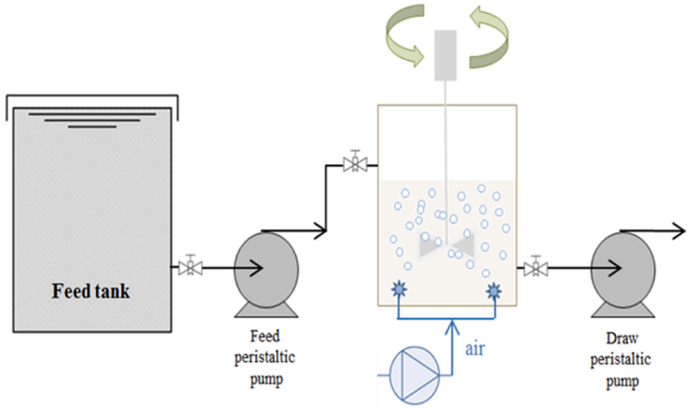

Fig. 1. Scheme of laboratory plant (SBR) and operational parameters of biological treatment.

nitrogen (N_T), other nitrogen forms (NO_3-N; NO_2-N), total phosphorous (P_T) and phosphorous in phosphate form (PO_4-P), which were measured once a week. pH and conductivity were measured with GLP 21 + and GLP 31 + (both from Crison), respectively. The other parameters were quantified spectrophotometrically by means of reaction kits and Spectroquant NOVA 30 from Merck.

Soluble microbial products (SMP) were evaluated measuring proteins and carbohydrates concentrations, since these substances comprise between 70% and 80% of the total SMP [24]. BCA method [25] and anthrone method [26] were followed to measure proteins and carbohydrates, respectively.

4-NP was measured in SBR-NP effluent by high performance liquid chromatography (HPLC) equipment from Japan Spectroscopy Corporation and Kinetex C18 (1.7 μm; 50 mm × 2.1 mm) column from Phenomenex. Methanol (A) and Milipore-water (B) were used as a mobile phase with the following sequence: 5% A + 95% B from 0 to 4.5 min; 30% A + 70% B from 4.5 to 6.5 min; 100% A from 6.5 to 11.9 min; 5% A + 95% B from 11.9 to 12 min. The injected sample volume was 2 mL at a flow rate of 0.6 mL·min^{-1}. The limit of quantification was 0.50 μg·L^{-1}. Effluent samples were measured every week after filtration with 0.22 μm pore size syringe filter from Labbox.

2.3. Biomass analysis

MLSS and mixed liquor volatile suspended solids (MLVSS) were measured following APHA standard methods [27]. Sludge growth (ΔX; g SS·d^{-1}) was calculated as reported in previous research works [28,29].

To evaluate autotrophic biomass activity BM-Advance equipment from Surcis was used. Each test was performed at 20.0 ± 0.1ºC, air flow rate of 0.90 ± 0.01 L·min^{-1} and 2000 rpm of stirring rate, with 1 L of activated sludge in endogenous state. In these conditions, ammonium chloride (NH_4Cl) in a concentration of 150 mg·L^{-1} was added, measuring the dissolved oxygen (DO) continuously until substrate had been consumed.

Other parameters related to biomass were the microbial hydrolytic enzymatic activities (MHEA), and the cellular viability, which were measured every two weeks. In this work, three MHEA were analyzed in both reactors: Protease, α-Glucosidase and Dehydrogenase. The methodologies followed to MHEA and cellular viability analysis were explained in more detail in a previous work [28].

2.4. Bacterial community

MiSeq sequencings of 16 S rRNA genes was the technique used to evaluate the bacterial community in both reactors. In order to study its changes throughout experimental time, samples of mixed liquor (1 mL) were collected in days 24, 53, 66 and 94. SBR-B and SBR-N samples were centrifuged at 8000 rpm for 2 min, and the pellets were resuspended in 978 μL of sodium phosphate buffer. DNA from SBR samples was extracted in duplicate, as previously described [30]. Briefly, total microbial genomic DNA was extracted from each SBR sample using a FastDNA® SPIN kit for soil (MP Biomedicals, USA) following the protocol supplied by the manufacturer. All the extracted DNA SBR samples were purified by a OneStep™ PCR Inhibitor Removal Kit (Zymo Research, CA, USA). DNA concentration was measured using Qubit®dsDNA BR assay Kit (Molecular probes, Eugene, OR, USA). The hypervariable V3-V4 regions of bacterial 16 S rRNA gene was amplified in all samples by Fundación FISABIO sequencing service (Valencia, Spain) using the primers 341 F and 805 R. Raw data were analyzed using QIIME™ 1.9.1 [31] and Microbiome Helper virtual box v0.4 [32]. Finally, taxonomic assignment against MiDAS v3.6 [33] at 97% similarity of the most abundant sequence of OUT (Operational Taxonomic Units), were achieved. In addition, alpha diversity indices (ACE, Chao1, Jackniffe, Shannon and Phylogenetic) were calculated to assess microbial diversity in SBR-B and SBR-NP, using the EzBioCloud server [34].

2.5. Statistical analysis

Results of both physicochemical and biological parameters were evaluated statistically with one-way ANOVA (Statgraphics Centurion XVII), to study whether 4-NP additions had any statistical significance comparing SBR-NP with SBR-B. In this way, F-ratio and p-value were calculated with a confidence level of 95%.

3. Results and discussion

3.1. Influence of 4-NP on treated water quality

The addition of 4-NP had no effect on pH and conductivity, showing similar values in both reactors during the experimental period: 7.3 ± 0.2 and 1.09 ± 0.07 mS·cm^{-1} in SBR-B and 7.6 ± 0.4 and 1.08 ± 0.06 S·cm^{-1} in SBR-NP, respectively.

Regarding COD removal percentage, it can be seen in Fig. 2 that in the first 36 days similar values were measured in both reactors: 97.2 ± 0.6% in SBR-B and 96.9 ± 0.6% in SBR-NP. This fact indicates that 4-NP in a concentration of 1 mg·L^{-1} did not affect SBR-NP performance in this period.

Throughout experimental time, a decrease of mixed liquor temperature in both reactors was observed (Fig. 2), due to the seasonal period. This low temperature period (LowTP) occurred between days 36 and 68. The mean temperatures decreased from 14.4 ± 2.5ºC in the first 36 days, to 9.9 ± 1.3ºC in the next 32 days, increasing to 14.1 ± 2.5ºC for the last 26 days. It is known that low temperatures lead to lower treatment performance of activated sludge system [35,36]. This behavior was observed in both reactors between days 43 and 68, in which a slight decrease of average COD removal was observed. After this LowTP, the initial COD removal percentages were recovered in SBR-B, while decline of this parameter continued in SBR-NP until day 85, increasing from this day on. Statistically significant difference was observed in COD removal efficiency of both reactors only from day 69 on ($F = 5.06$; p-value = 0.0400). The increase in SBR-NP performance in the last operating days was related to several factors explained in Sections 3.2 and 3.3.

On the other hand, Fig. 3 shows the concentrations of N_T, NH_4-N, NO_3-N in the effluents of both SBRs. It can be observed that N_T values in SBR-NP were lower than those measured in SBR-B between the start of the 4-NP addition (day 15) and day 50, observing statistical significance between both reactors in this period ($F = 14.32$; p-value = 0.0026). Since denitrification could not occur (reaction step does not include anoxic phase), the only explanation for the lower nitrogen concentration in this period in SBR-NP is its assimilation in the biomass. This fact is in accordance with the results of biomass production, which was higher in SBR-NP than in SBR-B in the first 50 days: 0.64 ± 0.13 and 0.50 ± 0.12 g·d^{-1}, respectively. Regarding the nitrogen forms in this

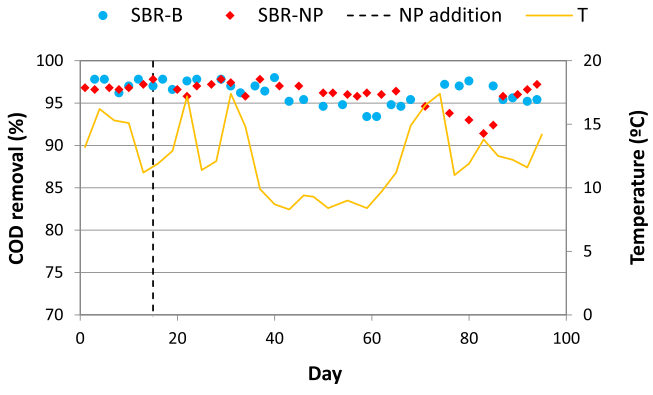

Fig. 2. COD removal (%) in SBR-B and SBR-NP. Dotted vertical line indicates the start of 4-NP addition in SBR-NP. Continuous line shown the mean temperature (T) of the reactors.

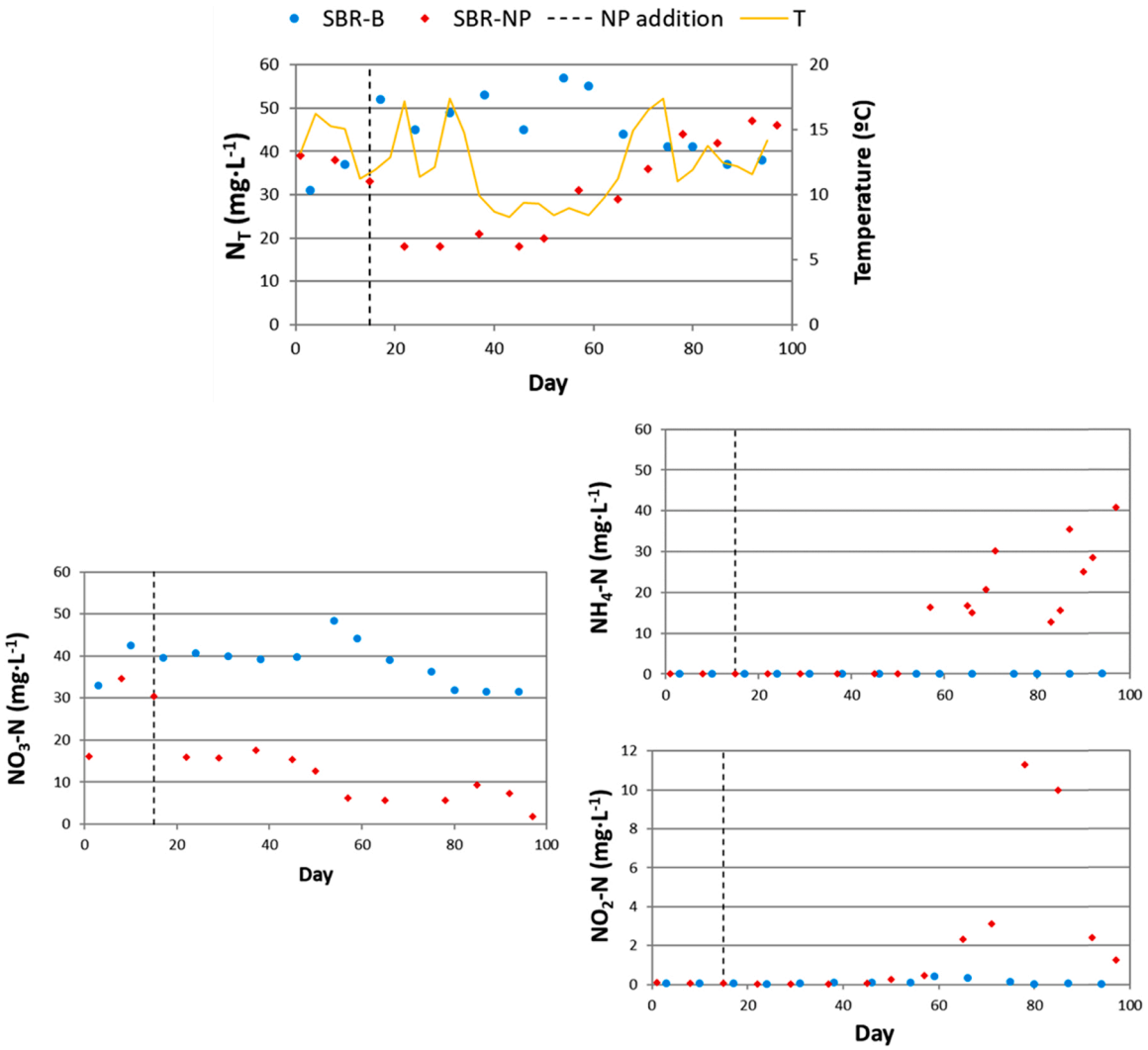

Fig. 3. Total nitrogen (N_T), nitrogen associated to nitrates and nitrites (NO_3-N and NO_2-N) and nitrogen associated to ammonium (NH_4-N) in SBR-B and SBR-NP. Dotted vertical line indicates the start of 4-NP addition in SBR-NP. Continuous line shown the mean temperature (T) of the reactors.

period (until day 50) in the SBRs (Fig. 3), it should be highlighted that total nitrogen (N_T) was found as NO_3-N in both reactors, since NH_4-N and NO_2-N were near to zero. This is the expected behavior when nitrification process is performed.

From day 51 on, a progressive increase of N_T occurred in SBR-NP, until this parameter reached values close to those measured in SBR-B. Concerning the nitrogen forms, NO_3-N decreased (from 17.5 to 1.9 mg·L^{-1}), and NH_4-N and NO_2-N increased in SBR-NP, reaching values of 40.8 mg·L^{-1} and 11.3, respectively. This behavior indicated that nitrification process had been affected in SBR-NP, since ammonia was not oxidized to nitrate by autotrophic bacteria, while this fact was not observed in SBR-B. To check it, respirometric techniques were performed in the last sampling day, under conditions explained in Section 2.3. Results confirmed that autotrophic biomass activity of SBR-NP was inhibited. This fact was due to the reduction of nitrifying bacteria abundance in SBR-NP (*Nitrosomonas* and *Nitrospira*), which was confirmed by the analysis of bacterial community presented in Section 3.3.

It is known that nitrifying bacteria growth can be affected mainly by temperature and pH or by an external agent [37,38]. As no variation of pH was observed in SBR-NP and LowTP did not affect nitrifying bacteria abundance of SBR-B, it should be concluded that 4-NP presence was the main cause of the nitrifying community decrease. Several authors reported that 4-NP in particular [23] and NP in general [39] have inhibitory effect on autotrophic bacteria. However, the same 4-NP concentration was added into the reactor throughout the experiment, and inhibitory effect only was observed from day 50. This fact could be explained by the gradual accumulation of 4-NP in the microbial flocs.

Among all effluent samples in which 4-NP was analysed, 4-NP was only detected in the sample of day 22 (the measured 4-NP concentration was 2.6 μg·L^{-1}). From this day on, 4-NP concentration was below the limit of quantification (0.5 μg·L^{-1}). Thus, residual non-biodegraded 4-NP had to be adsorbed on activated sludge. Taking into account the high octanol–water partition coefficient (log Kow: 4.48) and organic carbon partition coefficient (log Koc: 5.22 ± 0.38) [40] of 4-NP, a rapid adsorption of this substance can be expected. This fact was observed by other authors [41], who detected 4-NP on river sediments, while aqueous samples were free of this substance.

Nevertheless, there are authors who do report 4-NP concentrations in the soluble phase. Tanghe et al. [42] reported that there is a great dependence between efficiency of nonylphenol removal by activated sludge system and temperature. In a reactor fed with 8.33 mg·L^{-1} of NP, degradation of this substance decreased from 86% to 13% when temperature decreased from 28ºC to 10ºC. It must be highlighted that NP concentrations tested by these authors were substantially higher than those studied in this work.

Summarizing, it can be concluded that COD removal efficiency is not affected by the continuous addition of 4-NP. However, the addition of 4-

NP produced inhibition of nitrification after 50 days. In this way, it is produced a long-term effect. However, it is also important to highlight that toxicity of 4-NP can be also studied adding high concentrations during short periods of time (shock loading). Thus, Stasinakis et al. [23] reported concentrations of 50 mg/L of 4-NP for nitrification inhibition, which is considerably higher than concentrations tested in researches on the effect of 4-NP at long term. As an example, Bina et al. [43] reported that low concentration of 4 N (between 1 and 50 μg/L) caused an inhibitory effect of 4-NP on autotrophic bacteria. In general terms, nitrifying bacteria are also very sensitive to phenolic compounds and other potential pollutants like nanoparticles [44,45].

3.2. Influence of 4-NP on SMPs and on MHEAs

Regarding SMP production (as sum of proteins and carbohydrates) of SBR-B and SBR-NP (Fig. 4), LowTP also affected their values. SMP generation is related to substrate metabolism, biomass decay (BAP; biomass-associated products) and biomass growth (UAP; utilization-associated products) [46]. In this case, the substrate is the same throughout the experiment (except for the 4-NP) and the sludge production (related to UAP) decreased in SBR-NP and remained constant in SBR-B (0.36 ± 0.08 and 0.46 ± 0.46 from day 50, respectively). In this way, the increase of SMP concentrations in LowTP should be due to biomass decay.

Regarding Fig. 4, it can be concluded that there was hardly influence of 4-NP on SMP generation. Once 4-NP began to be added (day 15), SMPs did not increase significantly from day 24. However, SMP increased in both reactors in LowTP. Later, once the temperatures had increased again, SMP decreased progressively in SBR-B, achieving values very similar to the initial ones. However, this decrease was not observed at the same period of time in SBR-NP. This fact might be related to the decrease of autotrophic biomass from day 51 (explained in Section 3.1), which could have increased the SMP production as reported by Sepehri and Sarrafzadeh [47]. The SMP decrease of SBR-NP in the final experimental period was related to other phenomenon observed in this reactor, in which carbohydrates fraction of SMP decreased in the last sampling days, which explains the increase of COD removal in the same period (Fig. 2). In this way, the Prot/Carb ratio increased from 0.67 to 1.81 between the sampling days 71 and 94 in this reactor, while in SBR-B remained in similar values throughout the experiment (0.71 ± 0.14). This fact can be explained by increase of α-Glucosidase activity (Fig. 5), which enhanced the carbohydrates biodegradation.

As it can be seen in Fig. 5, in SBR-NP a sugar metabolism retardation occurred in comparison with SBR-B. Różalska et al. [21] reported the same behavior using the filamentous fungus *Gliocephalotrichum simplex*

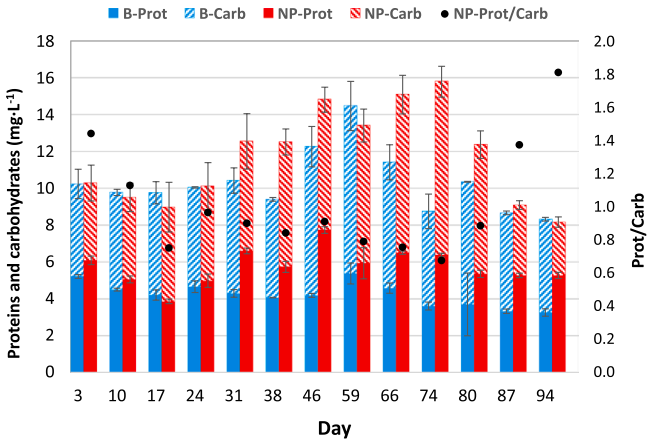

Fig. 4. SMP productions of SBR-B and SBR-NP as sum of proteins (Prot) and carbohydrates (Carb). Ratio Prot/Carb of SBR-NP (black dots).

to biodegrade 4-NP, and this inhibition depended on 4-NP concentration. Nevertheless, from sampling day 71, it was observed that α-Glucosidase activity increased, resulting in decrease of carbohydrates fraction on SMP and enhancement of COD removal. This fact could be due to the considerable increase of *Aquabacterium* genus in SBR-NP in the last experimental period, which enhanced 4-NP biodegradation, as explained in Section 3.3.

Regarding Dehydrogenase activity, it can be observed that values measured were lower in SBR-NP. This activity is related to oxidative activity cells and consequently may be an important indicator of microbial activity [48], which was reduced in presence of 4-NP. Protease activity remained constant in both reactors during the experimental period, which means that there is not influence of 4-NP on this MHEA.

Finally, it should be highlighted that the sludge growth in SBR-NP was also affected in LowTP, while no effect was observed in SBR-B. In this way, as commented in Section 3.1, ΔX was higher in SBR-NP than in SBR-B in the first 50 experimental days. However, from this day ΔX decreased in SBR-NP, achieving an average value of 0.36 ± 0.08 $g \cdot d^{-1}$ in the last experimental period, while this parameter remained stable in SBR-B (0.46 ± 0.08 $g \cdot d^{-1}$). This fact resulted in an increase of sludge retention time (SRT) in SBR-NP. In this way, in the first 50 days the SRT in SBR-NP and SBR-B were 28.5 and 30.7 days, respectively. However, from day 51 to the final of experiment the SRT in SBR-NP increased to 41.9 days, while in SBR-B remained in similar values to the initial ones (32.4 days).

3.3. Influence of 4-NP on biomass viability and bacterial community

Cellular viability decreased from day 50 in SBR-NP. In the first 50 days the viable cells were 85.7 ± 11.0%, decreasing to 63.0 ± 10.2% in the last 44 days (in SBR-B, cellular viability was 80.7 ± 8.5% during the experiment). In this way, it can be concluded that 4-NP had a negative impact on the viability of the biomass.

Diversity of a microbial community is often described using the total number of species (species richness), the relative abundances of the species (species evenness) or indices that combine these two dimensions [49]. The Operational Taxonomic Unit (OTU) allowed to obtain several parameters to measure bacterial species richness, as number of OTUs, ACE, Chao1 and Jackknife, and species evenness like Shannon index. Phylogenetic diversity is defined as the sum of the branch lengths of a phylogenetic tree connecting all species, which takes into account phylogenetic difference among species [50]. Regarding these indices, it can be shown in Table 2 that from day 50 was detected that both diversity and richness of species decreased in SBR-NP, in which all the evaluated indicators dropped in SBR-NP, while the variation in the SBR-B was minimal.

There were three predominant bacterial phyla in both reactors: *Proteobacteria*, *Bacteroidetes* and *Actinobacteria* (Fig. 6). These three phyla included 59.37 ± 0.03% of bacterial community in SBR-B during experimental time. In SBR-NP, this abundance was quite stable, remaining in 67.95 ± 0.01% in the three first sampling days. However, there was a significant increase at the end of the experiment, reaching 80.91% in the last sampling day. This fact also confirms that bacterial diversity decreased in SBR-NP due to presence of 4-NP.

Differences observed in both reactors in the last sampling day were due mainly to higher *Proteobacteria* abundance: 44.28% in SBR-NP and 25.88% in SBR-B. It should be commented that this phylum may play an active role in degrading phenolic compounds [51], and it was hypothesized that phenol-degrading microorganisms can degrade nonylphenols [52]. *Proteobacteria* differences between both reactors, which were observed in the last sampling day (18.40%), were due to *Betaproteobacteriales* order, and more specifically to *Aquabacterium* genus (13.00% and 0.00% in SBR-NP and SBR-B, respectively). With respect to this, it should be clarified that *Betaproteobacteriales* is now an order within *Gammaproteobacteria* class, due to taxonomic changes proposed in studies about microbial diversity of wastewater treatment systems

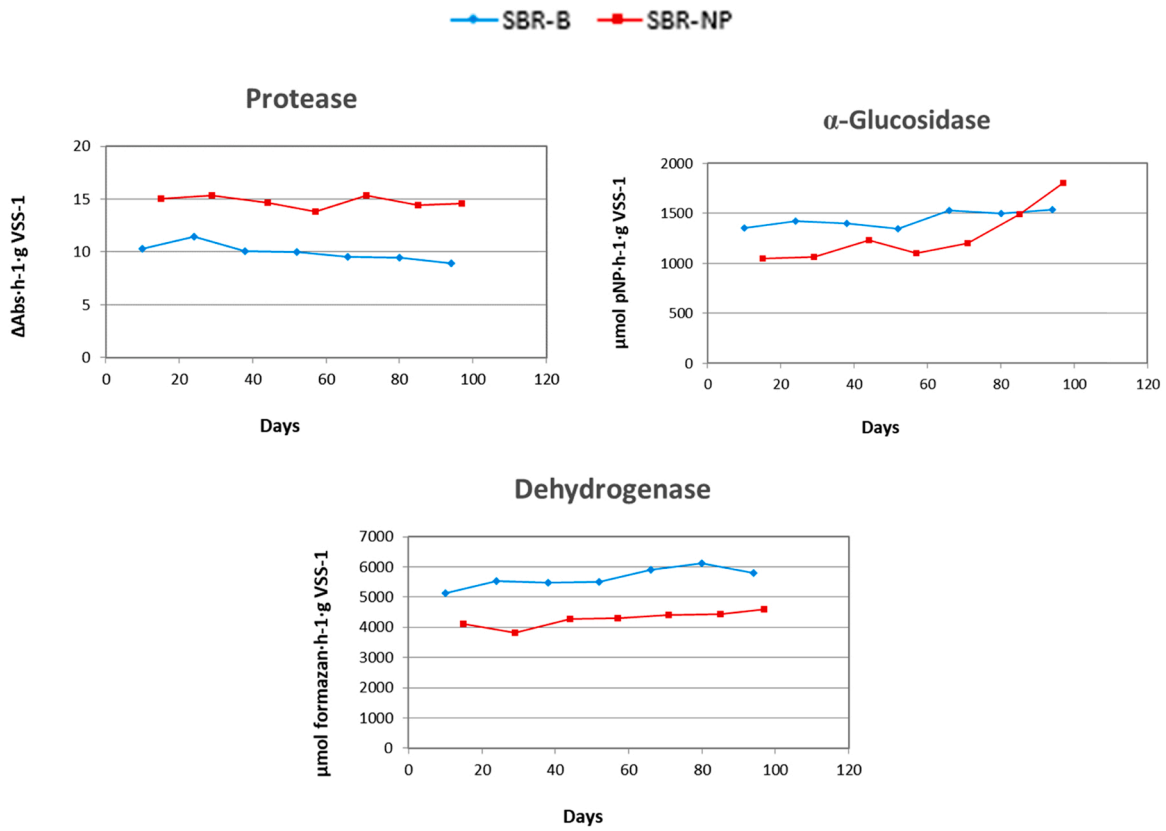

Fig. 5. Microbial hydrolytic enzymatic activities of SBR-B and SBR-NP.

Table 2
Microbial community diversity indices of 16S rRNA gene amplicon analysis.

Reactor	Day	Clean reads	OTU	ACE	Chao1	Jackknife	Shannon	Phylogenetic diversity	Coverage
SBR-B	24	92,980	1958	2139	2090	2281	5.466	2755	0.930
	53	93,049	1415	2221	2166	2355	5.790	2782	0.930
	66	63,348	1425	1838	1791	1962	5.604	2425	0.921
	94	93,196	2423	1999	1945	2128	5.562	2629	0.932
SBR-NP	24	93,431	1796	1925	1857	2012	5.271	2506	0.998
	53	93,232	1652	1782	1727	1870	5.424	2335	0.998
	66	93,358	1611	1715	1667	1799	5.380	2273	0.998
	94	92,935	1300	1404	1358	1476	4.726	1882	0.998

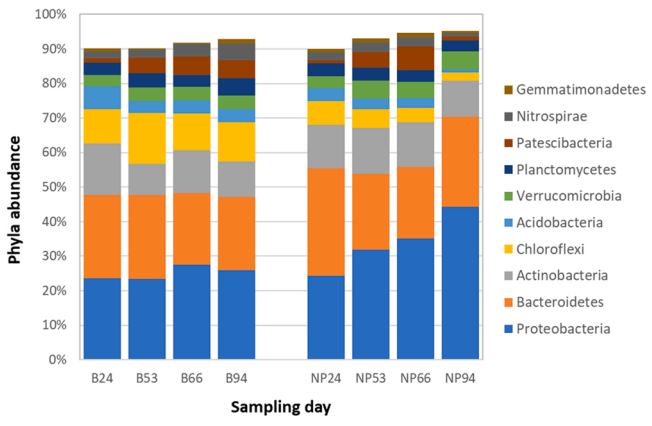

Fig. 6. Percentage of bacterial community using the individual number of OTUs at phylum level by MiSeq sequencing (B = SBR-B; NP = SBR-NP samples). The number before B or BPA indicates the sampling day.

[33]. *Betaproteobacteriales* is reported as dominant order in the activated sludge from wastewater contaminated with phenol [53]. Additionally, *Aquabacterium* seems to play an important role in NP degradation. Some authors reported that microplastics from polyethylene terephthalate, high-density polyethylene, and polyvinylchloride (which contain NP), are quickly colonized by biofilms. *Aquabacterium* is a common member of these biofilms which have potential for plastic degradation [54,55].

The relative abundance of *Actinobacteria* was similar in both reactors in the last sampling day (10.58% in SBR-NP and 10.31% in SBR-B). However, *Bacteroidetes* abundance was 4.78% higher in SBR-NP. This behavior was also reported by other authors in bacterial community of river sediments contaminated with nonylphenol [56], and in activated sludge treating wastewaters containing phenolic compounds [57].

Massive data at order (Fig. S1) and genus (Table S1 and spreadsheet) levels from both SBRs can be consulted in Supplementary Material. Regarding this information, it should be highlighted that evaluating bacterial community as genus level, several heterotrophic aerobic bacteria showed in Table S1, increased their abundance in SBR-NP throughout experimental days, remaining constant or decreasing in SBR-B. These bacteria (Fig. 7) are related to the phenolic compounds biodegradation.

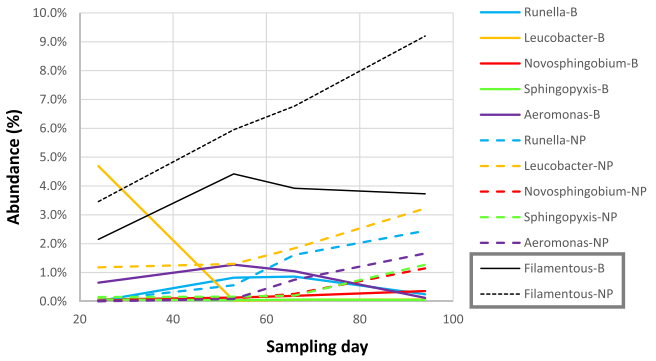

Fig. 7. Abundance of heterotrophic aerobic bacteria, at genus level, in SBR-B and SBR-NP related by phenolic compounds degradation. Filamentous group included *Nocardioides*, *Haliscomenobacter*, *midas_g_3144*, *midas_g_65*, *Ca Alysiosphaera* and *Thiothrix*.

Leucobacter and *Runella* contain genes encoding enzymes involved in the degradation of aromatic compounds [58,59]. *Aeromonas* have been reported as 4-nonylphenol degraders in anaerobic fluidized bed reactor [60]. *Sphingopyxis* and *Novosphingobium* belong to *Sphingomonadaceae* and members of this family have been recognized as microorganisms involved in NP removal [61,62].

In Fig. 7, the total abundances of six filamentous species (sum of *Nocardioides*, *Haliscomenobacter*, *midas_g_3144*, *midas_g_65*, *Ca Alysiosphaera* and *Thiothrix*; data in spreadsheet) in both reactors are also presented. In this figure, it can be seen that filamentous community, at genus level, increased in SBR-NP. This fact can be due to phenol presence. In this way, some authors have reported that *Nocardioides* presented phenol and p-nitrophenol degrading activity [63], and *Thiothrix* were largely produced in activated sludge from phenol wastewater treatment [64].

In contrast, the relative abundance of nitrifying bacteria decreased in SBR-NP while increased in SBR-B. *Nitrosomonas* and *Nitrospira* abundance in SBR-B increased from 0.56% to 1.10%, and from 1.95% to 4.73%, respectively, between the first and the last experimental day (Table S1). Contrary, these genus level abundances decreased in SBR-NP from 0.62% to 0.45%, and from 2.39% to 1.01%, respectively. This fact explains the inhibition of nitrification capacity of SBR-NP commented in Section 3.1.

4. Conclusions

4-NP is one of the most relevant endocrine-disrupting compounds that can be found in wastewaters. In this way, the evaluation of its impact on the activated sludge processes of the wastewater treatment plants is an important issue.

Results showed that 1 $mg{\cdot}L^{-1}$ of 4-NP did not affect process performance in the first 36 operational days, since similar COD removal percentages were achieved in both reactors ($97.2 \pm 0.6\%$ in SBR-B and $96.9 \pm 0.6\%$ in SBR-NP). However, 4-NP accumulation, which was enhanced by a low temperatures period, resulted in a decrease of SBR-NP performance (97.0% in SBR-B and 91.4% in SBR-NP in day 83). Finally, the COD removal efficiency in SBR-NP increased again up to the initial values, which coincided with the microbial population shift. In this way, an increase of *Proteobacteria* abundance, and in particular a considerably increase of the *Aquabacterium* genus, whose abundance increased from 0% to 13%, occurred in SBR-NP. These bacteria are able to degrade phenolic compounds.

On the contrary, nitrification process was completely inhibited. This phenomenon was observed by ammonia-nitrogen accumulation in the reactor, and it was corroborated both by respirometric techniques and by the significant reduction of *Nitrosomonas* and *Nitrospira* in the mixed liquor. This was produced in spite of the absence of 4-NP (< 0.5 μg/L) in the aqueous phase. It means that sludge analysis is necessary for assessing the cause of nitrification problems in an activated sludge reactor, since substances like 4-NP can accumulate in the sludge leading to bacteria inhibition.

From these results, it can be concluded that no toxic effects on heterotrophic microbial population were produced by the addition of 1 $mg{\cdot}L^{-1}$ of 4-NP to the wastewater. However, the continuous dosing of 4-NP led to toxic effects on autotrophic bacteria, inhibiting nitrification.

CRediT authorship contribution statement

E. Ferrer-Polonio: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Writing – review & editing **J. Fernández-Navarro:** Investigation, Writing – original draft, **J.A. Mendoza-Roca:** Conceptualization, Methodology, Validation, Writing – original draft, Writing – review & editing, Supervision, Project administration, Funding acquisition, **A. Bes-Piá:** Conceptualization, Validation, Writing – original draft, Writing – review & editing, Supervision, Project administration, Funding acquisition. **J.L. Alonso-Molina:** Formal analysis, Investigation, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

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

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jece.2022.107249.

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