

Research article

Simultaneous nitrogen and dissolved methane removal from an upflow anaerobic sludge blanket reactor effluent using an integrated fixed-film activated sludge system

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ABSTRACT

One of the main drawbacks of upflow anaerobic sludge blanket (UASB) reactors that treat low-strength sewage at room temperature is related to the low quality of their effluents in terms of dissolved methane, organic matter, and nitrogen content. The present study aims to evaluate the feasibility of using an integrated fixed-film activated sludge (IFAS) system as an alternative post-treatment technology to mitigate the environmental impact of such effluents. For this purpose, a pilot plant composed of a UASB (120 L) followed by an IFAS (66 L) system was operated for 407 days. Special attention was paid to the suspended biomass retention capacity and the dissolved methane and nitrogen removal potential of the IFAS post-treatment system. Furthermore, the role of carriers on denitrification and nitrification processes and the microbial communities present in the biofilm were also analyzed. Average total chemical oxygen demand (CODT) and ammonium removal efficiencies of $92 \pm 3\%$ and around $57 \pm 16\%$ were attained throughout the entire operation, respectively. During a first period in which biomass was maintained in both biofilms and suspension, and nitrite was the main electron acceptor, maximum nitrogen removal and methane removal efficiencies of 32.5 mg TN L⁻¹ and 93% were observed in the IFAS system, respectively. However, throughout the second period, in which suspended biomass was completely washed out from the IFAS system, and nitrate became the main electron acceptor, these values decreased to 18 ± 4 mg TN L⁻¹ and $77 \pm 12\%$, respectively. Surprisingly, throughout the entire operation, it was observed that around 50 and 41% of the total nitrogen and methane removals observed in the IFAS system, respectively, were carried out in the aerobic compartment. Aerobic methane oxidizers and anammox were detected with significant relative abundances in the biofilm carriers used in the anoxic and aerobic compartments using 16S rRNA gene amplicon sequencing analysis. Therefore, the use of an IFAS system could be suited to diminish greenhouse gas emissions and nutrients concentration for those sewage treatment plants that used UASB systems, especially in countries with temperate and warm climates.

1. Introduction

Anaerobic wastewater treatment technologies are widely used in warm climate countries for treating low-strength streams at room temperature. Indeed, there are hundreds of upflow anaerobic sludge blanket (UASB) reactors operating in different parts of (semi)tropical regions due to low or even zero energy demand (Chernicharo et al., 2015). One of the main constraints of the UASB technology is related to its insufficient removal of organic matter and nitrogen. Given the low C/N ratios commonly observed in anaerobic effluents, the addition of expensive external carbon sources (e.g., ethanol and methanol) is frequently

necessary to improve nitrogen removal through heterotrophic denitrification processes. In addition, Noyola et al. (2006) estimated that up to 50% of the methane produced in methanogenic reactors that treat domestic sewage at 20 °C could remain dissolved in their effluents. Methane is a strong greenhouse gas (GHG) which contributes to aggravate the climate change problem. However, this dissolved methane present in UASB effluents can be biologically used as an inexpensive electron donor to denitrify to reduce both GHG emissions and nitrogen content of the treated wastewater. So far, different simultaneous nitrogen and methane removal bioprocesses are distinguished. First, in aerobic methane oxidation coupled to denitrification (AMO-D), strict aerobic methanotrophs oxidize methane into methane oxidation

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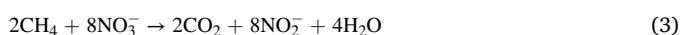
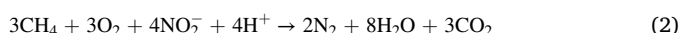
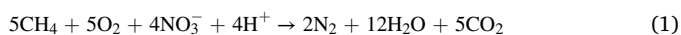
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Abbreviations

AMO-D:	aerobic methane oxidation coupled to denitrification
Anammox	anaerobic ammonium oxidation
CODS	soluble chemical oxygen demand
CODT	total chemical oxygen demand
DO	dissolved oxygen
FA	Free ammonia
GHG	greenhouse gas
HYME-D:	hypoxic methane oxidation coupled to denitrification
IFAS	Integrated fixed-film activated sludge
MBR	membrane bioreactor
MLTSS	mixed liquor total suspended solids

MLVSS	mixed liquor volatile suspended solids
MRR	methane removal rate
N-damo archaea	nitrate-dependent anaerobic methane oxidation archaea
N-damo bacteria	nitrite-dependent anaerobic methane oxidation bacteria
NOB	nitrite-oxidizing bacteria
NRR	nitrogen removal rate
OLR	organic loading rate
R	recycling ratio
TN	total nitrogen
UASB:	upflow anaerobic sludge blanket

products (Modin et al., 2007). Afterward, conventional heterotrophic denitrifiers can use these oxidation products as an electron donor to reduce nitrate and/or nitrite to dinitrogen gas (Equations (1) and (2)). Second, in the anaerobic pathway two main microorganisms are involved: nitrate-dependent anaerobic methane oxidizers (n-damo archaea), such as “*Candidatus Methanoperedens nitroreducens*” (Haroon et al., 2013), which are able to reduce nitrate to nitrite using methane as electron donor (Equation (3)), and nitrite-dependent anaerobic methane oxidizers (n-damo bacteria), such as “*Candidatus Methyloirabilis oxyfera*” (Ettwig et al., 2010), which are able to reduce nitrite to dinitrogen gas using also methane as electron donor (Equation (4)). Third, Cao et al. (2019) also demonstrated an innovative pathway for removing methane and nitrogen in extreme oxygen-limited conditions, which consist of hypoxic methane oxidation coupled to denitrification (HYME-D), where, instead of using oxygen as the final electron acceptor, aerobic methanotrophs directly utilize nitrate as electron acceptor to oxidize methane and produce organic compounds that could be subsequently utilized by conventional heterotrophic denitrifiers to denitrify.



Therefore, to minimize the environmental impact of effluents from UASB reactors that treat low-strength sewage at room temperature, and to comply with the increasingly severe effluent discharge regulations, further low-footprint post-treatment systems are required. On this ground, authors such as Cai et al. (2015), Van Kessel et al. (2018) and Liu et al. (2019) proposed the use of dissolved methane as an inexpensive electron donor to denitrify. In previous studies carried out in our laboratories, a hybrid MBR system was proposed as an alternative post-treatment system to minimize the impact of effluents from UASB reactors that treat domestic sewage at room temperature (Silva-Teira et al., 2017; Alvarino et al., 2019). In the present research, the same idea of using dissolved methane as an electron donor to denitrify is considered. However, the utilization of an integrated fixed-film activated sludge (IFAS) with a secondary settler instead of a membrane bioreactor (MBR) system was evaluated. Unlike the MBR technology, which requires a high energy demand associated with membrane operation, IFAS systems can result in a less-energy consuming alternative, but probably with lower effluent quality. The use of biofilm technologies such as the IFAS system, which combines the use of both attached and suspended biomass, has arisen as an alternative for the treatment of domestic or industrial sewage, leading to more compact systems than the conventional activated sludge ones. The IFAS technology enables to increase the effective biomass concentration, improving biomass diversity without the need to enlarge the size of the different compartments of

treatment plants. Furthermore, biofilm carriers enhance the retention of slow-growth microorganisms, such as nitrifiers (Randall and Sen, 1996) or aerobic/anaerobic methane oxidizers, preventing them from being washed out through the IFAS final effluent. Besides, biofilms are spatially heterogeneous, which makes possible the coexistence of anaerobic, anoxic, and aerobic bioprocesses in them (Leyva-Díaz et al., 2016).

The present study aims to evaluate the feasibility of using an IFAS system as an alternative post-treatment technology to mitigate the environmental impact of two of the main pollutants present in UASB effluents, such as dissolved methane and nitrogen. To this end, a low-footprint bench-scale plant (186 L), made up of a UASB (120 L) followed by an IFAS (66 L) system, was operated. In a previous study, within the same integrated system operation, Arias et al. (2018) analyzed the removal efficiencies achieved for a wide range of organic micropollutants. However, in the present study, special attention was given to the suspended biomass retention capacity and the potential for removal of dissolved methane and nitrogen removal of the IFAS post-treatment system. Furthermore, the role of carriers in the processes of denitrification and nitrification and the microbial communities present in the biofilm were also analyzed. Finally, the results achieved in this research were compared with previous studies, where similar operating conditions were applied (Silva-Teira et al., 2017; Alvarino et al., 2019). Nevertheless, instead of using an IFAS, a hybrid MBR was proposed as a post-treatment alternative to treat effluents from UASB reactors.

2. Materials and methods

2.1. Reactor configuration and operation

A 186 L pilot plant (Fig. 1 and S1) was used for 407 days in order to treat domestic sewage at room temperature. The pilot plant was first composed of a 120 L methanogenic reactor, using the UASB configuration with granular sludge. The obtained UASB effluent was subsequently led to a pre-anoxic IFAS system composed of three different compartments: a first anoxic compartment (36 L) with mechanical mixing, in which simultaneous nitrogen and methane removal bioprocesses were intended, using suspended biomass and also biomass attached to polyurethane sponge biofilm carriers (Levapor); a second aerobic compartment (20 L), where ammonium oxidation processes were carried out, with the presence of suspended biomass and biofilm adhered to rigid polyethylene supports (Biochip); and finally, a settler (10 L) in which the physical biomass/effluent separation was carried out.

Three different types of biomass conformation (granular, attached to carriers, and suspended) and three redox conditions (anaerobic, anoxic, and aerobic) were present in the suggested integrated system. The two different biofilm carriers used, Levapor and Biochip (Fig. S2), presented an external specific surface of 486 and 2174 m² m⁻³, and a volume of

2.8×10^{-3} and 4.15×10^{-4} L, respectively. The amount of carrier used for both varied throughout the experimentation period. The initial apparent volume percentage for Levapor was of 20%. However, this value was raised up to 23% on day 250. As a consequence of the higher difficulty in keeping Biochip carriers homogeneously mixed, the initial apparent volume percentage was only 7%. Nevertheless, this value was stepwise increased to 12, 16, and 19% on days 135, 247, and 309, respectively. Biochip was utilized in the aerobic compartment, instead of Levapor, due to its 4.47 times higher external specific surface, which favors the nitrifying capacity per unit of carrier volume, where oxygen transfer usually limits biological conversions (Henze et al., 2002). Conversely, Levapor carriers were used in the anoxic compartment because of their higher void volume, which could favor the development of anaerobic environments.

The UASB was continuously fed with synthetic wastewater, mimicking a typical composition for domestic sewage: skimmed milk (diluted with tap water); NaHCO_3 (960 mg L^{-1}); NH_4Cl (18 mg N L^{-1}) and trace elements with the same composition as proposed by Silva-Teira et al. (2017).

Two different recirculation streams were applied in the post-treatment system: an internal recycling ratio (R) of 1.5 was applied from the aerobic to the anoxic compartment to provide an electron acceptor, and an external recirculation from the settler to the anoxic chamber to return the activated sludge to the post-treatment system (R = 1.5). Therefore, a total recirculation value of 3 was fixed throughout the operation. This crucial parameter also governs the contact time in each post-treatment compartment and the quantity of O_2 that is recycled to the anoxic compartment. The UASB reactor was inoculated with active biomass from the brewery industry, while the post-treatment system was seeded using stored flocculent biomass obtained from the MBR post-treatment system operated by Silva-Teira et al. (2017).

Two main periods were distinguished during this study. In Period I (days 0–167), suspended and attached-to-carriers biomass were observed in the IFAS post-treatment system. Period II (days 168–407) was characterized by the presence of only attached-to-carriers biomass because all the suspended biomass was unintentionally washed out from the IFAS system.

2.2. Batch tests experimental procedure

In order to better understand the processes of denitrification and oxidation of methane in the proposed IFAS system, batch assays were performed in 500 mL flasks on day 407, using only biofilm adhered to Biochip and Levapor biofilm carriers. Thus, biomass conditions were specific to that given period. Processes such as anaerobic ammonium oxidation (anammox; $\text{NH}_4^+ + \text{NO}_2^-$), conventional heterotrophic denitrification (Acetate + NO_3^-), and anaerobic methane oxidation coupled to denitrification processes, using nitrate and nitrite ($\text{CH}_4 + \text{NO}_3^-/\text{NO}_2^-$) as electron acceptors, were evaluated. In the Levapor assays, the flasks were filled with 324 mL of a phosphate buffer solution (143 mg KH_2PO_4 L^{-1} and 740 mg K_2HPO_4 L^{-1}) and 27 foam biofilm carriers (76 mL of volume, 19% apparent volume). In the Biochip assays, the bottles were filled with 221 biofilm carriers (92 mL of volume, 23% apparent volume) and 302 mL of the same buffer solution. After adding the carriers into the flask bottles, they were flushed for 5 min with nitrogen or methane, according to the conditions of the tests. For the $\text{CH}_4 + \text{NO}_2^-/\text{NO}_3^-$ tests, the headspace of the flasks was saturated in CH_4 . Stock solutions with NH_4Cl , NaNO_2 , and $\text{CH}_3\text{COONa} \cdot 3\text{H}_2\text{O}$ were prepared. The flasks were incubated at 25 °C and shaken at 150 rpm for 8 h.

2.3. Liquid and gas samples analysis

Nitrogen species, Total (COD_T) and soluble chemical oxygen demand (COD_S), mixed-liquor total and volatile suspended solids (MLTSS and MLVSS), pH and temperature were measured according to Standard Methods (Rice et al., 2012). The dissolved oxygen (DO) concentration in

both compartments was also periodically measured by a multi-parameter meter (Hach HQ40d), with a luminescent optical probe (IntelliCAL LDO101). The dissolved methane concentration in the liquid phase of the anoxic and the aerobic compartments was measured according to the procedure described by Sánchez et al. (2016). A Milli-Gascounter type MGC-10 (Ritter) was used to measure the biogas production, while the biogas composition, including CO_2 , N_2 , and CH_4 , was analyzed using a gas chromatograph 5890 series II (Hewlett-Packard) with a thermal conductivity detector and a column of Porapak Q 80/100 2 m x 1/8" (SUPELCO), with column and detector temperatures of 34 °C and 110 °C, respectively, and helium as carrier gas.

2.4. DNA extraction and 16S rRNA gene amplicon sequencing

Biomass samples were taken from Levapor packing materials on days 175, 265, and 368. Furthermore, the attached biomass composition was also analyzed in Biochip carriers, on day 368. Unfortunately, suspended biomass communities were not evaluated due to complete washing of biomass detected from day 167 onwards. Microbial communities of the two carriers were monitored by sequencing the V3V4 and V2V3 regions of the 16S rRNA gene for bacteria and archaea, respectively. Amplicon libraries, sequencing, and data analysis were performed as described in the Supplementary Material. The sequences obtained in this study are already accessible in the Sequence Read Archive database (BioProject ID: PRJNA588584).

3. Results and discussion

3.1. General results

The pilot plant was continuously operated over a period of 407 days, with an average temperature value of 21.1 ± 1.4 °C. A daily feed flow of 138.3 ± 21.9 L d^{-1} was treated, with COD_T and COD_S concentrations of 891 ± 214 and 782 ± 204 mg COD L^{-1} , which resulted in an average organic loading rate (OLR) of around 1 kg COD $\text{m}^{-3} \text{d}^{-1}$ (referred to the UASB system). Average HRT values of 21.1 ± 3.1 and 9.8 ± 1.5 h were applied for the UASB and IFAS post-treatment systems, respectively. Considering both the external and internal recirculation in the IFAS system, a constant R of 3 ± 0.6 was applied throughout the operation. A pH value of 7.26 ± 0.26 was observed in the UASB throughout the operation, while values of 7.6 ± 0.1 and 7.9 ± 0.2 were determined in the anoxic and aerobic compartments of the IFAS system, respectively. During the entire experimentation period, a high average COD_T removal efficiency of $93 \pm 2\%$ was detected in the first methanogenic stage. From the beginning of the operation, high COD removals were observed in the UASB reactor due to the large amount of anaerobic granules used as inoculum. Approximately 75% of the overall COD inlet was methanized in the UASB reactor, producing a biogas flow of 37.3 ± 8.5 L d^{-1} , with the following composition: $75 \pm 2.5\%$ CH_4 , $15 \pm 2.8\%$ CO_2 , and $9 \pm 3\%$ N_2 . Nevertheless, not all methane produced was present in the gas phase, but 17.6 ± 2.9 mg CH_4 L^{-1} dissolved in the anaerobic effluent were detected, representing the $14 \pm 6\%$ of the total methane produced. Low COD_T and COD_S concentrations were detected in the anaerobic effluent, with values of only 57 ± 15 and 43 ± 12 mg COD L^{-1} , respectively, excluding in both measurements the portion related to the dissolved methane COD. This organic matter could be used as an electron donor in the pre-anoxic compartment of the IFAS system, promoting heterotrophic denitrification processes. Nevertheless, COD_T was also detected in the final effluent of the system, at values of 77 ± 21.4 and 53.8 ± 12 mg COD L^{-1} , during Periods I and II, respectively. During Period I, due to the continuous VSS washout in the IFAS system, COD concentrations in the final effluent were even higher than those detected in the UASB effluent. Regarding Period II, COD concentrations in the UASB outlet and in the final effluent were similar. This fact indicates that the biodegradable COD fraction of the UASB effluent is very low.

3.2. Impact of biofilm carriers' addition on the nitrification potential

Most of the total nitrogen (TN) of the UASB effluent was present in the form of ammonium, with an average concentration value of 54 ± 8 mg $\text{NH}_4^+\text{-N L}^{-1}$ (Fig. S3). Oxygen was continuously provided in the aerobic compartment to allow nitrification processes, and average DO concentration values of 3.2 ± 0.7 and 4.4 ± 0.6 mg $\text{O}_2 \text{ L}^{-1}$ were measured during Periods I and II, respectively. Whenever the Biochips' apparent volume was higher, the ammonium oxidizing rates, referred to the aerobic compartment, accordingly increased. Average values of 157.1 ± 61.5 , 165.4 ± 34.7 , 191.5 ± 47.9 , and 240.6 ± 55.5 mg $\text{NH}_4^+\text{-N L}^{-1} \text{ d}^{-1}$ were attained for the aforementioned periods, respectively (Fig. S4). Nevertheless, despite the supports addition, full ammonium oxidation was not accomplished during the study and concentrations of 25.3 ± 12.3 , 23.2 ± 5.2 , 23.2 ± 6.7 , and 19.3 ± 6.5 mg $\text{NH}_4^+\text{-N L}^{-1}$ were measured (Fig. S5).

Although the suspended biomass was totally washed-out from the IFAS system on day 167, significant ammonium oxidation rates were achieved during Period II (without the presence of suspended biomass in the IFAS system), indicating that biofilms were responsible for the removal of ammonium observed. This highlights the essential role of biofilm carriers in nitrification processes. In order to accomplish better nitrification rates, a higher surface area of biofilm carriers is required by increasing either the aerobic compartment volume or the carrier added volume. A second strategy is to generate higher DO concentrations in the aerobic compartment, which is usually the limiting substrate in biofilm processes.

Partial nitrification processes were observed in the aerobic compartment during most of Period I. In fact, throughout the first 119 days of operation, nitrite was the main available electron acceptor, with an average concentration of 3.5 ± 1.5 mg $\text{NO}_2^-\text{-N L}^{-1}$ (Fig. S5). From that day onwards, the nitrite concentration considerably declined due to the nitrite-oxidizing bacteria (NOB) activation. As a result, nitrate became the most abundant oxidized nitrogen species in the aerobic stage. During most of Period I, the estimated average free ammonia (FA) concentration of 0.81 mg $\text{NH}_3\text{-N L}^{-1}$ may have partially inhibited the NOB activity. It has been reported in the literature that FA values between 0.1 and 1.0 mg $\text{NH}_3\text{-N L}^{-1}$ initiated the inhibition of NOB activity (Anthonisen et al., 1976). Nevertheless, over time, the biomass colonization of the packing media could have provided a more suitable environment for NOB activity to tolerate the inhibitory effect of FA (Villaverde et al., 2000). During Period II, a nitrite concentration of only 0.25 ± 0.17 mg $\text{NO}_2^-\text{-N L}^{-1}$ was detected.

3.3. Suspended biomass washout in the IFAS system

One of the main concerns of the present study was related to the suspended biomass retention capacity of the IFAS system when treating low-C/N ratio UASB effluents, mainly due to the reports regarding the poor settling properties of suspended biomass when treating effluents with these characteristics (Metcalf and Eddy et al., 2013). At the beginning of the operation, the MLTSS and MLVSS concentrations measured in the pre-anoxic compartment were of 3.5 and 3.1 g L^{-1} (Fig. 2), respectively.

Nevertheless, from day 47–89, as a consequence of the low mixing in the anoxic compartment, large amounts of biomass gradually accumulated in the bottom part, resulting in a dramatic drop in the suspended solids concentration. Once the mechanical mixing was increased on day 83, the mixed liquor was immediately resuspended, obtaining a homogeneous mixture, which increased the MLVSS concentration. Between days 109 and 167, the MLVSS concentration declined in the IFAS system from 2.62 to only 0.05 g L^{-1} . During that period, sludge washout was observed in the effluent of the IFAS system, with MLTSS and MLVSS concentrations of approximately 61 ± 11 and 45 ± 12 mg L^{-1} , respectively. The average liquid up-flow velocity in the settler compartment was $0.31 \text{ m}^3 \text{ m}^{-2} \text{ h}^{-1}$ throughout the entire operation. Curiously, the biomass washout was concomitant with the period in which nitrite began to oxidize in the aerobic compartment, making nitrate the main electron acceptor. From day 167 onwards, the MLTSS and MLVSS observed in the final effluent were around 39.9 ± 11.4 and 33.7 ± 9 mg L^{-1} , respectively.

The low average OLR applied to the IFAS system throughout the entire operation, $0.14 \text{ kg COD m}^{-3} \text{ d}^{-1}$, and the poor organic removal rate observed in the IFAS system due to the low biodegradable COD content of the UASB effluent could be the main causes responsible for the biomass washout. Consequently, the only way of treating these kinds of anaerobic effluents with low C/N ratios is by promoting biofilm growth adhered to supports. Therefore, if the complete retention of suspended biomass is intended, a pre-anoxic MBR system, such as that done by Alvarino et al. (2019), must be used.

3.4. Methane removal in the IFAS post-treatment system

Methane removals in the anoxic compartment of the IFAS system were calculated using mass balances (Supplementary Material). During Period I, an upward trend in methane removal was clearly observed, attaining a maximum volumetric methane removal rate (MRR) and methane removal efficiency of $52.5 \text{ mg CH}_4 \text{ L}^{-1} \text{ d}^{-1}$ and 72.7% on day 126, respectively (Fig. 3), both values referred only to the anoxic

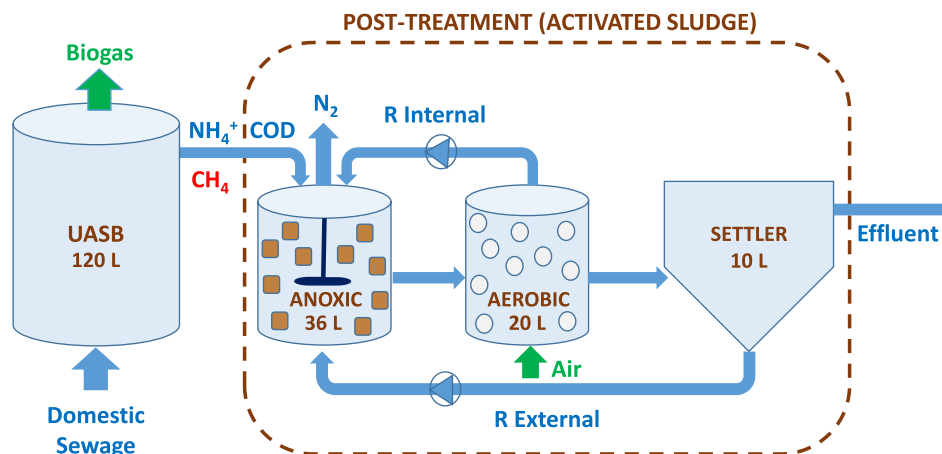


Fig. 1. Schematic diagram of the UASB methanogenic reactor coupled to an IFAS post-treatment system.

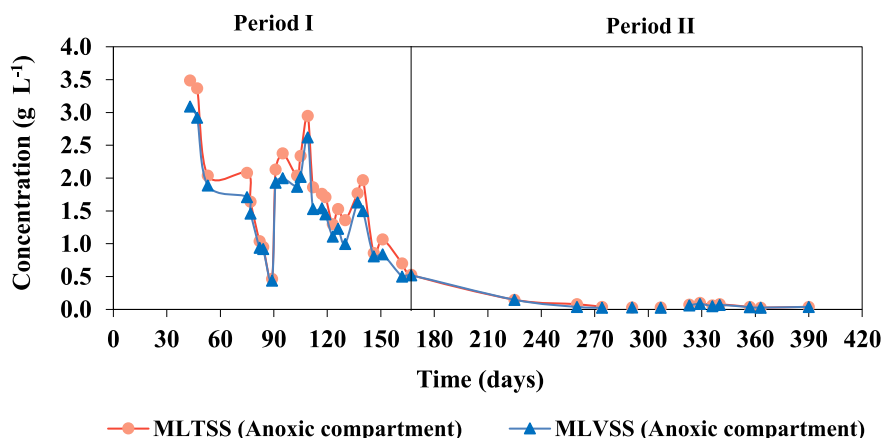


Fig. 2. Evolution of the mixed-liquor total (MLTSS) and volatile (MLVSS) suspended solids concentration in the anoxic compartment.

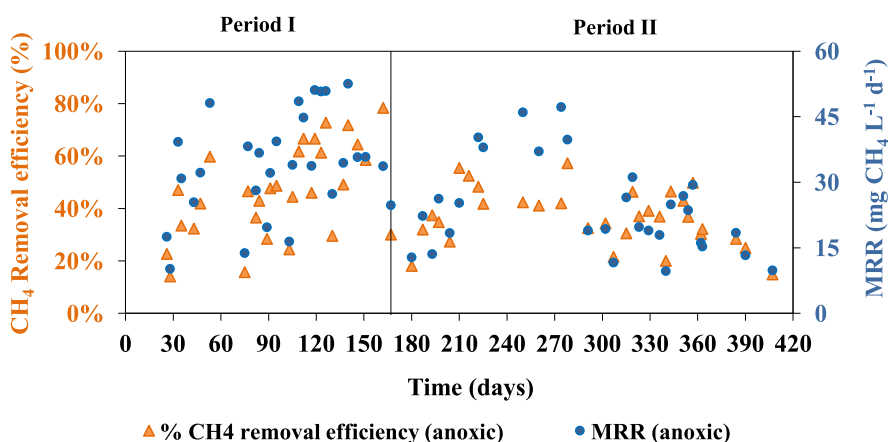


Fig. 3. Evolution of methane removal efficiencies and volumetric methane removal rates (MRR) in the pre-anoxic compartment of the IFAS system.

compartment. Nevertheless, once the suspended biomass from the IFAS system was lost, and nitrate became the main electron acceptor detected, methane removals significantly dropped into the anoxic compartment. During Period II, values of $23.9 \pm 10.4 \text{ mg CH}_4 \text{ L}^{-1} \text{ d}^{-1}$ and $36.6 \pm 10.5\%$ were attained, respectively. Thereby, the complete loss of suspended biomass in the IFAS system led to a reduction of around 50% in both parameters. In spite of the negative effect of the suspended biomass washout, the methane removals detected during Period II, highlight the important role of the adhered biomass in the methane oxidation bioprocesses.

The recirculations applied in the post-treatment system will determine the amount of DO provided to the anoxic compartment, which could cause the proliferation of aerobic methanotrophs. If it is assumed that all oxygen reduced in the anoxic stage during Period I and II, 32.5 ± 9.3 and $41.8 \pm 5.9 \text{ mg O}_2 \text{ L}^{-1} \text{ d}^{-1}$, respectively, was consumed through aerobic methanotrophs, and considering its stoichiometry ($2 \text{ g O}_2 \text{ g}^{-1} \text{ CH}_4$; Equations (1) and (2)), these microorganisms could explain up to 48% and 87% of the average MRRs observed in the anoxic compartment during Period I and II, respectively. Besides aerobic methane oxidation processes, some other metabolic pathways could be involved, especially during Period I, such as anaerobic methane oxidation and even the possibility of other lesser-studied metabolic pathways, such as the HYME-D suggested by Cao et al. (2019).

In previous studies, Silva-Teira et al. (2017) treating the same type of domestic sewage, under similar operating conditions, but using an MBR instead of an IFAS system, achieved a maximum MRR of $195 \pm 17 \text{ mg}$

$\text{CH}_4 \text{ L}^{-1} \text{ d}^{-1}$ in the anoxic compartment. This value is almost four times higher than the maximum attained in the current research ($52.5 \text{ mg CH}_4 \text{ L}^{-1} \text{ d}^{-1}$). This fact could be attributed to the presence of an ultrafiltration membrane, which ensured the complete retention of suspended biomass in the MBR. Furthermore, they observed that MRRs depended on the applied feed flow, which was approximately three times higher in the previous study ($355 \text{ L}_{\text{feed}} \text{ d}^{-1}$) than in the current one ($138 \text{ L}_{\text{feed}} \text{ d}^{-1}$). However, the highest methane removal efficiencies observed in the anoxic compartment during this research, with the presence of nitrite and suspended biomass (78%), were similar to the maximum attained by Silva-Teira et al. (2017). This suggested that the MRR observed during Period I, with the presence of microorganisms growing in biofilms and in suspension, was limited by the hydraulic load applied to the system. During Period II, in which only biofilms were responsible for the methane oxidation, MRRs could be limited by the transfer of either methane or oxygen to the biofilms.

The dissolved methane that was not oxidized in the first anoxic compartment was led to the aerobic one. This methane could be biologically oxidized or stripped-off into the atmosphere. Throughout the entire operation, dissolved methane was detected in the aerobic compartment, with values ranging from 2.10 to $0.01 \text{ mg CH}_4 \text{ L}^{-1}$. Average concentrations of 0.25 ± 0.2 and $0.32 \pm 0.4 \text{ mg CH}_4 \text{ L}^{-1}$ were measured during Periods I and II, respectively. The continuous detection of methane allowed to estimate the amount of this compound that was either desorbed or biologically oxidized in the aerobic compartment (Supplementary Material).

Average MRRs of 53.1 ± 33.4 and 43.5 ± 20.3 mg $\text{CH}_4 \text{ L}^{-1} \text{ d}^{-1}$ were estimated in the aerobic compartment during Period I and II, respectively. This indicates the crucial role of the aerobic compartment to minimize GHGs emissions from the process. In fact, the average MRRs observed in the aerobic compartment for Period I are similar to the maximum achieved in the anoxic compartment, and almost double the average values achieved during Period II. Considering the methane removals observed in the anoxic compartment and those estimated in the aerobic compartment, high overall removal efficiencies of $89.1 \pm 10\%$ and $77 \pm 12\%$ were achieved in the whole IFAS post-treatment system during Periods I and II, respectively. The remaining fraction of methane that was not biologically oxidized was considered to be stripped off into the atmosphere. Due to the high oxygen concentration measured in the aerobic compartment, a high fraction of the methane was surely completely oxidized to carbon dioxide. However, the development of AMO-D processes should not be discarded due to the presence of anoxic environments inside the Biochip.

3.5. Nitrogen removal performance in the IFAs post-treatment system

Similarly, methane mass balances in the anoxic and aerobic compartments, and in the whole IFAS post-treatment system were carried out. An average value of 54 ± 8 mg $\text{NH}_4^+\text{-N L}_{\text{feed}}^{-1}$ was observed in the UASB throughout the research. Due to the low organic matter content observed in anaerobic effluents, the use of a coexisting alternative electron donor to denitrify, such as ammonium or dissolved methane, is required to accomplish the denitrification processes. From the beginning of the study, the overall TN removal progressively increased throughout the whole IFAS system, reaching a maximum removal of $32.5 \text{ mg TN L}_{\text{feed}}^{-1}$ on day 109 (Fig. 4). Nevertheless, nitrogen removals dropped between days 126 and 167. This decrease was concomitant to the suspended biomass loss and also with the lower presence of nitrite in the aerobic compartment. However, although only attached biomass was available throughout Period II, an important average nitrogen removal of $18 \pm 3.9 \text{ mg TN L}_{\text{feed}}^{-1}$ was attained.

Regarding the nitrogen removal rate (NRR), a maximum value of $119.6 \text{ mg TN L}^{-1} \text{ d}^{-1}$ and an average value of $29.9 \pm 17.1 \text{ mg TN L}^{-1} \text{ d}^{-1}$ for Periods I and II, respectively, were observed in the first anoxic compartment of the IFAS system. Curiously, denitrification processes were not only restricted to this anoxic compartment but were also continuously detected in the aerobic compartment from the beginning of the study. This fact could be demonstrated because the average TN concentration in the final effluent was $2.9 \pm 1.7 \text{ mg N L}_{\text{feed}}^{-1}$ lower than the measured in the anoxic compartment. Differences in the TN concentrations between these two streams were permanently detected throughout the study (Fig. S3). Using mass balances, it was calculated

that 45.5 ± 24.4 and $54.9 \pm 24.2\%$ of the TN removal achieved was surprisingly carried out in the aerobic compartment in the IFAS system during Period I and II, respectively. The existence of different DO concentration gradients, which is characteristic of biofilm systems, may promote anoxic environments in the inner layers of the Biochip carriers, explaining the observed nitrogen removals in the aerobic compartment. This evidences that simultaneous nitrification-denitrification processes were conducted in the Biochip carriers.

The COD concentration observed in the final effluent of the post-treatment system was higher or similar than in the UASB effluent. This could indicate a minor role of conventional heterotrophic denitrifiers in the nitrogen removal achieved in the IFAS system. Therefore, other compounds have been alternatively used as an electron donor to denitrify. Part of the nitrogen removal could be explained by means of conventional heterotrophic denitrifiers, using the methane oxidation products produced by aerobic methanotrophs (AMO-D) in the anoxic and aerobic compartments, or by other methane oxidation pathways coupled to denitrification processes, such as anaerobic methane oxidation, or HYME-D. Furthermore, the coexistence of nitrite and ammonium under anoxic conditions could induce the development of anammox microorganisms in the anoxic and aerobic compartments, contributing to improving the nitrogen removals.

The maximum nitrogen removals observed in this research ($32.5 \text{ mg TN L}_{\text{feed}}^{-1}$) were similar to the maximum achieved using a hybrid MBR system ($35.1 \text{ mg TN L}_{\text{feed}}^{-1}$; Alvarino et al., 2019). In both cases, nitrite was present and enhanced nitrogen removals. Furthermore, even without suspended biomass in the IFAs post-treatment system, high average TN removals were attained ($18 \pm 3.9 \text{ mg TN L}_{\text{feed}}^{-1}$), similar values to those achieved by Silva-Teira et al. (2017; $15 \text{ mg TN L}_{\text{feed}}^{-1}$) using the MBR system. In both cases, the nitrite concentration was negligible, with nitrate being the main electron acceptor. Therefore, the current outcomes emphasize the key role performed by the aerobic compartment, with the presence of biofilm carriers, in nitrogen removal processes. It should be emphasized that the use of an IFAS system, with lower MRR, but similar dissolved methane and total nitrogen removal than the MBR system, could be simpler and cheaper, to improve those sewage treatment plants that treat municipal wastewater with UASB systems, as occurs in some warm and temperate countries of the world.

3.6. Microbial community analysis

During Period II, the microbial community was monitored in the two types of free-moving biofilm carriers presented in the reactor (Levapor and Biochip), by means of partial amplicon sequencing of the 16S rRNA gene. Three samples of the attached biomass in Levapor (operating days 175, 265, and 368) and a sample of the Biochip (day 368) biomass were

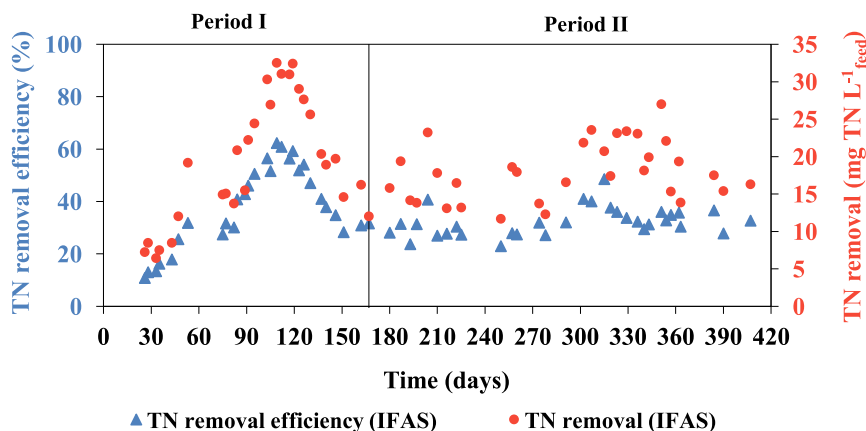


Fig. 4. Evolution of TN removal efficiencies and TN removal observed in the IFAS post-treatment system throughout the operating period.

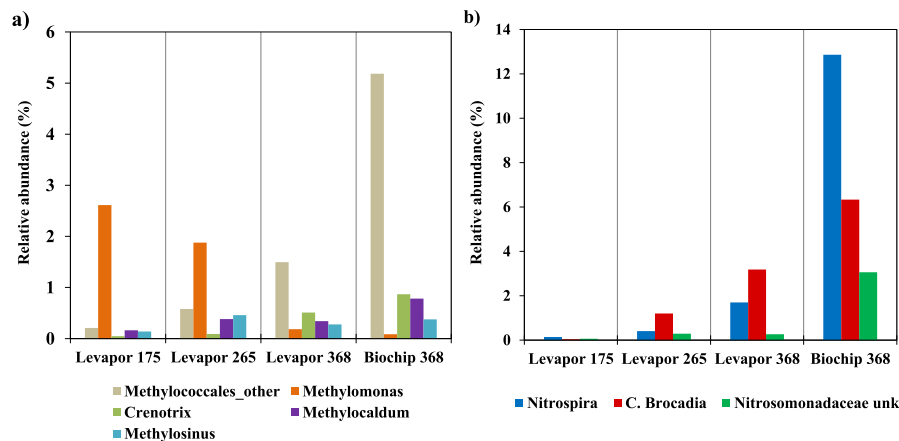


Fig. 5. Relative abundances of aerobic methanotrophs (a) and of the most abundant nitrifying and anammox bacteria (b) in the Levapor (days 175, 265 and 368) and Biochips (day 368) carriers. Only the aerobic methanotrophs with relative abundances over 0.5% in at least one observation are shown.

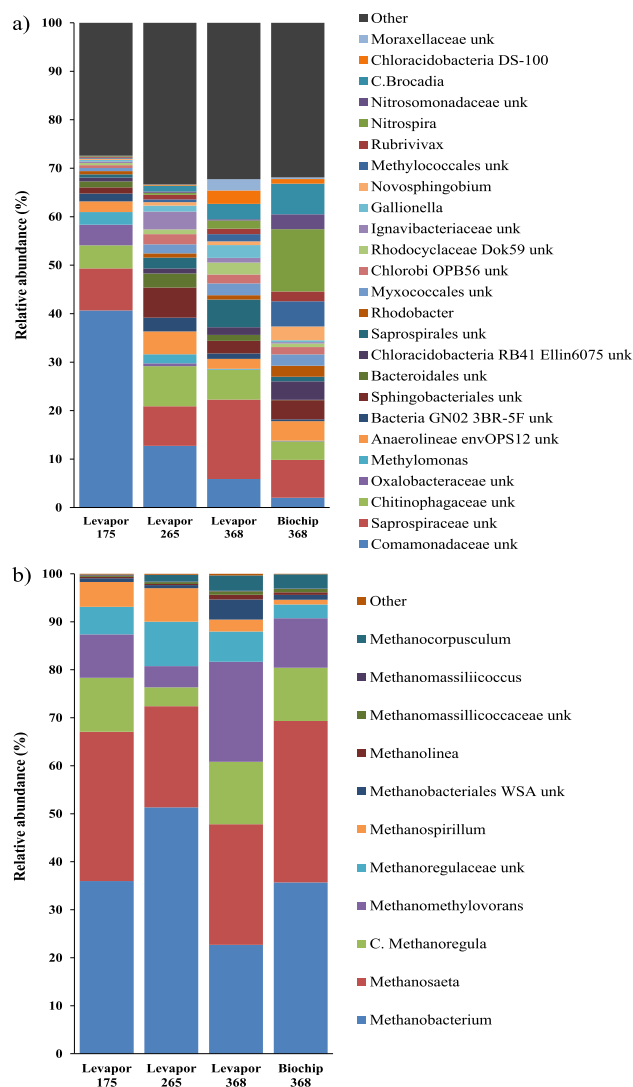


Fig. 6. a) Relative abundances of the 25 most dominant bacteria in the Levapor carriers on days 175, 265 and 368; and in the Biochip carriers on day 368 and b) Composition of the archaeal community in the Levapor and Biochip carriers. Numbers indicate the operational day.

analyzed. Due to the observed biomass washout, suspended biomass could not be studied. On days 175, 265, and 368, several aerobic methanotrophs (*Methylomonas*, *Methylocaldum*, *Crenotrix*, *Methylosinus*, and a putative methanotroph from the *Methylococcales* order) were detected in Levapor carriers with total relative abundances of 3.20, 3.48, and 2.90%, respectively (Fig. 5a). On day 368, its total relative abundance in Biochip carriers was even higher (7.40%), which could explain the high methane removals observed in the aerobic compartment of the IFAS system.

In contrast, n-damo bacteria (*Ca. Methyloirabilis*) were only detected in Levapor carriers (anoxic compartment) in the last days of operation (day 368), with a relative abundance of 0.02%, while n-damo archaea were not detected. Biochips carriers were quite enriched in nitrifying organisms during the last days of operation when more than 10% of the bacteria belonged to *Nitrospira* genus (Fig. 5b). Furthermore, ammonium-oxidizing bacteria of the *Nitrosomonadaceae* family were detected in Biochip carriers with a relative abundance of 3%. As ammonia and low concentrations of nitrite were available, conditions for the growth of anammox bacteria were present in the system. Indeed, *Ca. Brocadia* was present with a relative abundance of 0.03%, 1.20%, and 3.18% in Levapor carriers on days 175, 265, and 368, respectively, and with a relative abundance of 6.5% in Biochip carriers on day 368. During Period II, the rest of the community was composed mostly of bacterial families of heterotrophs considered saprophytic microorganisms, such as *Saprospiraceae* or *Moraxellaceae* (Martins and Carreria, 2014). There also species able to carry out nitrate reduction, such as the *Comamonadaceae* family, although their relative abundance decreased through the operation of the IFAS system (Fig. 6a). Most Archaea present in both, Levapor and Biochip, were well known methanogenic genera, such as *Methanosaeta*, *Candidatus Methanoregula*, *Methanobacterium*, and *Methanomethylivorans*, among others (Fig. 6b).

These data indicated that during Period II, methane removal was surely achieved in both compartments of the IFAS system through aerobic methanotrophs. Meanwhile, the nitrogen removals observed in Levapor carriers could have been carried out by anammox bacteria or conventional heterotrophic denitrifiers that use methane oxidation products released by aerobic methanotrophs. Moreover, the consortium of nitrifying and anammox bacteria probably contributed to the nitrogen removals observed in the adhered-to-Biochip biomass.

3.7. Batch assays

Batch experiments were performed under anaerobic conditions at the end of Period II to determine whether anaerobic ammonium oxidation (anammox), conventional heterotrophic denitrification, and anaerobic methane oxidation along with denitrification processes were

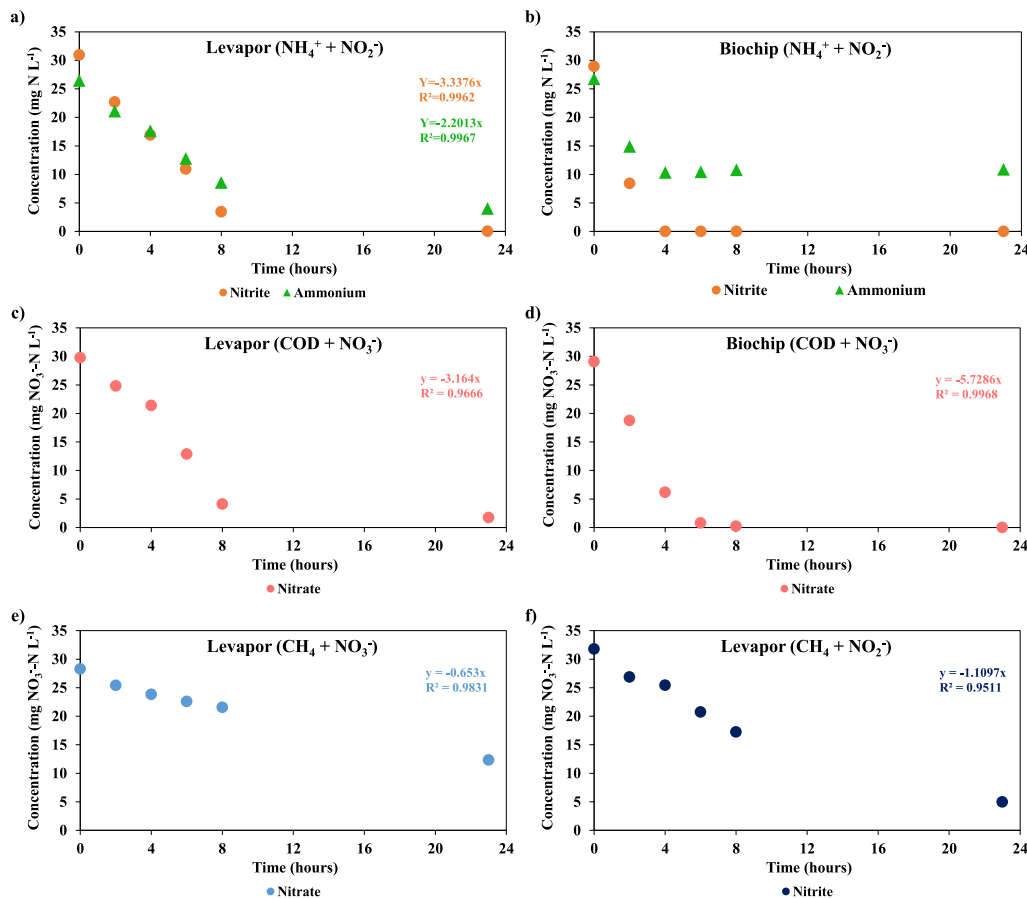


Fig. 7. Batch denitrification tests results for anammox process in Levapor (a) and Biochip (b) carriers media; for conventional heterotrophic denitrification in Levapor (c) and Biochip (d); and anaerobic methane oxidation processes in Levapor using nitrate (e) and nitrite (f) as electron acceptor.

Table 1

Nitrogen removal rates per unit of Levapor and Biochip surface for the different batch tests.

Batch test (type)	Nitrogen removal rate (g N m ⁻² d ⁻¹)	
	Levapor	Biochip
NO ₂ ⁻ + NH ₄ ⁺	NO ₂ ⁻ -N: 0.87 NH ₄ ⁺ -N: 0.58	NO ₂ ⁻ -N: 0.49 NH ₄ ⁺ -N: 0.29
Acetate + NO ₃ ⁻	0.83	0.27
CH ₄ + NO ₃ ⁻	0.17	–
CH ₄ + NO ₂ ⁻	0.29	–

feasible in both biofilm media carriers (Levapor and Biochip). The batch experiments containing NO₂⁻ and NH₄⁺ in the liquid, and N₂ in the gas phase, showed high denitrification in both carriers' media. Regarding Levapor, high volumetric NRRs of 80.1 and 52.8 mg N L⁻¹ d⁻¹ for nitrite and ammonium were observed, respectively (Fig. 7a). The resulting NO₂⁻-N/NH₄⁺-N ratio (1.5) is similar to that required for the anammox process (1.32). The nitrite and ammonium removal rates per unit of Levapor surface were 0.87 and 0.58 g N m⁻² d⁻¹, respectively (Table 1).

In the Biochip carriers, higher volumetric NRRs were observed. In fact, nitrite was totally consumed in less than 4 h. Given the lack of nitrite as electron acceptor between 4 and 23 h, the ammonium concentrations remained constant with values of around 10.5 mg N L⁻¹ (Fig. 7b). The maximum volumetric removal rates of nitrite and ammonium achieved were 246.2 and 142.4 mg N L⁻¹ d⁻¹, respectively. The nitrite and ammonium surface removal rates for Biochip carriers were 0.49 and 0.29 g N m⁻² d⁻¹, respectively. Therefore, the presence of anammox processes in both biofilm carriers' media was clearly

confirmed and even with remarkable activities.

With respect to the conventional heterotrophic denitrification tests using acetate as electron donor, nitrate removal rates of 75.9 and 137.5 mg NO₃⁻-N L⁻¹ d⁻¹ were achieved for Levapor and Biochip, respectively (Fig. 7c and d). The nitrate removal rates per unit of support surface were 0.83 and 0.27 g NO₃⁻-N m⁻² d⁻¹, respectively. These results pointed out the presence of conventional heterotrophic denitrification processes in both biofilm carriers. However, the low concentration of organic matter provided to the IFAs post-treatment system possibly limited the denitrification rates achieved during the continuous operation.

In the anaerobic methane oxidation tests performed only with Levapor carriers and using nitrate as an electron acceptor, a low NRR of 15.7 mg NO₃⁻-N L⁻¹ d⁻¹ was observed (Fig. 7e), which corresponds to a removal rate per unit of Levapor surface of 0.17 g NO₃⁻-N m⁻² d⁻¹. At the same time, using nitrite as an electron acceptor instead of nitrate, removal rate values of 22.6 mg NO₂⁻-N L⁻¹ d⁻¹ and 0.29 g NO₂⁻-N m⁻² d⁻¹ were attained (Fig. 7f). Therefore, the NRRs achieved using nitrite as an electron acceptor were 1.44 times higher than for nitrate. For the nitrate test, if the total CH₄ and NO₃⁻-N consumed are considered, a ratio of 0.61 mol CH₄ mol⁻¹ NO₃⁻-N was attained. Regarding the nitrite tests, the same ratio was achieved (0.61 mol CH₄ mol⁻¹ NO₂⁻-N). These ratios are similar to those of anaerobic methane oxidation or HYME-D processes (Cao et al., 2019).

4. Conclusions

The feasibility of using an IFAS system as a post-treatment alternative to mitigate the environmental impact of UASB treating sewage, in

terms of greenhouse gas emissions and nutrients, was evaluated. High overall COD removal efficiencies were achieved during the whole experimentation period, with COD_T concentrations in the final effluent that never exceeded 77 mg L⁻¹. Regarding ammonium removal, an average 57 ± 15.7% was achieved. The accomplished ammonium oxidation rates were improved by the gradual addition of Biochip carriers in the aerobic compartment. Therefore, a larger volume of the aerobic compartment, a higher biofilm carrier surface, or higher DO concentrations would have been required in order to achieve complete nitrification.

The maximum nitrogen removal and methane removal efficiencies achieved throughout the IFAS system, 32.5 mg TN L⁻¹_{feed} and around 93%, respectively, were detected during the period in which suspended biomass and nitrite were present. Once the suspended biomass was completely washed out, and nitrate became the main electron acceptor, this value progressively decreased. However, despite the absence of suspended biomass, average nitrogen removals and methane removal efficiencies of 18 ± 3.9 mg TN L⁻¹_{feed} and 77.2 ± 12.2% were attained, respectively, in the IFAS system. Therefore, the use of an IFAS system could be an alternative to reduce the problems related to high nutrient concentrations and greenhouse emissions of UASB systems used in many warm and temperate countries as a secondary sewage treatment method.

During the study, dissolved methane and nitrogen were not only removed in the anoxic compartment, but also in the aerobic one, and with significant importance. The results obtained pointed out the importance of both biofilm carriers, Levapor and Biochip, in the methane and nitrogen removals observed in the IFAS system. The significant detection in Biochip carriers of aerobic methane oxidizers and *Ca. Brocadia* confirmed the existence of methane and nitrogen removal processes in the aerobic compartment of the IFAS system. Both groups of microorganisms were also detected in the Levapor carriers present in the anoxic compartment.

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.

CRediT authorship contribution statement

T. Allegue: Investigation, Writing - original draft, Formal analysis, Conceptualization. **M.N. Carballo-Costa:** Investigation, Methodology. **N. Fernandez-Gonzalez:** Methodology, Formal analysis, Investigation. **J.M. Garrido:** Conceptualization, Funding acquisition, Supervision, Project administration.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jenvman.2020.110395>.

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