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Nitrogen Removal by an Anaerobic Iron-Dependent Ammonium Oxidation (Feammox) Enrichment: Potential for Wastewater Treatment

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Abstract: Nitrogen pollution in water is a growing concern. Anthropogenic activities have increased the amount of nitrogen released into watercourses, which harms human health and the environment, and causes serious problems, such as eutrophication. Feammox is a recently discovered biological pathway associated with the nitrogen cycle that has gained scientific interest. This process couples anaerobic ammonium oxidation with iron reduction. This work presents a study on the Feammox mechanism from the enrichment of an activated sludge obtained from a sewage treatment plant. The enrichment was carried out at neutral pH to study the N_2 pathway, that is, the Feammox process with the oxidation of ammonium (NH_4^+) directly to N_2. In addition, different sources of iron were studied: iron chloride ($FeCl_3$); ferrihydrite; and goethite. The characterization of the sludge showed the genes associated with ammonia monooxygenase, nitrate and nitrite reductases processes, along with relevant microbial species. The enrichment, carried out for 42 days and monitored every 14 days, showed that $FeCl_3$ as a source of Fe was more effective for the coupled process of oxidation of NH_4^+ and the reduction of Fe(III) to Fe(II). At the end of the enrichment period, a removal of 31% and 32.2% of NH_4^+, and an increase in Fe(II) concentration by 52.4 and 63.9 times regarding the initial value were achieved in aerobic and anaerobic sludge, respectively. This study provides information on the potential of Feammox in the removal of N from wastewater, and the oxidation/reduction yields in the initial enrichment phase.

Keywords: Feammox; ammonium removal; iron reduction; microbial community; functional genes

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1. Introduction

Nitrogen is one of the elements essential for life, building key cellular components, such as, nucleic acids, proteins, amino sugars, and amino lipids [1]. Nitrogen is found in nature mainly as dinitrogen (N_2), being the main constituent (~78%) of the earth's atmosphere [2]. However, N_2 is fora chemical form that cannot be used by most organisms. Nitrogen fixation is a prokaryotic process with high energy expenditure, in which N_2 is reduced to ammonium (NH_4^+) by N_2-fixing bacteria and archaea [3]. Other prokaryotes and eukaryotes require fixed nitrogen (also known as reactive nitrogen) which corresponds to nitrogen forms as nitrate, ammonium, or organic nitrogen [2]. Microorganisms are also responsible for nitrification and denitrification processes. Nitrification is carried out by nitrifying bacteria which oxidize NH_4^+ to nitrite (NO_2^-) and nitrate (NO_3^-) under aerobic conditions. Denitrification corresponds to an anaerobic process in which facultative heterotrophs reduce NO_3^- to nitrite and nitric oxide (NO) [4,5].

Although nitrogen compounds are essential nutrients for life, their substantial release into bodies of water, such as lakes and rivers, can cause eutrophication and severe damage to aquatic ecosystems [6–8]. Biological processes have commonly been the major focus for ammonium removal [8,9]. Among the most common biological processes, nitrification/denitrification and anammox stand out [9–13]. However, recently, adsorption technologies have been studied for the removal of ammonium from polluted waters [14]. Feammox is a recently discovered pathway for nitrogen loss in different ecosystems. Spe-cifically, Feammox mechanism couples anaerobic ammonium oxidation with iron reduc-tion, in the absence of nitrate or nitrite. Clément et al. [15] reported for the first time this ni-trogen loss pathway from a forested riparian wetland. Currently, treatment systems using Feammox has been primarily evaluated in laboratory-scale systems [11,16]. However, its use in large-scale treatment systems is promising, as it could be combined with other nitrogen removal techniques or replace other traditional methods that require aeration since Feammox has the advantage of being able to oxidize NH_4^+ under anoxic conditions [17].

Iron (Fe) is the fourth most abundant element on earth, occurring in different environments, typically in the redox states as Fe(II) (ferrous iron) and Fe(III) (ferric iron) [18]. This process, denominated Feammox (as anaerobic ammonium oxidation coupled to Fe(III) reduction) [19,20], generates the process of anaerobic oxidation of NH_4^+ coupled to the reduction of iron (Fe(III)) in the absence of oxygen, nitrate (NO_3^-), or NO_2^-, which are required by annamox [21], as previously mentioned, and theoretically could occur abiotically or be microbially mediated [22]. In this reaction, Fe(III) is reduced to ferrous iron Fe(II), while NH_4^+ is transformed to NO_2^-, nitrogen gas (N_2), or other forms of nitrogen as NO_3^-, with iron oxides [Ferric iron, Fe(III)] as the electron acceptor [23]. Figure 1 illustrates the different general processes for the cycling of nitrogen species, as with Feammox, where Fe(III) is used and reduced together with the oxidation of NH4+ for the production of the different nitrogen species.

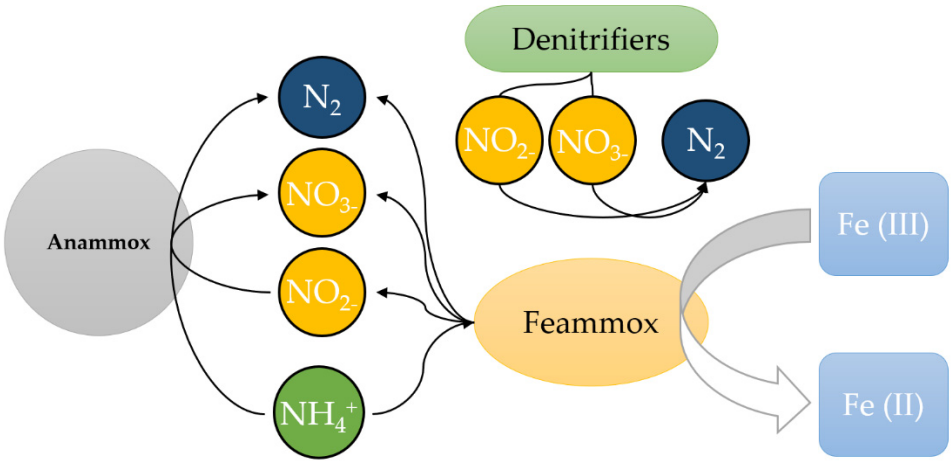

Figure 1. Illustrative diagram of the Anammox, denitrifiers, and Feammox process.

The Feammox process has been shown to produce either N_2, NO_2^-, or NO_3^- as the end-product in several environments, as shown in Equations (1)–(3) [18]. Among these reactions, the N_2 pathway is energetically more favorable than NO_2^- or NO_3^- pathways, and can occur over a wider range of conditions [22].

$$3Fe(OH)_3 + 5H^+ + NH_4^+ \rightarrow 3Fe^{2+} + 9H_2O + 0.5N_2 \; \Delta rG_m = 245 \text{ kJ} \times \text{mol}^{-1} \quad (1)$$

$$6Fe(OH)_3 + 10H^+ + NH_4^+ \rightarrow 6Fe^{2+} + 16H_2O + NO_2^- \; \Delta rG_m = 164 \text{ kJ} \times \text{mol}^{-1} \quad (2)$$

$$8Fe(OH)_3 + 14H^+ + NH_4^+ \rightarrow 8Fe^{2+} + 21H_2O + NO_3^- \; \Delta rG_m = 207 \text{ kJ} \times \text{mol}^{-1} \quad (3)$$

NH_4^+ conversion to NO_2^- or NO_3^- is more likely to occur in acidic environments (pH < 6.5) [11], and stoichiometrically requires more Fe(III). In addition, NO_2^- or NO_3^- pathways produce less energy compared with the N_2 reaction under the same conditions [22].

As for the microorganisms involved in the Feammox process, researchers have found that *Acidimicrobiaceae* sp. A6 [21] and other categories of iron-reducing bacteria (IRB), including *Geobacter*, *Shewanella*, *Geothrix*, *Desulfosporosinus*, *Dechloromonas*, unclassified *Pelobacteraceae*, and *Anaeromyxobacter* [24], are responsible for electron transfer in this reaction. In a recent study by Zhu et al. [24], they reported that Feammox activity is associated with the higher abundance of iron-reducing bacteria, especially *Clostridium_sensu_stricto_12*, *Desulfitobacterium*, *Thermoanaerobaculum*, *Anaeromyxobacter*, and *Geobacter*.

In this work, we study the Feammox process under different iron sources using an aerobic and an anaerobic sludge as inoculum obtained from two different treatment plants. The study focuses on the determination of ferric iron reduction coupled with the oxidation of NH_4^+. The NH_4^+, Fe(II), and Fe(III) were monitored in batch culture systems for 42 days. Also, we used PCR analysis to corroborate the presence of genes involved in this process. This study will allow for exploring the possible presence of Feammox activity in water treatment systems. In addition, the enrichment carried out at neutral pH could allow the identification of other microorganisms able of carrying out the Feammox process through the N_2 pathway (Equation (1)), and, thus, increase knowledge about this process.

2. Materials and Methods

2.1. Sludge Sample Collection

The aerobic activated sludge sample was obtained from the "La Farfana" sewage treatment plant (33°28′34.29″ S, 70°47′37.93″ W), and the anaerobic sludge was obtained from the yeast producer "Lefersa" (33°20′34.07″ S, 70°42′53.99″ W), both located in Santiago, Chile. The sample was collected in 1 L HDPE bottles, and transported at 4 °C under dark until chemical and molecular analysis, and the pre-incubation process.

2.2. Chemical Characterization of Sludge

Chemical analyses were performed to characterize the sludge samples used in batch enrichment experiments. The total solids (TS) and volatile solids (VS) were determined according to Method 2540 G from Standard Methods [25]. Also, the parameters of pH, electrical conductivity (EC), and ammonium (NH_4^+) were measured directly from the sludge using HACH probes (PHC301, CDC401, ISENH4181, HACH, Loveland, CO, USA). Anions, iron species, chemical oxygen demand (COD), and soluble chemical oxygen demand (sCOD) were determined colorimetrically in a HACH spectrophotometer (DR3900, HACH, CO, USA). The COD concentration was measured from a homogeneous sample of sludge. The anions, iron species, and sCOD were determined from filtered (0.22 μm) supernatant fraction obtained from the sludge centrifugation at 6000 rpm for 15 min (Hermle Z206A, Hermle Labortechnik GmbH, Wehingen, Germany). Figure 2 illustrates the procedure for obtaining samples used for the measurement of COD, sCOD, anions, and cations by spectrophotometry.

2.3. DNA Isolation and PCR Analysis

DNA samples were obtained from the aerobic and anaerobic sludges. DNA extraction from this sludge was carried out from 1000 μL of sample using a DNeasy®® PowerSoil®® Pro Kit (QIAGEN) as described by the manufacturer, except that pure nuclease-free water was used as a replacement for C6 solution. The quality of the DNA obtained was evaluated by agarose gel (1.5%). After DNA extraction, the DNA samples were stored at −20° until polymerase chain reaction (PCR) analysis.

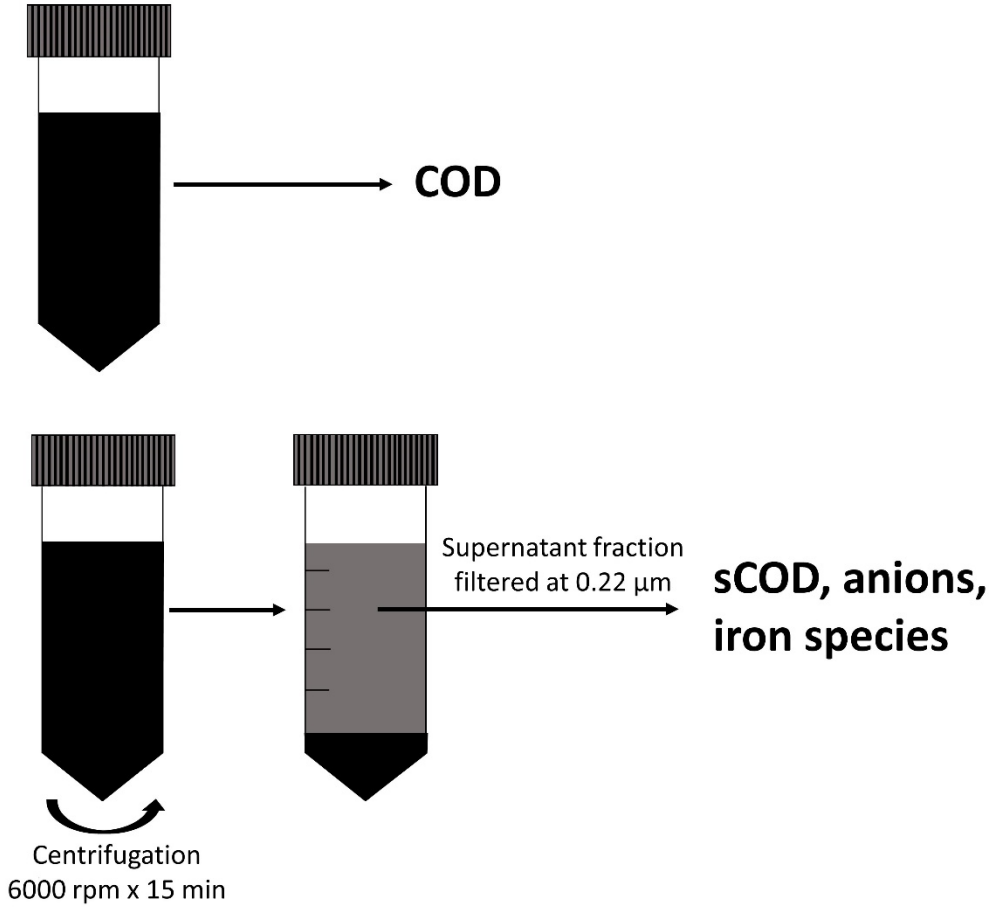

Figure 2. Procedure for obtaining samples for sludge characterization analysis.

PCR analysis was performed to evaluate the presence of genes involved in the Feammox process, such as functional genes and markers of relevant microbial species. The primer sets and the target genes evaluated are shown in Table A1, together with their melting temperature (MT) and the cycles used [26,27]. After each PCR program, the respective PCR products were evaluated by 1.5% agarose gel (run time 30 min at 90 V. GeneRuler 1 kb (Cat. No: SM0311, Thermo Fisher Scientific Inc., MA, USA), and a 100 bp DNA ladder (Car. No: N3231S, New England BioLabs, MA, USA) was used to corroborate band size.

2.4. Sludge Pre-Incubation

The sludges were pre-incubated anaerobically at room temperature in the dark using CO_2 as the purge gas, to remove any pre-existing electron acceptor, and to consume the organic carbon sources present. The pre-incubation was performed in a 100-mL culture flask for 24 h statically. 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 the sCOD. For this, the supernatant was previously replaced with deionized water. The procedure was repeated four times, and the resulting sludge was used as the inoculum for batch experiments.

2.5. Enrichment Culture in Batch Experiments

For the enrichments, the pre-incubations were used as Feammox culture to be added. The base culture media contained 208 mg/L NH_4Cl, 600 mg/L KH_2PO_4, 112 mg/L $CaCl_2{\cdot}2H_2O$, 400 mg/L $MgCl_2{\cdot}6H_2O$, 2520.21 mg/L $NaHCO_3$. After autoclaving these media, 1 mL of vitamins (1000×) and trace metals (1000×) were added (filtered (0.22 µm)) under a laminar flow cabinet. The pH was adjusted to 7.0 depending on the iron source by adding KOH or HCl drop-wise. Then, the Fe source was added to these culture media

considering four conditions: (i) without iron (-Fe); (ii) iron chloride ($FeCl_3$); (iii) ferrihydrite; and (iv) goethite. The cultures containing iron were adjusted to an iron concentration of 1 g/L. Ferrihydrite and goethite were ex-situ synthesized [28,29].

The media were transferred to 100 mL glass vials in duplicates for each condition. The cultures were incubated in the dark at room temperature using CO_2 as the purge gas.

For each case, biotic and abiotic controls were evaluated. The enrichments were monitored every 14 days, for a total of 42 days. On each occasion, samples were collected, and ammonium, pH (ISENH4181, PHC301 HACH, Loveland, CO, USA), nitrate, total iron, and ferrous iron (spectrophotometer DR3900, HACH, CO, USA) were measured.

2.6. Quality Assurance/Quality Control (QA/QC)

Quality assurance/quality control (QA/QC) procedures were performed for all the analyses in this study to ensure the quality, reproducibility, and accuracy of the obtained results. The equipment used in this study was calibrated before its use according to the instrument guidelines. All the chemical reagents used in this study were of analytical grade. Also, all materials used in the experiments were neatly cleaned and rinsed with Milli-Q water, and properly autoclaved. The accuracy and precision of the measurements were checked and compared against blank samples and synthetic standard samples of known concentration.

3. Results and Discussion

3.1. Sludge Characterization

To evaluate the potential of Feammox processes in wastewater treatment systems, a sample of activated sludge was used as inoculum for microbial enrichment. The activated sludge sample used in this study was characterized prior to conducting the enrichment experiments.

3.1.1. Chemical Characterization

Table 1 shows the chemical parameters in the activated sludge samples. pH had neutral values, with values of 6.93 and 7.85 for the aerobic and anaerobic sludge, respectively. The EC was 10.14 mS/cm in the aerobic sludge, and a slightly lower value of 7.85 mS/cm for the anaerobic sludge, which presented a higher value than that reported for other activated sludge [30,31], showing a higher concentration of ions. Ammonium concentration was very high in the aerobic sludge, with a value of 1420 mg/L, which can be explained by the high NH_4^+ concentrations of the influent sewage [32]. The ammonium concentration was much lower in the case of anaerobic sludge, with a value of 279 mg/L. The total iron concentration in both sludges was quite low, which would not favor the activity of Feammox in the treatment system. Besides, the COD and sCOD concentrations presented high values because of the large amount of organic matter. These values made the preincubation stage necessary to reduce the initial COD conditions to evaluate the Feammox activity (autotrophic anaerobic ferric ammonium oxidation).

3.1.2. Molecular Biology Characterization

Molecular microbial analysis was used to explore the Feammox-related pathways in both activated sludges samples in order to determine the potential of the sample to enrich Feammox processes. PCR analyses are shown in Figure 3. The functional gene observed was archaea ammonia monooxygenase (amoA), whereas the relevant microbial species found were *Geobacter* spp., *Shewanella* spp., *Ferrovum myxofaciens*, *Albidiferax ferrireducens*, and *Anammox bacteria*.

Table 1. Characterization of the aerobic and anaerobic sludge samples.

	Parameter	Aerobic Sludge	Anaerobic Sludge
	pH	6.93	7.85
	EC [mS/cm]	10.14	6.50
	NH_4^+ [mg/L]	1420.6	279
Anions	SO_4^{2-} [mg/L]	0	110
	Cl^- [mg/L]	0.22	0.02
	NO_3^- [mg/L]	20.4	53.6
	NO_2^- [mg/L]	0.1	0
Iron species	Total Fe [mg/L]	0.52	0.35
	Fe^{2+} [mg/L]	0	0.1
	COD [mg/L]	76,100	51,303
	sCOD [mg/L]	14,019	3567
	TS	2.74%	5.23%
	VS	2.11%	4.45%

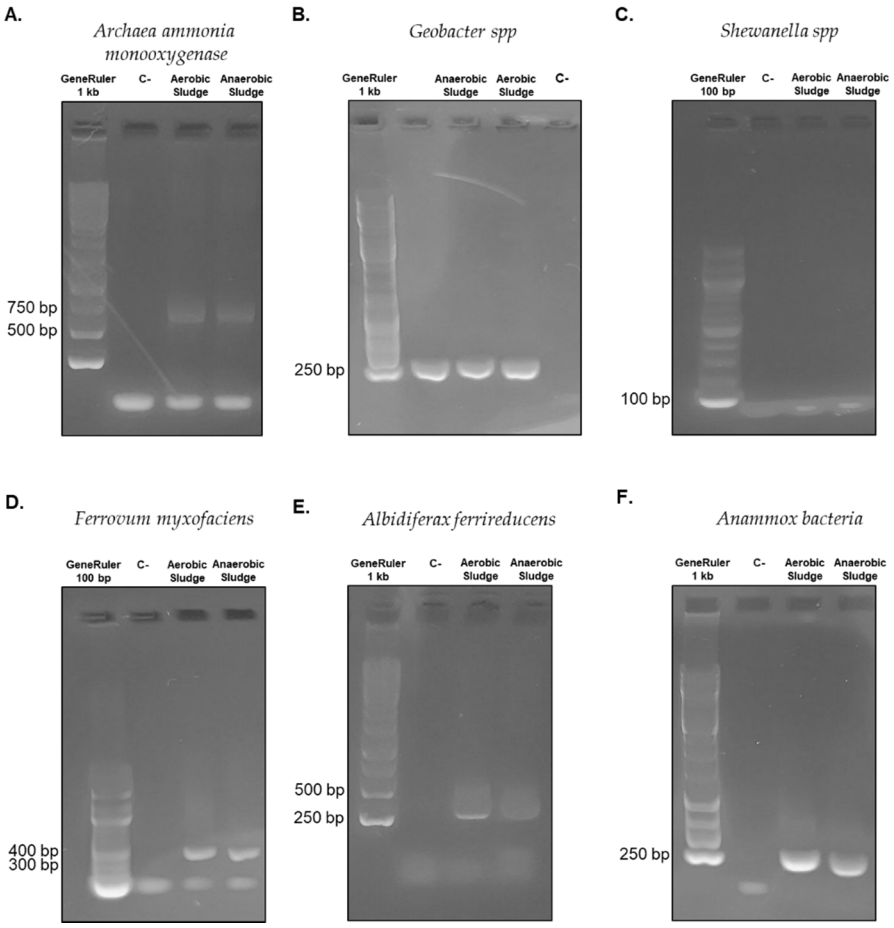

Figure 3. Electrophoresis gels PCR products to check for the presence of relevant Feammox genes. (**A**). amoAF AOA—amoAR AOA primers (*Ammonia-oxidizing archaea*, 635 bp), (**B**). Geo546F—Geo840R primers (*Geobacter* spp., 276 bp), (**C**). She120F- She220R primers (*Shewanella* spp., 100 bp), (**D**). Ferrovum643F—Uni-338F-RC primers (*Ferrovum myxofaciens*, 323 bp), (**E**). Uni-907R—RdoR-RC primers (*Albidiferax ferrireducens*, 312 bp), (**F**). AMX809F—AMX1066R, respectively (*anammox bacteria*, 239 bp). The PCR conditions are shown in Table A1 (Appendix A). The list of all primer sets tested is shown in Table A1 (Appendix A).

The aforementioned genes and their targets are related to Feammox processes. The results showed the presence of archaea ammonia monooxygenase gene, a key enzyme for

nitrification and for the global nitrogen cycle [33,34]. The beginning of the nitrification process occurs through the oxidation of ammonia (NH_3) to hydroxylamine (NH_2OH), a reaction catalyzed by the enzyme ammonia monooxygenase [33]. Ammonia-oxidizing archaea are closely associated with denitrifiers, and play a critical role in coupled nitrification–denitrification [35]. Bacterial AmoA was not amplified, which is explained because archaeal AmoA is more widespread than bacterial AmoA in sedimentary environments [35].

Regarding *Geobacter* and *Shewanella*, their presence may be associated with a Feammox process because these two were among the first bacteria studied in a pure culture that can obtain energy for growth by coupling the oxidation of organic matter with iron reduction, and are considered the model iron reducers [36,37]. Similarly, *Albidiferax ferrireducens*, previously recognized as *Rhodoferax ferrireducens* [38], is classified as a FeRM (iron-reducing microorganism). Therefore, it grows in Fe(III)-rich environments, and reduces it to ferrous iron [39,40]. At the same time, *Ferrovum myxofaciens* uses only ferrous iron as electron donor, and oxygen as electron acceptor. It is found exclusively in iron-rich acidic waters [41].

Finally, the anammox bacteria are typical of wastewater sludge [42], and are bacteria that anaerobically oxidize ammonium with nitrite to N_2 [43], which explains its presence in the sample. This is consistent with the presence of simultaneous Feammox processes.

From these results, the diversity of microorganisms and reactions related to Feammox can be seen. The presence of these genes in the initial sludges samples suggests a high potential to find Feammox activity from the enrichments, and to select those microorganisms that exhibit potential for NH_4^+ oxidation and Fe(III) reduction under conditions of high Fe concentration.

3.2. Sludge Pre-Incubation

The sCOD values were monitored daily during the pre-incubation process. In the aerobic sludge, during the first two days, sCOD decreased by 73%, and then stabilized between days 3 and 4 of pre-incubation. After this period, a 90% reduction in the initial sCOD was achieved. For the anaerobic sludge, sCOD continuously decreased to a 90% reduction on the fourth day. These results are shown in Figure 4.

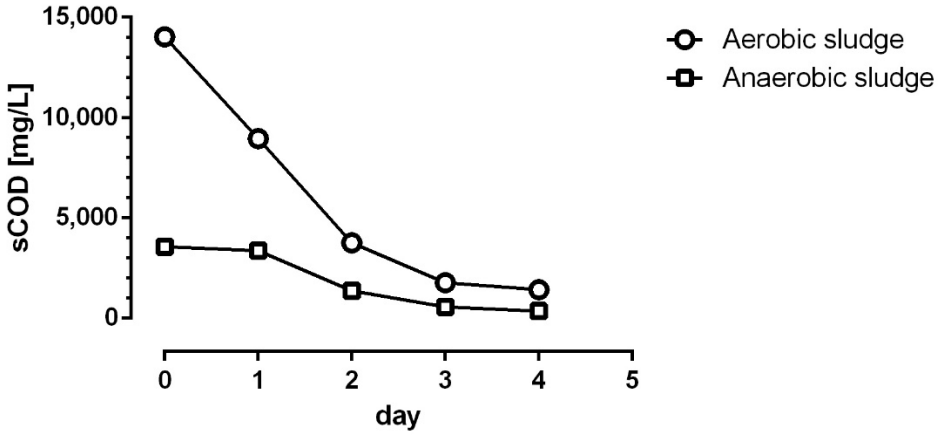

Figure 4. The concentration of sCOD during the preincubation process.

3.3. Enrichment Culture in Batch Experiments

Feammox enrichment was carried out using aerobic and anaerobic sludge samples. A microbial enrichment was able to enhance a Feammox culture capable of oxidizing NH_4^+ and reducing Fe(III) in 42 days. A decrease in NH_4^+ levels and an increase in Fe(II) were observed. Figure 5A–F shows that in the three cases with different iron sources in the aerobic sludge, a decrease in NH_4^+ levels was observed, together with an increase in Fe(II) and nitrate (NO_3^-) levels. When $FeCl_3$ was used (Figure 5A,B), the initial NH_4^+ concentration was decreased by 31% over the enrichment period. As for the Fe(II) concentration, it was possible to increase it by 52.4 times the initial concentration (from 0.0069 to 0.3616 mmol), and NO_3^- by 3.3 times. With ferrihydrite (Figure 5C,D), the total NH_4^+ was consumed by

20.5%, the initial value of Fe(II) was increased by 41.6 times (from 00025 to 0.1041 mmol), and the value of NO_3^- by 2 times. With goethite (Figure 5E,F), it was possible to decrease by 37.5% of the total initial NH_4^+. Fe(II) was increased by 11.4 times its initial value (from 0.0025 to 0.0285 mmol), and $NO3^-$ by 1.5 times. The Fe(III) concentration was relatively stable, which can be explained by its higher initial concentration. In the enrichment's case, carried out using an anaerobic activated sludge, a similar behavior to the aerobic sludge could be observed in terms of the parameters measured. When $FeCl_3$ was used (Figure 5A,B), the initial ammonium concentration was decreased by 32.23%. As for Fe(II), the initial concentration increased by 63.9 times (from 0.0069 to 0.4414 mmol), and $NO3^-$ by 2.2 times. With ferrihydrite (Figure 5C,D), the initial NH_4^+ concentration was consumed by 21.25%, and the Fe(II) concentration was increased by 57.62 times compared to the initial concentration (from 0.0025 to 0.1440 mmol). The $NO3^-$ was increased by 2.34 times. When goethite was used (Figure 5E,F), the initial NH_4^+ concentration was reduced by 36.12%. The initial concentration of Fe(II) was increased by 31.7 times (from 0.0025 to 0.0792 mmol), and for $NO3^-$ by 1.3 times. In Figure 5G, it can be seen that without Fe, the NH_4^+ and $NO3^-$ remain stable over time, which supports that the variation in the concentrations of these nitrogen species is associated with the presence of iron-dependent microorganisms. For each culture condition, abiotic controls were monitored in which the initial concentration of NO_3^- and Fe(II) was kept constant over time, whereas the sludge increases these levels. This is related to the expected Feammox behavior.

The results obtained for the aerobic and anaerobic sludge were analyzed by analysis of variance (ANOVA) to determine if the differences obtained in the measurements for each type of sludge have statistical significance. For this analysis, a significance level of $\alpha = 0.05$ was applied, using p-value < 0.05 for comparing cases. This analysis showed that for cultures with $FeCl_3$ and ferrihydrite as iron sources, the differences between each inoculum did not have statistical significance for the measurements of NH_4^+, NO_3^-, and Fe(II), so it cannot be deduced that one of the sludges is better than the other in its performance. For the case of goethite, the ammonium measurements did not have a significant difference. However, the measurements of NO_3^- and Fe(II) had differences with statistical significance, with a p-value of 0.028 and 0.019, respectively, so it can be concluded that the anaerobic sludge was significantly better in the production of NO_3^-, and the reduction of Fe(III) to Fe(II) than aerobic sludge. This can be explained because of the microbial community present in the anaerobic sludge, which is naturally acclimated to anoxic conditions as used in the experimental setup. Therefore, it would not require an anaerobic adaptation step.

As previously mentioned, Feammox produces the anaerobic oxidation of NH_4^+ to NO_3^-, NO_2^- or N_2, together with the reduction of Fe(III) to Fe(II) [44]. This behavior is consistent with our results, where the decay of NH_4^+ together with the rise of Fe(II) were observed. Other studies have shown similar trends [11,21,45–48]. Similar behavior was found for the concentrations of NH_4^+, NO_3^-, and Fe(II and III), with different values for the removal rates and days of residence. As for the iron source, Huang et al. [21] and Huang et al. [46] studied the effect of the iron source on Feammox enrichment from isolates, and proposed that the best iron source in terms of NH_4^+ removal and Fe(III) reduction was ferrihydrite, followed by goethite, and finally, $FeCl_3$. This can be explained due to the higher thermodynamic feasibility of ferrihydrite compared to goethite as a source of Fe(III), since ferrihydrite has a higher negative Gibbs free energy than goethite [18]. However, our results showed that when using goethite, the highest NH_4^+ removal was obtained, followed by $FeCl_3$ and ferrihydrite in both cases, anaerobic and aerobic. The highest Fe(III) to Fe(II) reduction occurs when using $FeCl_3$, followed by ferrihydrite and goethite, respectively, in both sludges. This can be explained due to the ability of the activated sludge to adapt to use different iron sources. As for nitrate (NO_3^-), an increase can be observed in all the different conditions, as in the process described in equation 3, although the direct production to N_2 is the most favorable and, therefore, the most expected. However, the different production rates in the studies are not always consistent with the theory, as Feammox to NO_3^- was also thermodynamically feasible under these conditions [18,22]. Furthermore, ammonium

reduction under Fe-rich anaerobic conditions is consistent with what has been previously reported for Feammox processes in natural systems.

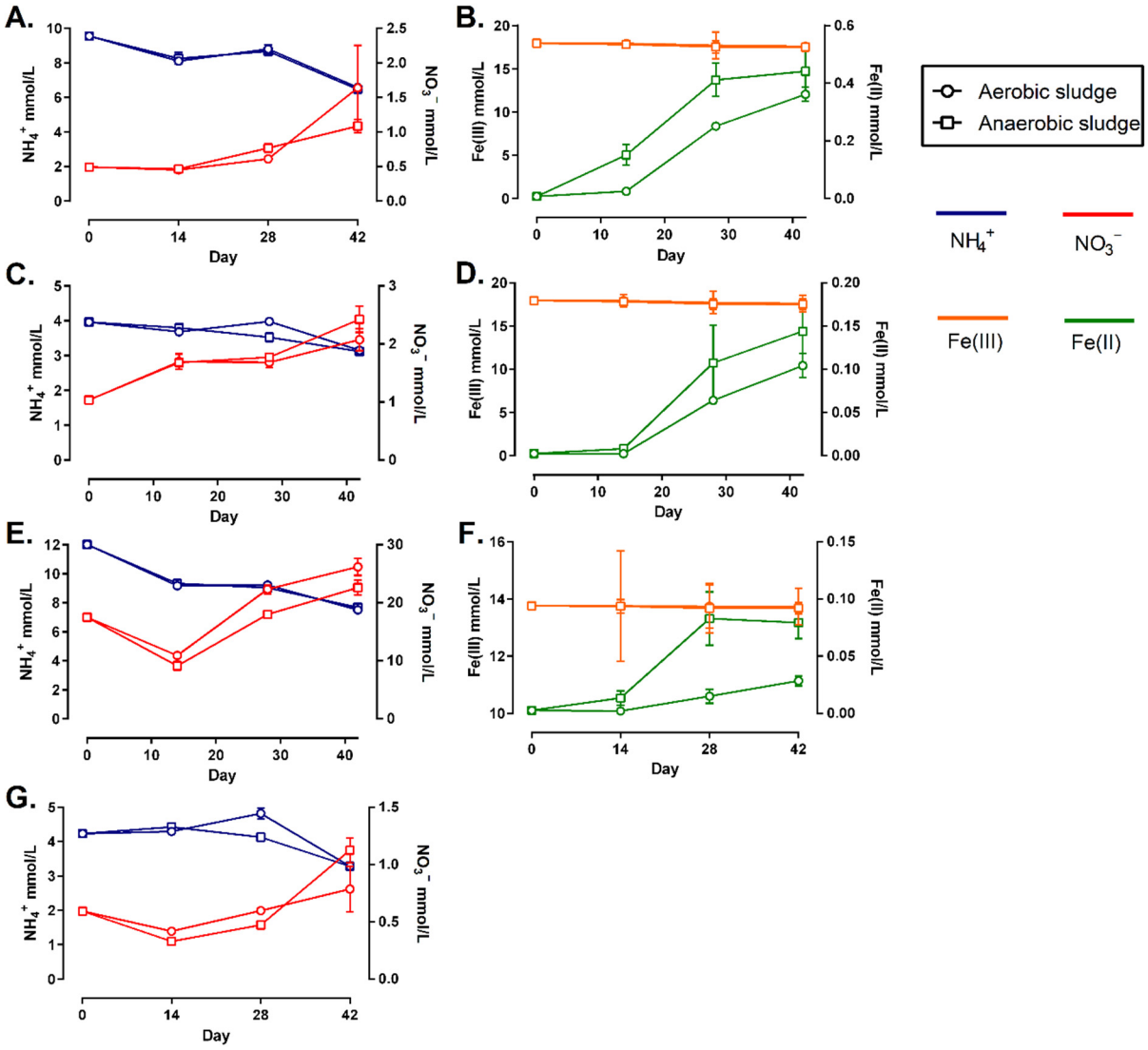

Figure 5. Concentration of NH_4^+, NO_3^-, Fe(II), and Fe(III) over 42 days of incubation, and measurements using different iron sources: (**A**,**B**) $FeCl_3$; (**C**,**D**) ferrihydrite; (**E**,**F**) gothite; and (**G**) without Fe (control). The concentrations of the different parameters were grouped on the right and left y-axes, in order to be easily visualized.

4. Conclusions

The study of the presence of Feammox activity from the enrichment of an activated sludge showed successful results in terms of ammonium removal by the Feammox process. The enrichment was able to enhance a Feammox culture capable of oxidizing NH_4^+ and reducing Fe(III) in 42 days. PCR analysis revealed the presence of certain relevant genes associated with Feammox activity, such as archaea ammonia monooxygenase (amoA), and relevant microbial species, such as *Geobacter* spp., *Shewanella* spp., *Ferrovum myxofaciens*, *Albidiferax ferrireducens*, and *Anammox bacteria*. The enrichment using different iron types showed that the best iron source in terms of NH_4^+ removal, along with the reduction of Fe(III) to Fe(II), was $FeCl_3$. Although goethite presents a higher percentage of NH_4^+ removal, it is the source with the lowest Fe(III) reduction rate, so a very direct relationship to Feammox could not necessarily be established.

Our preliminary findings confirm the presence of the Feammox process in a wastewater sludge sample, which can contribute to understanding how these processes can be

enhanced in order to improve nitrogen removal processes in wastewater. Thus, this article contributes to the gaining of knowledge of the Feammox process by providing more information for a better understanding of the process, along with the use of activated sludge from aerobic and anaerobic sources. It also tests the different iron sources and their reduction capacity, along with the oxidation of NH_4^+. These results can be critical for scaling these processes to more realistic systems by understanding where Feammox processes are most efficient or in which environments they are most prevalent. Even so, additional efforts are necessary to fully understand this process and the conditions that promote its prevalence.

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Conflicts of Interest: The authors declare no conflict of interest.

Appendix A

Table A1. Primer sets, characteristics, and PCR cycles.

Primers	Tm (°C)	Sequence	Band Lenght	PCR Cycles	Target
ACD840	53.5	CGA CAC TGA AGT GCT AAG C	505 bp	Pre-heating at 50 °C for 2 min, pre-denaturation at 95 °C for 10 min, denaturation at 95 °C for 15 s, annealing at 61 °C for 45 s, and extension at 72 °C for 45 s	FeRB (*Acidiphilium* spp.)
Uni-338F-RC	59.4	ACT CCT ACG GGA GGC AGC			
amoAF AOA	52.6	STA ATG GTC TGG CTT AGA CG	635 bp	3 min at 94 °C, followed by 40 cycles of 30 s at 94 °C, 1 min at 53 °C, and 1 min at72 °C	Archaea ammonia monoooxygenase (AOA-amoA)
amoAR AOA	57.5	GCG GCC ATC CAT CTG TAT GT			
amoAF AOB	54.1	GGG GTT TCT ACT GGT GGT	471 bp	3 min at 94 °C, followed by 40 cycles of 30 s at 94 °C, 30 s at 55 °C, and 45 s at 72 °C	Bacteria ammonia monoooxygenase (AOB-amoA)
amoAR AOB	59.2	CCC CTC KGS AAA GCC TTC TTC			
AMX1066R	59.5	AAC GTC TCA CGA CAC GAG CTG	239 bp	10 min at 95 °C, followed by 35 cycles of 60 s at 95 °C, 60 s at 60 °C, and 45 s at 72 °C	Anammox Bacteria
AMX809F	58.2	GCC GTA AAC GAT GGG CAC T			
AMX818F	60	ATG GGC ACT MRG TAG AGG GGT TT	228 bp		

Table A1. *Cont.*

Primers	Tm (°C)	Sequence	Band Lenght	PCR Cycles	Target
Geo564F	49.8	AAG CGT TGT TCG GAW TTA T	276 bp	Pre-heating at 50 °C for 2 min, pre-denaturation at 95 °C for 10 min, denaturation at 94 °C for 30 s, annealing at 57 °C for 30 s, and extension at 72 °C for 30 s (40 cycles)	FeRB (*Geobacter* spp.)
Geo840R	57.6	GGC ACT GCA GG GGT CAA TA			
napA3F	56.9	CCC AAT GCT CGC CAC TG	130 bp	5 min at 95 °C, followed by 35 cycles of 60 s at 95 °C, 60 s at 60 °C, and 45 s at 72 °C	Functional genes (napA) Dissimilarity nitrite reducing bacteria
napA3R	55.1	CAT GTT KGA GCC CCA CAG			
narG2F	53.8	CTC GAY CTG GTG GTY GA	89 bp	5 min at 95 °C, followed by 35 cycles of 60 s at 95 °C, 60 s at 55 °C, and 45 s at 72 °C	Functional genes (narG) Dissimilarity nitrite reducing bacteria
narG2R	53.2	TTY TCG TAC CAG GTS GC			
nirKF	59.9	ATY GGC GGV AYG GCG A	165 bp	5 min at 95 °C, followed by 35 cycles of 60 s at 95 °C, 60 s at 57 °C, and 45 s at 72 °C	Functional genes (nirK) Denitrifying bacteria
nirKR	52.8	GCC TCG ATC AGR TTR TGG			
nirSnF	69.1	TAC CAC CCC GAG CCG CGC GT	164 bp	5 min at 95 °C, followed by 35 cycles of 60 s at 95 °C, 60 s at 63 °C, and 45 s at 72 °C	Gnes funcionales (nirS) Denitrifying bacteria
nirSnr	61.6	GCC GCC GTC RTG VAG GAA			
nosZ1F	59.5	WCS YTG TTC MTC GAC AGC CAG	259 bp	5 min at 95 °C, followed by 35 cycles of 60 s at 95 °C, 60 s at 63 °C, and 45 s at 72 °C	Functional genes (nosZ) Denitrifying bacteria
nosZ1R	56.8	ATG TCG ATC ARC TGV KCR TTY TC			
nrfA2F	59.1	CAC GAC AGC AAG ACT GCC G	67 bp	5 min at 95 °C, followed by 35 cycles of 60 s at 95 °C, 60 s at 60 °C, and 45 s at 72 °C	Functional genes (nrfA) Dissimilarity nitrate reducing bacteria
nrfA2R	59.6	CCG GCA CTT TCG AGC CC			
F1norA	58.7	CAG ACC GAC GTG TGC GAA AG	322 bp	Pre-heating at 50 °C for 2 min, pre-denaturation at 95 °C for 10 min, denaturation at 95 °C for 15 s, annealing at 57 °C for 30 s, and extension at 72 °C for 30 s	Functional genes (nxrA) Nitrite oxidizing bacteria
R1norA	55.4	TCY ACA AGG AAC GGA AGG TC			
Uni-907R	49.9	CCG TCA ATT CMT TTG AGT TT	312 bp	Pre-heating at 50 °C for 2 min, pre-denaturation at 95 °C for 10 min, denaturation at 95 °C for 15 s, annealing at 52 °C for 45 s, and extension at 72 °C for 45 s	FeRB (*Albidiferax ferrireducens*)
RdoR-RC	54.3	GAC CTG CAT TTG TGA CTG YA			
She 120F	61.9	GCC TAG GGA TCT GCC CAG TCG	100 bp	Pre-heating at 50 °C for 2 min, pre-denaturation at 95 °C for 10 min, denaturation at 95 °C for 15 s, annealing at 60 °C for 60 s, and extension at 72 °C for 30 s (40 cycles)	FeRB (*Shewanella* spp.)
She 220R	53.2	CTA GGT TCA TCC AAT CGC G			

Table A1. *Cont.*

Primers	Tm (°C)	Sequence	Band Lenght	PCR Cycles	Target
Ferrovum643F	55.7	ACA GAC TCT AGC TTG CCA GT	323 bp	Pre-heating at 50 °C for 2 min, pre-denaturation at 95 °C for 10 min, denaturation at 95 °C for 15 s, annealing at 57 °C for 45 s, and extension at 72 °C for 45 s	FeOB (*Ferrovum myxofaciens*)
Uni-338F-RC	59.4	ACT CCT ACG GGA GGC AGC			
Uni-907R-RC	49.9	AAA CTC AAA KGA ATT GAC GG	110 bp	Pre-heating at 50 °C for 2 min, pre-denaturation at 95 °C for 10 min, denaturation at 95 °C for 15 s, annealing at 52 °C for 45 s, and extension at 72 °C for 45 s	FeOB (*Acidimicrobium*)
Amf995	60.5	CTC TGC GGC TTT TCC CTC CAT G			

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