

Article

Effect of Fe₂O₃ Nanoparticles on the Efficiency of Anammox Process

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Abstract

Nanotechnology plays an increasingly important role in the economy and human life, which means that more and more amounts of nanosubstances, including nanoparticles of metal oxides, together with wastewater, end up in the environment. This study aimed to study the impact of iron(III) oxide nanoparticles (n-Fe₂O₃), which have magnetic properties, on the efficiency of the Anammox wastewater treatment process. The results indicate that n-Fe₂O₃ in the range of low concentrations may have a positive effect on nitrogen metabolism, increasing the efficiency of NH₄-N removal to 98% in 120 min and at 30 °C. During the first 30 min of the process, when almost anaerobic conditions arose, nanoparticles of Fe₂O₃, stabilized the system by producing ROS. However, a constant control of TOC and pH is necessary because of the constant increase in the amount of organic compounds and H⁺ ions during the reaction. However, a longer contact of n-Fe₂O₃ with biomass causes the efficiency to decrease, and, as a result, the efficiency is lower compared to the system containing only Anammox.

Keywords: Fe₂O₃ nanoparticles; anammox; efficiency



Academic Editor:
Jesus Gonzalez-Lopez

Received: 6 June 2025

Revised: 2 July 2025

Accepted: 9 July 2025

Published: 14 July 2025

Citation: Rabajczyk, A.; Qiu, S.; Zhan, X. Effect of Fe₂O₃ Nanoparticles on the Efficiency of Anammox Process. *Water* **2025**, *17*, 2100. <https://doi.org/10.3390/w17142100>

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1. Introduction

Anammox is a competitive process in relation to nitrification and denitrification processes, widely carried out in biological sewage treatment plants as well as reclamation and environmental protection processes. However, there are many factors that determine the proper course of this process, including biocenosis composition, concentration and form of organic and inorganic compounds (especially the NH₄⁺:NO₂[−] ratio), pH, and temperature. Individual transformations can take place only under specific environmental conditions (Figure 1), taking into account the presence of appropriate micro-organisms or plants.

The presence of metals, including iron, is important for the biological purification processes. Some of the micro-organisms need this element to produce the enzyme necessary to transform nitrogen. Literature data indicate that the presence of iron is essential for the proper functioning of some micro-organisms, while for others it can act as an inhibitor or even determine toxicity. Therefore, for biological removal of pollutions, it is necessary to know the form of iron and the optimal conditions for the proper conduct of environmental

processes occurring in the presence of iron. Literature reports indicate that the iron-assisted anammox process may be an effective solution. Dai et al. [1] reviewed the effect of iron in three different forms, i.e., ionic iron, zero valence iron and iron-containing minerals, on the performance of the anammox process. They pointed out that Fe(II) is the most important as an essential substrate element for the growth of anammox bacteria. The right amount of Fe^{2+} can significantly determine the activity of anammox bacteria, the speed of initiation of the anammox process, or the ability of anammox bacteria to adapt in the event of unfavorable conditions [2]. The results of the work of Choi et al. [3] indicate the important role of Fe_3O_4 , too. The iron-rich environment created by the addition of magnetite allowed for the significant growth of *Ignavibacteria* (a Feammox bacteria) [3].

	T [°C]	Oxygen [mg O ₂ ·dm ⁻³]	pH	Microorganisms	Characteristic
Nitrification	10–35	1–1.5	6.45–8.95	Ammonia oxidizing bacteria (AOB: <i>Nitrosomonas</i> , <i>Nitrospira</i> , <i>Nitrosovibrio</i> , <i>Nitrosolobus</i>) and nitrite oxidizing bacteria (NOB: <i>Nitrobacter</i> , <i>Nitrospina</i> , <i>Nitrococcus</i>)	bind CO ₂ in the Calvin cycle; mineral environment; inhibitors: high concentration of NH ₃ , HNO ₂ ; high sensitivity to the presence of toxic substances
Denitrification	5–35	< 0.5	6.0–7.5	group of microorganisms very systematically diverse: <i>Proteobacteria</i> (eg. phototrophic <i>Rhodospseudomonas Rhodobacter</i> ; lithotrophic <i>Beggiatoa</i> , <i>Thiobacillus</i> , organotrophic <i>Bacillus</i> , <i>Pseudomonas</i> , <i>Paracoccus</i> , <i>Flavobacterium</i>), <i>Archaea</i> (halophile: <i>Haloarcula</i> , <i>Halobacterium</i> , <i>Haloferax</i> , hyperthermophile: <i>Pyrobaculum</i>); heterotrophic <i>Bacillus</i> , <i>Pseudomonas stutzeri</i> , <i>Ps. aeruginosa</i> , <i>Ps. celsis</i> , <i>Achromobacter denitrificans</i> ; <i>N.europea</i> , <i>N.eutropha</i> ,	BOD ₅ :N-NO ₃ ⁻ > 3.5; BOD ₅ :N _{org} > 4–6; using organic compounds is accompanied by an increase in the alkalinity of wastewater by an average of 3 g CaCO ₃ · g ⁻¹ N-NO ₃ ⁻ ; inhibitory effect: Cl ⁻ and cations of Cu, Cd, Co, Cr, Pb, Zn, Ni, Hg
Anammoxidation	40–60	Aerobic/ anaerobic	6.5–8.5	<i>Bacillus</i> sp., <i>Clostridium</i> sp., <i>Proteus</i> sp., <i>Pseudomonas</i> sp., <i>Serratia</i> sp., <i>Escherichia coli</i> , <i>Sprosaricina ureae</i> , many species of <i>Clostridium</i>	increase in alkalinity at the level 71.4 mval·g ⁻¹ N
Anammox	6–43	Anaerobic	6.7–8.3	<i>Candidatus Scalindua</i> , <i>Candidatus Jettenia</i> , <i>Candidatus Brocadia</i> , <i>Candidatus Anammoxoglobus</i> , <i>Candidatus Kuenenia</i> , and <i>Candidatus Anammoximicrobium</i> affiliated with the phylum <i>Planctomycetes</i> ; <i>Candidatus Brocadia anammoxidans</i> , <i>Candidatus Kuenenia stuttgartiensis</i> , <i>Candidatus Scalindua sorokinii</i>	process inhibitor: oxygen, phosphates and nitrates(III) in quantity 100 g·m ⁻³ ; ratio of NH ₄ :NO ₃ ⁻ 1:1.3; pH control

Figure 1. Conditions necessary for environmental processes responsible for nitrogen transformation in the environment (where: AOB—Ammonia Oxidizing *Bacteria*; NOB—Nitrite-Oxidizing *Bacteria*; BOD₅—five-day Biochemical Oxygen Demand at 20 °C).

While iron can come from various sources, however, in recent years, nanotechnology-based processes have become increasingly important as a source of emissions of this element. Nanoparticles of iron oxides (IONPs), Fe_2O_3 , Fe_3O_4 , FeO , depending on the degree of iron oxidation, grain size and shape, surface characteristics, and synthesis methods, including the concentration of the precursor used [4,5], have various physicochemical properties, including optical and magnetic, and thus biological activity. Such huge diversity causes a very wide use of IONPs in industry, pharmacy, and medicine, as well as everyday products. One of the iron oxides with a wide application is $\text{n-Fe}_2\text{O}_3$.

Depending on the method of synthesis, nanoparticles of iron(III) may have grains of various sizes and shapes, including spherical, uneven, quasi-cube, parallel hexahedron, irregular sphere, nanosheet, and hexagon shapes [6,7] and thus possess ferromagnetic, catalytic, oxidizing, and sorption properties of varying intensity. It has also been found that iron oxide nanoparticles, including Fe_3O_4 and Fe_2O_3 , have intrinsic enzyme-like activity and are now considered new enzyme mimetics, so-called nanozyme [8]. Therefore, it is necessary to determine the role of individual forms of “nanoiron” on biological processes commonly used in various areas of environmental engineering. Research work has been undertaken in various research centers to determine the effect of such nanoparticles on anammox as TiO_2 [9], Cu and CuO [10], or ZnO [11]. In recent years, the results of works attempting to determine the mechanism of the impact on the life of selected microorganisms or the biodiversity of the biocenosis of selected anammox structures have also been published, works on the influence of iron nanoparticles at various oxidation states, including zero valent iron nanoparticles (nZVI), n-Fe or FeO , Fe_2O_3 , or Fe_3O_4 nanoparticles,

on the efficiency of the anammox process in terms of nitrogen removal [3,5,12–15]. It should be noted that under appropriate conditions, as shown by the results of studies conducted by Weng et al. [5], n-Fe release Fe^{2+} upon contact with water, and some of it was bound to functional groups in extracellular polymeric substances (EPS), and the rest penetrated the bacteria, creating highly absorbable substances. Some of the n-Fe, on the other hand, aggregated and settled at the bottom of the reactor, eventually transforming into Fe_3O_4 and permanently existing in the anammox system.

Therefore, this study attempted to determine the impact of the presence of iron(III) oxide nanoparticles on the effectiveness of conducting the anammox process under various conditions of temperature or reaction environment. Taking into account the fact that iron present in the environment affects individual micro-organisms present in the environment, responsible for nitrogen transformations, in a different way, it is necessary to determine the impact of nanoparticles of iron(III) oxide on the effectiveness of work of a specific group of organisms used in a given technology, and not a single micro-organism, in terms of not only nitrogen removal, but also other wastewater quality parameters. Since the aim of the experiment was not to destroy biomass, but to check whether anammox, which is also an environmental process, can occur under conditions of solar radiation and n- Fe_2O_3 .

2. Materials and Methods

2.1. Biomass of Anammox

For the research work, an SBR with a working volume of 8 L and a height-to-diameter ratio of 1.4, composed of an acrylic cylinder, was used. The biomass used in this study was collected from a partial nitrification-anammox reactor [16]. The reactor was run as intermittently aerated sequencing batch reactor to treat synthetic wastewater containing 300–900 mg/L $\text{NH}_4\text{-N}$ (ammonium sulfate) and 100 mg/L chemical oxygen demand (COD) (glucose), 58 mg/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 111 mg/L KH_2PO_4 and 170 mg/L $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$, in accordance with Qiu et al. (all chemicals were bought from Fisher Scientific Ireland Limited, Dublin, Ireland) [16]. Five groups of nitrogen-conversion micro-organisms were detected in the reactor: (1) three ammonium-oxidizing bacteria (AOB) genera with *Nitrosomonas* as the most abundant genus; (2) four NOB genera with *Nitrospira* as the most abundant genus; (3) three ammonium-oxidizing archaea (AOA) genera with *Candidatus Nitrososphaera* as the predominant genus; (4) three anammox genera, including *Candidatus Kuenenia* and *Candidatus Brocadia* and *Candidatus Anammoximicrobium*, and (5) potential heterotrophic denitrifiers.

2.2. Sample Preparation

The processes were carried out in optimal temperature (20 and 30 °C) and pH (7 and 8) conditions for anammox, in order to eliminate the possibility of other factors. Samples were taken from the reactor, washed twice with phosphate buffer of appropriate pH (7 or 8) and then centrifuged. The pH of the solutions limited the possibility of dissolving Fe_2O_3 nanoparticles in the prepared samples. Nanoparticles of Fe_2O_3 (Sigma-Aldrich, Darmstadt, Germany), crystalline (primarily γ), with nominal sizes of <50 nm and surface area 50–245 m^2/g were used for the work.

After the last centrifugation, phosphorus buffer was added to the precipitate, and thus the prepared anammox biocenosis precipitate was divided into 8 samples of 50 cm^3 each (two samples for each system). To the samples prepared in this way were added 2.5 cm^3 $\text{NH}_4\text{-N}$ at 1000 $\text{mg}\cdot\text{dm}^{-3}$, 0.25 cm^3 $\text{NO}_2\text{-N}$ at 4000 $\text{mg}\cdot\text{dm}^{-3}$, and 1 cm^3 NaHCO_3 at 10.0 $\text{mg}\cdot\text{dm}^{-3}$, and different volumes of the n- Fe_2O_3 standard solution, prepared in accordance with Rabajczyk et al. [17], to obtain three solutions with different concentrations of n- Fe_2O_3 (0.5, 1.0, and 2.5 $\text{mg}\cdot\text{dm}^{-3}$). It should be added that the concentration range was selected based on the results of the authors' research on the effectiveness of removing

impurities using activated sludge in the presence of $n\text{-Fe}_2\text{O}_3$ [17], and concentrations for which a visible impact on the efficiency of nutrient reduction was selected for this study.

2.3. Analysis

The samples were placed in a temperature-controlled thermostat with a shaker and without UV radiation or with solar radiation. Samples for testing were taken at time points: 0, 30, 60, 90, 120, and 1440 min. Dissolved oxygen (DO), conductivity (cond), and pH were determined with a portable meter (Multi3620, WTW, Weilheim, Germany). Prior to analysis, effluent water samples were filtered through syringe filters with a pore size of $0.45\ \mu\text{m}$ (Sarstedt Ltd., Nümbrecht, Germany). Concentrations of $\text{NH}_4^+\text{-N}$, $\text{NO}_2^-\text{-N}$ and $\text{NO}_3^-\text{-N}$ were measured using a nutrient analyzer (Konelab 20, Thermo Clinical Labsystems, Waltham, MA, USA). UV_{254} absorption was determined using a Varian (Cary 100) UV-Vis Spectrometer. The material collected on the filter was analyzed using a Hitachi scanning electron microscope HBB for elemental (point and area) analysis and imaging of embedded material.

The size of particles present in the solutions and their zeta potential were analyzed using Zetasizer Nano ZS90 (Malvern Panalytical, Malvern, UK) and Litesizer 500 (Anton Paar, VA, USA). Zeta potential Reference Material 175,108 AntonPaar was used to calibrate the device. The PDI index was calculated according to the equation:

$$\text{PDI} = (\text{D90} - \text{D10})/\text{D50} \quad (1)$$

Free ammonia (FA) concentration was calculated according to the equation:

$$\text{FA as } \text{NH}_3 [\text{mg}\cdot\text{dm}^{-3}] = (17/14) \cdot \text{TN-NH}_4/1 + (K/10^{\text{pH}}), \quad (2)$$

where

TN-NH_4 —total ammonium nitrogen [$\text{mg NH}_4\text{-N}\cdot\text{dm}^{-3}$]

$$K = e^{6344/(273 + T)}; T = 20\ ^\circ\text{C}/30\ ^\circ\text{C}.$$

The assessment of the purification process stability was based on the verification of the purification efficiency in terms of $\text{NH}_4\text{-N}$ reduction within 1 min, calculated according to the formula:

$$K_{\text{NH}_4} = [\text{NH}_4\text{-N}_0 - \text{NH}_4\text{-N}_x]/t [\text{mg}/\text{min}],$$

where

$\text{NH}_4\text{-N}_0/\text{NH}_4\text{-N}_x$ — $\text{NH}_4\text{-N}$ concentration at the beginning of the process and after the analyzed time. The process was considered stable when the K_{NH_4} values did not differ by more than $\pm 30\%$.

3. Results

The results of studies on the influence of Fe_2O_3 nanoparticles on the efficiency of the anammox process at temperatures of 20 and $30\ ^\circ\text{C}$, at selected pH, radiation, or oxygen saturation values, depending on the concentration of iron(III) oxide nanoparticles, are presented in Tables S1–S5 (Supplementary Materials).

4. Discussion

The anammox process is used in sewage treatment plants to remove nitrogen. In biological treatment, micro-organisms are used, whose enzymes participate in the nitrogen assimilation process. The effect is a change in the form of nitrogen occurrence or its binding in bacterial cells [18–20]. The proper functioning of bacteria is often determined by the presence of appropriate metals in the environment, such as molybdenum, vanadium,

or iron, which are necessary for the production of various groups of metalloenzymes, including nitrogenases [21,22]. The form of occurrence of metals and their bioavailability for micro-organisms is of great importance.

4.1. Agglomeration

The presence of nanoparticles of metals and metal oxides (NPMOs) in wastewater is a growing problem in terms of the efficiency of biological water and wastewater treatment processes. NPMOs show a diverse inhibitory effect on the anammox process. It depends not only on the size and shape of the grains, but also on the structure of the nanoparticles, whether it is a metal nanoparticle or a metal oxide nanoparticle, and the degree of oxidation of the metal in the compounds.

Fe₂O₃ nanoparticles, which belong to substances with magnetic properties, undergo very rapid aggregation in aqueous solutions. Analysis of the particle size in the initial solutions (distilled water, no ionic substances, pH ~ 7) and analysis of the particle size in real solutions, which included micro-organisms as well as inorganic and organic substances determining the ionic strength of the solution, indicate that the aggregation of nanoparticle-micro-organism occurs very quickly, right after introducing nanoparticles into the real solution. Not only the particle size changes, but also the zeta potential that defines the activity of the resulting systems. Fe₂O₃ nanoparticles in the initial solution, where redistilled water was used, had a zeta potential of −36.00 mV and an average particle size of 98 nm, while in solution containing buffer and inorganic ions, the zeta potential was between −33 and −31 mV, and particle size depended on the concentration (Table S1).

However, in the case of real solutions containing micro-organisms, but without nanoparticles, the particle size ranged between 805 and 1186 nm (at 20 and 30 °C, respectively), while the zeta potential was approximately −13.80 mV. As a result of the addition of nanoparticles to the real solution, the values of zeta potential were and ranged from −31.41 to −33.19 mV, for the most diluted system and the most concentrated solution, respectively. Systems containing only biomass or only iron(III) oxide nanoparticles were characterized by values lower than −30 mV, which indicates stable systems in which the agglomeration process should not take place. As a result of the connection, the value of zeta potential increases (Table S1), which indicates the formation of very dynamic systems with unstable dispersion. Particles present in solutions may agglomerate and disintegrate.

The change in the nature of the solution is also indicated by the values of diffusion coefficients, which increase as a result of mixing the solutions.

As a result of processes such as absorption, dissolution, and biochemical transformations occurring in real solutions, where dissolved organic and inorganic substances are present, as well as micro-organisms, the zeta potential of particles present in solutions has changed significantly, depending on the time of contact, as well as pH, access to oxygen, and UV radiation, up to −11.78 mV (Table S1). The particle sizes present in the solutions also changed. Iron(III) oxide nanoparticles, immediately after being introduced into the biomass solution, lay around and on the biomass surface, which is visible in the SEM photo taken for the sample just after the introduction of n-Fe₂O₃ into the solution (Figure 2). As a result of the reaction after 120 min, scattered n-Fe₂O₃ agglomerates and partially damaged biomass fluff structures can be seen.

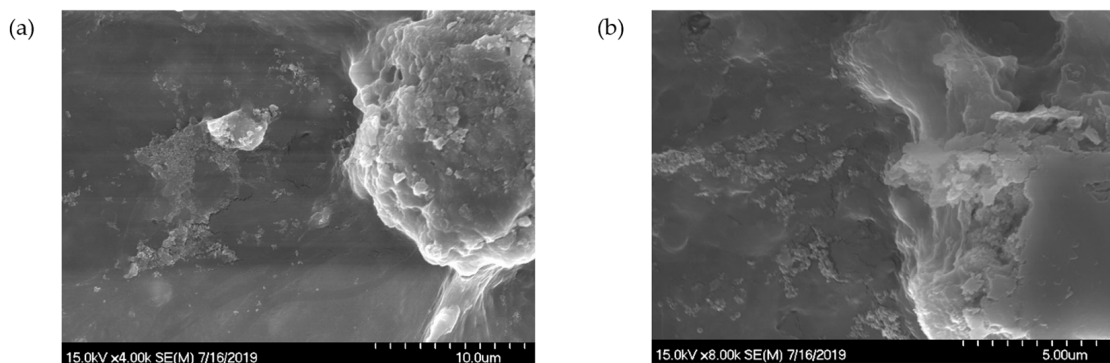


Figure 2. System of biomass and $n\text{-Fe}_2\text{O}_3$ with a concentration of $2.5 \text{ mg}\cdot\text{dm}^{-3}$ at pH 8, temperature 30°C (a) after mixing the reactants, time 0 min, (b) after 120 min of reaction time.

The presence of $n\text{-Fe}_2\text{O}_3$ reduces the agglomeration of micro-organisms and the formation of large biomass agglomerates in the form of activated sludge flocs, as evidenced by changes in the size of particles present in solutions per unit of time. It is an active and dynamic system in which the particle size changes. However, nano-sized systems are not observed. This is probably related to the change in the zeta potential as a consequence of sorption occurring in the biomass— $n\text{-Fe}_2\text{O}_3$ system.

It should be added that the temperature and pH of solutions are also important for the interaction of Fe_2O_3 nanoparticles—biomass (Figures 2 and 3). Comparing the structure of the agglomerate obtained at pH 8 and $T\ 30^\circ\text{C}$, with the agglomerate formed at pH 7 and $T\ 20^\circ\text{C}$, it was found that the lower temperature promotes the construction of accumulated agglomerates, with an irregular structure, but close to a spherical shape. On the other hand, higher temperature and higher pH value affect the formation of agglomerates with a more irregular, loose structure, with numerous perforations between organic particles, with a blurred shoreline.

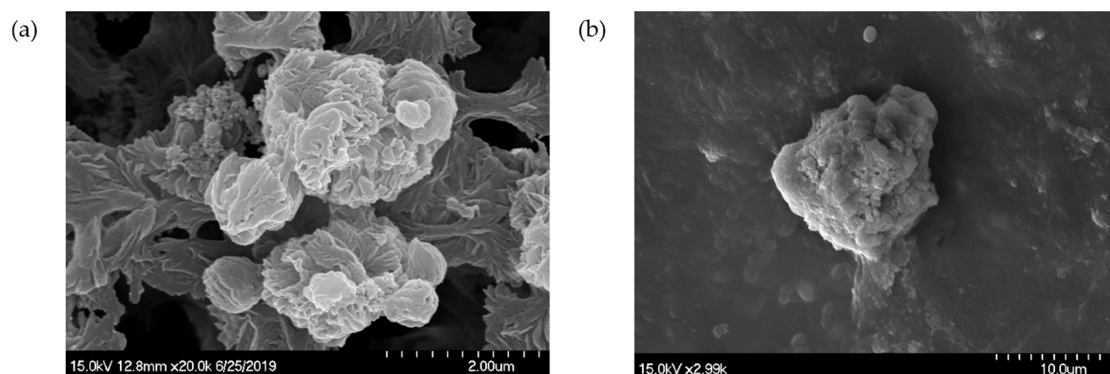


Figure 3. System at pH 7, 20°C after 24 h (a) biomass— $n\text{-Fe}_2\text{O}_3$ with a concentration of $2.5 \text{ mg}\cdot\text{dm}^{-3}$, (b) only biomass.

The structure of the particle created as a result of agglomeration is very important for the effectiveness of environmental cleaning works. In a situation where we are dealing with an irregular, loose structure, sedimentation properties deteriorate [7,23,24]. There is a close relationship between the floc properties in the biocenosis used and the properties of this system. Structures with small pores show low dropout, while large and dense ones show high dropout. Poorly sloping structures are very difficult to dehydrate, which is why obtaining the right structures is important to ensure adequate flocculation, which affects the course of wastewater treatment [25–27].

4.2. Physicochemical Parameters and the Efficiency of the Anammox Process

In the case of conducted works, it should be stated that Fe_2O_3 nanoparticles, depending on the duration of the reaction, solution pH, and temperature, affect the effectiveness of the anammox process in various ways (Figures 4 and 5).

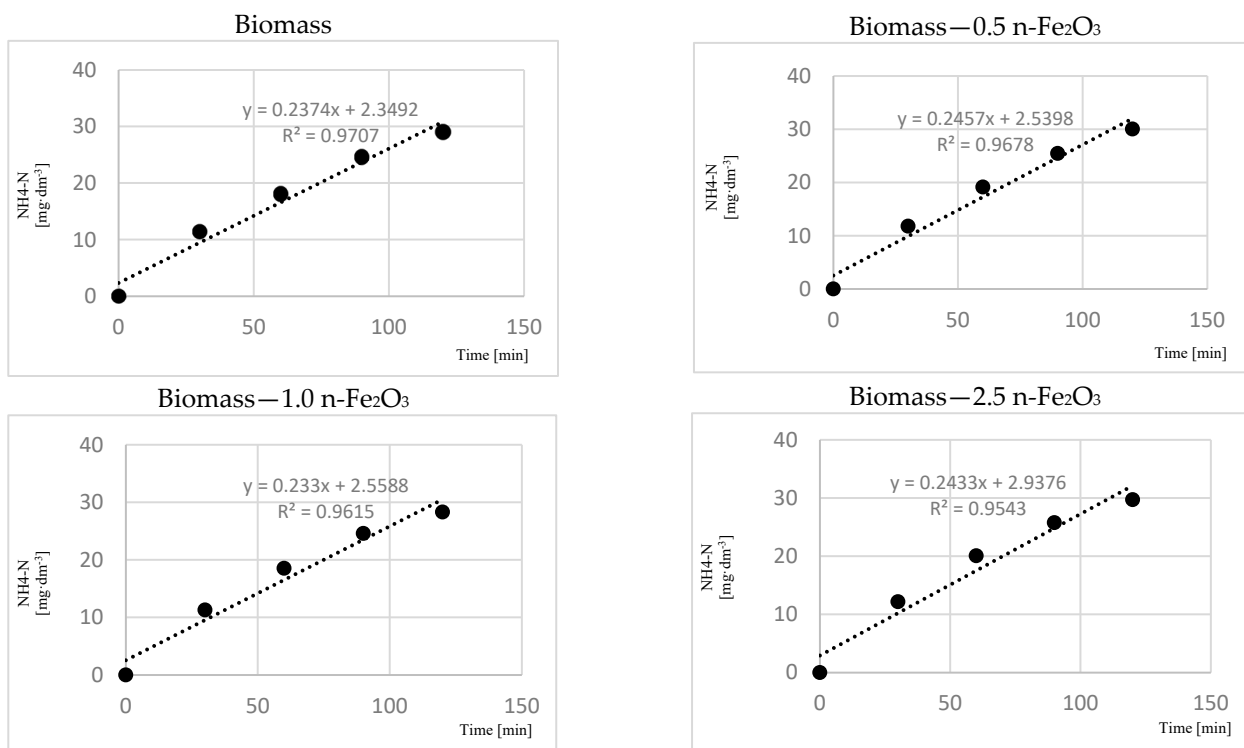


Figure 4. Dependence of the reduction in $\text{NH}_4\text{-N}$ concentration for the system at pH 8 and 20 °C for 24 h.

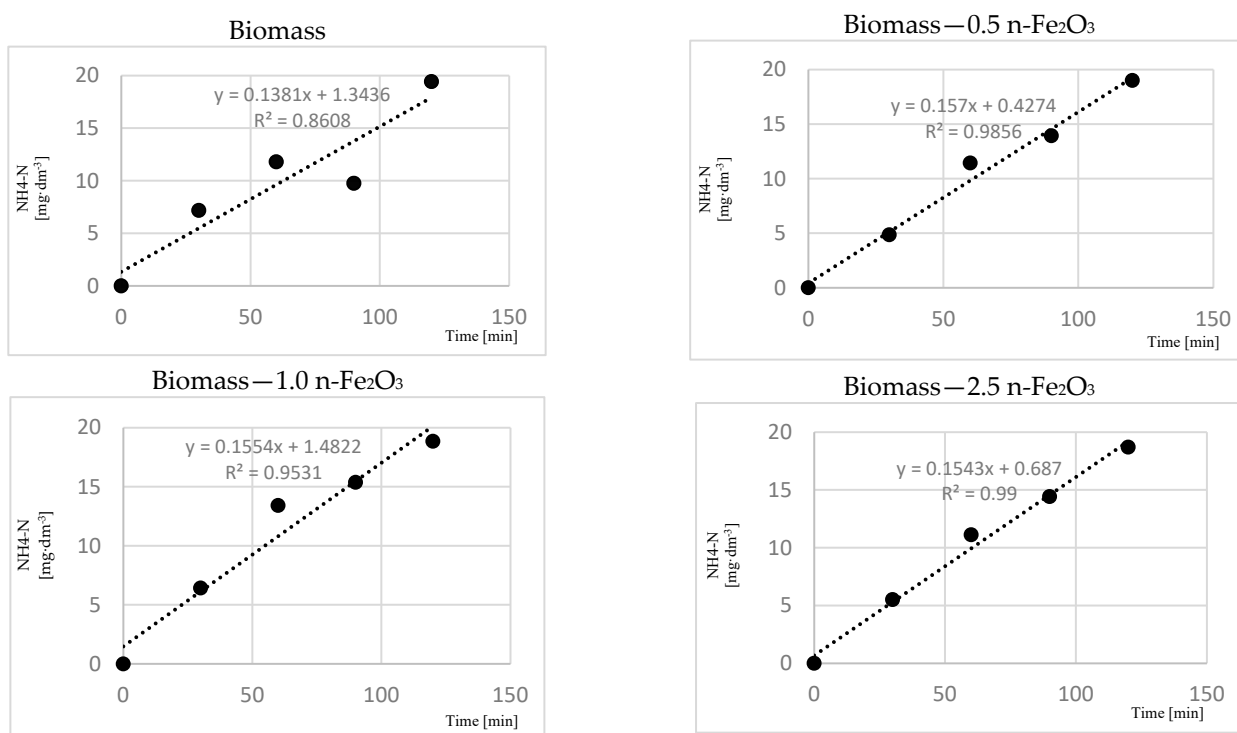


Figure 5. Dependence of the reduction in $\text{NH}_4\text{-N}$ concentration for the system at pH 7 and 30 °C for 24 h.

The most important for anammox processes was the first 30–60 min when increased $\text{NH}_4\text{-N}$ removal efficiency was observed for $\text{n-Fe}_2\text{O}_3$ 1.0 and $2.5 \text{ mg}\cdot\text{dm}^{-3}$, while for $\text{n-Fe}_2\text{O}_3$ $0.5 \text{ mg}\cdot\text{dm}^{-3}$ no significant changes were observed compared to the system containing only biomass in each of the analyzed systems. However, after 120 min there was a reduction in the efficiency of $\text{NH}_4\text{-N}$ reduction from approximately 1% to 2–3% as the reaction time was extended in systems containing $\text{n-Fe}_2\text{O}_3$, the higher the concentration of $\text{n-Fe}_2\text{O}_3$ used, the more effective the anammox process was.

The exception is a system with a pH of 7 and a temperature of 30°C . The results obtained show the lowest $\text{NH}_4\text{-N}$ removal efficiency of up to 40% in 120 min and almost 70% in 24 h using only biomass. The addition of the highest concentration of Fe_2O_3 nanoparticles to the system allowed a slight increase in the amount of ammonium nitrogen removed to almost 42% in 120 min and improved the work of anammox (Figure 5). This is important because at 30°C the efficiency of $\text{NH}_4\text{-N}$ removal is greater for solutions with a pH of 8 (approximately 98% after 24 h) than for solutions with a pH of 7 (approximately 70% after 24 h).

An important element determining the effectiveness of biochemical processes is the presence of oxygen. In the case of aerobic micro-organisms, for which this gas is necessary, the presence of iron(III) oxide nanoparticles may be a factor determining their proper functioning, and thus determine the correct, effective process of nitrogen transformation. In the case of micro-organisms living in conditions with limited oxygen access, $\text{n-Fe}_2\text{O}_3$, due to the production of ROS, may generate toxic conditions. Song et al. [28], while conducting research on the effects of ZnO NP, found that ROS does not directly affect the release of LDH dehydrogenase and there is no damage to the integrity of the cell membrane, and therefore bacterial DNA damage and subsequent induction of cell death may occur [29]. However, Li et al. [29] found that for Fe_3O_4 nanoparticles, which also have the ability to produce ROS, a higher concentration of nanoparticles makes the anammox process more efficient [29,30].

It can therefore be concluded that the presence of iron nanoparticles and their role in the functioning of micro-organisms are important and key elements. In the case of Fe_2O_3 nanoparticles, the first 30-min period of interaction of $\text{n-Fe}_2\text{O}_3$ —micro-organisms forming biocenosis in the bioreactor (Figure 6) was important. During this time, in each case analyzed (pH 7/8, temp. $20/30^\circ\text{C}$), a sharp drop is observed, followed by an increase in the oxygen content in the system, which may indicate acclimatization of micro-organisms and self-organization of the system in the presence of $\text{n-Fe}_2\text{O}_3$. After 30 min, the oxygen content was already slightly fluctuating, remaining at a similar level, slightly higher than at the beginning of the reaction. A similar situation is also in the case of systems isolated from UV light, but this jump is definitely greater, because in the initial phase anaerobic conditions occur.

In the case of systems containing only biomass, the level of oxygen saturation during 120 min changed from an average of 0.81 at the beginning of the reaction to $8.14 \text{ mg O}_2\cdot\text{dm}^{-3}$ after 24 h for a system with solar radiation access and from 1.25 to 7, 92 $\text{mg O}_2\cdot\text{dm}^{-3}$ without UV access. The presence of Fe_2O_3 nanoparticles with a concentration of 1.0 and $2.5 \text{ mg}\cdot\text{dm}^{-3}$ allowed the system to react faster to changes in oxygen conditions. It is probably related to the possibility of ROS release by Fe_2O_3 nanoparticles. However, it should be noted that despite the stabilization of the system, the efficiency of $\text{NH}_4\text{-N}$ reduction was lower than for a system without nanoparticles. Such a small difference may be the effect not only of the size of the sample subjected to analysis and the concentrations of nitrogen and nanoparticles used, but also of the use of solar radiation instead of UV-C, which is used for disinfection and destruction of micro-organisms.

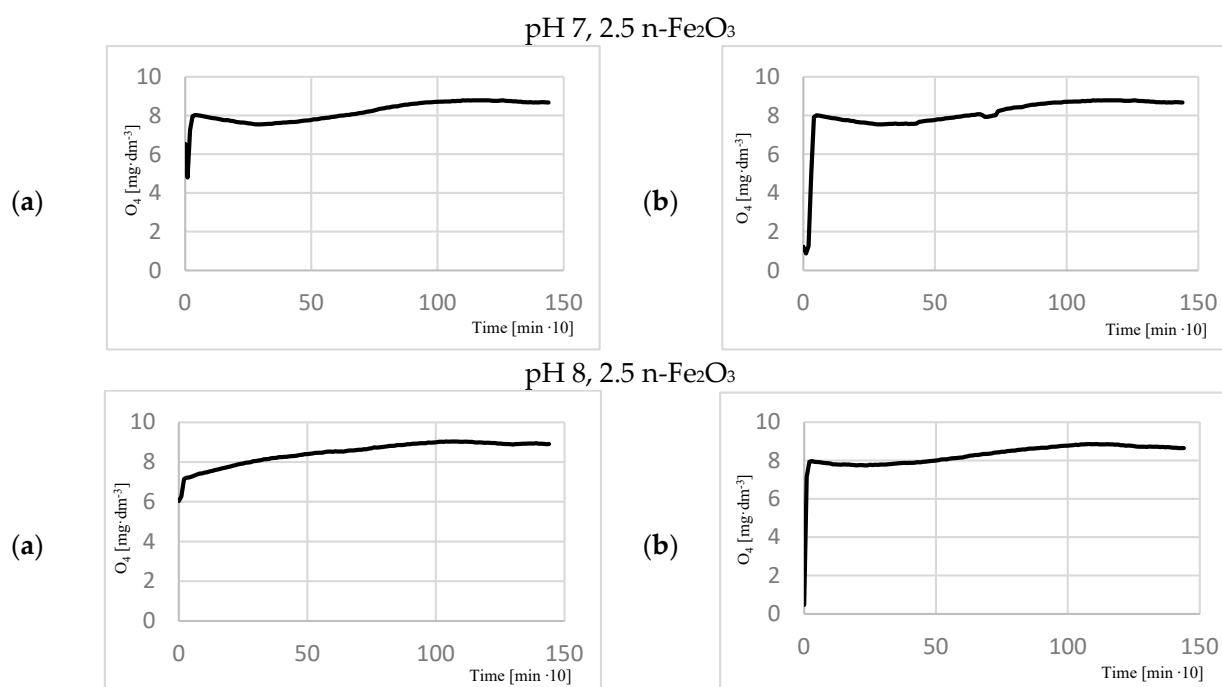


Figure 6. Changes in oxygen concentration during 24 h for the system operating at 20 °C (a) with UV access and (b) without UV access.

It is also worth pointing out that the anaerobic oxidation of ammonium, which is associated with the reduction in iron(III), is referred to as Feammox. The results of a study by Yang et al. [2] showed that *Geobacter* and *Anammox* bacteria such as *Brocadia*, *Kuenenia*, and *Jettenia* may play an important role in nitrogen removal in this process. Thus, the presence of $n\text{-Fe}_2\text{O}_3$ can significantly contribute to the effective process of nitrogen removal.

4.3. MONPs and the Anammox Process

Depending on the purpose of the conducted environmental works, due to the presence of Fe_2O_3 nanoparticles, temperature, and pH value, we can therefore influence the efficiency and direction of processes occurring in the system. It should also be added that elemental analysis clearly indicates that iron is an important part of the anammox biomass used in this study, which means that among the biocenosis-forming micro-organisms there are organisms in which iron was found (Figure 7).

The presence of such elements as C, N, O, K, Ca, and P is a consequence of the structure of micro-organisms and the composition of the solution, which included such compounds as phosphate buffer, bicarbonates, nitrates(III), and ammonium ion. Analysis of selected areas also showed the presence of such an element as Nb, however, it is at a trace level, less than 0.2%, which may indicate contamination of reagents used in the work. This is confirmed by the analysis carried out for the entire surface observed in the photo (Figure 2), which showed that Nb is outside the analytical range and represents less than 0.1% of the mass of the surface analyzed. On the other hand, quantitative analysis of iron for the same sample showed a content of almost 2.5% by weight of the entire analyzed surface. In the elemental analysis of the biomass— $n\text{-Fe}_2\text{O}_3$ system, the amount of iron increases by up to 7.25% by mass for the entire analyzed surface and zero content in the case of niobium, while for selected areas, including biomass— $n\text{-Fe}_2\text{O}_3$ agglomerates, the amount of iron oscillates within 10.5% by mass (Figure 8).

The increase in iron content may be a consequence of biomass- $n\text{-Fe}_2\text{O}_3$ sorption processes, as well as the utilization of iron for functional purposes and the incorporation of iron into the biological material of microbial cells.

Nanoparticles of metals, particularly Ag, Cu, CuO, TiO₂ as well as iron oxide nanoparticles (IONPs), have antibacterial properties, which can determine the biological processes occurring in the anammox process [29,31–37]. Nanoparticles of CuO and Cu reduce the number of *Candidatus kuenenia* and *Candidatus brocadia* forming biocenosis, thereby reducing the efficiency of the nitrogen removal process. However, it was noticed that the presence of Cu NPs elevated the expression of the copA metal resistance gene from 3.56×10^{-4} in the control to 12.4×10^{-4} , while CuO nanoparticles did not show such a process [38]. Additionally, research by Zhang et al. [32] showed a diverse influence of CuO and Cu nanoparticles on the anammox process.

Comparing the effect of CuO and ZnO nanoparticles on the anammox process, it was found that n-CuO did not affect anammox activity even at high doses, while n-ZnO, even at low doses, severely inhibited anammox activity. However, this was due to the dissolution of n-ZnO in water rich in ammonium ion and, thus, increased release of zinc ions. It is therefore possible to reduce the negative effect on anammox caused by the presence of ZnO nanoparticles by increasing the concentration of NH₄⁺ [28]. Additionally, in the case of CuO nanoparticles, a toxic effect of Cu²⁺ ion released from nanoparticles has been observed [39]. However, in the case of n-CuO, the addition of EDTA to the solution as a complexing substance released Cu²⁺ ions. The reduction in bioavailability allows a reduction in toxicity to micro-organisms [39].

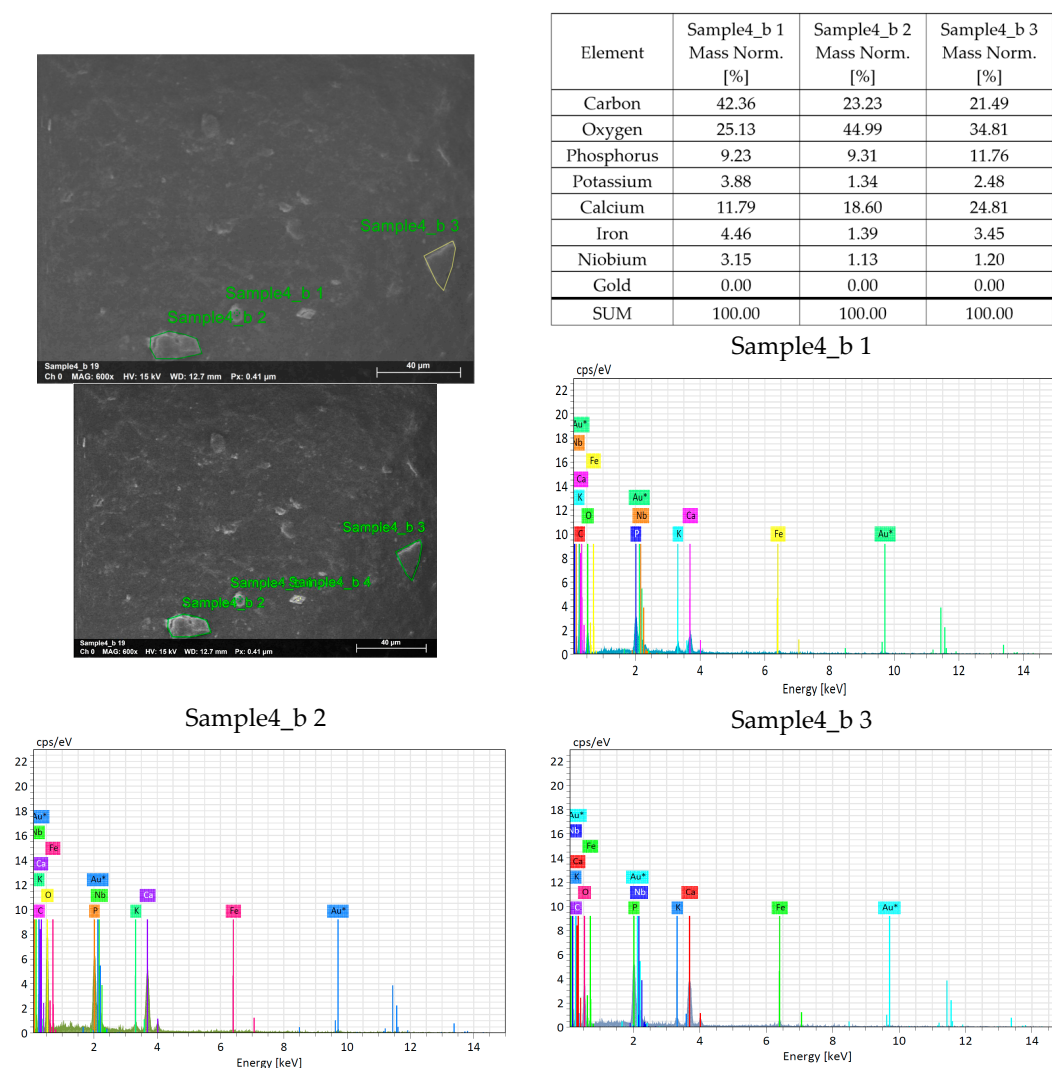


Figure 7. Elemental analysis of biomass at pH 8, temperature 30 °C (where Au*—analysis background).

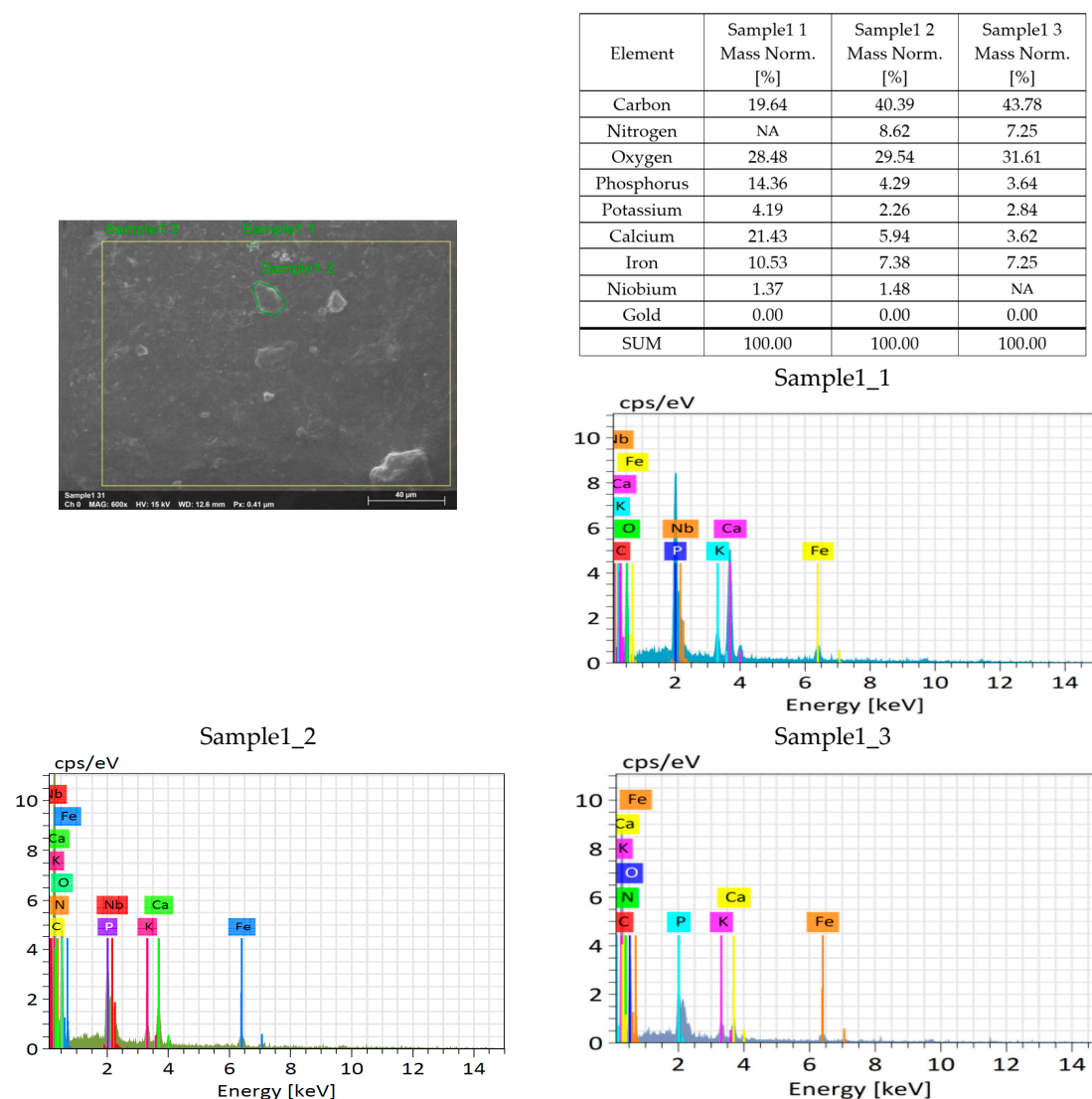


Figure 8. Elemental analysis of the biomass-n-Fe₂O₃ system at pH 7, temperature 20 °C, after 120 min of reaction time.

Results of research conducted by Zhang et al. [35] also showed a negative impact of ZnO and CuO nanoparticles on the anammox process. It was found that, already at the concentration of nanoparticles of the order of 1 mg·dm^{−3}, nanoparticles significantly inhibit nitrogen removal. At higher concentrations of 5–20 mg·dm^{−3} and 10–50 mg·dm^{−3} for ZnO and CuO, respectively, the biocoenoses were characterized by self-adaptation to these nanoparticles. In contrast, TiO₂ nanoparticles, at low concentrations, contributed to greater efficiency in nitrogen removal, while for concentrations > 1 mg·dm^{−3} inhibition of anaerobic ammonia-oxidizing bacteria (AAOB) bioactivity was observed. At higher concentrations, on the order of 50 mg·dm^{−3}, n-TiO₂ no longer had an effect on anammox, due to aggregation of nanoparticles [35].

Additionally, in the case of other metal oxide nanoparticles, such as n-SiO₂, n-Al₂O₃, and n-CeO₂, the presence of nanoparticles in the concentration range of 1, 50, 200 mg·dm^{−3}, did not show a visible effect on the efficiency of nitrogen removal from anammox reactors. However, high concentrations of nanoparticles, of the order of 200 mg·dm^{−3}, had a clear impact on shaping the anammox community. Prolonged exposure to MONP caused different responses in the relative abundance of *Ca. Kuenenia*, the level of the functional *HzsA* gene, and the activity of the three key enzymes involved in anammox metabolism,

but no significant inhibitory effect on specific anammox activity was detected. In general, the impact of MONP on the structure of the anammox community and sediment properties depended on their types and levels and was consistent with the order of $\text{SiO}_2 > \text{CeO}_2 > \text{Al}_2\text{O}_3 > \text{TiO}_2$ [33].

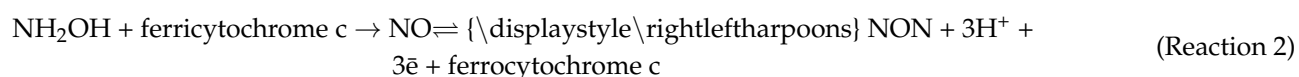
4.4. Anammox Process and $n\text{-Fe}_2\text{O}_3$

The enzyme contains an iron-sulfur center and molybdenum, less often vanadium, and is composed of two types of protein units. The larger unit, the so-called FeMo protein, consists of 4 polypeptide chains and contains a cofactor containing iron and molybdenum atoms, i.e., FeMo. On the opposite sides of the FeMo protein, two smaller iron proteins are attached [21,22,40–43].

According to literature data, the FeV metalloprotein was found in gram-negative aerobes such as *Azotobacter saliestris* and *Azotobacter chroococcum* or *Anabaena variabilis*, a filamentous cyanobacterium. There are also micro-organisms that have been observed to lose the ability to bind atoms of two different metals in favor of iron. Fe metalloprotein is found in anaerobic, gram-positive *Clostridium pasteurianum*, gram-negative *Rhodobacter capsulatus*, facultative anaerobic gram-negative *Rhodospirillum rubrum* or aerobic gram-negative bacterium *Azomonas macrocytogenes* [44,45].

It should be added that the factor limiting the enzyme's activity is oxygen. Nitrogenase is active under anaerobic conditions or in the presence of low O_2 concentrations. Higher concentrations of this element cause its inactivation. If a substance with ROS production capacity is present in the system, this enzyme can also be inactivated when no oxygen is supplied to the system. Thus, the process may be disturbed, and the production of ammonia may be inhibited.

The enzyme, which also contains iron in its structure, is Hydroxylamine Oxidase or Hydroxylamine Oxidoreductase (HAO) [46,47]. This enzyme is a catalyst for the process of converting hydroxylamine into other forms of nitrogen, as recorded:



This enzyme has, among others, the heterotrophic nitrifying bacterium *Paracoccus denitrificans*. It is built with three to five non-heme, non-iron-sulfur iron atoms as prosthetic groups and requires molecular oxygen [48].

Similar non-heme HAOs have been partially purified from *Arthrobacter glubiformis* (gram-negative bacterium species from the genus of *Arthrobacter*) [49], gram-negative bacterium *Thiosphaera pantotropha* [50], gram-negative bacterium *Alcaligenes faecalis* [51], an aerobic denitrifying bacterium *Bacillus* sp. [52] and gram-negative bacterium *Pseudomonas* sp. [53]. Anaerobic oxidation of ammonia using nitrite as an electron acceptor is carried out by some *Planctomycetales* bacteria. *Planctomycetales* are mostly aerobic, chemoorganotrophic organisms. However, in the case of anammox bacteria, we are dealing with anaerobic organisms and chemolithoautotrophs. Three types of anammox bacteria are known: *Brocardia* and *Kuenenia* (isolated from wastewater) and *Scalindua* (isolated from the Black Sea) [54,55]. In addition, anammox bacteria have a low specific growth rate compared to nitrifying bacteria. In addition, the biomass doubling time is very slow, depending on the type of anammox bacteria [56,57].

In anammox bacteria, the enzyme hydroxylamine oxidoreductase constitutes as much as 10% of the cellular protein and is located in the organelle associated with the cell

membrane—anammoxosome. Hydroxylamine oxidoreductase catalyses the oxidation of: hydroxylamine (NH_2OH) and hydrazine (N_2H_4). In anaerobic bioreactors and biological membranes also nitrifying bacteria, e.g., *Nitrosomonas europaea*, which under the conditions of oxygen limitation are able to carry out the denitrification process survive [58].

Only a part of the denitrifying bacteria is capable of full denitrification, some of them lead to nitrates (V) transformations to intermediate products [59]. These include, for example, *Roseobacter denitrificans*, for which the final stage of denitrification is the formation of nitric oxide (I) or *Comamonas testosteroni*, which only reduce nitrates (V) to nitrates (III) [59,60]. Another group of bacteria, in turn, uses intermediates as substrates to carry out subsequent stages, e.g., *Alcaligenes*, for which the substrate is nitrates (III). Hence, the biomass structure may be one of the reasons for the accumulation of intermediate denitrification products, which usually occur at very low concentrations, and their high content is undesirable. This applies to nitric oxide (II), nitric oxide (I), and above all nitrates (III), which are inhibitory to microorganisms, especially in the pH range of 4.5–6.0 [59].

Iron oxide nanoparticles are often used to combat micro-organisms, including bacteria commonly found in the environment, such as *Bacillus subtilis* and *Escherichia coli*. It should be noted, however, that *Bacillus subtilis* is used in biological preparations used in wastewater treatment processes to counteract, among other negative effects of low temperatures on microorganisms such as *Nitrosomonas* and *Nitrobacter* [61]. Arakha et al. [37] showed that the concentration of IONP < 50 μM has little antimicrobial activity against *Bacillus subtilis* and *Escherichia coli*, however, the chitosan coating can cause an increase in ROS production and thus an antimicrobial effect [37]. That is why the method of producing iron oxide nanoparticles is so important.

Research results obtained by Li et al. [29] indicate that long-term exposure to nanoparticles of Fe_3O_4 of varying concentrations improves the removal of total nitrogen from ecosystems. Results of research conducted by Li et al. [29] showed that the number of anaerobic *Candidatus Anammoxoglobus* increased significantly at a concentration of n- Fe_3O_4 at 10 $\text{mg}\cdot\text{dm}^{-3}$, while when exposed to n- Fe_3O_4 at a concentration of 1 $\text{mg}\cdot\text{dm}^{-3}$ n- Fe_3O_4 is reduced. In contrast, the number of anaerobic, gram-positive *Desulfosporosinus* and *Exiguobacterium* at an n- Fe_3O_4 concentration of 1 $\text{mg}\cdot\text{dm}^{-3}$ increased only to some extent, suggesting that Fe-anammox played an important role in removing TN. It should be noted, however, that the relative activity of the two key enzymes of the anammox bacteria, i.e., nitrite reductase (NIR) and hydrazine dehydrogenase (HDH), as a result of prolonged exposure to different concentrations of n- Fe_3O_4 , was different. For HDH, a decrease compared to the control was observed in proportion to the concentration of n- Fe_3O_4 , while NIR activity remained stable for n- Fe_3O_4 and was not significantly changed [29,62]. Despite a significant decrease in HDH activity, no changes in nitrogen removal processes were found. Some bacteria can oxidize $\text{NH}_4\text{-N}$ in combination with the simultaneous reduction of iron (Fe^{3+}), called Fe-anammox [29]. This group of bacteria, therefore, plays a very important role, however, the presence of Fe(III) is required. Peng et al. [14] showed that iron-rich nanoparticles are present in anammoxosomes. The iron-rich nanoparticles were identified as $\alpha\text{-Fe}_2\text{O}_3$ and referred to as bacterioferritin.

It should be noted that anammox bacteria have a relatively high demand for iron, however, the form of iron occurrence and its degree of oxidation play an important role. For example, high concentrations of iron nanoparticles (n-Fe) have a toxic effect on bacteria [15,63]. Nanoparticles cause the destruction of the cell membrane, mediate in the accumulation of toxic intermediates, and interfere with metabolism. However, research results indicate that adding the right amount of n-Fe can positively influence the process and increase the nitrogen removal activity in anammox systems [15,64,65]. Iron storage proteins in anammox bacteria have also been shown to regulate cellular iron balance [15]. When iron is deficient

in the environment, bacteria release stored iron. However, when the iron concentration was adequate for the needs of microorganisms, Fe^{2+} was converted to Fe^{3+} by the ferredoxin protein and then, in this form, was stored in iron storage proteins. Providing appropriate Fe^{2+} concentrations was conducive to ribosome metabolism and protein synthesis of AnGS bacteria, especially anammox bacteria [15]. In this, for example, it provided a redox reaction or the presence of iron-storing proteins in anammox bacteria, which were involved in the regulation of the anammox metabolic process and iron balance, which ensured the growth and nitrogen removal capacity of anammox bacteria. It was also found that the presence of a special structure of protein envelopes makes Anammox bacteria more resistant to Fe nanoparticles and can sequester n-Fe toxicity [15]. The acclimatization process has been observed in many cases. It allowed the achievement of adaptation of biocenosis to harmful factors. Two mechanisms explaining acclimatization are presented. In one case, microorganisms acquire the biodegradability of inhibitors, especially in long-term exposure to the inhibitor. The second mechanism is based on the ability of microorganisms to acquire the ability to regenerate new proteins in inactive cells, thanks to which their activity is restored [66,67].

The impact of the presence of nanoparticles is noticeable primarily in the analysis of the course of processes. Chemical analysis of the solutions in 30-min intervals showed that for solutions with a pH of 7, the course is different after the addition of n- Fe_2O_3 , especially with respect to the presence and concentration of dissolved organic compounds analyzed as the UV-Vis254 parameter (Figure 9).

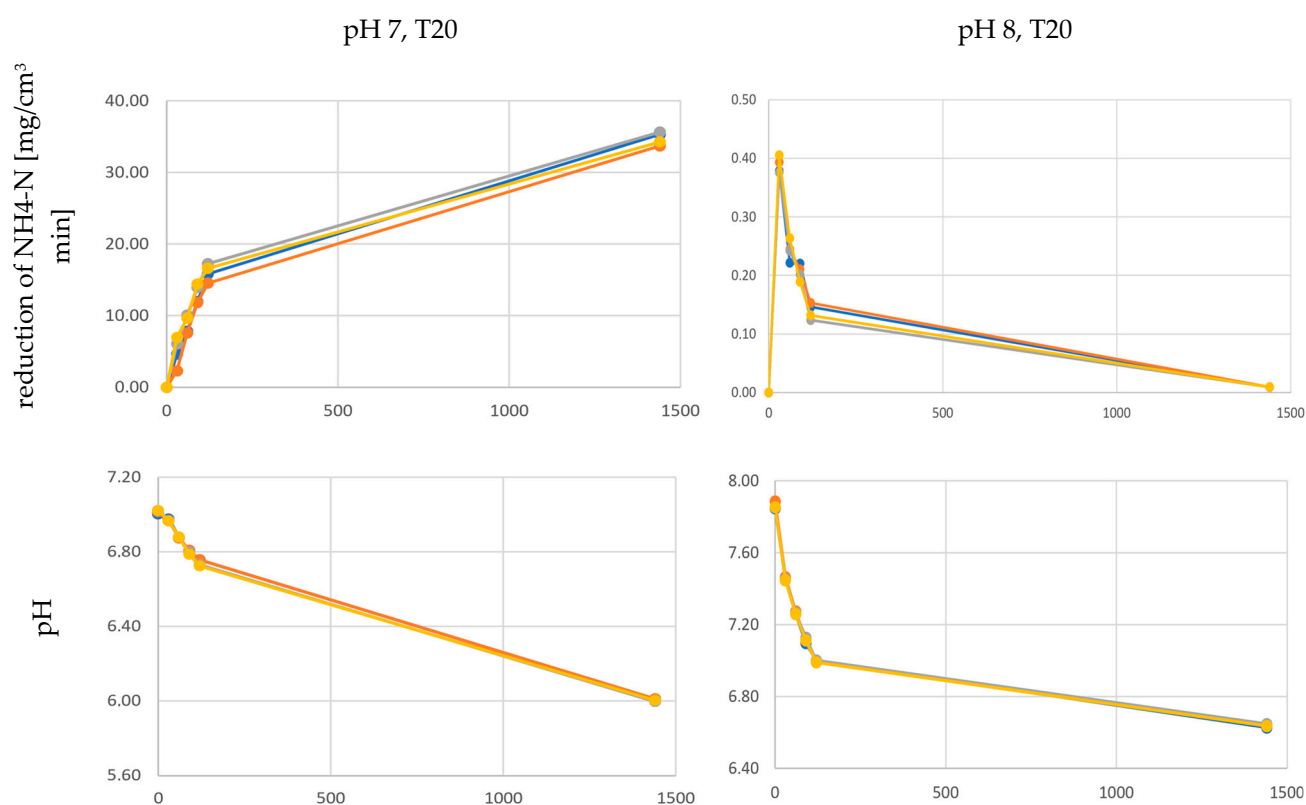


Figure 9. Cont.

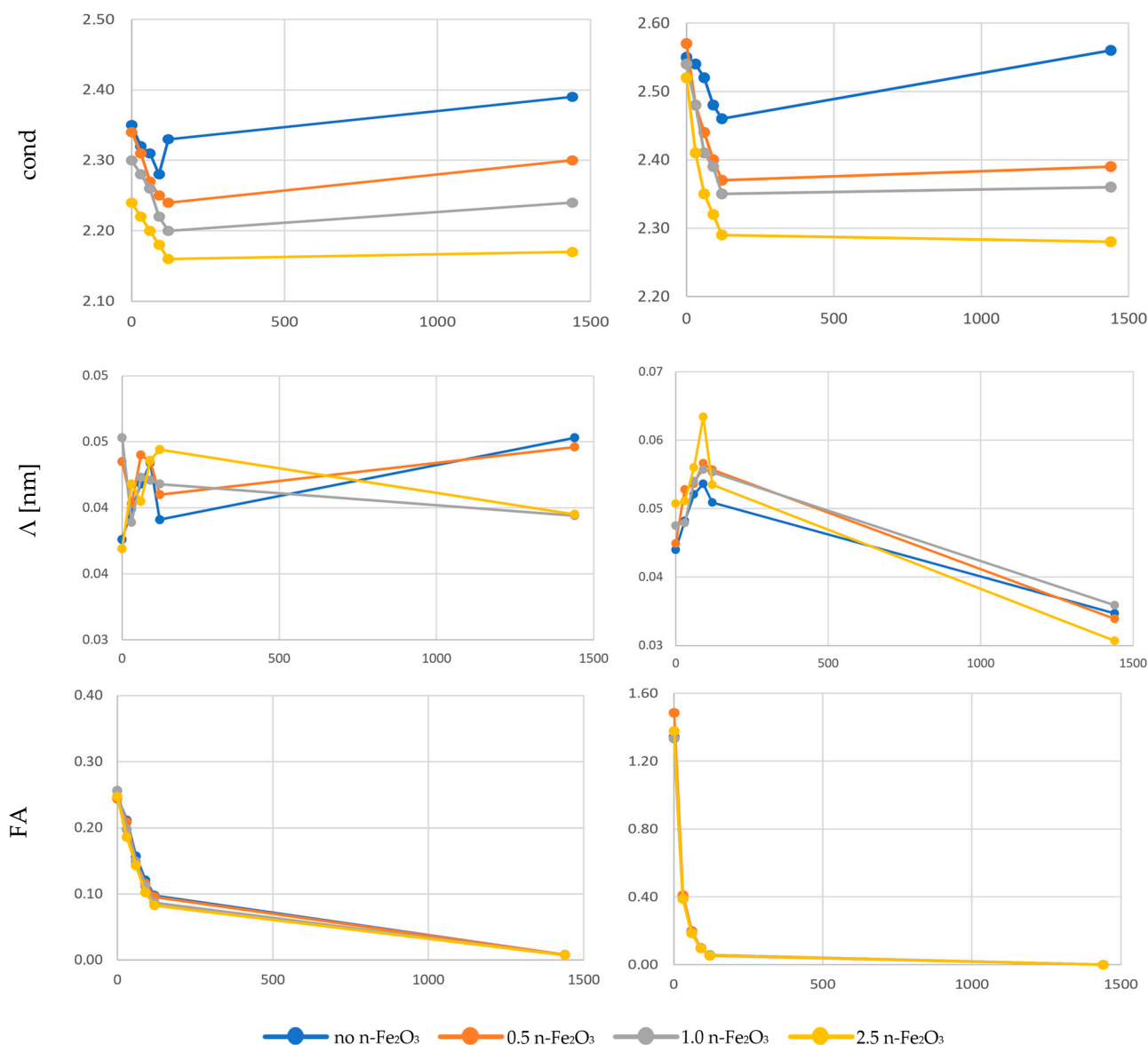


Figure 9. Change in time [min] of average values of concentrations of analytes and indicators covered by the analysis for various systems.

UV absorption spectroscopy enables the determination of the total amount of organic compounds in water. Absorbance in UV_{254} does not show sugars, ethers, alcohols, or saturated carboxylic acids, in contrast to humic acids, phenols, lignins, and other compounds having aromatic rings in their composition [68,69]. The most significant changes were observed for the system with pH 7 and temperature 20°C . The addition of $n\text{-Fe}_2\text{O}_3$ with a concentration of 1.0 and $2.5\text{ mg}\cdot\text{dm}^{-3}$ caused an increase in the amount of organic compounds having aromatic groups. This type of situation was not observed in the other systems. This is important because organic compounds with an aromatic structure are considered precursors of disinfection or oxidation by-products. In the case of biological pollution removal processes, among which Fe_2O_3 nanoparticles may be present, there may be a risk of the formation of compounds dangerous to humans, which will then be introduced into the receiver.

Indicators for which changes showed the same trend, regardless of the presence and concentration of $n\text{-Fe}_2\text{O}_3$, are pH and FA parameter (free ammonia). For each system, the addition of nanoparticles did not change the trend. The same process was observed

associated with gradual acidification of the solution and decreasing the value of the parameter characterizing free ammonia as the reaction proceeded. The initial pH value is important for the course of changes in these parameters over time, especially in relation to the FA parameter. Free ammonia can exert an inhibitory or biocidal effect on some biocenosis-forming micro-organisms [70], causing changes in the biomass structure and thus affecting the effectiveness of biological treatment. It should be added, however, that the Anammox inhibition process resulting from FA is reversible, and in approximately 1 month, anammox may return to its pre-FA state, as FA does not fundamentally change the physical properties of Anammox [71].

5. Conclusions

Nanoparticles of Fe_2O_3 , thanks to their property, can be an additional tool for effective management of biological processes of water and wastewater treatment or reclamation of degraded areas. During the first 30–60 min, Fe_2O_3 nanoparticles allow achieving a higher level of $\text{NH}_4\text{-N}$ removal efficiency than in a system without n- Fe_2O_3 . However, a longer effect causes the effectiveness to decrease over the course of the reaction and, as a result, after 120 min/24 h the efficiency is lower compared to a system containing only anammox. However, iron nanoparticles allow the system to react faster to partially anaerobic conditions, which probably contributes to higher yields in less time. In contrast, extending the contact time of micro-organisms— Fe_2O_3 nanoparticles reduces the effectiveness of anammox. The temperature and pH value determine the interaction of Fe_2O_3 nanoparticles—micro-organisms that translate into the effectiveness of reducing $\text{NH}_4\text{-N}$ in the system, the formation of aromatic compounds. It is therefore necessary to carry out further work to determine the optimal conditions for the interaction of n- Fe_2O_3 —anammox, allowing for an increase in the efficiency of $\text{NH}_4\text{-N}$ removal.

In conclusion, it should be added that the presence of n- Fe_2O_3 :

- encourages better process efficiency, but only in the first 30–60 min of biomass—n- Fe_2O_3 contact and faster reaction of the system to the occurrence of partially anaerobic conditions,
- facilitates sedimentation and flocculation processes under pH 7 and a temperature of 20 °C, thanks to participation in the agglomeration process and creation of appropriate structures,
- reduces the agglomeration process of the biomass itself through biomass-n- Fe_2O_3 sorption,
- depending on the temperature, determines the efficiency of $\text{NH}_4\text{-N}$ removal and at a temperature of 30 °C the efficiency of $\text{NH}_4\text{-N}$ removal is higher for solutions with pH 8 (approx. 98% after 24 h) than for solutions with pH 7 (approx. 70% after 24 h).

However, further research is needed to determine the groups of micro-organisms for which Fe_2O_3 nanoparticles are necessary for proper functioning in the environment and process optimization. It is also necessary to determine the groups of organisms for which Fe_2O_3 nanoparticles may constitute a toxin, preventing their development and affecting processes, including the proper course of nitrogen transformation processes in the environment. For practical reasons, it is very important to analyze the behavior of NPMOs in real systems, because only then are we able to actually observe the behavior and processes occurring in the presence and participation of nanoparticles.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/w17142100/s1>, Table S1: Selected value of Zeta potential values and particle size distribution in the analysed solutions; Table S2: Selected analysis results of systems without nanoparticles and containing n- Fe_2O_3 , for pH 7, T 20 °C; Table S3: Selected analysis results of systems without nanoparticles and containing n- Fe_2O_3 , for pH 8, T 20 °C; Table S4: Selected analysis

results of systems without nanoparticles and containing n-Fe₂O₃, for pH 7, T 30 °C; Table S5: Selected analysis results of systems without nanoparticles and containing n-Fe₂O₃, for pH 8, T 30 °C; Figure S1: Biomass—various magnifications; Figure S2: System of biomass—n-Fe₂O₃ with a concentration of 2.5 mg·dm^{−3} at: pH 8, temp. 30 °C; Figure S3: System of biomass—n-Fe₂O₃ with a concentration of 2.5 mg·dm^{−3} at: pH 7, temp. 20 °C.

Author Contributions: Conceptualization, A.R.; methodology, A.R., S.Q. and X.Z.; validation, A.R.; formal analysis, A.R., S.Q. and X.Z.; investigation, A.R. and X.Z.; resources, A.R. and X.Z.; data curation, A.R.; writing—original draft preparation, A.R.; writing—review and editing, A.R., S.Q. and X.Z.; visualization, A.R.; supervision, A.R.; project administration, A.R.; funding acquisition, A.R. All authors have read and agreed to the published version of the manuscript.

Funding: This research was co-funded by The Polish National Agency for Academic Exchange NAWA, The Bekker Programme, no. PPN/BEK/2018/1/00397/DEC/1.

Data Availability Statement: The original contributions presented in this study are included in the article/Supplementary Materials. Further inquiries can be directed to the corresponding author.

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

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