An innovative wastewater treatment technology based on UASB and IFAS for cost-efficient macro and micropollutant removal

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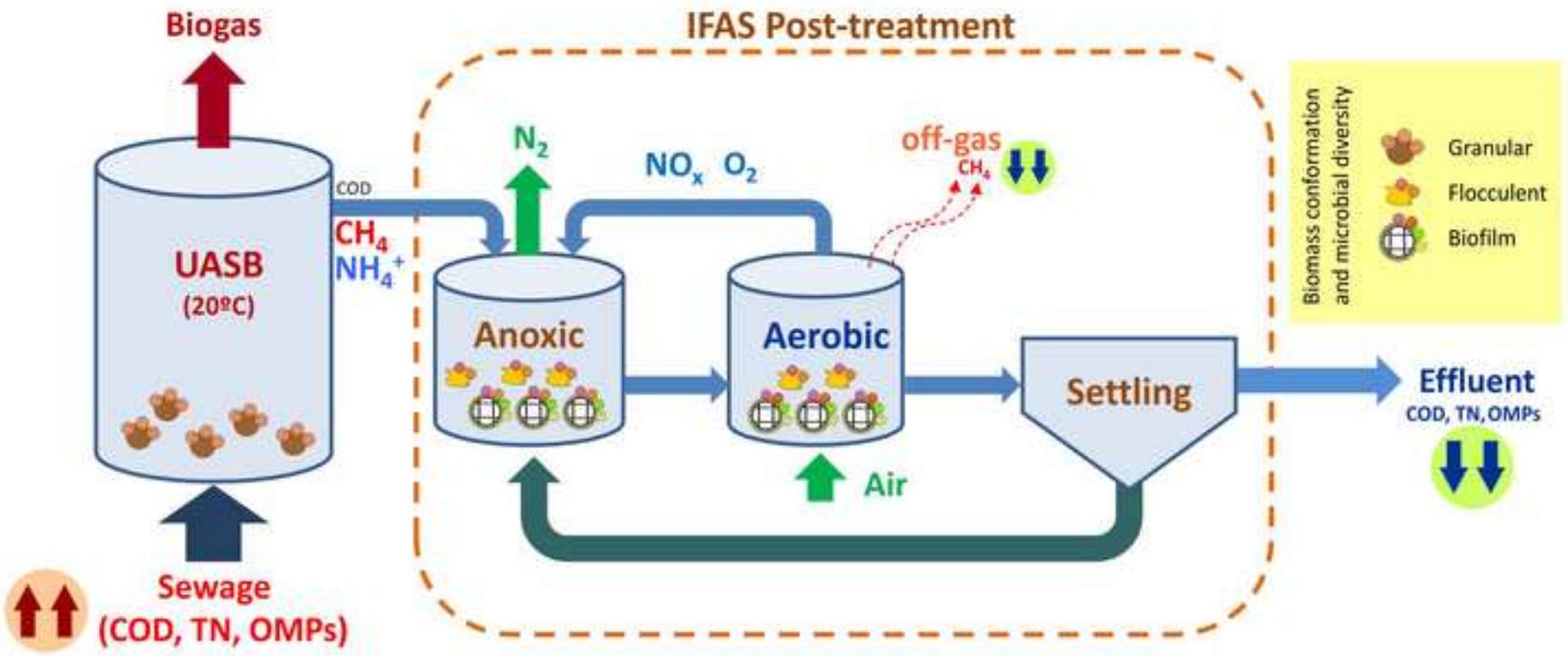
Highlights

- The combination of redox potentials improves organic micropollutant (OMP) removal
- OMP removal is also influenced by biomass activity and conformation
- Bisphenol A and ethinylestradiol removal depends on nitrifying-denitrifying activity
- The UASB+IFAS system upgrades anaerobic treatments with lower N and GHGs emissions
- Biofilms highly enhances microbial diversity and the growth of anammox microorganisms

Graphical Abstract



Abstract

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Abstract

An innovative process based on the combination of a UASB reactor and an IFAS system is proposed in order to combine different redox conditions and biomass conformations to promote a high microbial diversity. The objective of this configuration is to enhance the biological removal of organic micropollutants (OMPs) as well as to achieve the abatement of nitrogen by using the dissolved methane as an inexpensive electron donor. Results showed high removals of COD (93%) and dissolved methane present in the UASB effluent (up to 85%) was biodegraded by a consortium of aerobic methanotrophs and heterotrophic denitrifiers. Total nitrogen removal decreased slightly along the operation (from 44 to 33%), depending on the availability of electron donor, biomass concentration, and configuration (floccules and biofilm). A high removal was achieved in the hybrid system (>80%) for 6 of the studied OMPs. Sulfamethoxazole, trimethoprim, naproxen, and estradiol were readily biotransformed under anaerobic conditions, whereas ibuprofen or bisphenol A were removed in the anoxic-aerobic compartment. Evidence of the cometabolic biotransformation of OMPs has been found, such as the influence of nitrification activity on the removal of bisphenol A, and of the denitrification activity on ethinylestradiol removal.

Keywords: Organic micropollutants, pharmaceuticals, redox conditions, methane emissions, hybrid process.

ABBREVIATIONS

AMO: ammonia monooxygenase

BPA: bisphenol A

COD: chemical oxygen demand

COD_S: soluble chemical oxygen demand

COD_T: total chemical oxygen demand

damo: denitrifying anaerobic methane oxidation

DCF: diclofenac

DO: dissolved oxygen

ERY: erythromycin

E1: estrone

E2: β-estradiol

EE2: α-ethinylestradiol

FA: free ammonia

FISH: fluorescence *in situ* hybridization

GHG: greenhouse gas

IBP: ibuprofen

IFAS: integrated fixed-film activated sludge

Kd: sorption coefficient

MBR: membrane bioreactor

MLTSS: mixed-liquor total suspended solids

MLVSS: mixed-liquor volatile suspended solids

NPX: naproxen

OLR: organic loading rate

OMPs: organic micropollutants

ORP: oxidation-reduction potential

R: recirculation ratio

ROX: roxithromycin

SMX: sulfamethoxazole

TMP: trimethoprim

UASB: upflow anaerobic sludge blanket

1. INTRODUCTION

Nowadays, wastewater treatment should address not only the removal of conventional pollutants, such as nitrogen or organic matter but also the presence of organic micropollutants (OMPs), an issue of increasing concern in the society. There is a consensus in considering the effluents from conventional wastewater treatment plants as the main entrance of these substances into the aquatic environment since common treatment processes are not able to effectively remove many of them [1,2]. New advanced treatment processes, in many cases based on the combination of biological processes with physico-chemical post-treatment stages, have been developed in the last years to overcome the existing limitations [3,4,5]. High OMP removal efficiencies can be achieved by the application of biological configurations upgraded with tertiary stages with oxidants or adsorbents such as ozone or activated carbon. Nevertheless, these upgraded wastewater treatment systems increase operational expenses [6,7]. An alternative approach would be to develop enhanced biological processes for the removal of macro and micropollutants. For this purpose, recent studies have shown the interest of new biological configurations which combine different redox conditions (anaerobic, anoxic, aerobic) [8], different biomass physical conformations (granular, flocculent, biofilm) and wide microbial diversity [9].

OMPs biotransformation is determined by their chemical structure and clearly influenced by redox conditions [10]. For instance, sulfamethoxazole can be biotransformed at a high extent under anaerobic conditions, due to the presence of electron-withdrawing groups, such as sulfonyl, which is easily degraded under reductive conditions [11]. On the other hand, ibuprofen is a readily biotransformable OMP under aerobic conditions [12], especially favored when nitrification takes place due to its hydroxylation by the action of the enzyme ammonia monooxygenase (AMO) to produce 2-hydroxy-ibuprofen [13]. However, the branched substitutions existing on the para-position of the aromatic ring hampers its biotransformation under anaerobic redox potentials [11]. Normally, the combination of aerobic and anaerobic stages leads to the enhancement of the removal of several OMPs, such as venlafaxine or tramadol [14].

The use of carriers enhances the retention of slow-growing bacteria in biofilms, such as nitrifiers, allowing the increase of the microbial diversity [15,16]. The presence of nitrifying bacteria enhances several OMPs removal, such as the hormone estradiol, whose removal is correlated with the nitrifying activity [11]. Falas et al. [17] studied the removal of OMPs by biofilms and suspended biomass and measured a higher removal rate per unit biomass in the biofilm for most of the OMPs, as in the case of ketoprofen or gemfibrozil. Biomass conformation also influences OMP removal. In the case of lipophilic OMPs, such as musk fragrances, higher sorption coefficients (Kd) were observed in a membrane bioreactor (MBR) compared to a conventional activated sludge (CAS) plant due to the lower biomass particle size developed in MBRs [18]. De la Torre et al. [19] compared the removal of reactors operated either with suspended biomass or biofilms, as well as with the combination of both. They observed the best results in terms of OMP removal for the integrated fixed-film activated sludge (IFAS)-MBR, which combines both suspended biomass and biofilms, while the worst results were obtained for the reactor operated only with biofilms. The use of membranes and/or supports allows the retention of slow-growing bacteria [8,20]. This enhances the microbial diversity and the types of enzymes present in the biomass and, consequently, the removal of macro- and micropollutants [10].

Other of the key issues in wastewater treatment is the operational cost related to energy consumption. With the aim of reducing this important contribution to the overall operational expenses, innovative approaches are under development. First an anaerobic methanogenic step in the water line, as roughing stage, would allow the reduction of aeration costs, as well as a valorization of the organic matter in terms of energy production. Nevertheless, the effluent of a methanogenic stage should be treated to reduce the remaining organic and nitrogen compounds, being chemical oxygen demand (COD) levels still high. Part of the methane remains dissolved in the liquid phase (up to 25-50%) especially at temperatures below 20ºC [21]. On one hand, although aerobic treatment is the common post-treatment alternative, part of the dissolved methane is released into the atmosphere by stripping, increasing greenhouse gas (GHG) emissions of wastewater treatment [22]. On the other hand, the use of conventional nitrification-denitrification is often limited by the low remaining organic matter after anaerobic treatment, which limits the denitrification efficiency. Aerobic and anaerobic consortia of bacteria/archaea (methanotrophs) are able to use methane as a carbon source for

denitrification [23]. Aerobic methanotrophs are able to convert methane into oxidized species that can be employed as an organic carbon source by the denitrifying heterotrophic bacteria [24]. Under anoxic conditions, denitrification coupled to anaerobic methane oxidation (damo) could be carried out by either damo bacteria (*Candidatus* Methylomirabilis oxyfera) or damo archaea (*Candidatus* Methanoperedens nitroreducens) with nitrite or nitrate as electron acceptor, respectively [25].

The aim of this study was to investigate the removal of micro- and macropollutants in a combined upflow anaerobic sludge blanket (UASB)-IFAS system treating low-strength wastewater. The system combines anaerobic, anoxic and aerobic redox conditions in a sequence of reactors, where biomass with different conformations (granular, flocculent and biofilm) is developed. The promotion of methanotrophic denitrification allowed overcoming problems related to methane emissions by low temperature anaerobic processes.

2. MATERIALS AND METHODS

Experimental set-up

The system combined a first UASB unit of 120 L volume, coupled to an IFAS polishing stage of 56 L (Figure S1). The anaerobic reactor was inoculated with anaerobic granular biomass from an internal circulation reactor treating brewery wastewater. Biomass concentration in the UASB was 30 g L^{-1} of total suspended solids (TSS). No purges were carried out in the anaerobic reactor. The IFAS was started up on operating day 26. The IFAS system consists of two biological compartments (anoxic, 36 L and aerobic, 20 L) and a secondary settler (10 L). Two types of carriers were used in order to promote the growth of biofilms and retain the slow-growing bacteria: Synthetic porous foams (Levapor GmbH, Germany) in the anoxic compartment and porous semi-flexible carriers (Mutag Biochip, Multi Umwelttechnologie A.G., Germany) in the aerobic compartment. The IFAS was started-up using an apparent volume of 20% of Levapor and 7% of Biochip carriers. At the operating day 257, these volumes were raised up to 23 and 20%, respectively. The specific external surface of the products, were of 486 and 2,174 m^2 m^{-3}, for the outer boundary of the Levapor and Biochip carriers, respectively.

The system was operated during 407 days at room temperature (21 ± 1ºC) with an overall COD loading rate of 680 ± 190 mg L^{-1} d^{-1}. A synthetic medium-low strength wastewater was feed to the UASB composed by diluted skimmed milk as a carbon

source (COD_T and COD_S of 891 ± 214, 782 ± 204 mg L^{-1}, respectively), sodium bicarbonate for maintaining alkalinity (960 mg L^{-1}) and ammonium chloride as N source (18 mg N L^{-1}). HRT was 20 ± 2 h in the UASB and 9 ± 1 h in the IFAS post-treatment. A total recirculation ratio (R) of 3 was applied in the IFAS stage to achieve high nitrogen removal, combining both an internal and an external recirculation to the anoxic compartment.

Analytical methods

Mixed-liquor total and volatile suspended solids (MLTSS and MLVSS, respectively), ammonium, nitrite, nitrate, total and soluble chemical oxygen demand (COD_T and COD_S, respectively) were analyzed according to Standard Methods [26]. Temperature, dissolved oxygen (DO) and pH were monitored with a Hach HQ40d multi-parameter digital sensor. Biogas composition was measured in a gas chromatograph HP 5890 Series II using a column of Porapack Q 80/100 (SUPELCO). Dissolved methane concentration was measured following the method described by Sánchez et al. (2016).

Eleven OMPs, representative of several widely consumed pharmaceuticals and chemicals with different physico-chemical behavior, which are known to be present in sewage, were spiked to the synthetic wastewater: three anti-inflammatories at concentrations of 10 ppb (ibuprofen IBP, naproxen NPX, diclofenac DCF), four antibiotics at concentrations of 10 ppb (sulfamethoxazole SMX, trimethoprim TMP, erythromycin ERY, roxithromycin ROX) and four endocrine disruptors (bisphenol BPA, estrone E1, β-estradiol E2, α-ethinylestradiol EE2) at a concentration range of 1-10 ppb. These values can be considered as "environmental levels" in accordance to the amounts detected in sewage, an also to the limits of quantification of the analytical methods applied. The removal of the selected OMPs was followed through the different stages of the system in order to assess the effect of the redox conditions and the different microbial communities. Samples were taken with a time delay of one hydraulic retention time (HRT) in four sampling points: the influent and effluent of the UASB reactor, the anoxic compartment, and final effluent. Seven sampling campaigns were carried out over the operation to follow up the removal of the macro and micropollutants. For the detection of OMPs, samples were prefiltered (AP3004705, Millipore), preconcentrated by a solid-phase extraction (SPE) with OASIS HLB cartridges. Anti-inflammatories and BPA were analysed by gas chromatography-mass spectrometry (GC/MS) and antibiotics and hormones by liquid chromatography-tandem

mass spectrometry (LC/MS/MS). The analytical procedure, limits of quantification and recoveries were described by Alvarino et al. [4].

Molecular biology techniques

Microbial community was monitored by using fluorescence *in situ* hybridization (FISH) and 16S rRNA gene amplicon sequencing analysis by using Illumina MiSeq. This last analysis was carried out at Parque Científico de Madrid, Spain. For FISH analysis, biomass samples were collected during the operation, disrupted and fixed according to the procedure described by Amman et al. [27]. FISH probes and Illumina procedure are described in Supplementary Material. FISH samples were collected at the operating days 50, 99, 265 and 343. Illumina samples were collected at the operating days 175, 265 and 368 (3 samples for Levapor and 1 for Biochip biofilms).

3. RESULTS AND DISCUSSION

3.1 Reactor performance

The anaerobic stage achieved a COD_T removal of 93 ± 2% in the UASB reactor, which coincides with the overall removal achieved in the combined system (Table S1). The measured biogas production in the UASB was 37 ± 9 L d^{-1} with a methane percentage of 75 ± 2%. Most of the COD (74%) was methanized under anaerobic conditions, reducing energy consumption by saving aeration and producing biogas. A fraction of the produced methane remained dissolved in the effluent of the UASB, due to methane solubility at low temperatures [22, 28]. Presence of dissolved methane is still a concern because it increases GHG emissions. Thus, it should be post-treated in order to decrease its environmental impact.

Measured dissolved methane concentration in the UASB effluent was 18 ± 3 mg L^{-1} throughout the whole operation. Methane mass balances showed that dissolved methane corresponded to 10 - 20% of the overall methane generated in the UASB reactor.

The effluent from the UASB reactor contained low COD_T and COD_S values of 57 ± 2 mg L^{-1} of and 43 ± 1 mg L^{-1}, respectively, without considering dissolved methane. Ammonia concentration of 54 ± 8 mg N L^{-1} was measured. The IFAS stage contributed

to achieving a higher quality effluent, being its total nitrogen concentration of 36 ± 11 mg L^{-1}.

Considering the evolution of nitrification, two different periods of operation were identified in the IFAS system: Period 1 (P1), from day 0 to 167, in which only partial nitrification was observed and nitrite was the main electron acceptor under anoxic conditions. At the end of P1, the development of nitrite-oxidizing bacteria (NOB) was detected; and Period 2 (P2), from day 168 to 407, when nitrite oxidation to nitrate was achieved and no nitrite accumulation was observed.

During P1 the reactor contained suspended biomass as well as biofilms in the anoxic and aerobic compartments. MLTSS and MLVSS concentrations in the anoxic compartment were 1.5 ± 0.9 and 1.3 ± 0.8 g L^{-1}, respectively. DO and ORP parameters were monitored, obtaining values between 0.15 and 0.31 mg L^{-1} and 0 and -150 mV, respectively, and pH was 7.6 ± 0.2. DO in the aerobic compartment was 3.2 ± 0.9 mg L^{-1}. At the end of P1, worse settling properties of the suspended biomass were observed, which led to an almost complete washout of the suspended biomass present in the IFAS system (days 146 to 167).

Along P2 suspended biomass concentration was lower than 50 mg L^{-1} and biofilm growth onto the two types of carriers was observed. Thus, the system was operated as a biofilm system from day 167 onwards. In the anoxic compartment, the DO and ORP values ranged from 0.25 to 0.45 mg L^{-1} and 0 to -75 mV, respectively.

The quality of the effluent of the IFAS system, in terms of COD_T, was lower in P1 than in P2, with values of 77 ± 55 and 52 ±19mg L^{-1}, respectively. CODs was 24±13 mg·L^{-1} during the whole experimentation. VSS in the effluent was lower in P2 (32 ± 11 mg L^{-1}) compared with P1, 50 ± 40 mg L^{-1}. On the contrary, the observed TN concentration in the effluent in P1 was lower than in P2, with values of 30 ± 7 and 37 ± 7 mg L^{-1}, respectively.

3.2 Methane and nitrogen removal in the IFAS system.

The UASB effluent contained 54 ± 8 mg N L^{-1} of ammonia. In P1, nitrogen ions concentration present in the final effluent were 28 ± 16 mg NH_4^+-N L^{-1} and 2.5 ± 1.8 mg NO_2^-N L^{-1}. After operating day 28, TN was 30 ± 7 mg L^{-1} and TN removal achieved 44%, corresponding to a nitrogen removal rate of 55 ± 18 mg N L^{-1} d^{-1},

referred to the whole IFAS system. Nitrate was hardly detected and ammonia was partially oxidized to nitrite, with a nitrification rate of 158 ± 84 mg NH_4^+-N L^{-1} d^{-1}, observed in the aerobic compartment. The IFAS system was not able to achieve complete nitrification of the NO_2^- generated due to free ammonia (FA) accumulation of 1.1 mg NH_3-N L^{-1} that inhibited nitrite oxidizers [29]. Moreover, overall nitrogen loading rate applied to the IFAS system was high, around 135 mg TN L^{-1} d^{-1}. After operating day 28, TN measured in the samples taken in the anoxic compartment were 2±2 mg·L^{-1} higher than those observed in the final effluent. This fact indicated the possible existence of simultaneous nitrification-denitrification in the biofilms of the aerobic compartment.

In P2, nitrite was below 0.2 mg NO_2^--NL^{-1} in the final effluent. The system could not achieve full ammonia oxidation. Nitrogen ions concentration in the final effluent were 22 ± 7 mg NH_4^+-N L^{-1} and 15 ± 5 mg NO_3^--N L^{-1}, with a denitrification rate of 43 ± 10 mg N L^{-1} d^{-1}. TN fed was around 54 ± 8 mg L^{-1}. Despite operating with biofilm, the fraction of ammonia oxidised was completely oxidised to nitrate, achieving a nitrification rate of 202 ± 60 mg NH_4^+-N L^{-1} d^{-1}. Although FA was still high, 1.6 mg NH_3-N L^{-1}, the development of nitrifiers inside biofilms is a more adequate environment than flocculent biomass to better tolerate this inhibitor [30]. Average TN concentration was in 39 ± 10 mg N L^{-1} in the anoxic compartment and 36 ± 10 mg N L^{-1} in the final effluent. TN removal decreased to a 33%, as result of the higher electron donor requirements of nitrate denitrification and by the lower denitrification rate of this nitrogen ion.

Denitrification was carried out by a heterogeneous group of bacteria comprising aerobic methanotrophs and nitrate/nitrite denitrifying heterotrophs (Figure 1B). The aerobic methanotrophs contributed to reducing the dissolved methane concentration. In P2 the growth of anammox bacteria in biofilm was observed. The fact of operating the system with biofilm in the aerobic compartment could enhance denitrification due to anoxic/anaerobic environments within the internal layers of the biofilms.

Dissolved methane was biologically removed in the IFAS along P1 in a high extent (85%), corresponding the remaining 15% to stripping. Around 50% of dissolved methane was removed in the anoxic compartment, and some additional 35% in the aerobic stage, considering k_La of methane and the measured dissolved methane concentration in the aerobic compartment. Methane biodegradation decreased in P2,

down to 69%, with 37% and 32% corresponding to the anoxic and aerobic compartments, respectively. This decrease in dissolved methane biodegradation is very likely related to the washout of the suspended biomass in which methane-oxidizing bacteria were abundant.

The support selected in the anoxic compartment of the IFAS, pads of Levapor, is a polyurethane foam, with high porosity (75-90%) and a size of 20x20x7 mm. This carrier was selected in order to promote the growth of anaerobic and anoxic microorganisms in the inner pores of this small rectangular prism. On the other hand, the carrier used in the aerobic compartment, Biochip, is a round form polyethylene support with a diameter of 25 mm and a thickness of 1.1 mm. The surface presents a great number of holes and cavities where microorganisms grew adhered. This support, with the higher specific external surface, was selected in order to promote the growth of a thin biofilm layer with a large fraction of aerobic microorganisms.

3.3 Microbiological analysis

In Levapor and Biochip biofilm 27 and 22 different genera of bacteria were found, respectively, with more than 1% of relative abundance.

Experimental results indicated that nitrite oxidation took place mainly in P2. Heterotrophic denitrification is not the only biological way of nitrogen removal, so other microorganisms, as anammox, might coexist in the suspended biomass and the carriers. FISH analysis and 16S rRNA gene sequencing allowed elucidating the main biological mechanisms responsible for nitrification, as well as for removal of methane and of nitrogen.

Microbiological analysis of suspended biomass and biofilms in the aerobic compartment showed that among nitrifiers, only ammonia oxidizing bacteria were present in the system, with a small presence of nitrite oxidizers, in P1, confirming the partial nitritation in this period. In P2, *Nitrospira* spp. growth was observed, likely the responsible for the nitrite oxidation to nitrate.

Type I methanotrophs, aerobic methane oxidizers, were detected in large quantities in suspended biomass during P1, and in both type of biofilms during all the operation by FISH analysis (Figure S2). On day 368 (P2), Illumina analysis indicated that type I methanotrophs relative abundance in Levapor and Biochip biofilms were 2.10% and

6.15%, respectively. In contrast, 0.02% of damo bacteria were only detected in the anoxic biofilm. No damo archaea were detected.

Ammonium and nitrite were available, so anammox growth operating conditions were present. They were detected by FISH analysis on day 265 in both anoxic and aerobic biofilms (Figure S2), with a relative abundance on the day of operation 368 of 3.18% in Levapor biofilm and 6.33% in Biochip biofilm, according to the results obtained by using Illumina analysis.

These data indicate that methane removal was achieved mainly by type I methanotrophs and methane removal decreased in P2 due to suspended biomass washout. Furthermore, the presence of aerobic methanotrophs in the aerobic biofilm, detected by both FISH and Illumina analysis, indicated that some biological methane removal took place in the aerobic chamber. Nitrogen removal was carried out by heterotrophic denitrifiers in P1, but in P2 anammox pathway was also contributing to nitrogen removal, present in the anoxic/anaerobic inner part of Biochip biofilms. Thus, the aforementioned lower TN observed in final effluent, could be explained among others by the anammox process, carried out by the biofilms of the aerobic compartment.

3.4 OMP removal

The removal of the selected OMPs was studied in the overall hybrid system, as well as in each compartment. Figure 2 shows the removal efficiencies obtained from mass balances applied to the both the UASB and the IFAS reactors. Sorption was not significant since the studied OMPs are not lipophilic compounds. Even though some of the studied OMPs can be partially present in the mixed liquor in its positively charged form (e.g. cationic carbamazepine) and interact electrostatically with the negatively charged surfaces of the microorganisms (known as adsorption) [31]. It was not relevant in the present study in agreement with previous studies about the long term operation of UASB reactors [8,11]. Consequently, OMPs removal was associated with biotransformation [32], as confirmed by mass flow calculations. In the anaerobic reactor, four substances were substantially removed: the antibiotics SMX, TMP, the estrogen estradiol and the anti-inflammatory NPX (> 80%). The other OMPs were only removed at a partial or low extent reaching the anoxic-aerobic IFAS (Figure 2). Their anaerobic biotransformation can be partially explained in terms of their chemical

structure. One of the functional groups of the antibiotic TMP is a substituted pyrimidine ring which is biodegradable under anaerobic conditions [33], whereas the biotransformation of SMX is related to the presence of the sulfonyl group (an electron-withdrawing group) [11]. NPX can be biotransformed by o-demethylation of the aromatic methoxy group [34]. The metabolite produced (o-desmethylnaproxen) is recalcitrant under anaerobic conditions, although an aerobic post-treatment could further improve its biotransformation [34,35]. No significant differences were observed in the results during the UASB operation (Figure 2), since P1 and P2 were different only concerning the anoxic-aerobic post-treatment stage. These results are in agreement with previous studies carried out in UASB reactors used for treating medium-low strength sewage [8,36,37].

Apart from the removal of COD and nitrogen, the importance of the polishing step is crucial to obtain higher biotransformation efficiencies of most of the OMPs and particularly of those which were not affected by the UASB reactor. Some compounds such as BPA, E1, and IBP were mainly removed in the polishing stage, whereas there is another group of OMPs for which the combination of the three redox conditions was positive for their removal (ROX or EE2). This is an evidence of how hybrid systems considering different redox environments can improve the possibilities of biological reactors to enhance the biotransformation of many of the compounds present in the complex mixtures of OMPs detected in sewage [10,36,38]. In fact, although higher removal of OMPs is usually reported under aerobic than anoxic conditions, Torresi et al., [39] showed that in absence of a cometabolic limitation, the biotransformation of several OMPs can be more advantageous under anoxic than aerobic conditions.

Figure 3 shows the overall removal efficiencies obtained for the OMPs considering the whole UASB + IFAS process among periods P1 and P2. Since the operation of the UASB reactor suffered no operational changes, the differences between both periods are related to the changes observed in the IFAS polishing stage. The most relevant operational differences that could influence the OMP behavior were those related to nitrification and biomass conformation: P1 only partial nitrification followed by denitrification took place and co-existence of suspended biomass and biofilm in the reactors; P2 complete nitrification and a biological process only driven by biofilm. Despite the wash out of most of the suspended biomass, the OMP removal efficiencies were maintained during P2. In the case of some OMPs, such as ERY, and to a lesser

extent ROX and IBP, the removal efficiencies in P2 even improved. Other authors also observed a higher activity in biofilm systems compared to suspended biomass in terms of the removal of OMPs [40], confirming the importance of the biomass physical conformation. One of the reasons indicated is the higher capacity of biofilms to protect the bacteria against adverse conditions such as the presence of inhibitors [41,42]. Moreover, the longer sludge retention time of the attached biomass facilitates the development of the slow-growing bacteria (such as nitrifiers) enhancing the microbial diversity [15,19]. In this way, Alvarino et al. [8] observed a clear enhancement in the removal of IBP and natural estrogens by incorporating carriers inside a bioreactor, which was correlated with the increase of the nitrification rate.

In the case of the natural hormones E1 and E2, removal efficiencies above 90% were observed in both periods of operation. The presence of strong electron donating functional groups (-OH) in their chemical structures explains their removal under aerobic conditions [43]. This was very relevant in the case of E1 that was totally biotransformed along the anoxic-aerobic stages. However, E2 was mainly removed in the UASB (above 80%), with the remaining fraction degraded in the polishing stage (Figure 2). This behavior is in accordance with Joss et al. [44] who compared the removal of E1 under aerobic and anaerobic conditions and observed a higher biotransformation rate with the positive redox conditions. The removal of the remaining E2 in the anoxic-aerobic compartment was not affected by the shift of bacterial populations observed among both periods (in P2 NOB and anammox bacteria were developed). This is in accordance with Peng et al. [45] who observed that AOB-linked bacteria biotransformation is dominant over the biotransformation carried out by other bacteria.

The removal of the synthetic hormone EE2 was below 70% throughout all periods of operation (Figure 3). A positive contribution of the different redox conditions was observed in the overall removal of this hormone. Unlike in the case of the natural hormones, the removal of EE2 decreased slightly along the operation when the polishing compartment worked as a biofilm reactor and the denitrification rate decreased (P2). In fact, a linear relationship was found between the denitrification rate and the EE2 removal (Figure 4), supporting its removal by cometabolism as previously reported by Su et al.,[46]. They observed that the EE2 removal capacity is driven by heterotrophic denitrifying activity. Moreover, heterotrophic bacteria can contribute to

the removal of EE2, for instance, *Sphingobacterium* is able to biotransform EE2 to E1 under aerobic conditions [47].

A recalcitrant behavior of the anti-inflammatory IBP was observed under anaerobic conditions, while it was readily biotransformed under aerobic conditions (Figure 2). Previous studies showed the influence of nitrification in IBP removal [8,48]. This is a result of the availability of secondary and tertiary carbons in linear alkyl chains in its chemical structure to be hydroxylated by AMO, as well as to the action of NOB [13]. In the reactor, a biotransformation enhancement was observed in P2 for IBP (Figure 3), that might be related to the presence of NOB bacteria when the total nitrification was achieved. In fact, Fernandez-Fontaina et al. [13] determined higher biotransformation rates of IBP when the NOB activity was the predominant in the nitrification. The contribution of other bacteria in IBP removal, such as heterotrophic bacteria, has to be considered, even though these bacteria influence to less extent IBP biotransformation than autotrophic bacteria [13,48].

The removal of the antibiotics ERY and ROX was clearly enhanced throughout the second period of operation (Figure 3). In fact, the behavior of ERY along P1 was quite recalcitrant (removals below 25%), whereas in P2 it was almost completely removed (higher than 95%). ROX also suffered a moderate removal increase along P2. In this case, the development of NOB leading to total nitrification and the growth of anammox bacteria could be factors explaining these observations. In fact, Torresi et al. [49] observed a positive correlation of ERY removal rate and the microbial diversity. Alvarino et al. [8] reported a direct correlation between the ammonium degradation rate and the removal of both antibiotics. Additionally, the influence of the anammox activity on ERY removal in nitritation-anammox reactors was also reported [50].

As shown in Figure 3, DCF was the only compound that exhibited higher removal efficiency in P1 than in P2. The explanation seems to be related to the presence of nitrite in the aerobic compartment (Figure 5a), since only partial nitritation/denitritation was taking place. In fact, a correlation was observed between the nitrite concentration and DCF removal (Figure 5a). Osorio et al. [51] studied the removal of DCF in a conventional nitrification-denitrification process and detected the metabolites nitroso-DCF and 5-nitro-diclofenac in the presence of nitrite. Nitroso-DCF is produced by the nitrosation of the nitrogen atom present in the molecule of DCF, whereas the formation

of 5-nitro-diclofenac is related to the transfer of a nitro group by an electron-deficient group on the aromatic ring of the phenylacetic acid [52].

The fate of the endocrine disruptor BPA shows that it is recalcitrant under anaerobic conditions but readily biotransformed during the anoxic-aerobic stage in both periods of operation (Figures 2 and 3). Its degradation can be related to the nitrification rate in the polishing step [53,54]. In the case of the BPA removal efficiency, a correlation with the nitrate concentration in the aerobic chamber was observed (Figure 5b), which evidences the role of the nitrite oxidation bacteria (NOB) in this transformation. Even though, the BPA removal efficiencies were quite stable during the whole operation of the hybrid system (Figure 3). This indicated that also other bacteria (e.g. heterotrophic bacteria) have played a role in its biotransformation [55].

4. CONCLUSIONS

In the context of developing more sustainable processes for low-strength wastewater treatment, anaerobic digestion based processes are becoming more attractive. The innovative process proposed in this work, based in the combination of an anaerobic step (UASB reactor) and an IFAS system, is especially suited to minimize problems derived from this approach, such as nitrogen and methane emissions, as well as to achieve high removal efficiencies for a wide range of OMPs. In this system, high removals of COD were achieved (93%), whereas nitrogen was removed by a consortia of heterotrophic denitrifiers, methane oxidation, and anammox microorganisms. Methanotrophs present in suspended biomass and biofilms in the anoxic-aerobic compartments were responsible for the reduction of dissolved methane up to 85%. The anaerobic stage enhanced the removal of several OMPs, such as the antibiotics TMP and SMX with respect to the conventional anoxic-aerobic processes. The combination of several biomass conformations enhanced the microbiology of the reactor, mainly under anoxic conditions. The growth of anammox bacteria in the biofilm influenced positively the removal of the antibiotic ERY. A correlation between the presence of nitrite in the aerobic compartment and the removal of DCF was observed, while the removal of BPA was slightly dependent on the nitrite oxidation. The biomass washout episode did not have a significant effect on OMP removal (showing the crucial role of biofilm), although methanotrophs were clearly affected. The maintenance of biomass

concentrations rich in these organisms would be a tool to optimize the system in terms of nitrogen and methane abatement.

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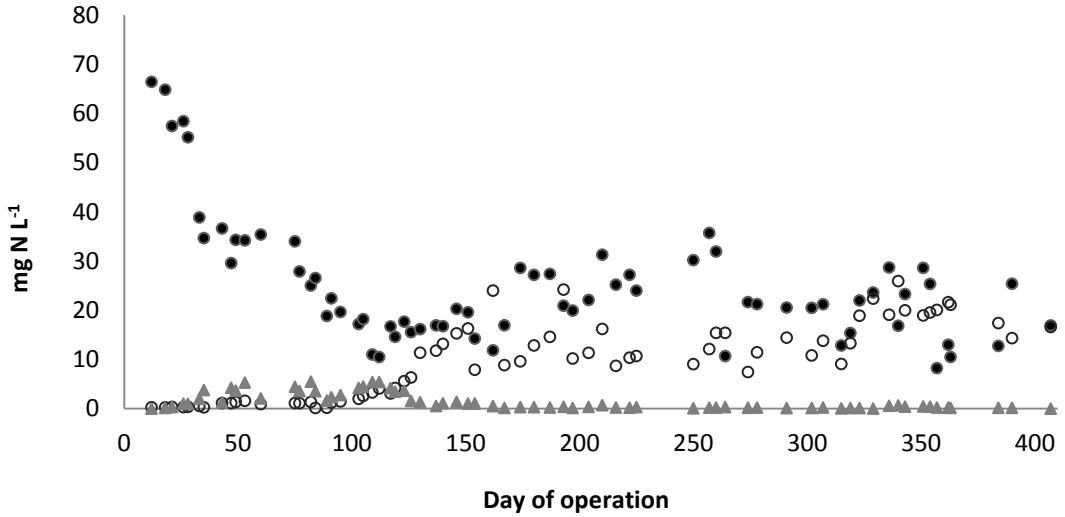
Figure

A)



B)

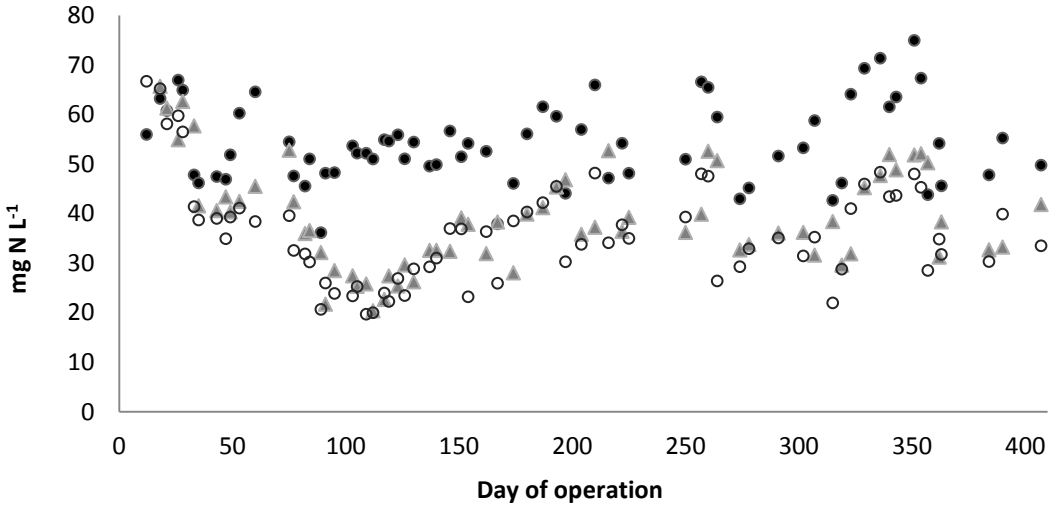


Figure 1.Evolution of A) the nitrogen ions concentration in the final effluent: N-NH_4^+ (●), N-NO_2^- (▲) and N-NO_3^- (○); and B) the comparison of the TN in the three stages: anaerobic (●), anoxic (▲) and aerobic (○) stages.

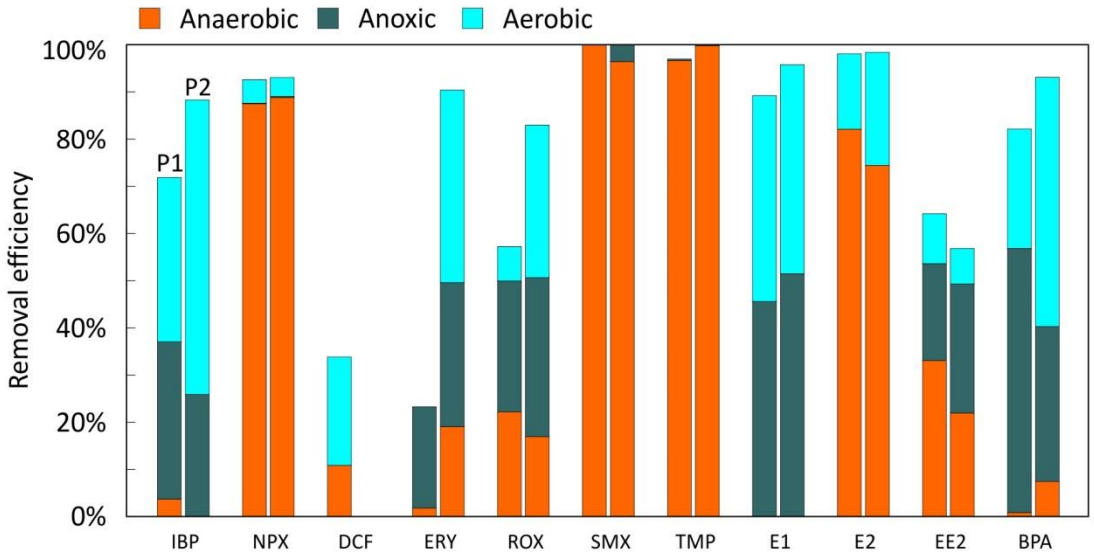


Figure 2. OMPs removal efficiencies attained in each compartment: anaerobic, anoxic and aerobic along both periods of operation (P1 and P2).

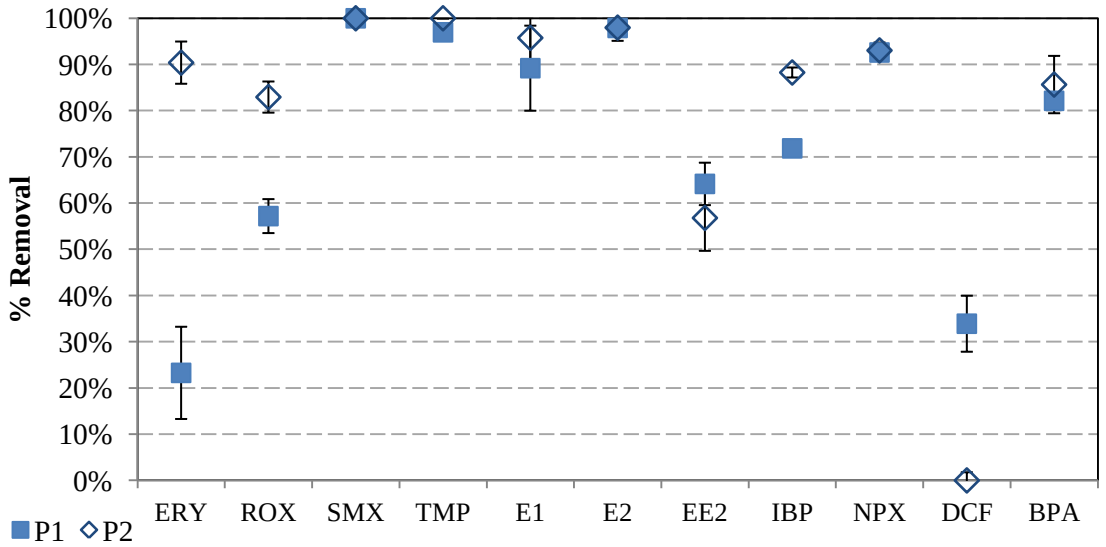


Figure 3. Comparison among both periods of operation in terms of OMPs removal attained in the overall system.

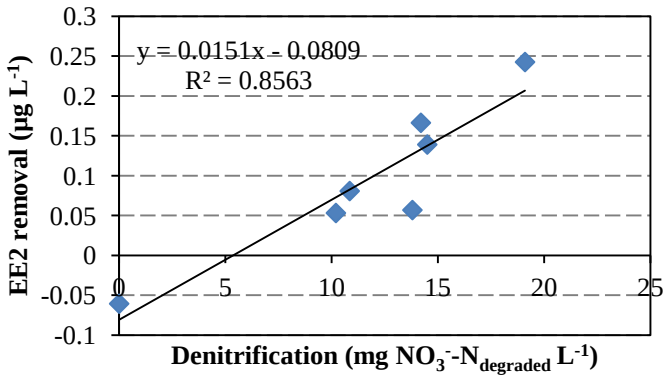


Figure 4. Influence of the denitrification rate in the removal of the hormone EE2.

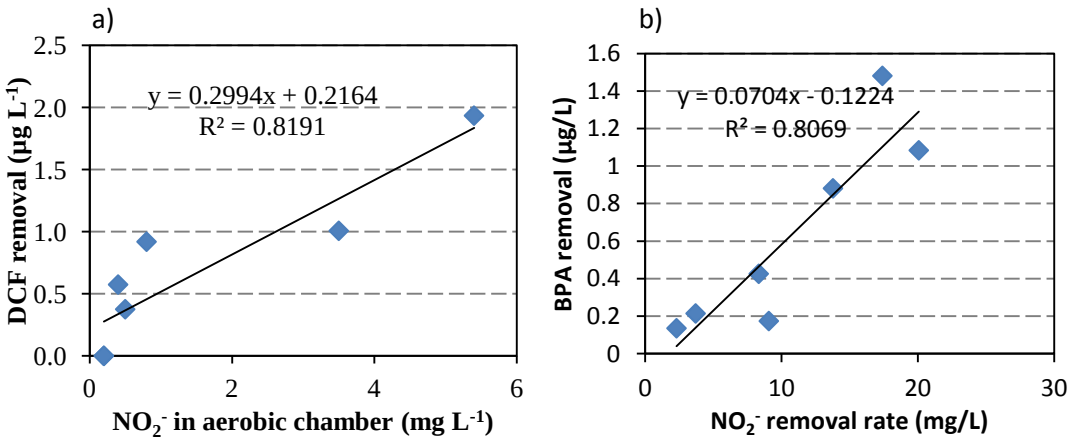


Figure 5. Influence of presence of nitrite in the aerobic compartment in the removal of DCF (a) and the nitrite oxidation in the removal of BPA (b).

Materials and Methods

Molecular biology techniques

Ntspa712 (most members of phylum Nitrospirae), Nso1225 (Ammonio-oxidizing-β-Proteobacteria), NIT3 (Nitrobacter spp.), Amx368 (all anammox bacteria), MG705 and MG84 (Type I methanotrophs), DBACT193 and DBACT1027 (damo bacteria) and DARCH872 (damo archaea) were the probes selected for FISH analysis. All the details of each probe (formamide percentage, sequence and target organism) can be found in the probe-Base database (http://www.microbial-ecology.net/probebase/). Fluorescence signals were recorded with an acquisition system (Coolsnap, Roper Scientific Photometrics) coupled with an Axioskop2 epifluorescence microscope (Zeiss, Germany).

For the sequencing of the 16S rRNA gene, the protocol described by Regueiro et al. [1] was followed with few modifications. In brief, after extracting the bulk DNA, the V3V4 and V2V3 regions of the 16S rRNA gene were amplified for Bacteria [2] and Archaea [3] domains respectively, and sequenced in an Illumina MiSeq platform. Raw sequences were filtered to remove low-quality reads and then clustered into Operational Taxonomic Units (OTU) at 97% of sequence similarity using QIIME v1.9.1 [4]. Community diversity was analyzed to measure the compositional complexity of reactor microbiome. Richness was evaluated by the estimated the number of species (S) and the Gini-Simpson index (HGS) while community evenness was measured with the Simpson evenness index (E). The community similarity was determined by Bray-Curtis dissimilarities and explored by non-metric multidimensional scaling (NMDS). All statistical analyses were performed in R [5]. Finally, a co-occurring microbial network was built for the abundant OTUs (relative abundances over 0.1%) with CoNet v.1.1.1 [6].

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