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Feammox Bacterial Biofilms as an Alternative Biological Process for the Removal of Nitrogen from Agricultural Wastewater

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Abstract: The excessive deposition of ammonium (reactive nitrogen) in the environment has led to losses of biodiversity and the eutrophication of ecosystems. Anthropogenic sources contribute twice the natural rate of terrestrial reactive nitrogen and provide about 45% of the total amount of it produced annually on Earth. Recently, a biological process that anaerobically metabolizes ammonium and facilitates iron reduction, termed Feammox, was discovered. The use of Feammox activity together with hollow fiber membrane bioreactors (HFMB), for which the latter are based on the formation of biofilms of bacterial communities, constitutes an efficient and sustainable method for the removal of ammonium from agriculturally derived wastewater. To implement the use of HFMB with Feammox activity, the formation of Feammox bacterial biofilms from wastewater sludge samples from a brewery was evaluated. The cultures were enriched with two different carbon sources, namely, sodium acetate and sodium bicarbonate; then, ferrous iron and ammonium concentrations, which were used as indicators of reactive nitrogen removal, were measured. The measurements revealed that the ammonium removal level reaches 20.4% when sodium acetate is used as carbon source. Moreover, an increase in the ferrous iron concentration of +Δ84.6 mg/L was observed, indicating that Feammox activity had been generated. Biofilm formation was observed under Feammox conditions on the hollow fibers. These results showed that Feammox bacteria can form biofilms and efficiently remove ammonium from wastewater, constituting an essential feature with which to scale up the process to HFMBs. Overall, these results contribute to a better understanding of the Feammox process that can be used to implement these processes in agriculture and thus progress towards a more sustainable industry.

Keywords: Feammox; biofilms; nitrogen removal; reactive nitrogen; ammonium; iron; agricultural nitrogen

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1. Introduction

Nitrogen (N) is a key cellular component and an essential element for all forms of life [1]. The reactive nitrogen forms that are commonly found in ecosystems (mainly aquatic) are ammonium (NH_4^+), nitrite (NO_2^-), and nitrate (NO_3^-) [2]. However, N can also be present in the form of ammonia (NH_3), nitrous oxide (N_2O), and gaseous nitrogen (N_2) incorporated into organic substances [3]. In recent decades, human activities, together with the growth of industrialization, have altered the N cycle and generated high levels of pollution via reactive nitrogen (Nr) in the form of NH_4^+ [4].

One of the main causes is the excessive use of nitrogen fertilizers in agriculture, mainly though the seeding of nitrogen-fixing crops (legumes) and the burning of fossil fuels, which have significantly increased the Earth's Nr stock [5,6]. In 2010, it was estimated that anthropogenic sources contribute twice the natural rate of terrestrial Nr and provide about 45% of the total Nr produced annually on Earth [7]. Consequently, excessive Nr deposition

in the environment has caused serious environmental problems, such as biodiversity loss, eutrophication, and soil acidification [8]. Biological N removal is one of the most widely used technologies for N removal due to its many advantages, including its easy operation, high efficiency and stability, and the use of environmentally friendly products [9]. N removal processes applied to wastewater are based on the use of activated sludge, which corresponds to a heterogeneous set of microorganisms that are dominated by a series of bacterial groups categorized as bacterial consortia (BC) [10]. Conventional nitrification–denitrification processes consume large quantities of organics and oxygen [4,11]. Most denitrifying bacteria are heterotrophic, requiring organic carbon sources that have an important effect on cell growth and nitrate reduction [12]. Meanwhile, nitrifying bacteria are autotrophic, which means that the carbon source required for their bacterial processes is inorganic [13]. In low C/N wastewater, nitrogen removal is limited by the availability of organic compounds, and external carbon sources are required to improve nitrogen removal efficiency [14]. Thus, fully autotrophic processes for treatment of low C/N wastewater have received considerable attention in recent years [15,16].

Recently, microorganisms capable of anaerobically metabolizing NH_4^+ by reducing iron III (Fe^{+3}), a process known as Feammox, were discovered [17]. These were mainly identified in riverine sediments, tropical forest soils, and rice soils [6]. Since then, the Feammox process has been reported in many different environments, such as tropical rainforest soils [18], paddy soils [19], intertidal wetlands [6], river sediments [20], and marine environments [21], among others. Figure 1 shows the Feammox process and the chemical pathways that allow for the oxidation of NH_4^+ to N_2, NO_3^-, or NO_2^- coupled with the Fe (III) reduction [19]. Although the three Feammox pathways are thermodynamically favorable, the generation of dinitrogen yields more energy and remains energetically favorable over a wide pH range, while the generation of nitrite and nitrate, which occur only at a pH < 6.5, require more Fe^{+3} and yield less energy [18]. Recently, our research group found that the provision of iron chloride ($FeCl_3$) as a source of Fe^{+3}, pH 7, and room temperature (20 ± 2 °C) are optimal conditions for Feammox bacteria enrichment from sewage sludge in batch reactors [22]. However, it is necessary to evaluate whether these conditions are optimal for the growth of Feammox bacteria as biofilms, since this could favor their greater efficiency for N removal in scaled systems, thus reducing energy consumption and operating costs [18].

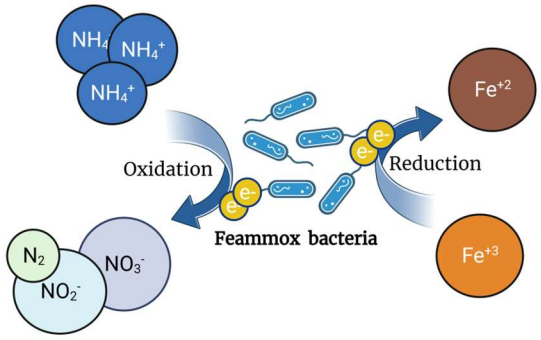

Figure 1. Illustrative diagram of the Feammox process. Created with BioRender.com (accessed on 15 December 2022).

In wastewater treatment, compact and efficient bioreactor systems have been used for the separation of matter [23]. Within these systems are batch and membrane bioreactors (MRB). The MRBs integrate a selective membrane (used as a filter) and a biological treatment based on the formation of biofilms of bacterial communities [24]. Hollow fiber membrane bioreactors (HFMBs) have fibers that correspond to narrow filaments, which function as a surface for the permeate flow to circulate [25]. In this way, HFMBs allow for a high packing density and a large contact surface to obtain reclaimed water with high physicochemical and microbiological quality [26]. The growth of Feammox bacteria in HFMBs could have

a significant impact on enhancing the autotrophic removal of nitrogen from wastewaters, improving removal efficiency, and reducing the energy costs (oxygen) and organic carbon requirements of conventional nitrification–denitrification processes. Even so, it is necessary to evaluate whether Feammox microorganisms form biofilms and the to ascertain the optimal conditions that maintain and facilitate their growth.

This study explores the formation of Feammox bacteria biofilms in an HFMB as an alternative method for NH_4^+ removal via the reduction of Fe^{+3} in wastewater. Ferrous iron (Fe^{+2}) and NH_4^+ concentrations were quantified as indicators of Nr removal by Feammox. A sludge sample from a wastewater treatment plant was used as the initial inoculum for the selective enrichment of Feammox bacteria in a batch reactor. The biofilm formation of Feammox bacteria in the hollow fibers was verified and determined by scanning electron microscopy (SEM) in order to explore future applications in wastewater treatment systems for N removal.

2. Materials and Methods

2.1. Sludge Sample Collection

The anaerobic sludge sample was obtained from "AB-inbev", a beer treatment plant (IC) (33°20′34,29″ S, 70°47′37.93″ W) located in Santiago, Chile. The sample was collected in 1L HDPE bottles and transported at 4 °C in the dark until chemical and molecular analysis and pre-incubation process were conducted.

2.2. Chemical Characterization of Sludge

Chemical analyses were performed to characterize the IC sludge sample used in the batch bioreactor experiments. The pH and NH_4^+ parameters were measured directly from the sludge using HACH probes (PHC301, ISENH4181, HACH, Loveland, CO, USA). Anions, iron species, chemical oxygen demand (COD), and soluble chemical oxygen demand (sCOD) were determined colorimetrically in a spectrophotometer (DR3900, HACH, Loveland, CO, USA). COD concentration was measured from a homogeneous sludge sample. Anions, iron species, and sCOD were determined from the filtered supernatant fraction (0.22 µm) obtained from the sludge centrifuged at 6000 rpm for 15 min (Hermle Z206A, Hermle Labortechnik GmbH, Wehingen, Germany). The results are described in Table 1.

Table 1. Characterization of the IC anaerobic sludge.

	Parameters	IC Sludge
Anions	NO_3^- [mg/L]	16.8
	NO_2^- [mg/L]	0
Fe species	Total Fe [mg/L]	1.46
	Fe^{+2} [mg/L]	0.36
Other	COD [mg/L]	14039
	sCOD [mg/L]	5649
	pH	7.35
	NH_4^+ [mg/L]	410.0

2.3. Sludge Pre-Incubation

The IC sludge sample was anaerobically pre-incubated in the dark with 20 ± 2 °C set as the growth temperature and using carbon dioxide (CO_2) as a purge gas to remove any pre-existing electron acceptors and to consume the organic carbon sources present. Preincubation was performed in a 100 mL culture flask for 24 h under static conditions. Then, the culture was centrifuged at 6000 rpm for 15 min (Hermle Z206A, Hermle Labortechnik GmbH, Wehingen, Germany), and the supernatant was used to measure sCOD and was replaced with distilled water. The procedure was repeated four times, and the resulting sludge was used as inoculum for experiments in the batch bioreactor.

2.4. Enrichment Culture in Batch Experiments

For Feammox pathway enrichment, base selective culture media were prepared in 1 L bottle with two different carbon sources: sodium bicarbonate ($NaHCO_3$) and sodium acetate ($C_2H_3NaO_2$). The culture media were composed of 208 mg/L of NH_4Cl, 600 mg/L of KH_2PO_4, 112 mg/L of $CaCl_2{\cdot}2H_2O$, 400 mg/L of $MgCl_2{\cdot}6H_2O$, 4840.7 mg/L of $FeCl_3$, 2520.21 mg/L of $NaHCO_3$, or 2460.9 mg/L of $C_2H_3NaO_2$. The pH was adjusted to 7.0 by dropwise addition of KOH or HCl. The culture media were autoclaved, and 1 mL of filtered water (0.22 µm) containing vitamins (1000×) and trace metals (1000×) were added under a laminar flow cabinet. Finally, the previously prepared sludge sample inoculum was added, and the media were incubated in the dark with 20 ± 2 °C set as growth temperature and purged with CO_2 in a stainless-steel glove anaerobic chamber (model TMAX-GBV). The culture media were monitored every 7 days for a total of 28 days; the procedure is detailed in Figure 2. For the monitoring cultures, water samples were collected and measured to determine the concentration of ammonium, the pH (ISENH4181, PHC301 HACH, Loveland, CO, USA), and the concentration of ferrous iron (Fe^{+2}) (DR3900, Hach, Loveland, CO, USA).

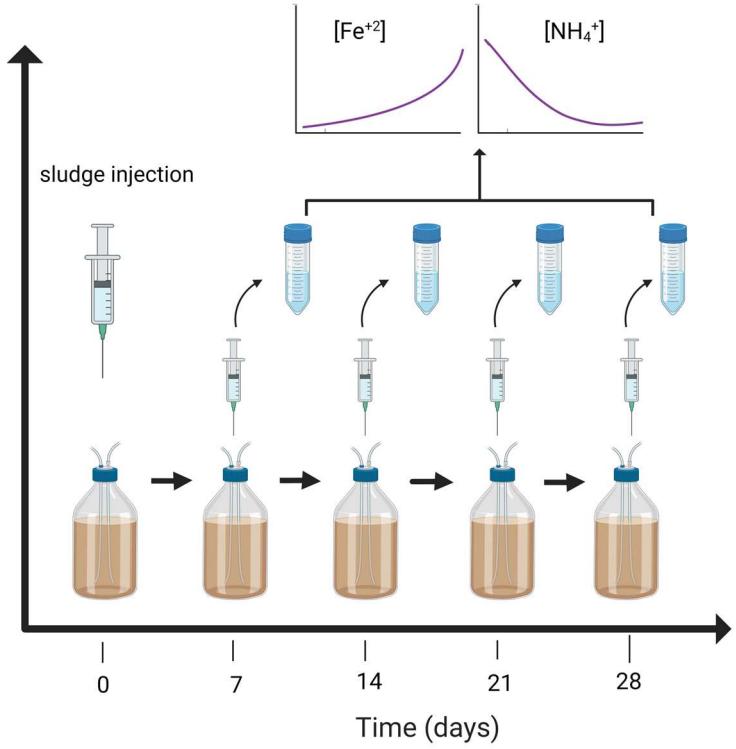

Figure 2. Batch reactor operating procedure. Created with BioRender.com (accessed on 15 December 2022).

2.5. Biofilm Formation on Hollow Fibers

At this stage, 10 cm pieces of hollow fibers (0.4–2.6 mm diameter) were incubated with 20 mL of the culture media inoculated with the enriched culture (after 28 days of growth) in a petri dish, under anaerobic conditions, at a growth temperature of 20 ± 2 °C, and in a stainless-steel glove chamber (model TMAX-GBV) for 10 days.

To analyze biofilm formation, scanning electronic microscopy coupled with energy-dispersive X-ray spectroscopy (SEM-EDS) were carried out. Specifically, the morphology of the biofilms growing on the hollow fibers and the analysis of the chemical composition of the surface were evaluated.

Prior to their observation, the samples were fixed, and critical point drying and shading were performed. For fixation, the samples were embedded in 2% glutaraldehyde

solution in a sodium cacodylate buffer (0.2 M pH 7.2) for ~4 h. Subsequently, critical point drying was performed, for which the samples were de-hydrated stepwise in a series of water/ethanol solutions (50, 70, 80, and 100%). Afterwards, the samples were treated for 45 min with a critical-point dryer (Autosamdri-815; Tousimis, Rockville, MD, USA). Then, the already-dried samples were shaded with gold in a desktop sputterer (Desk V; Denton Vacuum, Moorestown, NJ, USA). Finally, the observation of the samples was performed using a scanning electron microscope (JSM-IT300LV; JEOL Ltd., Tokyo, Japan) and considering several points of each sample for surface topography and compositional analysis (EDS).

3. Results and Discussion

3.1. Enrichment of Feammox and Ammonium Removal in Batch Reactors

The IC sludge sample was incubated in a selective medium to promote the Feammox process. The culture was performed in a batch reactor for 28 days, varying the carbon source for enrichment. Two samples were evaluated: (a) a combination of IC sludge, a Feammox selective medium, and $NaHCO_3$ as carbon source, and (b) IC sludge, a Feammox selective medium, and $C_2H_3NaO_2$ as carbon source. In both samples, $[Fe^{+2}]$ and $[NH_4^+]$ concentrations and pH were measured.

Figure 3 shows the concentrations of Fe^{+2} (3A) and NH_4^+ (3B) of the sludge samples with $NaHCO_3$ y $C_2H_3NaO_2$. It can be observed that both samples begin enrichment with similar Fe^{+2} concentrations, but from day 7 onward their behavior differs. Sample b (IC mud + $C_2H_3NaO_2$) shows an increase in the Fe^{2+} concentration, reaching a variation of +Δ84.6 mg/L of $[Fe^{+2}]$. Conversely, the Fe^{+2} concentration increases less for sample a (IC mud + $NaHCO_3$), and from day 14 to 28 it does not change significantly. Specifically, its increase was only +Δ20.4 mg/L of $[Fe^{+2}]$. Interestingly, in Figure 3B, it is possible to note that the behavior of the two samples was opposite. Indeed, an increase in the concentration of NH_4^+ was observed for sample a, reaching 143 mg/L, with a +Δ21 mg/L increment in $[NH_4^+]$. While for sample (b), there is a decrease in the concentration of NH_4^+, reaching up to 129 mg/L, with a −Δ33 mg/L decrement in $[NH_4^+]$.

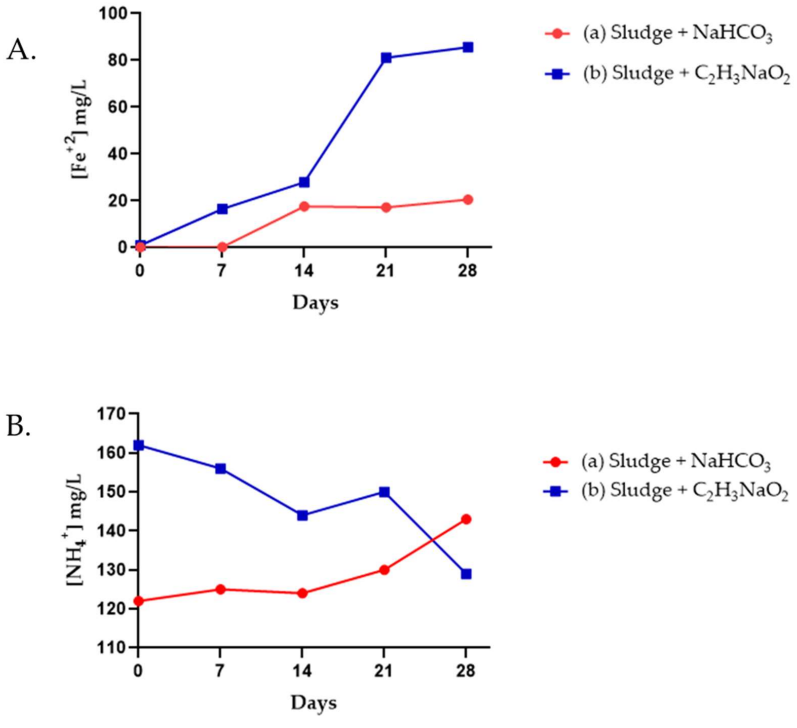

Figure 3. Concentration of Fe^{+2} (**A**) and NH_4^+ (**B**) during enrichment process with IC sludge sample and using inorganic ($NaHCO_3$) and organic ($C_2H_3NaO_2$) carbon sources.

The increase in the NH_4^+ concentration with $NaHCO_3$ as a carbon source (Figure 3B) may be because the added Fe^{3+} was not sufficient to enable NH_4^+ oxidation, as it can be absorbed by bacteria to perform other processes [27,28]. Another reason may be the death of bacteria due to the conditions of the culture media employed, which causes the protein to hydrolyze in the sludge, resulting in the increase in the NH_4^+ concentration [29]. Additionally, denitrification could also have occurred in the system, altering the balance of nitrogen species and, consequently, the NH_4^+ concentrations. Li et al. [6] have shown that denitrification occurs in intertidal sediments after the Feammox process, a reaction in which nitrate (NO_3^-) generated from Feammox may be being used as a substrate, thus shifting the equilibrium in the redox reaction that allows for the oxidation of NH_4^+.

The results of Figure 3 strongly suggest the influence of the Feammox pathway in the enrichment carried out with sample b, since a removal of 20.37% for NH_4^+ was observed, along with continuous Fe^{3+} reduction. Previous research indicated that the supply of organic carbon could influence the biological Nr removal process mediated by this new anammox-like process (Feammox), thereby promoting the transformation of crystalline iron oxide into amorphous iron oxide, which is more accessible to bacteria during electron transfer [30]. Yang et al. [30] indicate that the Feammox process is more practical and suitable for the in situ removal of high-concentration NH_4^+ compared to the common anammox process since it would only require a single anaerobic system. Particularly, this process may be relevant for the treatment of water bodies contaminated with high-concentration NH_4^+ released by diffuse pollution from agriculture; however, it is critical to achieve adequate Fe^{3+} regeneration because NO_x^- is a non-dominant product during the Feammox process, and this pathway can be stopped when there is no Fe^{3+} to reduce. On the other hand, the degradation of $C_2H_3NaO_2$ can release protons, thus reducing the pH of the medium, which implies a favorable environment for the Feammox reaction [31], which would explain the positive results obtained when $C_2H_3NaO_2$ was used as a carbon source.

3.2. Effect of pH on Feammox Enrichment in a Batch Reactor

Ammonium removal processes are highly sensitive to pH, temperature, and toxic substances; for this reason, controlling the pH conditions in the Feammox process is of great relevance [32]. Initially, the selective enrichment media were prepared at pH 7, because, based on previous work carried out on Feammox pathways, it was experimentally proven that at this pH value, the bacterial consortia can react efficiently, thus increasing the Fe^{+2} concentration and decreasing the NH_4^+ concentration [22].

In Figure 4, it is possible to observe that the pH values remain stable between pH 6–7 (approximately). In the case of sample (a), where $NaHCO_3$ was used as a carbon source, there is a greater variation in the pH values; however, these remain close to 7, which is logical given the pKa1 (6.3) of carbonic acid. In previous research, bicarbonate was shown to lead to a buffered environment for denitrifying bacteria, after NH4+ oxidation [33], providing a suitable environment to maintain the pH in the desirable range of 7–8.2 [34]. For the case of sample (b), where $C_2H_3NaO_2$ was used as carbon source, it can be observed that there is an increase in the pH value, reaching 7 on day 28. Indeed, it has been shown that the pH considered optimal for most environmental strains of denitrifying bacteria, which are related to the Feammox process, is between 7 and 8 [34], which supports our results (Figure 3). In addition, these results suggest that the Feammox route that may be favored is the direct conversion of NH_4^+ to N_2, which has been demonstrated as favorable at a neutral pH. Therefore, it is possible that both pathways are engaged by the consortia of bacteria that are selected with Feammox media, because it has been observed that the oxidation of ammonium to nitrate and/or nitrite by Feammox is followed by the denitrification process in an anoxic environment [35]. For this reason, a consensus has been reached that several routes of the nitrogen cycle will be favored at a neutral pH [36].

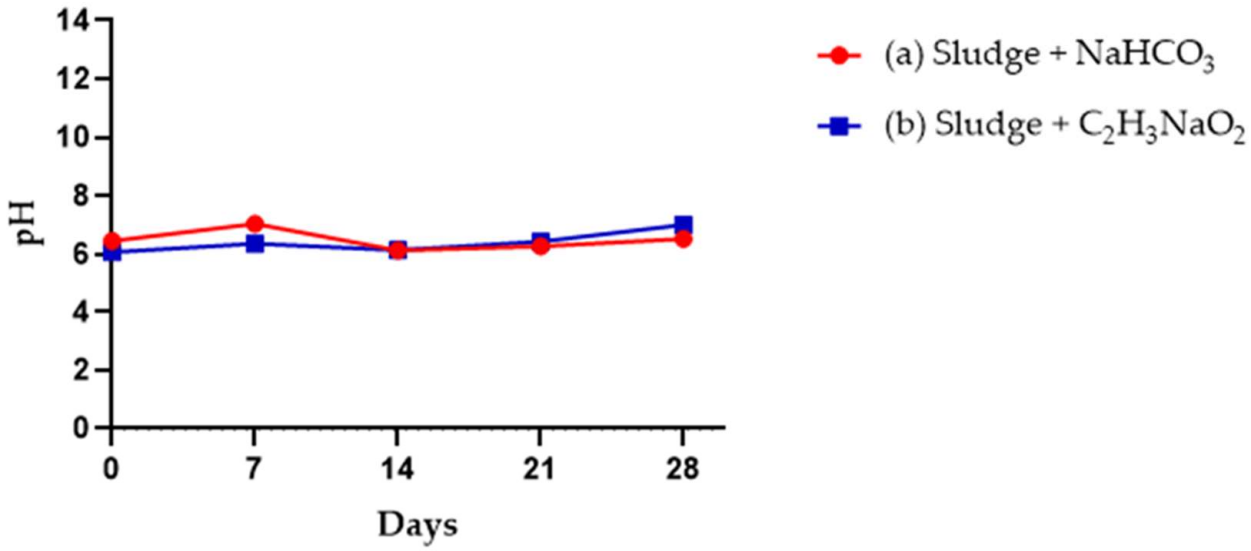

Figure 4. Evolution of the pH of the enriched sludge samples.

Sun et al. [36] indicated that there is a pH that favors the Feammox process, which is approximately 6.8, adding that Feammox has also been identified in intertidal areas with an alkaline pH. They also indicate that there are several potential Feammox microorganisms that have greater activity in neutral pH conditions, which supports our results. On the other hand, it has been observed that $C_2H_3NaO_2$ is capable of releasing protons into a medium through its decomposition, which would stabilize the pH when it becomes very alkaline, thereby favoring the Feammox reaction at a neutral pH [31].

In the various studies conducted on ammonium removal, it has been theorized that carbon limitation may be the main reason for the dramatic decrease in the growth and activity of nitrifying bacteria below a neutral pH [37]. In addition, higher pH values were able to delay nitrite removal more severely than nitrate removal, which would indicate that nitrite accumulation is associated with a high pH [38], so variations in pH would imply deviations in the Feammox process and decrease its efficiency.

3.3. Biofilm Formation

To verify that the IC sludge samples, in the presence of bacterial consortia, could form biofilms on hollow fibers, for the subsequent implementation in an HFMB, the enriched sludge samples were incubated for 28 days, and the biofilms were visualized through SEM.

Figure 5 shows SEM images of cultures (a) and (b). The results show the formation of biofilm networks between the Feammox bacteria on hollow fibers using $NaHCO_3$ (Figure 5A–D) and $C_2H_3NaO_2$ (Figure 5E–H) as carbon sources. Figure 5B,F show that the level of biofilm formation was not homogeneous, which may be due to the formation of iron mineralization or cell detachment. This was observed for both carbon sources ($NaHCO_3$ and $C_2H_3NaO_2$). On the other hand, in Figure 5C,D,G,H, cell adhesion, cell aggregation, and EPS production can be observed, revealing a biofilm-like structural arrangement on the surface of the hollow fibers. Several microorganisms have been reported to be involved in the Feammox pathway, including *Geobacteraceae* spp., *Shewanella* spp., *Exiguobacterium* spp., *Acidimicrobiaceae*, Nitrososphaeraceae and *Pseudomonas* [39]. *Acidimicrobiaceae* sp. strain A6 (A6), from the phylum Actinobacteria, was recently identified as the only Feammox strain isolated that can carry out anaerobic NH_4^+ oxidation coupled with iron reduction [24]. However, there are few works that have studied the formation of biofilms by Feammox bacteria. Such a study may be key to scaling this process to a wastewater treatment system.

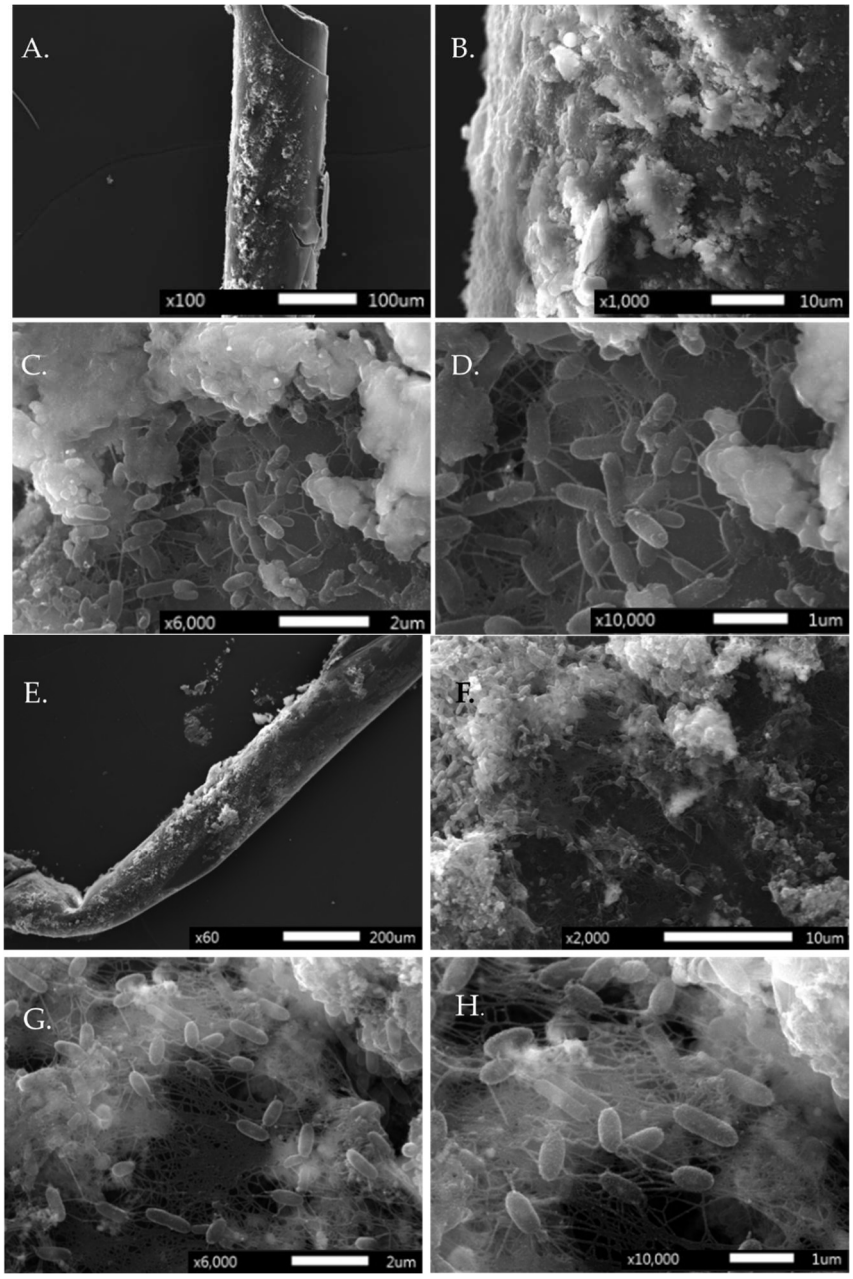

Figure 5. SEM images of hollow fibers incubated with enriched IC sludge samples. Sample (a) IC sludge with $NaHCO_3$ (100×, 1000×, 6000× and 10,000×) (**A**–**D**). Sample (b) IC sludge with $C_2H_3NaO_2$ (60×, 2000×, 6000× and 10,000×) (**E**–**H**).

Compared to activated sludge (suspended growth reactors), working with biofilm formation is more convenient, since it allows for the acquirement of a higher biomass concentration, a denser sample structure, and greater resistance to shock loads [40]. In addition, the three-dimensional architecture of the biofilms provides bacteria embedded in the biofilm with the potential to alleviate temperature-generated stress [9]. Ruiz-Urigüen et al. [24] demonstrated the ability of Feammox bacteria to colonize electrodes previously used for bioelectricity production. Therefore, the biofilm-forming capacity of Feammox bacteria would allow for the stable and efficient operation of biofilm-based bioreactors for wastewater treatment.

It has been demonstrated that Feammox microorganisms are autotrophic, meaning that they obtain energy from the oxidation of ammonium to fix inorganic carbon, which is

essential for their biological processes [41]. Therefore, inorganic carbon is essential for the growth and activity of Feammox microorganisms. However, several investigations have indicated that organic carbon, such as $C_2H_3NaO_2$, could play an important role during the Feammox reaction, such as promoting the release of structural Fe in clay minerals and supporting the formation of amorphous Fe (III) oxide, thereby stimulating the Feammox reaction [35]. In the same way, organic carbon could be used as a carbon and energy source by some heterotrophic microorganisms that coexist and cooperate with Feammox bacteria, such as denitrifiers, iron-oxidizing bacteria, and iron-reducing bacteria, among others microbial groups [39]. Thus, although organic carbon is not directly required for the Feammox pathway, it could have an important role in mediating Feammox growth rates through its participation in associated metabolic pathways.

In Figure 6, it is possible to observe the results of the EDX analysis; in (A) (IC sludge + $NaHCO_3$), the chemical characterization shows 20.1% of iron (Fe) atoms on the surface of the formed biofilm, while in (B) (IC sludge + $C_2H_3NaO_2$), there are 32% Fe atoms. The similar values of the %Wt of Fe found in both samples strongly suggest that the differences in performance (Fe^{3+} reduction and NH_4^+ removal, Figure 3) are a consequence of the formation of specific biofilms and not the availability of Fe for the Feammox process.

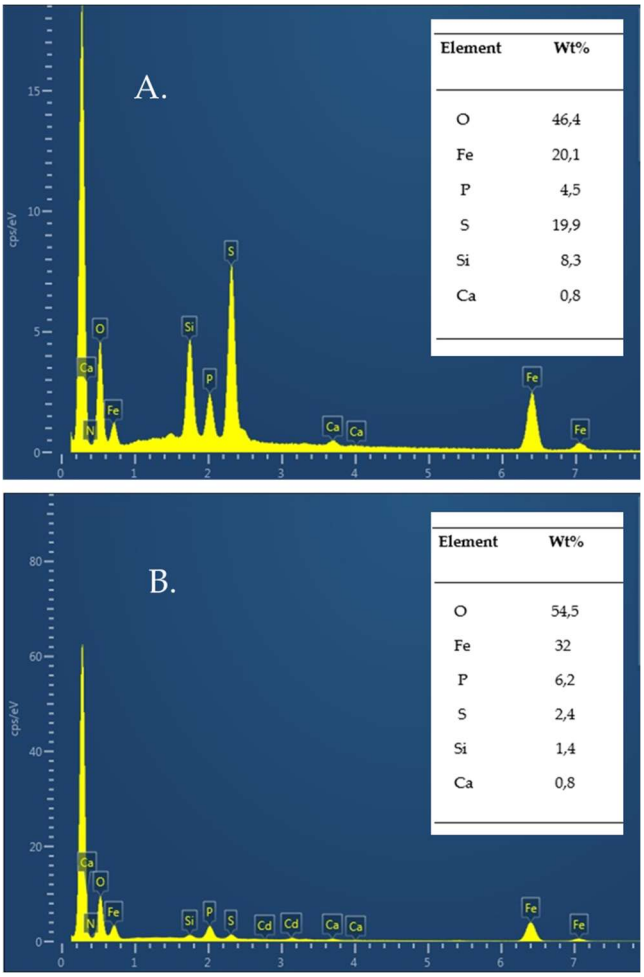

Figure 6. Energy-dispersive X-ray spectra (EDX) of hollow fibers incubated with enriched IC sludge samples. (**A**) Sample (a) IC sludge and $NaHCO_3$. (**B**) Sample (b) IC sludge and $C_2H_3NaO_2$. Magnification: 10,000×.

These results suggests that Feammox bacteria can form biofilms despite the presence of high levels of Fe. Furthermore, the formation of biofilms could favor the treatment of water with high-concentration NH_4^+, thus constituting a potential alternative for the treatment and remediation of wastewater contaminated by nitrate or ammonium-based fertilizers.

4. Conclusions

Based on the research carried out on the enrichment of the IC sludge, it was possible to prove the formation of biofilms in the hollow fibers under Feammox conditions. This is an essential characteristic for the scaling up of the process to HFMBs and for the generation of a sustainable process for the removal of Nr from the environment. In addition, it was possible to evidence that there is a 20.37% removal of Nr when using an organic carbon source, showing an increase in Fe^{2+} concentration of $+\Delta 84.6$ mg/L and a decrease in NH_4^+ concentration of $-\Delta 33$ mg/L.

Regarding the performance of sodium acetate, it can be remarked that this compound represents the most effective carbon source for bacterial metabolism in the electron transfer process, corresponding to a source of organic carbon, which allows for the growth of heterotrophic bacteria that are closely related to the Feammox processes. Therefore, the present study can set a precedent for the efficiency of the Feammox process for its subsequent implementation in the treatment and remediation of water bodies polluted by the excessive use of nitrogen fertilizers.

Author Contributions: The manuscript was written by Á.C. and E.L., but all the authors contributed to its preparation and review. Experiments were performed by Á.C. and M.G.; data analyses were carried out by Á.C. and M.G. in discussion with J.S., C.R. and E.L. All authors have read and agreed to the published version of the manuscript.

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