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Use of Denitrifying Sludge for the Removal of Acetaminophen in Water

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Abstract

Acetaminophen, more commonly known as paracetamol (APAP), is one of the most widely used analgesics and antipyretic drugs worldwide. Its presence in the environment poses a risk to the organisms it comes into contact with, which is why it has been classified as an emerging contaminant. Given its adverse effects and continuous discharge into water bodies, it is necessary to study efficient, environmentally sustainable processes for its complete removal. Denitrification is a biological process that has been studied for the biodegradation of recalcitrant compounds and certain pharmaceuticals such as 17β-estradiol and ampicillin, transforming them into harmless products such as N_2 and HCO_3^-. In the present study, the biodegradation of 6 mg L^{-1} of APAP-C was evaluated through a denitrifying process. Batch experiments were conducted, achieving acetaminophen (APAP) removal efficiencies ($E_{APAP\text{-}C}$) of 83.3 ± 0.86% and nitrate removal efficiencies ($E_{N\text{-}NO3^-}$) of 100%. The substrates were predominantly converted into HCO_3^- and N_2, with yields greater than 0.9, while intermediates such as NO_2^- were observed only transiently during the reaction. At the end of the experimental period, no secondary metabolites were detected, indicating that intermediates did not accumulate to quantifiable levels.

Keywords: acetaminophen; water; denitrification; biodegradation; mineralization

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1. Introduction

Acetaminophen [N-(4-hydroxyphenyl) acetamide, APAP] is a pharmaceutical compound widely used for its analgesic and antipyretic properties [1]. It is marketed as an over-the-counter drug, either as a single active ingredient or in combination formulations with other analgesics [2]. Its therapeutic use includes the treatment of mild to moderate pain associated with joint disorders, earaches, headaches, odontogenic pain, neuralgia, minor surgical procedures, as well as the treatment of fever caused by viral infections or post-vaccination fever, among others [1,3] in both humans and animals. During the COVID-19 pandemic, APAP was widely incorporated into therapeutic protocols, significantly increasing its global demand [4]. In 2023, the compound's estimated consumption was 145,000 tons, and it is projected to reach 235,000 tons by 2034 [5]. The structure of APAP ($C_8H_9NO_2$) consists of a benzene ring substituted with a hydroxyl group and an amide group in the para (1,4) position [6]. It has a molecular weight of 151.16 g mol^{-1}, a pKa of 9.38, a log P of 0.46, and a solubility of 14 g L^{-1} at 25 °C [7–9]. These physicochemical

properties, together with its high unregulated consumption and inadequate final disposal, have resulted in its presence in natural water bodies, leading to its classification as an emerging contaminant (EC) [10]. An emerging contaminant is a chemical compound that is not regulated and poses a risk to human health and the environment. The main pathways through which APAP enters the environment include effluents from the pharmaceutical industry, as well as hospital and domestic wastewater [3]. The presence of APAP has been reported in surface waters of 71 countries, with an average concentration of 0.161 μg L^{-1} and a maximum concentration of 230 μg L^{-1} [11]. In Mexico, concentrations ranging from 20.96 to 29.91 μg L^{-1} were detected in wastewater from the Tula Valley, Hidalgo [12]. In the Apatlaco River, in the state of Morelos, concentrations ranging from 0.35 to 14.9 μg L^{-1} have been reported [13], and in irrigation canals of the Mezquital Valley in the state of Hidalgo, levels between 0.61 and 67.20 μg L^{-1} have been found [14]. Likewise, traces of this analgesic have been identified in hospital effluents from Toluca, Cuernavaca, and Puebla at concentrations ranging from 2.66 to 38.74 μg L^{-1} [13,15,16].

The presence of acetaminophen (APAP) in water bodies, even at low concentrations, has been shown to induce toxic effects in aquatic biota, such as endocrine disruption, neurotoxicity, oxidative stress, enzymatic inhibition, genetic damage, and reproductive alterations [5,17]. Furthermore, an increase in the activity of antioxidant enzymes has been reported in mollusks (*Dreissena polymorpha*) [4], as well as oxidative stress in species such as *Oncorhynchus mykiss*, *Daphnia magna*, and *Mytilus galloprovincialis*, along with reproductive dysfunction and the development of abnormalities in zebrafish due to chronic exposure to APAP [18]. Additionally, it has been demonstrated that this drug and its metabolites may promote the development of genetic resistance to antibiotics [19].

Currently, conventional wastewater treatment technologies such as primary treatment; secondary biological processes, e.g., activated sludge systems; and tertiary treatments have not been fully efficient in removing APAP. Although these systems are effective for the removal of organic matter and nutrients, they are not specifically designed to eliminate emerging contaminants such as pharmaceuticals, resulting in only partial removal and, in some cases, incomplete mineralization, resulting in partial removal and, in some cases, incomplete mineralization [20,21], thereby allowing the persistence of the compound in treated effluents [22]. Consequently, a wide range of physicochemical and biological techniques, as well as hybrid processes, have been employed for APAP removal. Among physicochemical treatments, advanced oxidation processes (AOPs) such as ozonolysis, Fenton, and photo-Fenton have shown acetaminophen removal efficiencies close to 100%. Despite their high efficiency, most of these processes do not achieve complete mineralization of the compound, leading to the formation of toxic intermediates such as *p*-aminophenol, hydroquinone, and benzoquinone [23,24]. Ozonation presents disadvantages associated with the high cost of O_3 generators, its limited solubility in water due to the presence of organic matter, pH and temperature effects, as well as the short half-life of ozone, which reduces its efficiency and often requires combination with other technologies [25]. In a photo-Fenton process, 99.3% degradation and 69% mineralization of 40 mg·L^{-1} of APAP was achieved [23]. The treatment was carried out at pH 2.8, using iron as a catalyst, hydrogen peroxide as the oxidizing agent, and a UV lamp emitting radiation between 300 and 420 nm for 75 min. The authors reported the formation of hydroquinone and benzoquinone as degradation byproducts. In another study evaluating simple ozonation with initial APAP concentrations of 20, 30, and 50 mg·L^{-1} at pH 10 and 24 °C, with an ozone flow rate of 0.5 g·h^{-1} for 60 min, removal efficiencies lower than 80% were obtained, and the generation of potentially toxic byproducts was reported [24]. Likewise, Ling et al. [26] developed a photocatalytic ozonolysis technique using 4% Ag-g-C_3N_4 as a catalyst and a 500 W xenon lamp, with an initial APAP concentration of 10 mg·L^{-1} and an ozone flow rate

of 50 mg·h^{-1}, achieving a removal efficiency greater than 84%. In all the studies mentioned, none provide evidence of the final fate of the contaminant, and most reports only mention the reduction in APAP.

Several studies have reported the adsorption of pharmaceuticals onto activated carbon from various sources. While these techniques often achieve high removal efficiencies, they only transfer the contaminants from the aqueous phase to the solid adsorbent, without actually degrading them. Consequently, the underlying environmental problem remains unresolved, as the adsorbed compounds may still pose risks if not properly managed or further treated [27].

On the other hand, some biological processes have proven to be an efficient alternative for APAP treatment. A wide variety of microorganisms have been studied for APAP biodegradation, including bacteria, fungi, and microalgae [28]. For example, Hasan et al. [29] used a batch aerobic reactor with *Paracoccus* sp. and *Enterobacter* sp. bacteria isolated from hospital wastewater sludge, achieving 89–96% degradation of an initial APAP concentration of 10 mg·L^{-1} within 9.4 days; however, the authors did not mention the final degradation products. A study conducted by Chopra and Kumar [30] used an anaerobic reactor with *Bacillus drentensis* strain S1, with an initial APAP concentration of 400 mg·L^{-1} at pH 7.0, 40 °C, and 165 rpm for 30 days, achieving 94.5% degradation with mineralization to nitrites and nitrates; however, the study did not report the carbonaceous end products. Dionisi and Etteh [31] reported 80% degradation of 1000 mg·L^{-1} APAP in an aerobic sequencing batch reactor (SBR) over a 30-day period. Another study by Abdolvand et al. [32] implemented an upflow fluidized bed reactor (UFBR) using a mixture of *Pseudomonas* spp. and *Bacillus* spp. at pH 7.2 ± 0.2, room temperature, and a hydraulic retention time (HRT) of 0.25 days, achieved > 99% removal, and suggesting complete biodegradation of APAP. In all cases, however, none of the studies reported the final fate of the mineralization products.

Among the biological processes, denitrification has attracted attention as a potential pathway. This process consists of the reduction of nitrate to molecular nitrogen through the action of four enzymes, namely nitrate reductase (Nar), nitrite reductase (Nor), nitric oxide reductase (Nir), and nitrous oxide reductase, synthesized by different genera of bacteria [32]. Denitrification requires the presence of an electron donor, usually an organic substrate, under anoxic conditions. Several studies have demonstrated that denitrification can biodegrade emerging contaminants, including pharmaceuticals, phenols, and aromatic compounds [33–35]. For instance, Camacho-López et al. [34] evaluated the biodegradation of estradiol (E2) using 0.5 g volatile suspended solids (VSS L^{-1}) of denitrifying sludge and reported a 100% removal, which was attributed to completed biodegradation, with bicarbonate (Y_{HCO3^-}) and gaseous nitrogen (Y_{N2}) yields of 0.91 ± 0.03 and 0.96 ± 0.02, respectively. Similarly, ampicillin (AMP) biodegradation was reported, achieving 100% removal efficiency with 0.5 g VSS L^{-1} and yields close to 1 [36]. Other studies have examined compounds such as p-cresol, chlorophenol, and BTX, reporting biodegradation efficiencies ranging from 50% to 95%, associated with the denitrification process [37–39]. These results highlight the potential of denitrification not only for the biotransformation of nitrogen compounds but also as a viable strategy for the removal of emerging organic pollutants in aqueous matrices. However, the fate of acetaminophen (APAP) under denitrifying conditions has not yet been fully identified. In this context, this study addresses this limitation by evaluating APAP as an electron donor under anoxic conditions, integrating a mass balance approach to analyze the distribution of carbon and nitrogen during the process. Therefore, the objective of this study is to evaluate the performance of APAP as a carbon source in a denitrification process, compared to acetate, by analyzing removal efficiencies, yields, specific rates, and mass balance, in order to determine its fate and support its potential application in wastewater treatment systems.

2. Materials and Methods

2.1. Inoculum Source

A 1.4 L upflow anaerobic sludge blanket (UASB) reactor was installed and inoculated with 10.98 g VSS L^{-1} of anaerobic sludge of methanogenic origin, obtained from the Wastewater Treatment Plant of Atotonilco de Tula, Hidalgo, México. Prior to its use, the sludge was repeatedly washed with a physiological NaCl solution (9 g L^{-1}) to remove residual substrates and soluble organic matter. The inoculum consisted of a mixed microbial consortium, and no specific microbial characterization at the species level was performed. Under the experimental conditions, the system was operated to promote denitrifying activity. The reactor was continuously fed with two mineral media supplied independently and acidified to prevent precipitation of compounds. The composition of the carbon source was (mg L^{-1}): CH_3COO^--C (1000), KH_2PO_4 (1000), $Na_2MoO_4{\cdot}2H_2O$ (120), $CaCl_2$ (60), and $MgSO_4{\cdot}7H_2O$ (400); and that of the nitrogen source was (mg L^{-1}): $NO_3{}^-$-N (555.5), $FeCl_3{\cdot}6H_2O$ (100), and $CuSO_4{\cdot}5H_2O$ (20). The reactor operated at a hydraulic retention time (HRT) of 1.92 d, 30 ± 0.1 °C, and pH 7.98 ± 0.19. Under these conditions, the denitrifying steady state (DSS) of the sludge in the UASB reactor was reached after 80 days of operation, achieving $NO_3{}^-$-N removal efficiencies (E_{NO3^-N}) of 97.48 ± 0.76%, while the biodegradation efficiency of CH_3COO^--C was 98.38 ± 0.067%. The corresponding yields were Y_{N2} of 0.93 ± 0.03, Y_{HCO3^-C} of 0.76 ± 0.02, and $Y_{Biomass}$ of 0.25 ± 0.06. Similarly, volumetric rates of bicarbonate production (Q_{HCO3^-C}) and nitrogen production (Q_{N2}) of 186.5 ± 8.6 mg L^{-1} d^{-1} and 92.29 ± 4.59 mg L^{-1} d^{-1}, respectively, were obtained. Since the reactor was monitored for 180 days, the denitrifying sludge was considered to have reached denitrifying steady state (DSS). This conclusion was supported by 22 measurement points (the last 22 days), with three samples per point and the arithmetic mean calculated, resulting in a coefficient of variation (CV) < 6% for the response variables. The sludge was then used as inoculum in both abiotic batch and denitrifying assays; in all cases, it was withdrawn from the UASB reactor and washed three times with a 0.9% NaCl solution before use.

2.2. Batch Assays

For the batch assays, 60 mL serum bottles were used, with a working volume of 80% and a 20% headspace. Each bottle was filled with mineral medium composed of (mg L^{-1}): KH_4PO_4 (500), $MgSO_4{\cdot}7H_2O$ (100), $FeCl_3{\cdot}6H_2O$ (50), $CuSO_4{\cdot}5H_2O$ (10), $NaMoO_4{\cdot}H_2O$ (25), and $CaCl_2{\cdot}2H_2O$ (150). The carbon source was supplied either as CH_3COO^--C (6) or APAP-C (6), and the N-$NO_3{}^-$ concentration was adjusted to achieve a stoichiometric C N^{-1} ratio of 1.1 and 0.7, respectively, and the initial pH was adjusted to 7.0 ± 0.2.

It is important to note that all experiments were conducted at a pH of 7.0 ± 0.2, as this parameter critically influences the activity of denitrifying microorganisms and the overall efficiency of carbonaceous species degradation. Previous studies have reported that denitrifying bacteria exhibit maximum activity under neutral to slightly alkaline conditions (pH 7–8), whereas acidic (pH < 6) or strongly alkaline conditions (pH > 9) can inhibit enzymatic activity and microbial growth [40,41]. Additionally, the formation of $HCO_3{}^-$-C is also influenced by pH. Rosso and Vione [42] indicate that within a pH range of 6.3–10.3, $HCO_3{}^-$-C is the predominant chemical species. From an environmental perspective, this is highly relevant, as it reduces CO_2 emissions, a major greenhouse gas.

The bottles were inoculated with 0.5 g VSS L^{-1} of sludge from the UASB reactor. Subsequently, they were hermetically sealed with a septum and aluminum crimp cap, and the air was displaced with an Ar stream to ensure an anoxic atmosphere. The bottles were placed in an incubator and maintained at 34 °C under continuous shaking at 200 rpm. All

assays were conducted in duplicate. Liquid and gas samples were taken for analysis. Each bottle represented an independent experimental unit and was discarded after analysis.

2.3. Abiotic Assays

Two types of abiotic control assays were performed. The first consisted of mineral medium with carbon and nitrogen sources in the absence of denitrifying sludge, used in order to evaluate substrate loss due to chemical reactions and volatilization. The second included 0.5 mg VSS L^{-1} of previously sterilized denitrifying sludge (autoclaved at 120 °C for 1 h), used to assess potential substrate loss due to physical phenomena such as adsorption onto sludge. These assays were carried out with a concentration of 6 mg L^{-1} APAP-C and sufficient NO_3^--N to obtain a C N^{-1} ratio of 1.1.

2.4. Denitrification Assays with Acetate and Acetaminophen

Reference assays were performed using 6.00 mg L^{-1} of acetate (CH_3COO^--C) as the carbon source and 5.45 mg L^{-1} of nitrate (NO_3^--N) as the nitrogen source at a C N^{-1} ratio of 1.1. These assays were conducted to evaluate denitrification using a readily biodegradable carbon source, and the results served as a benchmark for assessing the respiratory process in the presence of acetaminophen (APAP). The denitrification tests with APAP were performed with 6 mg L^{-1} of APAP-C and sufficient NO_3^--N to maintain a C N^{-1} ratio of 0.7.

2.5. Analytical Methods

APAP-C concentrations were determined using a High-Performance Liquid Chromatography (HPLC) system (Perkin Elmer Series 200, Norwalk, CT, USA) equipped with a UV-visible detector at a wavelength (λ) of 220 nm and a BEH 2-EP column (Waters, Wexford, Ireland, 10 mm × 250 mm, 5 µm). The mobile phase consisted of HPLC-grade water (J.T. Baker, Phillipsburg, NJ, USA) at a flow rate of 1 mL min^{-1}, with an injection volume of 20 µL. Both the mobile phase and samples were filtered through 2 µm nylon membranes. NO_2^--N and NO_3^--N concentrations were quantified by high-performance liquid chromatography (HPLC) at a wavelength (λ) of 214 nm using an anion-exchange column (Dionex IonPac AS11-HC, Sunnyvale, CA, USA 4 µm, 0.4 × 250 mm). The mobile phase consisted of (mL): water (840), acetonitrile (120), butanol (20), and gluconate–borate solution (20), delivered at a flow rate of 1 mL min^{-1}. The injection volume was 20 µL. Samples were previously filtered and analyzed in triplicate. N_2 and CO_2 were quantified by gas chromatography (GC) (Perkin Elmer Autosystem XL, Norwalk, CT, USA) equipped with a thermal conductivity detector (TCD) and an HP-PLOT/Q capillary column (Agilent Technologies, Santa Clara, CA, USA; 30 m × 0.530 mm internal diameter × 40 µm film thickness). Ultra-high-purity argon was used as the carrier gas at a flow rate of 10 mL min^{-1}. The injection volume was 100 µL. The oven, injector, and detector temperatures were set at 60 °C, 100 °C, and 200 °C, respectively. All samples were analyzed in triplicate.

Total carbon (TC) and inorganic carbon (IC) were determined using a total organic carbon analyzer (Shimadzu TOC-L CSH, Suzhou, Jiangsu, China). Volatile suspended solids (VSS), total solids (TS), and fixed total solids (FTS) were determined gravimetrically according to [43].

Table 1 presents the key parameters obtained from the different calibration curves. For APAP, NO_2^-, and NO_3^-, at least five concentration levels ranging from 0 to 15 mg L^{-1} were used; for TC and IC, at least nine concentration levels ranging from 0 to 100 mg L^{-1} were applied; and for N_2 and CO_2, six concentration points were employed, covering 0–43 mg L^{-1} and 0–56.15 mg L^{-1}, respectively.

Table 1. Key parameters of the calibration curves for APAP, NO_2^-, NO_3^-, TC, IC, N_2, and CO_2, including the calibration range, LOD, LOQ, and the coefficient of determination (R^2).

Compound	Calibration Range (mg L^{-1})	LOQ (mg L^{-1})	LOD (mg L^{-1})	Equation of a Line	R^2
TC	0–100	5.20	1.71	y = 2.8669x + 1.0453	0.9998
IC	0–100	0.288	0.086	y = 3.0605x + 0.8977	0.9996
APAP	0–15	1.53	0.46	y = 57,194x – 22,075	0.9984
NO_2^-	0–15	2.77	0.83	y = 208,399x – 53,572	0.9934
NO_3^-	0–15	3.64	1.09	y = 229,356x – 87,329	0.9906
N_2	0–43	9.87	2.7	y = 150.36x + 315.68	0.9954
CO_2	0–56.15	4.21	1.26	y = 47.942x + 279.21	0.999

TC: total carbon; IC: inorganic carbon; LOD: limit of detection; LOQ: limit of quantification.

2.6. Evaluation of the Denitrifying Respiratory Process

To evaluate the batch denitrifying respiratory process, experiments were conducted at different time points to characterize the denitrification performance and establish kinetic profiles. Substrate consumption efficiencies (E) were calculated using Equation (1):

$$E = \left(\frac{C_0 - C_i}{C_0}\right) \times 100 \quad (1)$$

where Co (mg L^{-1}) is the initial substrate concentration and Ci (mg L^{-1}) is the substrate concentration at each sampling time.

Product yields (Y) were calculated using Equation (2):

$$Y = \frac{C_p}{C_0 - C_i} \quad (2)$$

where C_p (mg L^{-1}) is the concentration of the generated product.

Biodegradation kinetics were evaluated by calculating the specific rates (q), expressed as mg of substrate consumed or product formed per g VSS^{-1} h^{-1}, using a fit to the Gompertz model, which was selected due to its suitability for describing microbial growth-associated biodegradation processes involving lag, exponential, and stationary phases [44] (Equation (3)):

$$y = A\exp\{-\exp(B(t - C))\} \quad (3)$$

where y is the residual substrate concentration (mg L^{-1}), A is the initial substrate concentration (mg L^{-1}), B is the kinetic constant (h^{-1}), t is the time, and C is the time required to reach the maximum rate of consumption or product formation.

The Gompertz model was fitted to the experimental data, yielding a coefficient of determination (R^2) of 0.92, indicating an excellent goodness of fit. As this is a non-linear model, R^2 is interpreted as an indicator of how well the model describes the overall trend of the data.

2.7. Statistical Analysis

For the determination of the analytes of interest, all calibration curves were performed in triplicate. In all cases, the coefficients of determination (R^2) were higher than 0.99, indicating excellent linearity. Limits of detection (LOD) and quantification (LOQ) were also determined for each compound, ensuring the reliability and accuracy of the analytical methods across the evaluated concentration ranges. These results confirm that the calibration curves provide a robust basis for quantifying both substrates and products measured in this study.

It should be noted that the reported values correspond to the mean obtained from experimental assays, along with the standard deviation of two independent samples. In order to identify statistically significant differences among the response variables obtained (substrate consumption efficiencies, product yields, and the rates of each species) in abiotic and denitrifying assays, a two-way analysis of variance (ANOVA) was performed ($\alpha = 0.05$). Prior to the analysis, normality and homogeneity of variances were evaluated. When significant differences were detected, Tukey's post hoc test was applied for mean comparisons ($\alpha = 0.05$).

3. Results and Discussion

3.1. Evaluation of Abiotic Assays

The results of the abiotic assays (Figure 1), conducted in the absence of denitrifying sludge (Figure 1a), show that the APAP-C concentration decreased by 18.3% during the first 2 h of reaction and remained constant until 24 h. Subsequently, a recovery in concentration was observed, such that at 30 h the reduction was only 7%, and at 50 h only 3% below the initial value. No formation or presence of secondary metabolites of carbonaceous species was detected; therefore, concentration losses due to chemical reactions or APAP volatilization were ruled out. These results can be explained by the fact that APAP, being a phenolic compound with a pKa of 9.38 [45], is predominantly present in its neutral form at pH 7 [46], indicating that no reaction occurred with PO_4^{3-}, SO_4^{2-}, MoO_4^{2-}, Cl^-, Mg^{2+}, Fe^{2+}, Na^+, and Ca^{2+} ions present in the mineral medium. Therefore, the initial decrease in concentration observed in the assays was most likely due to a temporary reduction in APAP solubility, considering that its solubility is 14 g L^{-1} at 25 °C [7–9]. This solubility may be affected by ionic competition in the mineral medium. It can thus be concluded that, under the experimental conditions applied, continuous agitation (200 rpm) and a temperature of 34 °C, the pharmaceutical initially tended to precipitate but gradually became completely solubilized over time.

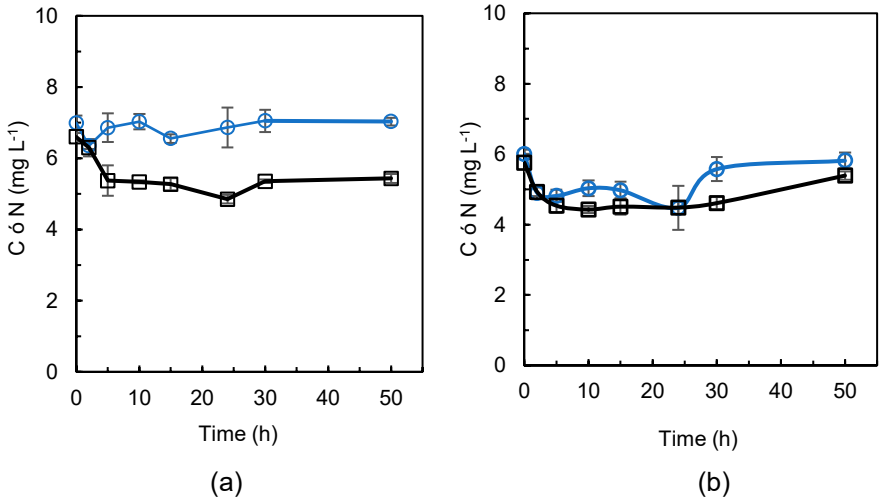


Figure 1. Kinetic profile of abiotic assays with 6 mg L^{-1} of APAP–C: (**a**) in the absence of denitrifying sludge and (**b**) in the presence of inactivated denitrifying sludge. (○) APAP-C, (□) NO_3^--N.

Regarding NO_3^--N, a 6% variation in concentration was recorded at the end of the abiotic assay conducted in the absence of denitrifying sludge. This variation falls within the coefficient of variation in the analytical method (<10%) and is therefore attributed to analytical variability and minor experimental deviations associated with sampling and measurement. Given the high solubility and stability of nitrate (921 g L^{-1} at 25 °C) [47],

losses due to chemical reactions or volatilization were considered negligible. Consequently, the observed decrease does not represent a real consumption process.

Regarding the results of the abiotic assays conducted in the presence of sterile sludge (Figure 1b), APAP-C concentration decreased by 9.1% after 2 h of experimentation. This decrease may be attributed to a temporary reduction in solubility, similar to that observed in assays performed in the absence of sludge (Figure 1a). At the end of the experiments, the difference between the initial and final concentrations was less than 1%, indicating that no adsorption, chemical reaction, or volatilization occurred. Other authors have reported that, in assays conducted with sterile sludge from a wastewater treatment plant, a natural attenuation or adsorption effect of 15.7% was observed after 28 days of reaction, suggesting that abiotic effects were responsible for the concentration decrease [48].

With respect to NO_3^-, a 12% decrease in concentration was observed after 5 h from the start of the assay. At the end of the experiment, the difference relative to the initial concentration was 14%, which may be partially attributed to the adsorption of NO_3^- ions onto the sludge. This interpretation is supported by the absence of detected metabolites and the lack of molecular nitrogen production; however, it cannot be conclusively confirmed based on the available data.

3.2. Evaluation of the Reference Assays

The reference assays conducted with 6.00 mg L^{-1} of CH_3COO^--C as the carbon source and 5.45 mg L^{-1} of NO_3^--N as the nitrogen source, at a C N^{-1} of 1.1 (Figure 2), showed that 61.1% of CH_3COO^--C was consumed during the first hour of the assay, reaching an E_{CH3COO^-C} del 97.35 ± 2.68% after 5 h. Simultaneously, HCO_3^--C was generated as the sole carbonaceous product, with a yield ($Y_{HCO3\text{-}C}$) of 0.95 ± 0.03.

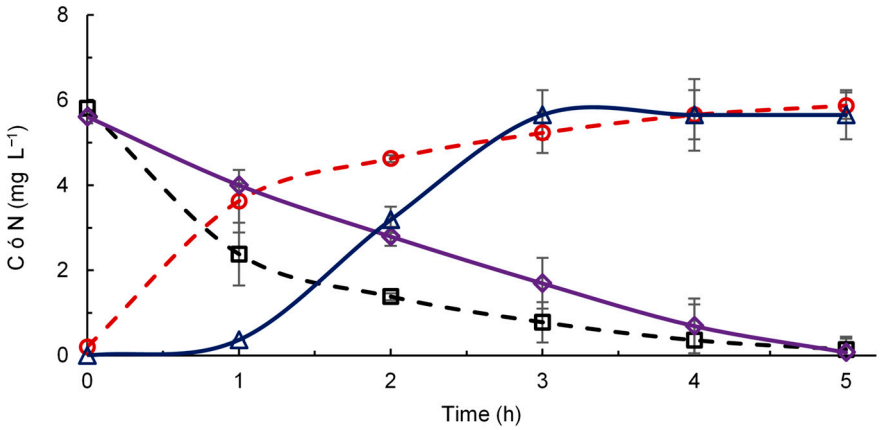


Figure 2. Kinetic profile of the reference test in the presence of 6.12 mg CH_3COO^-–C L^{-1} and NO_3^-–N at a C N^{-1} ratio of 1.1: (□) CH_3COO^--C, (○) HCO_3^--C, (◊) NO_3^--N and (Δ) N_2.

Regarding nitrogen species, 28.69% of NO_3-N was consumed during the first hour, achieving an $E_{NO3\text{-}N}$ of 98.39 ± 0.43% after 4 h of reaction. In parallel, N_2 was produced as the only nitrogenous product, with a Y_{N2} of 0.99 ± 0.02 (Table 2). These results indicate that CH_3COO^--C oxidation was coupled to the reduction of NO_3^--N to N_2, which is consistent with the well-established role of acetate as an electron donor in heterotrophic denitrification processes [49]. It should be noted that these results were used as a reference when active sludge was tested in the presence of APAP.

Table 2. Consumption efficiencies and yield values obtained in the experimental assays: denitrification reference (acetate and nitrate), in the presence of AMP (plus acetate and nitrate), and recovery assays.

Essay	% E			Y	
	NO_3-N	CH_3COO^--C	APAP-C	N_2-N	HCO_3-C
Denitrification reference	98.39 ± 0.43	97.35 ± 2.68	0	0.99 ± 0.02	0.95 ± 0.03
APAP	100	----	83.27 ± 0.86	1.018 ± 0.02	0.93 ± 0.007

E_{CH3COO^-C}, mg CH_3COO^--C consumed/mg CH_3COO^--C fed; $E_{NO3\text{-}N}$, mg $NO_{3_}$-N consumed/mg NO_3^--N fed; $E_{APAP\text{-}C}$, mg APAP-C consumed/mg APAP-C fed; $Y_{N2\text{-}N}$, mg N_2 produced/mg $NO_{3_}$-N consumed; $Y_{HCO3\text{-}C}$, mg $HCO_{3_}$−C produced/mg C consumed.

Studies conducted with 10 and 20 mg L^{-1} CH_3COO^-–C [34,50] as the sole carbon source and NO_3^- to achieve a C N^{-1} ratio of 1.1 reported that the consumption time for all species was 2 h, obtaining $E_{CH3COO\text{-}C}$ and de $E_{NO3\text{-}N}$ of 100%, as well as Y_{HCO3}^- and Y_{N2} values close to 1. Although the consumption time was longer in the present study, the efficiency and yield were very similar. Likewise, the results may be attributed to the origin of the sludge and its prior exposure to other contaminants [51,52]. It is worth noting that the production and accumulation of carbonaceous and nitrogenous intermediates were not observed in the reference tests, indicating that the sludge was capable of achieving a successful and complete denitrification process. The results obtained under these conditions were used as a reference to evaluate the denitrification process in the presence of acetaminophen.

3.3. Denitrification Assays with Acetate and Acetaminophen

The results for the performance of the denitrifying sludge in the biodegradation of APAP are presented in Table 2 and Figure 3. These show that during the first hour of the assay, an $E_{APAP\text{–}C}$ of 12.17% was obtained; as time progressed and up to 40 h, an $E_{APAP\text{–}C}$ of less than 20% had been achieved. The process was monitored up to 500 h, reaching an $E_{APAP\text{–}C}$ of 83.27 ± 0.86%. Simultaneously, HCO_3^--C was generated as the only carbonaceous compound, with a yield ($Y_{HCO3\text{–}C}$) of 0.93 ± 0.007. These results demonstrated the mineralization of acetaminophen under the conditions described in the present study. A study in which *Bacillus drentensis* S1 was used under anaerobic conditions to degrade 50 mg L^{-1} of APAP reported 94.5% degradation after 40 h of reaction [53]. The authors indicated that the major degradation products were 2-isopropyl-5-methylcyclohexanone and oxalic acid, but complete mineralization was not achieved. Another study employing *Paracoccus* sp. and *Enterobacter* sp. for the removal of 300 mg L^{-1} of acetaminophen achieved 88.96% and 85.92% biodegradation, respectively, after 192 h, identifying 4-aminophenol, hydroquinone, and 3-hydroxy-2,4-hexadienoic acid as process products [28]. In both studies, although APAP degradation was close to 100%, complete mineralization of the compound was not achieved.

Another study using *P. extremorientalis* CSW01 for the biodegradation of 10 mg L^{-1} APAP reported mineralization of 23.2–27.6% after 672 h; when the same bacterium was used in a consortium with *M. aubagnense* HPB1.1, 73.9% mineralization was achieved over the same period [48]. Although both studies state that mineralization occurred, they do not indicate the final destination of the contaminant, i.e., the products of mineralization.

Likewise, in a study using *P. aeruginosa* HJ1012, a CO_2 production rate of up to 71.4% was observed from a maximum tested APAP concentration of 2200 mg L^{-1}, indicating that the microorganisms were able to tolerate and mineralize high concentrations of APAP [54].

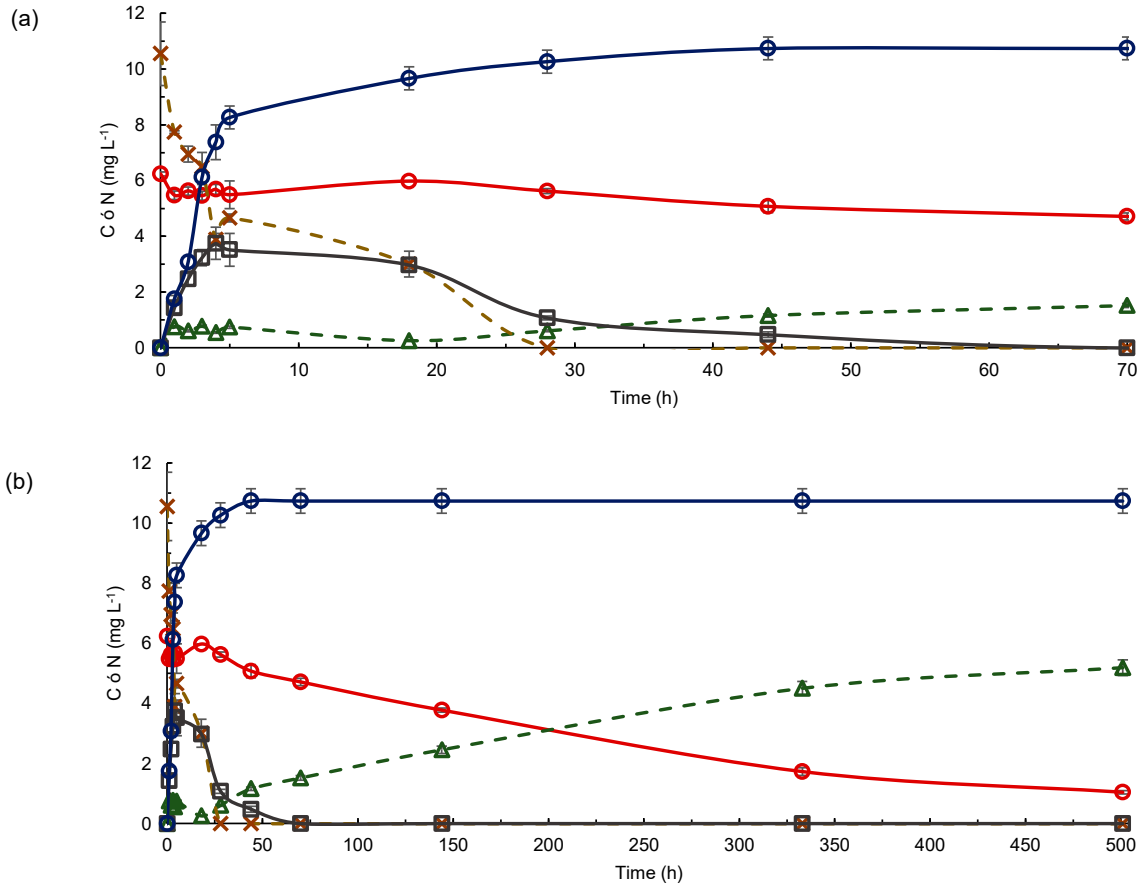


Figure 3. Kinetic profile of denitrifying assays with 6.0 mg L^{-1} of APAP–C as the sole electron donor and NO_3^-–N at a C N^{-1} ratio of 0.7: (**a**) time interval 0–70 h and (**b**) time interval 0–500 h. (○) APAP-C, (Δ) HCO_3^--C, (x) NO_3^--N, (□) NO_2^--N and (○) N_2.

In this study, a denitrifying bacterial consortium obtained from the inoculum source described in Section 2.1 was used under the experimental conditions detailed in Section 2.2, achieving biodegradation efficiencies at an APAP-C concentration of 6 mg L^{-1} comparable to those reported in previous studies. The results indicate that the final carbonaceous mineralization product was predominantly HCO_3^--C, with no CO_2 detected. This finding is consistent with the literature, which indicates that within a pH range of 6.3–10.3, HCO_3^--C is the predominant chemical species [40,41]. From an environmental perspective, this behavior is relevant, as it minimizes CO_2 emissions, a greenhouse gas, while simultaneously promoting greater activity of the denitrifying bacteria. On the other hand, different authors have identified metabolites such as 4-aminophenol, hydroquinone, and benzoquinone during APAP biodegradation [8,55]. At the end of the reaction time, no secondary metabolites were detected, indicating that the intermediates did not accumulate to quantifiable levels. This behavior can be attributed to their rapid conversion through sequential enzymatic pathways under denitrification conditions. Furthermore, the mass balance confirms that the substrate concentrations were stoichiometrically consistent with those of the products obtained (Table 3).

Table 3. Carbon and nitrogen mass balance obtained in the experimental assays.

Essay	Input (mg L^{-1})					Output (mg L^{-1})		
	APAP-C	CH_3COO^--C	NO_3^--N	HCO_3^--C	CH_3COO^--C	APAP-C	NO_3^--N	N_2-N
Denitrification reference	0	5.806 ± 0.29	5.608 ± 0.28	5.869 ± 0.29	0.136 ± 0.007	0	0.073 ± 0.004	5.646 ± 0.28
APAP	6.232 ± 0.07	0	10.549 ± 1.14	5.189 ± 0.12	0	1.042 ± 0.05	0	10.735 ± 0.54

On the other hand, Jan-Roblero et al. [56] reported that when using *Amycolatopsis* sp. Poz14, for the biodegradation of acetaminophen as the sole carbon source, the actinobacterium degraded APAP with an efficiency of 71% after 504 h of reaction. The results obtained in the present study show that the reaction time was very similar, whereas the efficiency was 17.28% higher than the 71% reported by those authors.

It is worth mentioning that most of the reviewed studies indicate that mineralization occurred; however, they do not report the mineral compounds obtained. On the other hand, most studies quantify metabolites formed after ring cleavage rather than directly measuring the amount of CO_2 or HCO_3^- produced. This suggests that true mineralization did not occur, but rather only biodegradation, in which the metabolites formed may even be more toxic than APAP itself.

The literature reports studies on pure bacterial strains, with promising efficiencies achieved. For example, *Bacillus licheniformis* PPY-2 was used for the biodegradation of different APAP concentrations (400, 200, and 2500 mg L^{-1}). The authors mention that complete biodegradation was achieved; however, they do not report the biodegradation products [53].

Another example is the study that employed *Pseudomonas moorei* KB4. This strain degraded glucose and 50 mg L^{-1} of APAP via co-metabolism. The authors reported the formation of intermediate products such as *p*-aminophenol and hydroquinone [57]. These studies demonstrate that none has achieved complete mineralization of APAP. Although complete mineralization of paracetamol under strictly anaerobic conditions has been less frequently reported, previous studies have demonstrated that its biodegradation proceeds through the formation of intermediates such as 4-aminophenol and hydroquinone under anoxic conditions [58]. These intermediates can undergo further transformation, including aromatic ring cleavage and oxidation through enzymatic pathways, ultimately leading to mineralization products such as CO_2/HCO_3^- [48]. Although these pathways have been mainly described under aerobic or mixed conditions, similar transformations may occur under denitrifying conditions, where organic intermediates are oxidized while nitrate serves as the terminal electron acceptor.

In the present work, it is demonstrated that 83.27% of APAP was mineralized to HCO_3^-, evidencing the capacity of the denitrifying bacterial consortium to biodegrade and mineralize APAP under the working conditions described. Some reports mention that among the bacteria identified as APAP degraders, only a few have been capable of mineralizing it [48]. In the present study, a bacterial consortium taken directly from a wastewater treatment plant (WWTP) and acclimated to denitrifying steady state conditions was used, and the results indicate the viability of the bacteria in the biotransformation of APAP.

Regarding the nitrogen species, the kinetic profile (Figure 3a,b) shows that during the first 5 h of reaction, an $E_{NO3\text{-}N}$ of 55% was reached, achieving 100% $E_{NO3\text{-}N}$ for 28 h of reaction. It is worth noting that a transient production of 4.15 mg L^{-1} of NO_2^-–N was quantified as the only process intermediate, with its appearance recorded from the first hour of the assay, as shown in Figure 3a. Despite this initial accumulation, the denitrification process continued, and NO_2^-–N was consumed after 44 h of reaction, becoming non-quantifiable by the end of the experiment. This behavior confirms that NO_2^-–N acted solely as an intermediate and was further reduced to gaseous end products, consistent with a complete denitrification pathway leading to N_2 [59].

Similar results were reported when ampicillin (AMP–C) and CH_3COO^-–C were used as carbon sources in a denitrifying system, in which total consumption of CH_3COO^-–C and partial consumption of AMP–C occurred during the first 10 h, associated with the complete depletion of NO_3^-–N. In addition, NO_2^-–N production was reported from the first hour

and was consumed after 100 h of testing [36]. In the present study, total consumption of the carbonaceous species was achieved at 70 h of reaction, and N_2 was the only product obtained from NO_3^-–N, with a Y_{N2} of 1.018 ± 0.02. Based on these results, it is assumed that APAP oxidation was coupled to the reduction of NO_3^-–N to N_2. Likewise, it has been reported that the oxidation of 5–15 mg AMP–C L^{-1} was coupled to NO_3^-–N reduction, with a Y_{N2} equal to 1 [50]. There is insufficient evidence in the literature on the use of denitrifying bacteria for APAP biotransformation; although denitrification is mentioned, the evidence is unclear because the final products are not reported.

The results for the specific rates of each substrate and product in the present study are shown in Figure 4 and Table 4. A comparison was made between the results obtained when CH_3COO^-–C and APAP–C were used as carbon sources in the reference assays. The results indicated that $q_{NO3\text{-}N}$ was 26.8% higher than with APAP-C, demonstrating that the presence of APAP exerts a negative effect on the kinetic activity of denitrifying bacteria. Regarding the carbonaceous species, the consumption rate $q_{CH3COO^-\text{-}C}$ was 94.78% higher than $q_{APAP\text{-}C}$ when CH_3COO^-–C was used as the sole carbon source. This behavior can be explained by the fact that CH_3COO^-–C is a simple molecule, easily assimilable, highly bioavailable, and low in toxicity. In contrast, APAP is an aromatic compound containing a phenolic ring and an amide group, which makes it more difficult to biodegrade and less readily available. Some authors have reported that during denitrifying respiration in the presence of APAP, various intermediates are formed throughout the biodegradation process, including 4-aminophenol, hydroquinone, and oxalic acid [4,54,60], thereby reducing the efficiency of electron transfer to nitrate.

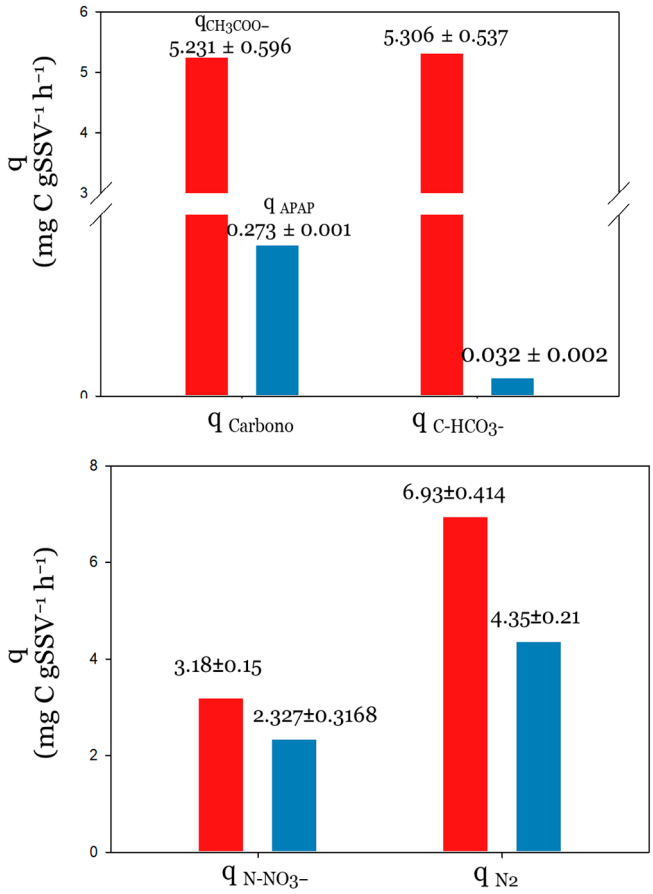


Figure 4. Specific rates in (■) reference assays and (■) denitrifying assays with APAP.

Table 4. Specific rate values obtained in the experimental assays: denitrification reference (acetate and nitrate) and APAP (APAP and nitrate).

Specific Rate (q)		Denitrification Reference	APAP
Consumption	NO_3^--N	3.18 ± 0.15	2.327 ± 0.3168
	CH_3COO^--C	5.231 ± 0.596	0
	APAP-C	0	0.273 ± 0.001
Production	N_2	6.93 ± 0.414	4.35 ± 0.21
	HCO_3^--C	5.306 ± 0.537	0.032 ± 0.002

Specific consumption (q, mg of substrate consumed/mg SSV h^{-1}, $q_{NO3\text{-}N}$, $q_{Acetate\text{-}C}$, $q_{APAP\text{-}C}$) and specific production (q, mg of product formed/mg SSV h^{-1}, $q_{N2\text{-}N}$, $q_{HCO3\text{-}C}$) rates.

To further elucidate the kinetic behavior of APAP biodegradation, the experimental data were analyzed using different kinetic approaches commonly applied in biodegradation studies. Initially, a first-order kinetic model was evaluated; however, it was not adequate to describe the experimental data, particularly due to the presence of an initial lag phase and a non-linear substrate consumption pattern. These deviations indicate that APAP biodegradation by the denitrifying consortium does not follow a simple exponential decay. In contrast, the Gompertz model showed a significantly better fit (R^2 = 0.92), accurately describing the lag, exponential, and stationary phases of the process. The observed lag phase suggests a period of microbial adaptation before active degradation, likely associated with the activation of specific metabolic pathways required for the transformation of aromatic compounds. Consistent with this, APAP exhibited a relatively low specific consumption rate ($q_{APAP\text{–}C}$ = 0.273 ± 0.001) compared to acetate ($q_{CH3COO^-\text{–}C}$ = 2.327 ± 0.3168), supporting the hypothesis that its chemical structure imposes kinetic limitations. Overall, these results confirm that the Gompertz model is suitable for describing APAP biodegradation kinetics under the evaluated conditions and provides relevant insights into the microbial dynamics governing the process.

In this sense, the kinetic profile (Figure 3a) shows transient accumulation of NO_2^-–N. This result indicates that the transient accumulation of NO_2^- observed during the experiments suggests a temporary imbalance in the denitrification pathway, likely associated with a limitation in the nitrite reduction step. Although this behavior could be consistent with a transient inhibitory effect of APAP, the current data do not allow the identification of the specific enzymatic target. Therefore, it is not possible to confirm whether nitrite reductase (Nir) was directly affected. This interpretation should be considered as a hypothesis, and further studies involving direct enzymatic activity assays would be required to elucidate the underlying mechanism. However, this effect was reversible as the APAP concentration decreased. Despite this, the denitrification process was complete, since 100% $E_{NO3^-\text{-}N}$ and $E_{APAP\text{-}C}$ of 83.27 ± 0.86, were achieved, as well as $Y_{N2\text{-}N}$ and $Y_{HCO3^-\text{-}C}$ values of 1.018 ± 0.02 and 0.93 ± 0.007, respectively (Table 2). In relation to these results and the material balance (Table 3), the fact that the molecular nitrogen yield was close to 1 and the bicarbonate yield was higher than 0.93, together with the remaining APAP–C determined (1.042 ± 0.053), demonstrates that the denitrification process was clearly dissimilatory in nature. According to the mass balance (Table 3), where most of the carbon and nitrogen were recovered as HCO_3^- and N_2, respectively, the process appears to be predominantly dissimilatory. Although biomass production was not directly measured, these results suggest that NO_3^-–N and APAP–C were primarily used as energy sources under the evaluated conditions. This observation supports the potential application of the denitrification process for the mineralization of pollutants with low biomass generation, which is relevant for its application in wastewater treatment systems, where excessive biosolids production represents an operational constraint.

Regarding reports in the literature, studies using 10 mg L^{-1} of 17β-estradiol (E2) reported a specific consumption rate ($q_{C\text{–}E2}$) of 1.44 ± 0.01 mg C $gSSV^{-1}$ h^{-1} [34], which is 5.3 times higher than the $q_{C\text{–}APAP}$ obtained in the present study. Similarly, assays with

5 mg L^{-1} of AMP–C reported a specific consumption rate ($q_{AMP–C}$) of 4.35 ± 0.14 mg C g SSV^{-1} h^{-1} [50], equivalent to 15.9 times higher than that obtained in this work. These results indicate that although denitrifying sludge can use various contaminants as electron donors and efficiently consume and mineralize them, differences in kinetic behavior suggest that the degradation rate strongly depends on the chemical structure of the electron donor [61]. In this study, APAP–C was used as the sole electron source; it remains to be investigated whether co-metabolism could enhance consumption rates, as observed in other studies [36].

Regarding the specific rates of the nitrogen species (Figure 4 and Table 4) obtained in the denitrifying assays with APAP–C, these were compared with those obtained in the reference assays. The results indicated that q_{NO3^-N} in the reference assays was 26.8% higher than $q_{NO3\text{-}N}$ with APAP-C, demonstrating a reduction in $q_{NO3\text{-}N}$ in the presence of APAP–C. The consumption rate of CH_3COO^-–C was 19 times higher than the consumption rate of APAP–C; however, the NO_3^-–N consumption rate was only 1.4 times higher in the reference assays with CH_3COO^-–C than in the assays with APAP–C. These results indicate that the presence of APAP–C exerts an inhibitory effect on the denitrification process of the sludge, which may be attributed to the complexity of the chemical structure of acetaminophen and to the formation of several intermediates during the biodegradation process, such as 4-aminophenol, hydroquinone, and oxalic acid, among others [4,54,60]. However, under the conditions reported in the present study, the denitrifying bacterial consortium was able to biodegrade up to 83% of the tested concentration (6.0 mg APAP–C L^{-1}) while simultaneously reducing NO_3^-–N to N_2. Therefore, it is concluded that the denitrification process was complete.

In agreement with other authors' reports on the acclimation and metabolic stimulation of activated sludge, they emphasize the need for further research to evaluate whether the use of more readily biodegradable energy sources could increase the co-metabolic rate in denitrifying sludge systems [62,63].

Several studies have demonstrated that the availability of rapidly assimilable substrates enhances enzymatic activity and microbial adaptation, which could translate into higher APAP degradation rates. Therefore, it is relevant to further explore this approach to assess its feasibility and optimize the process's operational conditions.

In this study, APAP–C was used as the sole electron source, demonstrating the denitrifying of the consortium's capacity to simultaneously biodegrade the carbonaceous substrate and completely reduce nitrate. The results of this study not only confirm the denitrifying consortium's capability but also highlight its potential application in biological treatment systems for effluents containing APAP and nitrates.

However, future research is recommended to explore co-substrate strategies, as well as to evaluate the post-treatment condition of the sludge and its potential for regeneration. Nevertheless, the results showed that the denitrifying consortium maintained its metabolic activity under the tested conditions. This behavior is consistent with previous studies reporting the functional stability and resilience of denitrifying sludge systems under various operational conditions [40,41]. Further research is required to assess changes in sludge characteristics after treatment, which will help expand the available knowledge and support the potential application of this process under real-world conditions.

4. Conclusions

The denitrifying sludge demonstrated the ability to successfully mineralize acetaminophen (APAP) under denitrifying conditions, converting 83% of the APAP carbon to bicarbonate (HCO_3^-–C) and completely transforming the nitrate-derived nitrogen into molecular nitrogen (N_2). These results confirm that denitrification can be directly linked

to APAP mineralization, highlighting its potential as a tool for removing APAP from wastewater.

The presence of APAP influenced substrate consumption and production rates, indicating that this compound affects microbial activity. While these findings were obtained using synthetic wastewater, future studies should evaluate performance in real wastewater to determine its practical applicability. Mechanistic studies are also needed to identify the enzymatic pathways involved and any potential inhibitory effects.

Further research is required to extend these findings to other emerging contaminants and optimize process efficiency under varying operating conditions.

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