

Article

Insights into Pyrite-Based Autotrophic Denitrification: Impacts of the Initial Addition of Organic Co-Substrates at a Low Concentration

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Abstract

Pyrite-based autotrophic denitrification is an effective method for nitrate removal. However, pyrite does not exist alone and is inevitably accompanied by the presence of organic matter in nature, and thus the influence of organic co-substrates on pyrite-based denitrification should be taken into consideration. Even in a circumstance where no addition of an exogenous organic carbon source is implemented, the introduction of pyrite into groundwater and sediment is capable of stimulating both autotrophic and heterotrophic denitrifying bacteria. In this study, the impact of the initial addition of organic co-substrates on the performance and dynamics of bacterial communities in pyrite-based denitrification processes was evaluated under low-concentration conditions. The findings suggest that the initial addition of organic co-substrates at low concentrations (6–48 mg L^{−1}) could enhance the efficiency of pyrite-based autotrophic denitrification. In contrast, the competitive effects of organic co-substrates became positive with increasing additions of initial organic co-substrates. When an organic co-substrate was added at an initial concentration of 96 mg L^{−1}, the competition between heterotrophic denitrification and pyrite-based autotrophic denitrification was found to be more pronounced than their promotion role as the majority of nitrate was consumed by heterotrophic denitrification. *Thiobacillus* was the most dominant bacterium in the denitrification system, where pyrite served as the sole electron donor. At the same time, the addition of organic co-substrate under low initial concentration, led to a different microorganism composition.

Keywords: pyrite; autotrophic denitrification; organic co-substrate; microbial community

Academic Editor: Wim De Vries

Received: 13 May 2025

Revised: 18 June 2025

Accepted: 26 June 2025

Published: 28 June 2025

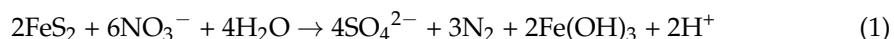
Citation: Xu, B.; Zhang, L.; Yuan, N.; Xiong, Y.; Fu, H. Insights into Pyrite-Based Autotrophic Denitrification: Impacts of the Initial Addition of Organic Co-Substrates at a Low Concentration. *Nitrogen* **2025**, *6*, 50. <https://doi.org/10.3390/nitrogen6030050>

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1. Introduction

Groundwater plays a pivotal role as a vital freshwater reservoir, serving as the cornerstone for ensuring global water and food security [1]. The presence of high concentrations of nitrates presents significant health risks to both humans, particularly infants, and livestock [2]. The presence of nitrate in groundwater can significantly restrict its suitability for drinking purposes [3]. The increasing prevalence of nitrate contamination in groundwater has raised widespread concerns, and actions must be taken to ensure the preservation of groundwater quality [4].

The utilization of pyrite-based denitrification has emerged as a reliable and cost-efficient approach for the remediation of nitrate-contaminated groundwater [5,6]. The functionality of this system relies on the ability of autotrophic denitrifying microorganisms to facilitate nitrate reduction reactions by utilizing pyrite as a solid electron donor. The overall reaction of pyrite-based autotrophic denitrification is described in Equation (1) [7].



The feasibility and durability of pyrite-based denitrification are initially assessed in a continuous flow experiment, resulting in a complete removal of nitrate that persisted for a duration of 180 days [8]. The utilization of pyrite in the denitrification process exhibits a substantial rate of nitrate removal and a minimal generation of sulfate, and also maintained a consistent pH level [5]. The bacterium *Thiobacillus denitrificans* has been shown to possess the capability for pyrite oxidation coupled with denitrification, although its mechanism remains unclear [5,9,10]. The analysis of 16S rRNA genes using high-throughput sequencing revealed that, in addition to *Thiobacillus*, other *Actinobacteria* genera such as *Rhodococcus* may also play a significant role in pyrite-based denitrification [11]. A PICRUSt2 analysis revealed a significant enrichment of genes involved in the biosynthesis of electron shuttles (specifically menaquinone-related redox-active molecules) during the process, indicating that microbe-derived electron shuttles may play a crucial role in driving pyrite oxidation [11]. The impact of particle dosage, pyrite pretreatment, and operating temperature on the performance of pyrite-based denitrification has been investigated [6,12].

The main challenges associated with pyrite-based autotrophic denitrification include the following: (1) a relatively low NO_3^- removal rate, which can be 1–2 orders of magnitude lower compared to the utilization of organic electron donors [7]; (2) a slow growth rate of autotrophic denitrifiers, leading to an extended start-up period for the bioreactor [13]; and (3) the generation of SO_4^{2-} , posing a potential risk of secondary contamination [14]. The integration of pyrite-based autotrophic denitrification and heterotrophic denitrification has been assessed in upflow packed-bed bioreactors, offering a promising approach to address these challenges and achieve enhanced NO_3^- removal efficiency while minimizing SO_4^{2-} accumulation [15]. The performance of pyrite-based autotrophic denitrification can be enhanced by incorporating natural organic substrates, such as sawdust and corncobs [16]. Another mixotrophic system employing pyrite and biodegradable polymer composites (PLA/PHBV/rice hulls) as electron donors was also investigated, and the average nitrate removal rate (16.3–40.6 mg-N/L/d) in this mixotrophic system was 37% higher than the combined rate in the single heterotrophic and autotrophic systems used for comparison [14]. The addition of an organic co-substrate at concentrations above 48 mg L⁻¹ suppressed pyrite-based autotrophic denitrification. Concurrently, both competitive and promotional effects on pyrite-based autotrophic denitrification manifested when the organic co-substrate was added at concentrations of 24 and 48 mg L⁻¹ [17]. Consequently, the function of the organic co-substrate in a mixotrophic system where pyrite and the organic co-substrate serve as electron donors remains incompletely understood.

In actuality, the existence of organic carbon in wastewater, rainwater, groundwater, and soils is unavoidable, and the influence of organic carbon on pyrite-based denitrification should be taken into consideration [17]. Even in the circumstance where no addition of an exogenous organic carbon source is implemented, the introduction of pyrite into groundwater and sediment is capable of stimulating both autotrophic and heterotrophic denitrifying bacteria [8]. As the effect of the continuous addition of organic co-substrates at diverse concentrations on the performance of the pyrite-based denitrification system has been inspected [17], the main purpose of this study is to deepen our comprehension of the role of an organic co-substrate on the growth of pyrite-based autotrophic denitrifying

bacteria, particularly when it is introduced merely at the beginning of the experiment at a low concentration. The specific objectives are the following: (1) To investigate the effect of the initial addition of an organic co-substrate (acetate) at a low concentration on the performance of the pyrite-based denitrification system. (2) To explore the functional bacterial populations by means of high-throughput sequencing technology when the organic co-substrate (acetate) is initially added at a low concentration. (3) To disclose the role of the initially added organic co-substrate (acetate) in the mixotrophic system.

2. Materials and Methods

2.1. Preparation of Materials

The particulate pyrite, synthetic groundwater, and paddy soil samples utilized in this study are detailed in our previous studies [6,17]. The particulate pyrite used in this study, obtained from Luanchuan Hengkai Metallurgical Materials Sales Co., Ltd. (Luoyang, China), had a composition of 47.09% (*w/w*) Fe, 39.96% S, 10.40% O, 0.969% Si, and 0.279% Al. Synthetic groundwater was prepared with 1010 mg L⁻¹ KNO₃, 680 mg L⁻¹ KH₂PO₄, and 840 mg L⁻¹ NaHCO₃, resulting in an initial nitrate concentration of 140 mg-N L⁻¹. The organic co-substrate (acetate) concentrations for each treatment are listed in Table 1. The paddy soil samples, collected from the upper layer (0–20 cm) of a farmland in Chongzuo, Guangxi Zhuang Autonomous Region, China (22°55' N, 106°44' E), were pre-activated at a 60% moisture content and 30 °C for 3 days before serving as the microbial inoculum in the batch experiments [18].

Table 1. The setup of the batch experiment.

	Soil	Nitrate—N (mg L ⁻¹)	Acetate—C (mg L ⁻¹)	NaHCO ₃ (mg L ⁻¹)	Pyrite (g)
Control	0	140	0	840	30
A0	0.1%	140	0	840	30
A6	0.1%	140	6	840	30
A12	0.1%	140	12	840	30
A24	0.1%	140	24	840	30
A48	0.1%	140	48	840	30
A96	0.1%	140	96	840	30

2.2. Experimental Procedure

The batch experiment was conducted in 500 mL anaerobic bottles in triplicates, with reference to a previous study [17]. Each anaerobic bottle was filled with 30 g of particulate pyrite and 400 mL of the synthetic groundwater of different acetate concentrations, and sterilized at 121 °C for 30 min. Six inoculated treatments, labeled as A0, A6, A12, A24, A48, and A96, were cultured at the initial acetate concentrations of 0, 6, 12, 24, 48, and 96 mg-C L⁻¹, respectively. Each inoculated bottle contained 0.4 g of the activated paddy soil samples. Meanwhile, un-inoculated bottles with an initial acetate concentration of 0 mg-C L⁻¹ and no addition of the paddy soil samples were used as controls. All the bottles underwent three rounds of vacuum extraction and helium re-filling to mitigate the impact of oxygen on denitrification and then were cultivated at 30 °C for 20 days.

2.3. Analysis

According to our previous study [17], water samples were collected every 48 h. The pH was measured promptly using a Potable Water Quality Analyzer (HQ40d, HACH, Loveland, CO, USA). Then, the water sample was filtered through a 0.45 µm membrane filter before conducting further water quality analyses. Ammonia nitrogen (NH₄⁺-N), total iron (Fe_{Total}), ferrous iron (Fe²⁺), nitrate (NO₃⁻-N), nitrite (NO₂⁻-N), sulfate (SO₄²⁻-S),

and total sulfate were determined using the same method as described in our previous study [17].

After the 20-day batch experiment, biomass samples were retrieved from each treatment (marked as A0, A6, A12, A24, A48, and A96, respectively). The activated soil sample serving as the microbial source was marked as Soil. All biomass samples were promptly sent to the laboratory for cryopreservation, and then sent to a gene sequencing company (Majorbio, Shanghai, China) for sequencing in time, as described in our previous study [17].

3. Results and Discussion

3.1. Pyrite-Based Denitrification Performance with the Initial Addition of Organic Co-Substrates at a Low Concentration

Different initial additions of organic co-substrates at a low concentration resulted in distinct denitrification performances (Figure 1). The organic co-substrate was exclusively added to the anaerobic bottles at the initial stage of the batch experiment. In the control treatments, no activated soils or organic co-substrates were added. Thus, the nitrate concentration decreased minimally. In the A0 treatments, activated paddy soil samples were inoculated as the microbial source, with no addition of organic co-substrates. The nitrate concentration declined to $96.1 \pm 0.7 \text{ mg-N L}^{-1}$, and merely 31.0% of the total nitrate was eliminated by the end of the experiments. The results of the control and A0 treatments were similar to those of previous studies [17] because these treatments had the same experimental conditions as the previous ones; therefore, the outcomes were the same. In the A6, A12, A24, A48, and A96 treatments, the organic co-substrate (acetate) was independently added at concentrations of 6, 12, 24, 48, and 96 mg L^{-1} at the beginning of the batch experiments. At the end of the experiments, nitrate removal efficiencies of 36.5%, 40.0%, 45.7%, 56.2%, and 76.0% were achieved in the respective treatments.

The nitrate reduction rate exhibited an increasing trend with the addition of varying concentrations of the organic co-substrate, acetate, at the commencement of the batch experiments. This phenomenon can be attributed to the introduction of acetate, which enhanced the heterotrophic denitrification in treatments A6, A12, A24, A48, and A96. As previously reported, all isolated neutrophilic bacterial strains capable of microbially driven nitrate-dependent iron oxidation are mixotrophic and necessitate the presence of an organic co-substrate for growth [19]. Therefore, the observed increase in the nitrate reduction rate may also be attributed to the enhancement of pyrite-based denitrification processes.

No nitrite was produced during the experiment in the control treatments, further demonstrating that no nitrate reduction occurred in these experiments. The accumulation of nitrite initially increased, reaching a maximum concentration of approximately $6.3 \pm 3.8 \text{ mg L}^{-1}$, before stabilizing at relatively constant levels in the A0, A6, A12, and A24 treatments. In comparison, the concentration of nitrite in the A48 and A96 treatments increased rapidly to $10.5 \pm 3.5 \text{ mg L}^{-1}$ and $27.4 \pm 3.4 \text{ mg L}^{-1}$, respectively, during the initial two days; however, it subsequently exhibited a gradual decline to $3.0 \pm 2.2 \text{ mg L}^{-1}$ and $19.7 \pm 1.6 \text{ mg L}^{-1}$. Through a comparative analysis of all the treatments, it can be concluded that the accumulation of nitrite increases with the rising initial concentration of the organic co-substrate (Figure 1b).

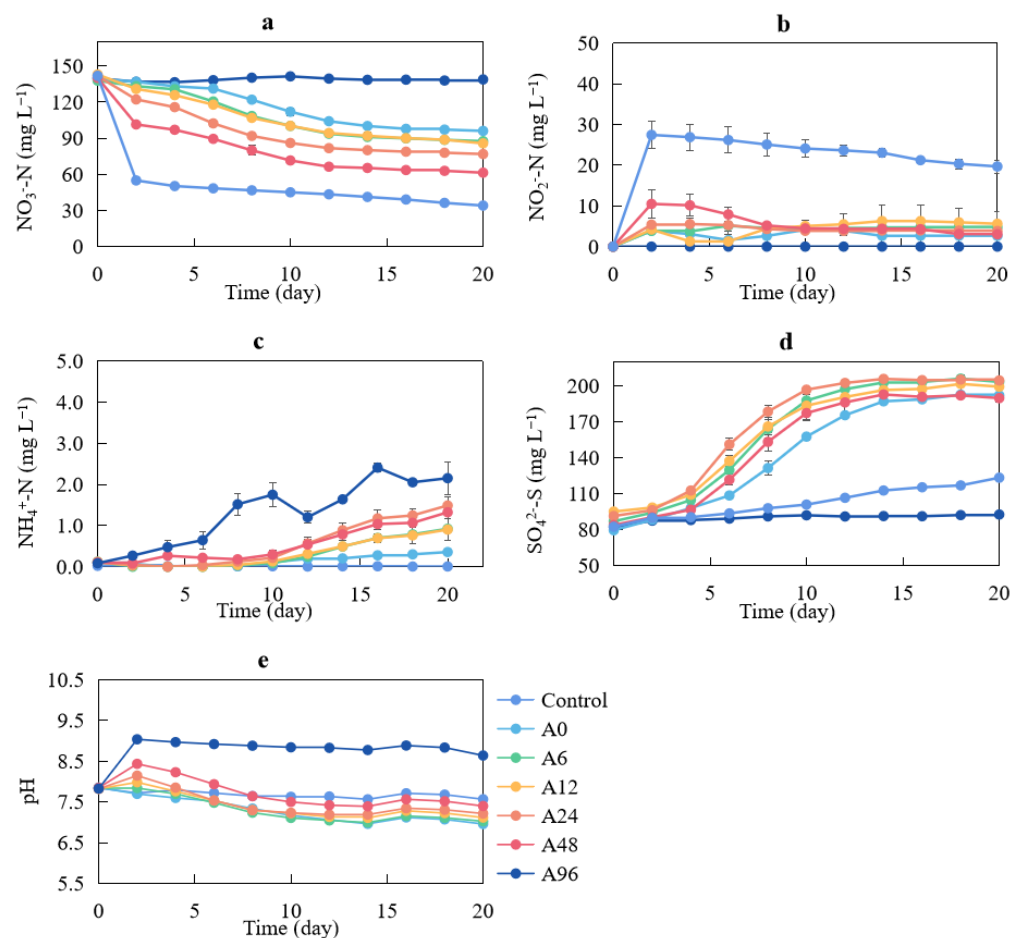


Figure 1. Changes in aqueous species concentrations during the incubation experiment. (a) Nitrate nitrogen, (b) nitrite nitrogen, (c) ammonia nitrogen, (d) sulfate concentration, and (e) pH variation with different initial organic co-substrate addition concentrations. (Un-inoculated bottles without initial organic co-substrate additions were used as control. A0, A6, A12, A24, A48, and A96 were cultured with acetate addition at the different initial concentrations of 0, 6, 12, 24, 48, and 96 mg-C L^{-1}).

In the control treatments, ammonia nitrogen production was negligible, whereas the experimental treatment utilizing activated soil as a microbial source exhibited relatively low levels of detected ammonia nitrogen. In the A0 treatments, the concentration of ammonia nitrogen was measured at only $0.4 \pm 0.4 \text{ mg L}^{-1}$, representing the lowest level observed among all experimental treatments. In a comparison of the various experimental treatments, the accumulation of ammonia nitrogen exhibited an increasing trend corresponding to the rise in the initial concentration of the organic co-substrate added. Simultaneously, the concentration of ammonia nitrogen exhibited a progressively increasing trend over time across all experimental treatments. The degradation of nitrate nitrogen was accompanied by a gradual accumulation of ammonia nitrogen, indicating the occurrence of dissimilatory nitrate reduction to ammonium (DNRA) or the ammonization of certain dead cells.

In the control treatments, the concentration of sulfate increased by only 10.6 mg L^{-1} during the incubation period, representing the lowest increment among all the treatment groups. No significant decrease in nitrate levels was observed in the control treatments; therefore, the increase in sulfate concentrations within these treatments is likely attributable to the chemical oxidation of pyrite facilitated by initially entrained air. In addition to the observed increase in sulfate, elevated levels of thiosulfate and sulfite were also detected in the control treatments. This finding supports our hypothesis that a biological denitrification process may not occur due to the absence of relevant microorganisms [17].

In the A0, A6, A12, A24, and A48 treatments, the sulfate concentrations increased by 112.7, 116.4, 104.3, 113.2, and 106.0 mg L⁻¹, respectively. Notably, sulfate accumulation remained relatively stable throughout the experiment. In contrast, both nitrate removal efficiency and total nitrate removal demonstrated a gradual enhancement corresponding to increasing initial organic co-substrate concentrations. This observation suggests that low-concentration organic co-substrate supplementation (0–48 mg L⁻¹) did not induce competitive inhibition on the pyrite-based denitrification process. The sulfate levels in the A6 and A24 treatments supplemented with the organic co-substrate exceeded those in the A0 control group, lacking such addition, demonstrating that an appropriate initial organic co-substrate concentration enhances both the heterotrophic denitrification and pyrite-based autotrophic denitrification processes. The organic co-substrate primarily functioned as a carbon source and electron donor for heterotrophic denitrification, while simultaneously stimulating pyrite-based autotrophic denitrification by supporting the proliferation of nitrate-dependent iron-oxidizing bacteria [19]. In the A96 experimental group, higher initial concentrations of the supplemented organic co-substrate correlated with maximal nitrate elimination efficiency while yielding the lowest sulfate generation rates (excluding the control group without a co-substrate), demonstrating that elevated organic co-substrate supplementation suppresses pyrite-driven autotrophic denitrification processes.

In prior investigations, supplemental organic co-substrates were introduced upon depletion, revealing dual inhibitory and stimulatory influences on pyrite-driven autotrophic denitrification, with competitive effects predominating over synergistic interactions at concentrations as low as 24–48 mg L⁻¹ [17]. Contrastingly, this study implemented single-dose organic co-substrate supplementation at experiment initiation. The results revealed that substoichiometric organic co-substrate levels could enhance pyrite-mediated autotrophic denitrification under favorable conditions. Specifically, supplementation stimulated heterotrophic denitrification through organic electron donation and carbon metabolism even at initial concentrations below 48 mg L⁻¹. Notably, low-level organic co-substrate supplementation preserved pyrite-mediated autotrophic denitrification efficacy, with an observable enhancement in the A6 and A24 systems. However, supplementation at elevated concentrations (96 mg L⁻¹) significantly suppressed this process, aligning with prior research findings [17]. Experimental verification confirmed that organic co-substrate supplementation exceeding 48 mg L⁻¹ suppressed pyrite-mediated autotrophic denitrification, attributable to the following, as established in previous mechanistic studies: (1) preferential nitrate depletion by heterotrophic denitrifiers under elevated organic carbon availability, thereby limiting substrate accessibility for autotrophic pathways; (2) the microbial toxicity of excessive acetate on lithotrophic bacterial consortia; (3) inhibitory nitrite accumulation from incomplete heterotrophic denitrification; and (4) pH elevation via acetate-driven electron donation, destabilizing pyrite dissolution dynamics.

Thiosulfate and sulfite accumulated in the unamended controls alongside sulfate, whereas these intermediate sulfur species were rapidly depleted to undetectable levels in the bioaugmented systems (Figure 2). This divergent accumulation pattern demonstrates accelerated autotrophic denitrification initiation in the soil-activated treatment groups through functional microbial consortium enrichment.

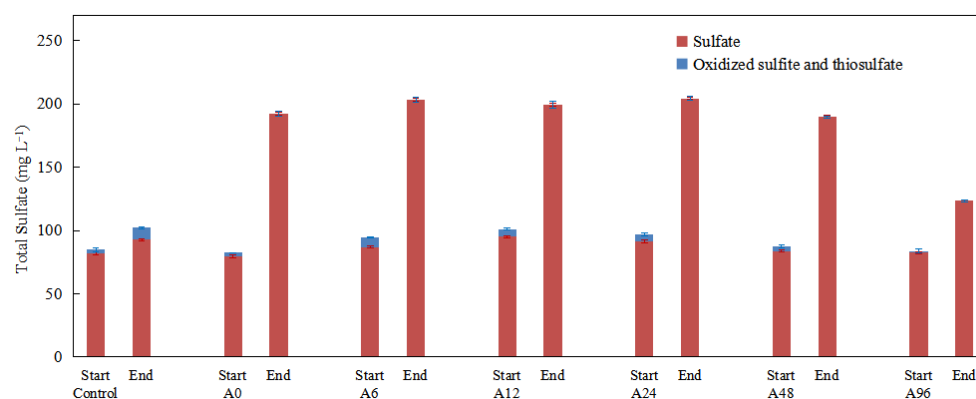
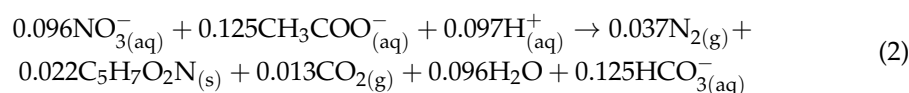


Figure 2. Changes in total sulfate (sulfate, oxidize sulfite, and thiosulfate) between the start and the end of the incubation experiments. (Un-inoculated bottles without initial organic co-substrate additions were used as control. A0, A6, A12, A24, A48, and A96 were cultured with acetate additions at the different initial concentrations of 0, 6, 12, 24, 48, and 96 mg-C L⁻¹).

Temperature is one of the primary factors affecting the performance of heterotrophic denitrification [20]. The optimal temperature for heterotrophic denitrification is between 25 and 35 °C [21]. Temperature has a much more sensitive influence on the denitrification process than phosphate and bicarbonate addition [6]. To our knowledge, the optimal temperature for autotrophic denitrification by pyrite has not been reported. Therefore, when studying the relationship between organic matter and pyrite, we set the temperature at 30 °C, which is relatively favorable, referring to the optimal temperature for heterotrophic denitrification. The optimal temperature for pyrite-based autotrophic denitrification will be further explored in our future research.

Divergent pH trajectories emerged across the experimental groups, exhibiting co-substrate dosage-dependent modulation. Progressive acidification characterized the unamended controls (A0) and low-dosage systems (A6), with the sharpest decline observed in A0. Conversely, the amended groups (A12–A96) exhibited an initial alkalinization peaking at day 2, followed by gradual acidification until termination. Maximum pH values demonstrated carbon supplementation dependency, with peak values positively correlating with supplementation levels (A12: 7.98 ± 0.01; A24: 8.14 ± 0.01; A48: 8.43 ± 0.08; A96: 9.04 ± 0.05). This biphasic pH evolution aligns with heterotrophic denitrification’s dual-phase alkalinity dynamics, consistent with the alkalinity generation mechanism outlined in Equation (2) [22]. In contrast, as shown in Equation (1), the autotrophic denitrification process consumes alkalinity, produces sulfate, and does not consume the organic carbon source [7]. The pH fluctuation mechanism was primarily governed by sequential metabolic phases: an initial alkalinization mediated through heterotrophic denitrification dominance, subsequently countered by acidification via pyrite-oxidizing autotrophy activation, forming characteristic geochemical cycling patterns.



Synergistic microbial interactions were observed wherein heterotrophic denitrification exhibited non-inhibitory coexistence with pyrite-oxidizing lithotrophic processes, demonstrating catalytic enhancement under optimal organic carbon supplementation (12–48 mg L⁻¹). pH trajectory analysis revealed metabolic succession dynamics: heterotrophic dominance prevailed during early-phase substrate abundance (>12 mg L⁻¹ organic carbon), transitioning to lithotrophic predominance post-depletion. Concurrent sulfate accumulation corroborated preserved pyrite dissolution activity, confirming that

low-level carbon supplementation maintains chemolithoautotrophic metabolic integrity. Biogeochemical profiling revealed that select amended systems exhibited sulfate yields exceeding the unamended controls. These elevated sulfate outputs indicate the substrate-dependent stimulation of pyrite-oxidizing chemolithotrophy, potentially mediated through microbial consortium enrichment favoring lithotrophic specialists. Stoichiometric analysis demonstrated threshold-dependent metabolic competition: at critical organic carbon loads (96 mg L^{-1}), heterotrophic denitrifiers initiated preferential nitrate scavenging, ultimately inducing chemolithotrophic activity suppression through electron acceptor limitation. This competitive dominance over synergistic facilitation emerged when initial carbon supplementation surpassed lithotrophic community metabolic capacity, as evidenced by redox partner exhaustion dynamics.

3.2. Microbial Community Analyses Under Different Organic Co-Substrate Additions in This Pyrite-Based Autotrophic Denitrification System

Through high-throughput sequencing, 422,485 effective sequences were obtained via 16S rRNA Illumina MiSeq sequencing. Considering a similarity of 97%, the index of the microbial diversity in the seven samples (activated soil, named Soil; water samples with co-substrate additions under different initial concentration after 20 days of incubation, named A0, A6, A12, A24, A48, and A96) is shown in Table 2.

Table 2. Species richness and diversity estimators of microbial populations in soil and water samples.

Sample ID	Reads	OTUs ²	Ace	Chao1 ³	Coverage	Shannon	Simpson
Soil ¹	50864	1420	1437.832	1431.336	0.998919	5.72763	0.014914
A0	44441	501	685.9039	652.2561	0.996786	3.911035	0.043122
A6	49162	375	738.9145	594.45	0.997007	3.705737	0.049191
A12	48249	337	685.5576	538.6667	0.997497	2.994198	0.180355
A24	48334	537	909.8316	775.9583	0.996145	3.922568	0.043082
A48	42202	517	1084.488	818.6429	0.99649	3.603932	0.060958
A96	58688	263	528.6979	395.4545	0.997773	3.06579	0.091909

¹ Activated soil is denoted as Soil, and water samples with addition of different concentrations of organic co-substrates after 20 days of incubation are denoted as A0, A6, A12, A24, A48, and A96. ² OTUs: Operational taxonomic units.

³ Chao1: a non-parametric estimator of species richness, commonly applied in biodiversity studies.

The Coverage index, number of OTUs, and ACE, Chao1, and Shannon indices of the seven samples can be seen in Table 2. Although slight variations exist, the overall trend matches our previous study [17]. The reason for these slight variations is that the organic co-substrates were continuously added in our previous study, whereas low concentrations of the initial organic co-substrate were added only at the beginning of the experiment in this study.

The taxonomic classification of pyrosequences from the bacterial communities in the soil samples and six water samples at the phylum level, class level, and genus level are shown in Figure 3. As presented in Figure 3a, the most dominant phyla (relative abundance > 3%) in the soil samples were Proteobacteria (28.5%), Bacteroidetes (13.4%), Actinobacteria (18.4%), Chloroflexi (17.3%), Firmicutes (9.1%), and Acidobacteria (7.6%). The most dominant phyla in the soil samples before incubation were similar to those in our prior study [17]. Compared with the soil samples, the microbial community of the pyrite-based autotrophic denitrification system changed significantly at the phylum level under different initial concentrations of co-substrate additions. For the A0 treatments, Proteobacteria, Bacteroidetes, and Firmicutes were the most dominant phyla, and accounted for 64.9%, 22.8%, and 4.8% of all microbial species, respectively. For the A6 treatments, Proteobacteria and Bacteroidetes were the most dominant phyla, and accounted for 77.2%

and 13.3% of all microbial species, respectively. For the A12 treatments, Proteobacteria, Bacteroidetes, Firmicutes, and Verrucomicrobia were the most dominant phyla, and accounted for 41.3%, 44.2%, 8.1%, and 3.1% of all microbial species, respectively. For the A24 treatments, Proteobacteria, Bacteroidetes, Firmicutes, Verrucomicrobia, and Cyanobacteria were the most dominant phyla, and accounted for 66.4%, 17.0%, 5.9%, 3.4%, and 3.2% of all microbial species, respectively. For the A48 treatments, Proteobacteria, Bacteroidetes, Firmicutes, Verrucomicrobia, and Cyanobacteria were the most dominant phyla, and accounted for 60.6%, 17.0%, 3.5%, 8.1%, and 7.0% of all microbial species, respectively. For the A96 treatments, Proteobacteria and Firmicutes were the most dominant phyla, and accounted for 91.3% and 4.3% of all microbial species, respectively.

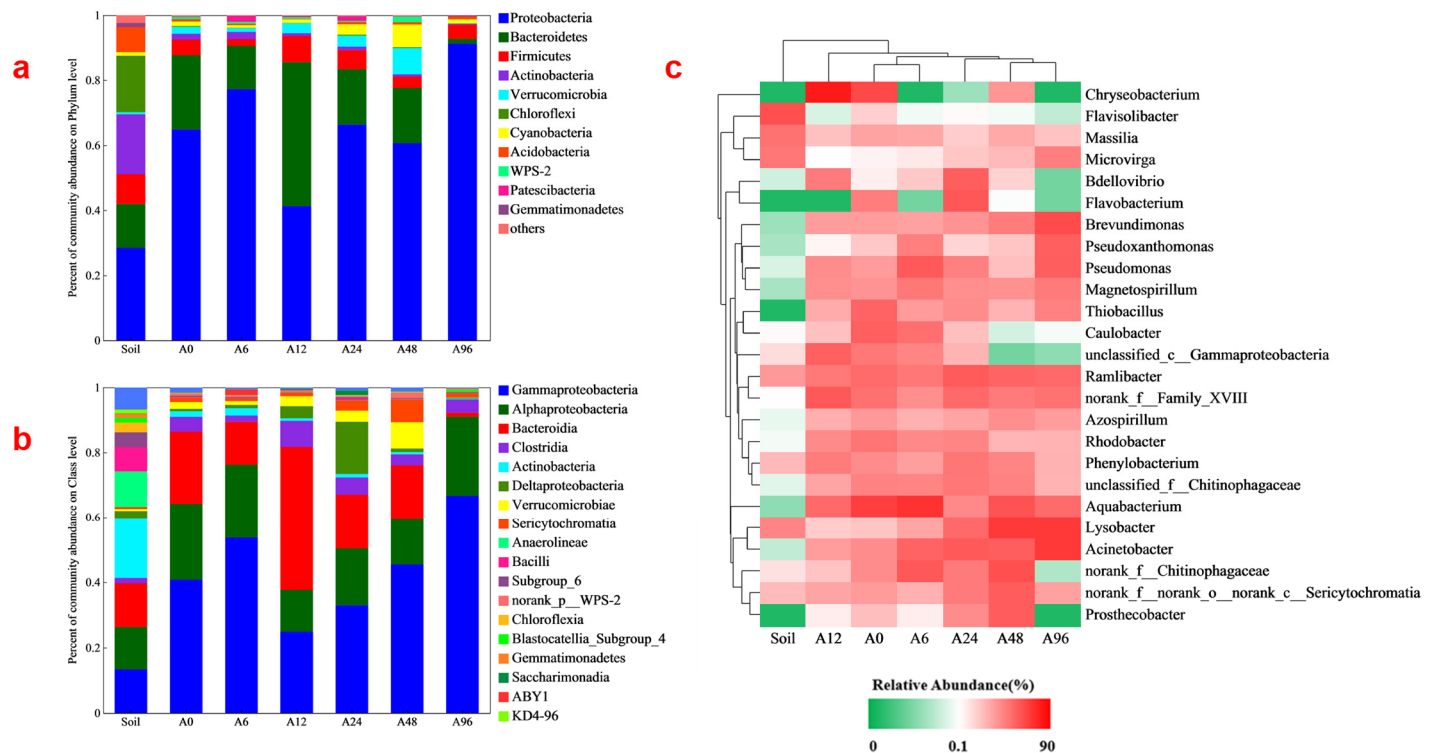


Figure 3. Microbial structure of activated soil and water samples at phylum level (a); class level (b); and genus level (c). Heatmap was drawn with relative abundances (%).

Proteobacteria, Firmicutes, Bacteroidetes, and Chloroflexi are the most dominant phyla in many denitrification systems [17,23,24]. The relative abundance of Proteobacteria in the A0, A6, A12, A24, A48, and A96 treatments was higher than that in the soil samples, suggesting that the microbial species from the Proteobacteria phylum may play an important role in the pyrite-based autotrophic denitrification system. The relative abundance of Bacteroidetes in the A0, A12, A24, and A48 treatments was higher than that in the soil samples, suggesting that the microbial species from the Bacteroidetes phylum may be beneficial to pyrite-based autotrophic denitrification under a proper initial concentration of organic co-substrate addition. Verrucomicrobia was found to be the dominant phylum in electrolysis-augmented constructed wetland and high-salinity lakes [25,26]. The relative abundance of Verrucomicrobia in the A0, A6, A12, A24, and A48 treatments was higher than that in soil samples, suggesting that the microbial species from the Verrucomicrobia phylum may be beneficial to pyrite-based autotrophic denitrification under a proper initial concentration of organic co-substrate addition. Cyanobacteria are nitrogen fixers, which could improve the nutrient uptake ability of associated plants [27]. Cyanobacteria was found to be the dominant phylum in electrolysis-augmented constructed wetlands [26]; the

reactivity of Cyanobacteria to nitrate would favor their proliferation within a context of physical and chemical shifts [28]. In this study, the relative abundance of Cyanobacteria in the A24 and A48 treatments was higher than that in the soil samples, suggesting that the microbial species from the Cyanobacteria phylum may be beneficial to pyrite-based autotrophic denitrification under a proper initial concentration of organic co-substrate addition.

As presented in Figure 3b, the most dominant classes (relative abundance > 3%) in the soil samples were Gammaproteobacteria (13.3%), Alphaproteobacteria (13.0%), Bacilli (7.5%), Bacteroidia (13.4%), Actinobacteria (18.4%), Anaerolineae (11.0%), Subgroup_6 (4.5%), and Chloroflexia (3.0%). Compared with the soil samples, the microbial community of the pyrite-based autotrophic denitrification system changed significantly at the class level under different initial concentrations of co-substrate additions. For the A0 treatments, Gammaproteobacteria (40.8%), Alphaproteobacteria (23.4%), Bacteroidia (22.2%), and Clostridia (4.5%) were the most dominant classes, and their relative abundances all increased compared to the soil samples. For the A6 treatments, Gammaproteobacteria (53.7%), Alphaproteobacteria (22.6%), and Bacteroidia (13.2%) were the most dominant classes, and the relative abundance of Gammaproteobacteria and Alphaproteobacteria increased compared to the soil samples. For the A12 treatments, Gammaproteobacteria (24.8%), Alphaproteobacteria (12.8%), Bacteroidia (44.1%), Clostridia (8.0%), Deltaproteobacteria (3.6%), and Verrucomicrobiae (3.1%) were the most dominant classes, and the relative abundance of Gammaproteobacteria, Bacteroidia, Clostridia, Deltaproteobacteria, and Verrucomicrobiae increased compared to the soil samples. For the A24 treatments, Gammaproteobacteria (32.9%), Alphaproteobacteria (17.4%), Bacteroidia (16.7%), Clostridia (5.3%), Deltaproteobacteria (16.0%), Verrucomicrobiae (3.4%), and Sericytochromatia (3.2%) were the most dominant classes, and their relative abundances all increased compared to the soil samples. For the A48 treatments, Gammaproteobacteria (45.4%), Alphaproteobacteria (14.2%), Bacteroidia (16.5%), Clostridia (3.4%), Verrucomicrobiae (8.1%), and Sericytochromatia (7.0%) were the most dominant classes, and their relative abundances all increased compared to the soil samples. For the A96 treatments, Gammaproteobacteria (66.7%), Alphaproteobacteria (24.3%), and Clostridia (4.1%) were the most dominant classes, and their relative abundances all increased compared to the soil samples.

Gammaproteobacteria, Alphaproteobacteria, Deltaproteobacteria, Bacteroidia, Clostridia, Verrucomicrobiae, and Sericytochromatia are the most dominant classes in many denitrification systems [17,23,29]. The relative abundances of Gammaproteobacteria in the A0, A6, A12, A24, A48, and A96 treatments were higher than that in the soil samples, suggesting that the microbial species from the Gammaproteobacteria class may play an important role in the pyrite-based autotrophic denitrification system with/without the addition of an organic co-substrate. The relative abundances of Alphaproteobacteria in the A0, A6, A24, A48, and A96 treatments were higher than that in the soil samples, while the relative abundance of Alphaproteobacteria in A12 was lower than that in the soil samples. The different performance of Alphaproteobacteria suggests that the microbial species from the Alphaproteobacteria class may play an important role in the pyrite-based autotrophic denitrification system in the A0, A6, A24, A48, and A96 treatments. The relative abundances of Deltaproteobacteria in the A12 and A24 treatments were higher than that in the soil samples and in the A0 treatments, suggesting that the microbial species from the Deltaproteobacteria class may play an important role in the A12 and A24 treatments for heterotrophic denitrification. The relative abundances of Bacteroidia in the A0, A12, A24 and A48 treatments were higher than that in the soil samples, suggesting that the microbial species from the Bacteroidia class may play an important role in the A0, A12, A24, and A48 treatments. The relative abundances of Clostridia in the A0, A12,

A24, A48, and A96 treatments were higher than that in the soil samples, suggesting that the microbial species from the Clostridia class may play an important role in the A0, A12, A24, A48, and A96 treatments. The relative abundances of Verrucomicrobiae in the A12, A24, and A48 treatments were higher than that in the soil samples and in the A0 treatments, suggesting that the microbial species from the Verrucomicrobiae class may play an important role in the A12, A24, and A48 treatments for heterotrophic denitrification. The relative abundances of Sericytochromatia in the A24 and A48 treatments were higher than that in the soil samples and in the A0 treatments, suggesting that the microbial species from the Verrucomicrobiae class may play an important role in the A24 and A48 treatments for heterotrophic denitrification.

As presented in Figure 3c, the most dominant classes (relative abundance > 3%) in the soil samples were Gammaproteobacteria (13.3%), Alphaproteobacteria (13.0%), Bacilli (7.5%), Bacteroidia (13.4%), Actinobacteria (18.4%), Anaerolineae (11.0%), Subgroup_6 (4.5%), and Chloroflexia (3.0%).

A total of 403 genera were detected in the six water and soil samples. The relative abundances of the top 25 genera across all samples were compared and displayed in a heatmap. Overall, both the dominant genera and their relative abundances exhibited significant variation across the samples. In the soil samples, *Flavisolibacter* (10.0%)—a member of the Bacteroidetes phylum known for its positive correlation with soil denitrification functional genes [30]—was the most abundant genus. In contrast, the dominant genera in the six water samples shifted to *Aquabacterium* (15.4–21.8%), *Chryseobacterium* (41.1%), *Bdellovibrio* (13.9%), and *Lysobacter* (18.1–18.7%).

In the soil samples, *Massilia* (4.1%) and *Microvirga* (3.5%) also exhibited relative abundances exceeding 3%. *Massilia* has been demonstrated to enhance denitrification under conditions of elevated carbon availability [31], whereas *Microvirga*, a well-characterized nitrogen-fixing bacterium, contributes to soil nitrogen enrichment through biological N₂ fixation [32]. The prevalence of these genera suggests a strong nitrification and denitrification potential in these soils.

In the A0 treatment, *Aquabacterium* dominated the microbial community (15.4%), a genus known for its autotrophic denitrification capability, particularly as a nitrate-dependent Fe²⁺ oxidizer under anaerobic conditions [33]. Its high relative abundance suggests the successful enrichment of Fe²⁺-dependent autotrophic denitrifiers from the paddy soil. Other prevalent genera included *Chryseobacterium* (12.3%), *Caulobacter* (6.6%), *Thiobacillus* (6.0%), *Ramlibacter* (4.8%), norank_f_Family_XVIII (4.3%), *Rhodobacter* (3.7%), unclassified_c_Gammaproteobacteria (3.5%), and *Flavobacterium* (3.0%). Members of the *Chryseobacterium* genus are capable of degrading organic waste and dead organic matter [34,35]. Furthermore, they contribute to the nitrogen cycle by utilizing NO₃[−]-N and NO₂[−]-N as substrates for denitrification, ultimately reducing them to N₂ [36]. *Caulobacter* (family Caulobacteraceae) is relatively abundant in granular sludge microbial communities [37]. This bacterium produces a strong adhesive gel, known as a holdfast, which facilitates microbial aggregation [38]. *Thiobacillus*, a key autotrophic denitrifier in sulfur-based systems, utilizes reduced sulfur compounds (e.g., elemental sulfur, sulfide, and thiosulfate) as electron donors for nitrate reduction [39]. *Ramlibacter* is linked to nitrification [40,41], while *Rhodobacter* performs aerobic denitrification [42]. Unclassified Gammaproteobacteria play a significant role in nitrogen removal [43]. Additionally, norank_f_Family_XVIII constituted a notable fraction in the A0 treatment, and needs to be further investigated.

In the A6 treatment, the most dominant genus was *Aquabacterium* (21.8%). In addition, the water samples also contained norank_f_Chitinophagaceae (8.0%), *Pseudomonas* (7.8%), *Acinetobacter* (6.0%), *Caulobacter* (4.4%), *Ramlibacter* (3.5%), and *Magnetospirillum* (3.4%). Norank_f_Chitinophagaceae, *Pseudomonas*, *Acinetobacter*, and *Mag-*

netospirillum were the newly emerging dominant species detected in the A6 treatments. Norank_f__Chitinophagaceae belongs to Chitinophagales, among which most microorganisms are aerobic or facultative anaerobic microorganisms [40]. Bacteria belonging to the *Acinetobacter* genus could conduct nitrogen assimilation, heterotrophic nitrification, and aerobic denitrification simultaneously [44]. *Pseudomonas* is a widely studied heterotrophic denitrifier bacterium [17], and this genus has been linked to the oxidation process of Fe^{2+} and Mn^{2+} and is also related to the NH_4^+ transformation [39,45,46]. *Magnetospirillum* contains genes capable of compiling nitrate reductase, nitrite reductase, nitric oxide reductase, and nitrous oxide reductase, with a complete denitrification capability [47]. The relative abundance of pyrite-based autotrophic denitrifying microorganisms was dominant, and was even higher than that in the A0 treatments. This was consistent with the higher denitrification performance in the A6 treatments than that in the A0 treatments.

In the A12 treatment, the most dominant genus was *Chryseobacterium* (41.1%). In addition, the water samples also contained norank_f__Family_XVIII (7.9%), unclassified_c__Gammaproteobacteria (6.5%), *Aquabacterium* (5.2%), *Ramlibacter* (3.4%), *Bdellovibrio* (3.3%), and *Phenylobacterium* (3.1%). *Bdellovibrio* and *Phenylobacterium* were the newly emerging dominant species detected in the A12 treatments. *Bdellovibrio* and similar organisms are ubiquitous in the environment and have been commonly found in estuaries, seacoasts, rivers, and activated sludge in WWTPs [48]. *Bdellovibrio* (1.249%) accounted for one of the highest proportions of denitrifying polyphosphate-accumulating organisms at the genus level in activated sludge [49]. *Phenylobacterium* was reported to contribute to N removal performance, enriching the substratum of subsurface wastewater infiltration systems [50].

In the A24 treatment, the most dominant genus was *Bdellovibrio* (13.9%). In addition, the water samples also contained *Flavobacterium* (8.2%), *Acinetobacter* (7.5%), *Ramlibacter* (7.4%), norank_f__Family_XVIII (5.2%), *Lysobacter* (5.1%), *Phenylobacterium* (3.6%), unclassified_f__Chitinophagaceae (3.6%), norank_f__Chitinophagaceae (3.3%), and norank_f__norank_o__norank_c__Sericytochromatia (3.2%). *Lysobacter*, unclassified_f__Chitinophagaceae, and norank_f__norank_o__norank_c__Sericytochromatia were the newly emerging dominant species detected in the A24 treatments. *Lysobacter*, which belongs to the Proteobacteria phylum, is a possible facultative autotrophic denitrifier [22,51]. Unclassified_f__Chitinophagaceae was reported to be a typical denitrification bacteria and utilizes complex less-degradable organic carbons as electron acceptors [52]. Norank_f__norank_o__norank_c__Sericytochromatia was isolated from the algae biofilm of a bioreactor; it belongs to a nonphotosynthetic clade of cyanobacteria and may promote biofilm formation [53].

In the A48 treatment, the most dominant genus was *Lysobacter* (18.1%). In addition, the water samples also contained unclassified_f__Chitinophagaceae (10.2%), *Aquabacterium* (9.4%), *Prostheco bacter* (7.1%), norank_f__norank_o__norank_c__Sericytochromatia (7.0%), *Acinetobacter* (6.9%), *Ramlibacter* (6.0%), norank_f__Family_XVIII (3.2%), and *Brevundimonas* (3.1%). *Prostheco bacter* and *Brevundimonas* were the newly emerged dominant genera detected in the A48 treatments. *Prostheco bacter* belongs to the Verrucomicrobia phylum and is closely related to the removal of nitrogenous pollutants in activated sludge systems with a denitrification function [54,55]. *Brevundimonas*, a denitrifying bacterium isolated from seabed sediments, has been successfully employed for hydrogen sulfide (H_2S) removal from swine manure, achieving an over 90% elimination efficiency [56,57].

In the A96 treatment, the most dominant genus was *Lysobacter* (18.7%). In addition, the water samples also contained *Acinetobacter* (18.6%), *Brevundimonas* (11.2%), *Pseudomonas* (6.9%), *Pseudoxanthomonas* (6.6%), *Ramlibacter* (5.2%), *Aquabacterium* (5.0%), norank_f__Family_XVIII (4.1%), and *Magnetospirillum* (3.2%). *Pseudoxanthomonas* was the

newly emerged dominant species detected in the A96 treatments, and was proven to show excellent performance in reducing nitrite. A *Pseudoxanthomonas* strain was isolated from a denitrification biofilter reactor. It showed a denitrification ability and could reduce nitrate and nitrite during the denitrification process in an anoxic environment [58].

Agricultural soils are typically characterized by diverse and abundant microbial communities that have been identified as potential sources of autotrophic denitrifying microorganisms for pyrite-based systems. In denitrification systems where pyrite is utilized as the sole electron donor, *Thiobacillus* was found to be the dominant genus, with sulfur compounds being used as electron donors. The addition of an organic co-substrate, however, led to a different microorganism composition. In the treatments with organic co-substrate additions under a low initial concentration, the denitrifying microorganisms were all most dominated by *Aquabacterium*, *Chryseobacterium*, *Bdellovibrio*, *Lysobacter*, and other heterotrophic/autotrophic denitrifying microorganisms among the treatments with different concentrations of the organic co-substrate.

3.3. Effects of Organic Co-Substrate on Pyrite-Based Autotrophic Denitrification System

Comparing the performance of denitrification and the microbial community structures among treatments with different concentrations of organic co-substrates, a conclusion was drawn that the addition of different concentrations of the initial organic co-substrate would result in different microbial community structures, but it would not negatively affect its denitrification performance. Suitable concentrations of initial organic co-substrate additions not only did not cause a decline in autotrophic denitrification but may have also enhanced the autotrophic denitrification driven by pyrite due to the promotion of the growth of autotrophic denitrifying microorganisms using Fe(II) as an electron donor for nitrate reduction.

Previous studies have indicated that the addition of an organic co-substrate at high concentrations could inhibit the process of pyrite-based autotrophic denitrification, and both competition and promotion effects on pyrite-based autotrophic denitrification exist when an organic co-substrate is added at a low concentration [17]. The difference in the results of this study may be attributed to different organic co-substrate replenishment patterns and concentrations. In the previous study, the organic co-substrate was added ceaselessly at a pretty high concentration, and so it presented a competition role between heterotrophic denitrification and pyrite-based autotrophic denitrification.

4. Conclusions

Agricultural soils are typically characterized by diverse and abundant microbial communities, which have been identified as potential sources of autotrophic denitrifying microorganisms for pyrite-based systems. In denitrification systems where pyrite is utilized as the sole electron donor, *Thiobacillus* was found to be the dominant genus, with sulfur compounds being used as electron donors. The effects of organic co-substrate addition under a low initial concentration were investigated in this study. And it was found that organic co-substrate additions with a low initial concentration do not have negative effects on pyrite-based autotrophic denitrification; on the contrary, they even promote pyrite-based autotrophic denitrification when the initial organic co-substrate is added at a low concentration of 6–48 mg L^{−1}. The competition role between heterotrophic denitrification and pyrite-based autotrophic denitrification was stronger than their promotion role when the initial organic co-substrate was added at a concentration of 96 mg L^{−1}. The addition of an organic co-substrate, however, led to a different microorganism composition.

Author Contributions: B.X.: Investigation, data curation, and writing—original draft. L.Z. and N.Y.: Investigation and validation. Y.X.: Investigation and validation. H.F.: Investigation and writing—review and editing. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Fundamental Research Funds for Central Public Welfare Research Institutes (Grant No. CKSF2023347/NY), National Natural Science Foundation of China (51522904 and 51629901).

Data Availability Statement: The raw data supporting the conclusions of this article will be made available by the authors on request.

Acknowledgments: We are grateful to all the members of our team for their helpful discussion on this project.

Conflicts of Interest: Lihong Zhang was employed by the company China Water Resources Beifang Investigation, Design and Research Company Limited, China. The remaining authors declare that this research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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