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Heterotrophic denitrification for the simultaneous reduction of nitrates using acorn cups as an energy source

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Nitrates are widely present in aquatic ecosystems, posing a threat to the environment and human health. Heterotrophic denitrification using plant-based biomaterials has emerged as an effective method for removing nitrates from contaminated waters. This study investigates the mechanisms and influencing factors of heterotrophic denitrification, using acorn cups as a plant-derived solid organic carbon source for denitrifying bacteria. The results highlight the distinctive characteristics of acorn cups, their effectiveness in supporting bacterial denitrification, and their notable physical stability. The results of analyses conducted using Fourier-transform infrared spectroscopy, atomic absorption spectroscopy, and X-ray fluorescence revealed a high carbon content and the presence of trace elements, which provide essential nutrients for denitrifying bacteria. In addition, scanning electron microscopy revealed a highly porous structure that enhances microbial adhesion. This low-cost, underutilised biomaterial often considered a by-product demonstrated strong denitrification efficiency, achieving a nitrate removal rate of 95.38 ± 0.13 % at neutral pH and up to 99.72 ± 0.17 % under high biomass conditions. Based on these results, it can be concluded that acorn cups represent a technically, economically, and environmentally promising solution for biological denitrification.

Keywords Acorn cups, Wastewater treatment, Physicochemical characterisation, Environmental biotechnology, Heterotrophic denitrification

Conventional methods for removing pollutants from wastewater include co-precipitation[1–7], electrochemical techniques[8], and adsorption[9].

In recent years, the doubling of the population (UNPF) and the subsequent demand for food, water, and energy have led to increased food production and extensive use of fertilisers and pesticides, which have dispersed into the environment. Excessive nitrogen fertilisation, defined as the application of nitrogen-based fertilisers in quantities beyond crop needs, along with inappropriate disposal of sanitary and industrial wastes, has resulted in widespread nitrate pollution in ground and surface waters through recharge, surface runoff, infiltration, and atmospheric deposition[10–12].

The most important sources of nitrate in groundwater are nitrogen contained in fertilisers and untreated or improperly treated industrial and domestic waste waters[13]. Consequently, the intake of excessive nitrate from nitrate-polluted drinking water promotes nitrite-induced methemoglobinemia and nitrosamine-induced malformations[14]. This is why nitrate is considered a priority pollutant due to its toxicity, as it is known to form carcinogenic compounds in the digestive system[15,16]. Nitrate contamination in groundwater is particularly serious in arid and semi-arid regions[17], as it can directly trigger surface algal blooms and indirectly harm human health[18–20]. Therefore, proper remediation is especially needed, as populations in these regions often depend on groundwater as their primary drinking-water source[21]. Thus, a standard of around 50 mg/L of (NO_3^-) in drinking water, according to Directive 98/83/EC of the Council of the European Union of 3 November 1998 was adopted in many countries[7]. To protect the aquatic environment, it is essential to use technically viable

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and economically feasible methods. Reverse osmosis, ion exchange, distillation, and electrodialysis are physicochemical techniques used for the removal of nitrate from drinking waters[22]. The main disadvantages of these processes are their high operational costs, low selectivity, and generation of secondary brine waste. Moreover, these processes are expensive and unsuitable for in-situ applications. For these reasons, biological denitrification is considered the most favourable approach for nitrate removal, due to its high efficiency and low cost[23–25]. Microbial denitrification is regarded as the dominant pathway for nitrogen loss in aquatic systems[26,27], with bacteria using nitrite (NO_2-N) as an electron acceptor and NH_4-N as an electron donor, and releasing N_2 as a product[28]. Denitrification and anammox processes are influenced by oxygen levels, temperature, salinity, pH, and organic matter availability[29–31]. In many carbon-deficient environments, such as sediments, groundwaters, and wetlands is limited[18,31–33], due to a low C/N ratio (<2), indicating insufficient organic carbon content to fully support the heterotrophic denitrification (HD) process[34]. Therefore, proper dosing of organic carbon is required for effective denitrification. A slight overdose of carbon has been suggested to ensure complete nitrate removal for the reliable treatment of nitrate-contaminated drinking water[35]. Although solid organic carbon-based denitrification systems have been validated, few studies have reported their practical application[34]. The carbon source to be added must meet certain criteria, such as low cost, non-toxicity, easy storage, and support of biomass production without microbial adaptation[36]. Natural biomass rich in carbon, mostly from agricultural or forestry co-products, offers both environmental and economic benefits when used as a supplementary carbon source in heterotrophic denitrification[37]. Therefore, this study aimed to select a carbon source from an original biomaterial used for the first time for heterotrophic denitrification. It possesses all the characteristics of an ideal biomaterial: inexpensive (co-product), biodegradable (environmentally safe), and requires no complex preparation equipment. Thus, biological denitrification using simple laboratory-scale batch reactors can be evaluated. Acorn cups, a co-product of acorns known for their tannin content[38], also provide natural dyes such as beige, grey, and black from the Valonia oak (Quercus macrolepis). Acorn cups are widely regarded as key materials for natural dyeing because of their high tannin content. Despite some toxicity, acorns have long been consumed dried, raw, or cooked and are nutritionally comparable to cereals, containing carbohydrates, fats, fibres, proteins, amino acids, and vitamins (A, C, and B6)[39]. They are also used as livestock feed[38]. In several Mediterranean countries, they are considered edible fruits used in desserts, liqueurs and ice cream. In Algeria, Morocco, and parts of the USA, acorn oil is produced, while in North Africa, acorns are directly consumed in products such as bread, cake, and coffee. Acorn bread was once baked in Italy, and acorn coffee is still produced in some countries such as Japan[40–43]. The structure and physical and chemical properties of the carbon source (acorn cups) were evaluated using FTIR, SEM, AAS, XRF, and BET analyses to assess their porous nature and nutritive potential for denitrifying bacteria. Additionally, parameters such as initial nitrate concentration, nitrite concentration, pH, and biomass content were analysed using UV–Visible spectrophotometry and a pH meter to study the behaviour of the denitrification process.

Materials and methods

The acorn cups used in this study were obtained from a herbalist's shop and sourced from coastal regions of Algeria. They were derived from Cork Oak (Quercus suber), one of the most characteristic tree species of Mediterranean vegetation in Algeria. The acorns produced by the Cork Oak are small and distinguished by woody cups and a hard shell.

Preparation of acorn cups sample and characterisation

The grinding step was essential for the evaluation and characterisation of the biomaterial, as the analytical instruments require finely powdered samples for accurate analysis. Initially, the acorn cups were rinsed and oven-dried at 200 °C for 10 min. The samples were then ground using an electric coffee grinder until a fine powder was obtained. Finally, the powder was sieved to ensure homogeneity.

Particle size distribution was assessed using a vibratory sieve shaker of the Retsch type (TAM-DCC-001) AS 200 with a stain less steel mesh. A sample mass of 100 g was used, and sieving was performed at 45 rpm for 20 min.

The particle size composition of the representative sample was evaluated using an electromagnetic sieve shaker (TAM-DCC-001, Retsch type AS 200). The agitator ensured uniform motion of the sample to obtain optimal sieving results. Each sieve was removed, and the retained acorn powder was carefully brushed out. The powder was then weighed using a Kern ABT 320-4 M analytical balance (320 g / 0.1 mg precision), model DSAE-001, using a watch glass, and the average values were recorded.

Moisture content is a key quality indicator for several essential reasons: it ensures optimal microbial activity, promotes effective nitrate adsorption, and contributes to a stable environment conducive to efficient denitrification. Additionally, accurate moisture assessment is crucial for the proper storage of biomaterials. This supports their stability, optimises storage conditions, prevents degradation, and facilitates logistical management. Effective moisture control not only maintains optimal denitrification performance, but also prolongs the durability and preserves the quality of biomaterials throughout their lifecycle. It also provides important information regarding the product's resistance to spoilage during storage.

According to AFNOR B.51004, moisture content is determined by weighing 3.09 g of the sample in a crucible. The sample was then placed in an oven at 105 °C for 3 h until a constant mass was achieved. Once dried, the crucible was cooled in a desiccator, and the final mass was recorded.

The moisture content was calculated by comparing the mass of the sample before and after drying. Sinhaneti et al.[44], reported that the water content is determined using the following formula (1):

$$\mathrm{MC}\,(\%) = \frac{m_0 - m_1}{m_0} \times 100 \tag{1}$$

where m_o and m_1 are the mass (g) before and after drying, respectively.

Various analytical techniques were employed to chemically characterise the acorn cups. These include Fourier-transform infrared (FTIR) spectroscopy, a phenotypic method used to quantify the absorption of infrared radiation by molecular components such as lipopolysaccharides, lipids, carbohydrates, nucleic acids, and proteins. This absorption generates a specific FTIR spectrum that reflects the overall biochemical composition of the sample[45]. In this study, the infrared spectra of the powdered acorn cups were obtained using a Perkin Elmer FTIR spectrometer, operated with Spectrum V5.3.1 software. A deuterated triglycine sulfate (DTGS) detector was employed to record the spectra, with a scanning frequency range of 650–4000 cm^{-1} and a resolution of 2 cm^{-1}. A representative spectrum of the sample is presented.

Atomic absorption spectrometry (AAS) was employed to determine the concentrations of chemical elements in environmental samples by analysing the radiation absorbed by the target element[46]. The method, as described in the *Atomic Absorption Data Book*, involves ashing the acorn cup samples under the combined effect of heat and nitric acid at a temperature not exceeding 450 °C for 8 h using a Carbolite furnace (model ESE/1210) to decompose the organic matter.

Subsequently, 1 g of the resulting inorganic residue was dissolved in dilute hydrochloric acid on a hotplate (model SHOT INSTRUMENT: SLK1) set at 150 °C. The ash solution was then diluted with 50 mL of deionised water and analysed using Vapour Graphite Furnace Atomic Absorption Spectrometry. According to the operating protocol of the equipment, standard solutions of the target metals (Co, Cu, Fe, Mn, Mg, Ni, Zn) were analysed using Air/Acetylene Flame Atomic Absorption Spectrometry, based on the Analytik Jena Zeenit 700P model. This instrument was also used for the analysis of additional metals (Li, Cr, Se, Cd, Ba, Pb, Na, and K).

The natural sample was crushed and compressed into a solid pellet using a hydraulic press to ensure a homogeneous surface for analysis and optimal interaction between the X-rays and the sample. This facilitated the analysis of major and trace elements using a Bruker-Axs Sequential Wavelength Dispersive Spectrometer (S8 TIGER) equipped with a rhodium anode and data-processing software (Spectra Plus). The resulting pellet was exposed to a primary X-ray source, which excited the atoms and caused the emission of secondary fluorescence X-rays characteristic of the sample's chemical composition.

Scanning Electron Microscopy (SEM) is primarily a high-resolution imaging technique. The use of appropriate detectors enables a wide range of material analysis applications. This approach integrates several complementary techniques, including elemental analysis, electron diffraction, and induced current measurements. Together, these techniques allow for the acquisition of comprehensive data, supporting to a deeper interpretation of the phenomena under investigation.

The specific surface area of the powdered sample was determined through nitrogen adsorption, measured at the boiling point of liquid nitrogen under atmospheric pressure.

The data were analysed using the Brunauer–Emmett–Teller (BET) method, employing a Quanta Chrome CHEM-BET 3000 instrument. As the sample (acorn cups) is organic, it was first oven-dried for 48 h at 60 °C to eliminate residual moisture. The dried sample was then weighed, with an initial mass of 0.5877 g. To preserve the structural integrity of the material, degassing was conducted at 80 °C. The katharometer detector baseline was adjusted, and the current was set to 120 A for analysis. Multiple measurements were performed for nitrogen adsorption using different signal attenuation settings to determine the most reliable output. The optimal attenuation level, within the range 2–32, was found to be 2. The final signal measurement yielded an average of 177 counts based on three replicates (170, 170, and 190 counts). The specific surface area was calculated using the single-point method according to Eq. (2):

The specific surface area (Ss) is given by the ratio of the total surface area (ST) to the sample mass (m):

$$Ss = \frac{\mathrm{S} \times \mathrm{T}}{\mathrm{m}} \tag{2}$$

The total surface area (ST) was calculated using the following equation:

$$\mathrm{ST} = \left(\frac{\mathrm{A}}{\mathrm{Acal}}\right) \times \mathrm{V_{cal}}\,\mathrm{Acs} \times (\mathrm{Pa} \times \mathrm{N/R} \times \mathrm{T}) \times \left(1 - \frac{\mathrm{P}}{\mathrm{P0}}\right) \tag{3}$$

The analysis was conducted under the following conditions: the sample was degassed for 50 h, and the first two adsorption measurements were recorded after 24 h. The calibration volume was set to 3 mL, with a calibration signal of 190 pulses.

Method of analysis of heterotrophic biological denitrification by acorn cups

Preparation of acorn cups sample for denitrification

For the heterotrophic biological denitrification of nitrate-laden water, acorn cups were crushed into coarse particles ranging from 1 to 5 mm, which were used as a carbon source for denitrifying bacteria. Fine powders were avoided due to their high tannin content, which could excessively colour the water during the washing stage. To minimise the release of coloured compounds and enable accurate nitrate monitoring, the crushed acorn cups were soaked in warm water for three days, with the water renewed every 24 h until it turned clear. This leaching process was conducted at a moderate temperature (approximately 35 °C) rather than boiling to facilitate the removal of tannins and pigments without significantly altering the organic carbon content. A preliminary chemical characterisation confirmed the material to be rich in potentially bioavailable organic compounds. Although no post-soaking chemical analysis was performed, this approach is supported by other studies involving plant-based materials. For example, Vítězová et al. (2019) demonstrated that spent coffee grounds retain sufficient organic matter to support microbial respiration and heterotrophic denitrification, even

after exposure to boiling water during coffee preparation. This finding reinforces the assumption that moderate soaking does not compromise the capacity of acorn cups to serve as a carbon source for denitrifying bacteria[47]. Nitrate concentrations in the samples were measured using UV–visible molecular absorption spectrophotometry, with sodium salicylate as the analytical reagent[48,49].

Effect of parameter variation

An experiment was conducted in batch mode using a beaker containing acorn cups and nitrate-contaminated water. The mixture was mechanically agitated at 600 rpm under ambient temperature. When initiating a biological reactor, the primary challenge is to develop an active sludge capable of degrading the target pollutant[50]. Denitrifying bacteria are ubiquitously distributed in various environments worldwide[51]. Therefore, inoculation was carried out using activated sludge specifically acclimated to the substrate and experimental conditions[50]. Denitrifying bacteria include species from various genera of Schizomycetes, with Halobacterium being a representative genus of the Archaebacteria[51]. As wastewater treatment plants typically harbour diverse and functionally competent microbial communities, a sample was collected from the Boumerdes Wastewater Treatment Plant, affiliated with the National Office of Sanitation (ONA) in Algeria. This sample contained denitrifying bacteria originating from the plant's activated sludge[50]. The protocol was adapted from the batch-mode denitrification methods described by Gibert et al.[52] and Greenan et al.[53], with modifications designed to enhance the proliferation of denitrifying bacteria. These adjustments were inspired by the methodologies described by Jeter et al.[54], Blaszczyk et al.[55], and Benbelkacem et al.[50], including minor changes in the composition of the added salts. The activated sludge used as the microbial inoculum was collected and handled in accordance with standard biosafety procedures, including the use of gloves, laboratory coats, and protective eyewear. No live microorganisms were released into the environment during the experiment. This approach of sourcing denitrifying bacteria from wastewater treatment plant sludge is commonly employed in laboratory-scale denitrification research. Similar batch-mode systems have been reported (e.g., Sahinkaya et al.[13], Jafari et al.[11], Benbelkacem and Benrachedi[50], Gibert et al.[52]) To prepare a solution with a nitrate concentration of 50 mg/L, 81.45 mg of KNO_3 was weighed and transferred into a 1000 mL volumetric flask. The following salts were then added: 1 mg of KH_2PO_4, 1 mg of NaCl, 0.20 mg of $MgCl_2$, 0.02 mg of $CaCl_2$, and 0.0001 mg of Na_2SO_4. Finally, distilled water was added to the flask until it reached the calibration mark. To identify the most favourable conditions for biological denitrification in the presence of crushed acorn cups, physical parameters were varied. In a 250 mL beaker, 100 mL of synthetic water (containing nitrate concentrations equal to or greater than 50 mg / L) was combined with 10 mL of wastewater rich in denitrifying bacteria. These bacteria were sourced from the activated sludge of the Boumerdes wastewater treatment plant (Algeria)[50]. Studies have estimated that approximately 10 % of the recoverable bacterial flora from soils, sediments, and aquatic environments are denitrifiers, with common genera including Pseudomonas and Alcaligenes[56,57]. Subsequently, a quantity of crushed, pre-soaked acorn cups of approximately 1 g / L was added, and the mixture was stirred at 600 rpm under neutral pH conditions. To evaluate the effect of acorn cups as a carbon source, a control experiment was also conducted, omitting any external organic material. This control followed the same protocol: 100 mL of synthetic nitrate-contaminated water (≥ 50 mg / L), 10 mL of inoculum from the Boumerdes treatment plant, stirred at 600 rpm under ambient conditions, and pH adjusted to neutral. The absence of carbon in this control allowed us to establish a baseline for denitrification performance under unsupplemented conditions. The preparation of the bacterial inoculum was adapted from the method proposed by Paixào et al.[58]. Activated sludge from Boumerdes was first homogenised and centrifuged at 3000 rpm for 10 min. The obtained pellet was washed twice with sterile saline solution (0.85 % NaCl) to eliminate impurities, resuspended in saline, and adjusted to an optical density (OD_{600}) of 0.5, corresponding to approximately 10^8 CFU / mL, was inoculated at 5 % (v/v) in batch reactors, as described by Zhang et al.[59]. This standardised inoculum ensured experimental consistency and enabled reliable comparisons, particularly with the control group. All experiments, including the controls, were conducted in triplicate to ensure repeatability. The parameters monitored included pH, nitrate concentration, nitrite concentration, and nitrate removal percentage, in relation to time, initial nitrate concentration, and acorn cup biomass. Analyses were conducted using UV–visible spectrophotometry and a pH meter. Nitrate quantification was performed using the sodium salicylate method[48,49]. 10 mL of the sample was mixed with 1 mL of sodium salicylate and 2 to 3 drops of 30 % NaOH, then evaporated to dryness at 75–88 °C. The resulting residue was treated with 2 mL of concentrated H_2SO_4 and left for 10 min before being diluted with 15 mL of distilled water and buffer solution. Absorbance was recorded at 415 nm[60–62]. For nitrite quantification, a mixed reagent was first prepared by dissolving 20 g of sulfanilamide and 1 g of N-(1-naphthyl) ethylenediamine in 50 mL of phosphoric acid (H_3PO_4). The final volume was adjusted to 500 mL using distilled water. Then, 1 mL of this reagent was added to 50 mL of the water sample. After 15 min of reaction, the development of a pink colour indicated the presence of nitrite ions (NO_2^-). Absorbance was measured at 543 nm using a spectrophotometer[60,63,64].

Statistical analyses

All quantitative measurements were processed and analyzed in R (version ≥ 4.5.0). Initially, the Shapiro–Wilk test was applied to model residuals to verify normality, and Levene's test (center = median) from the "car" R package was used to assess homogeneity of variances. In cases where both tests yielded $p > 0.05$, data were subjected to one-way ANOVA, followed by Tukey's HSD post-hoc comparisons via "agricolae" R package in order to assign statistically distinct groups. Whenever at least one assumption was violated ($p \leq 0.05$), a non-parametric approach was adopted: Kruskal–Wallis, succeeded by pairwise Wilcoxon rank-sum tests with Benjamini–Hochberg adjustment, and letter-grouping. Significance was defined at $\alpha = 0.05$. These group labels (a, b, c…) accompany means ± SD on Figs. 6 and 7.

Parameters	Gland cups result
pH	5.1
Dominant weight	30.65 g Particules between 200 and 355 µm
The finest particules	Less than 20 µm; smallest weight 0.021 g
Moisture content (H %)	5.7 %
BET SA	14 m^2 / g
Pore diameter	696 nm; 995 nm; 1.11 µm; 3.05 µm; 1.39 µm; 6.01 µm; 6.62 µm

Table 1. Summary of the physicochemical properties of the acorn cup powder used as a carbon source.

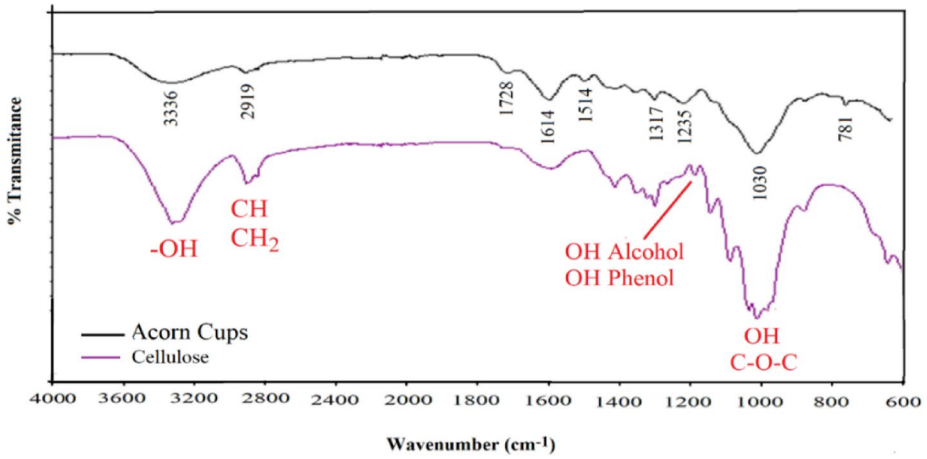


Fig. 1. FTIR spectral overlay of the acorn cup powder and reference cellulose, highlighting the common absorbance bands corresponding to key functional groups. (Sadtler IR spectral database)[65].

To evaluate the catalytic effect of acorn-cup biomass relative to abiotic controls, Welch's two-sample *t*-test was performed on triplicate data for each time point (NO_3^-, NO_2^-) and each initial NO_3^- level (removal %, pH). Differences were considered significant at $\alpha = 0.05$.

Results and discussion

Result of analysis of the carbon source (acorn cups)

Particle size distribution analysis was conducted on the powdered acorn cups to ensure suitability with the analytical instruments used for characterisation (Table 1). The results indicate that the majority of the particles fell within the 200–355 µm range, with the dominant weight recorded at 30.65 g. A minor proportion of the sample consisted of particles smaller than 20 µm, with the smallest fraction weighing only 0.021 g. The moisture content of the acorn cup powder was 5.7 %, indicating low water retention and high dry matter content. This low moisture level is advantageous for storage, as it enhances the stability of the material during handling and processing. Moisture content may vary depending on the acorn species or the drying intensity. Surface area analysis, performed using the Brunauer–Emmett–Teller (BET) method, revealed a specific surface area of 14 m^2 / g. This value suggests a sufficient degree of porosity and surface availability to support microbial adhesion, which is a crucial factor in promoting bacterial colonisation during denitrification. Pore diameter distribution was also assessed and revealed a range of pore sizes, including 696 nm, 995 nm, 1.11 µm, 1.39 µm, 3.05 µm, 6.01 µm, and 6.62 µm. The presence of.

both micro- and macropores likely facilitates water penetration and enhances microbial infiltration and colonisation within the structure of the biomaterial.

Fourier-transform infrared (FTIR) spectroscopy confirmed the presence of cellulose and starch in the acorn cups. Figure 1 shows an overlay of the FTIR spectrum of acorn cup powder and a reference cellulose sample, with strong similarities observed in the absorbance bands, particularly those corresponding to the O–H, C–H, and C–O–C functional groups.

Figure 2 shows a similar comparison between the acorn cup spectrum and that of the starch reference, further supporting the presence of plant-based polysaccharides. The FTIR spectra presented in this study were obtained from dried and ground acorn cups, with no chemical or thermal treatments applied apart from oven drying at 200 °C for 10 min. This ensured that the spectral data reflected the native functional groups of the untreated biomaterial.

Figure 3 displays the FTIR spectrum of the acorn cup powder, highlighting the characteristic absorbance bands: (O–H) at 3336.25 cm^{-1}, (C-H) and CH_2 at 2918.66 cm^{-1}, (O–H) from alcohols and phenols at 1235.04 cm^{-1}, and (C–O–C) at 1029.90 cm^{-1}. These findings indicate that acorn cups are rich in plant-based polysaccharides,

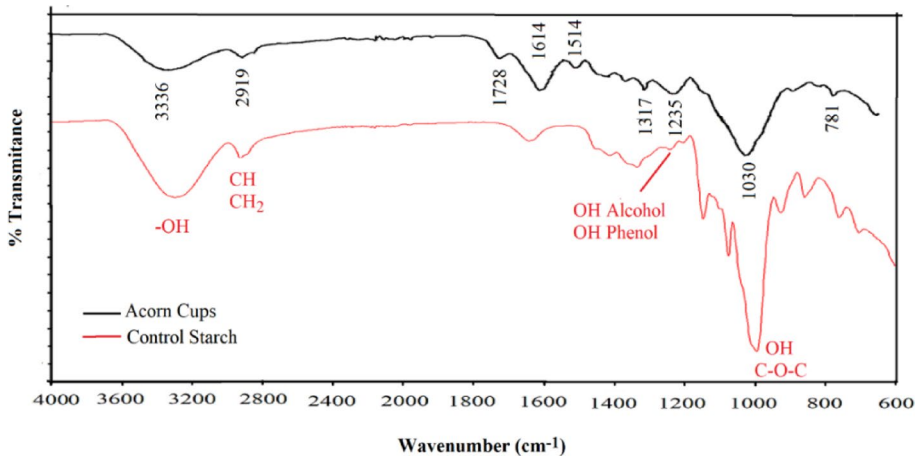


Fig. 2. FTIR spectral overlay of acorn cup powder and reference starch, showing similarities in the characteristic absorption peaks of polysaccharide structures. (Sadtler IR spectral database)[65].

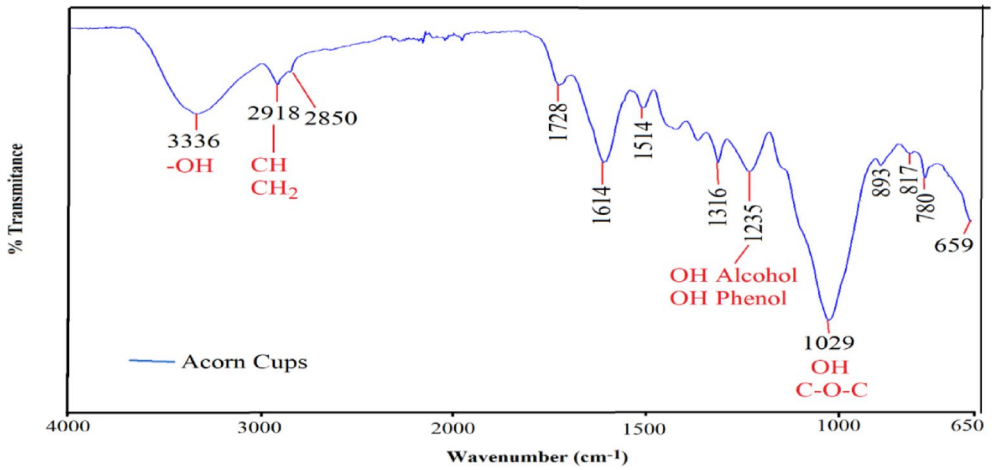


Fig. 3. FTIR spectrum of the acorn cup powder, indicating the main functional groups detected in the untreated biomaterial.

particularly cellulose and starch, which are relevant for biological applications. Cellulose, the most abundant renewable organic polymer, serves as a slowly degradable carbon source that supports sustained microbial activity during denitrification. Numerous studies have highlighted the importance of cellulose and lignin in facilitating bacterial attachment and promoting microbial colonisation in denitrifying systems. Cellulose is recognised as the most abundant renewable resource globally[66] and may provide a cost-effective alternative to refined carbon compounds commonly employed in the treatment of nitrate-contaminated water[67]. Although not directly quantified in this study, lignin is likely present in the acorn cups and contributes to the structural integrity and slow degradation of the biomass, thereby supporting a prolonged release of carbon. According to Zhang et al., lignin can hinder the enzymatic hydrolysis of polysaccharides, indicating that pretreatment or partial removal of lignin may enhance the biodegradability and overall efficiency of the denitrification process[68]. Furthermore, oak acorn cups contain hydrolysable tannins, such as ellagitannins, which are known for their antimicrobial properties and potential to modulate microbial growth. Yin et al., demonstrated that these phenolic compounds can interact with microbial enzymes and proteins, potentially influencing the structure and functionality of denitrifying microbial consortia[69].

Table 2 summarises the main wavenumbers and their corresponding functional group assignments[70]. This tabulated representation offers a clearer interpretation of the functional groups identified in the acorn cup powder. The spectrum confirms the presence of cellulose, hemicellulose, and lignin-derived compounds, as evidenced by the characteristic peaks at approximately 3336, 2918, 1728, 1514, and 1029 cm^{-1}. The presence of tannin-related and aromatic structures is also indicated by bands in the 1600–800 cm^{-1} region, supporting the chemical suitability of this biomass as a source of carbon for denitrifying bacteria.

The results obtained by atomic absorption spectroscopy (AAS) for certain metals (Co, Cu, Fe, Mn, Mg, Ni, and Zn) in our acorn cups sample are listed in Table 3. The sample was also analysed using Inductively Coupled

Wavenumber (cm^{-1})	Vibration Type	Functional Group/Assignment
3336.25	O–H stretching	Alcohols, phenols (hydrogen bonding)
2918.66	C–H / CH_2 stretching	Aliphatic groups (cellulose, starch)
2850.00	C–H symmetric stretching	CH_2 in long-chain fatty acids (waxes, lipids)
1728.00	C=O stretching	Carbonyl groups (acids, esters, hemicellulose, tannins)
1614.00	C=C stretching	Aromatic rings (lignin, polyphenols)
1514.00	C=C stretching	Aromatic skeletal vibrations (lignin)
1316.00	C–H bending	Cellulose, phenolic OH bending
1235.04	C–O stretching/O–H bending	Phenolic compounds and alcohols
1029.90	C–O–C stretching	Polysaccharides (cellulose, starch)
893.00	C–H deformation	β-glycosidic linkages in polysaccharides
817.00	C–H out-of-plane bending	Aromatic compounds
780.00	Ring bending	Aromatic skeletal modes
659.00	C–H out-of-plane bending	Aromatic substituents/lignin rings

Table 2. Main FTIR absorption bands identified in the acorn cup powder and their corresponding chemical bond assignments[70].

Elements	Concentration (mg/L)
Cr	0.280
Se	0.029
Cd	0.004
Ba	3.474
Pb	0.284
Na	46.89
K	730.5
Co	0.104
Cu	0.112
Fe	5.101
Mn	2.819
Mg	1.383
Ni	0.110
Zn	0.190

Table 3. Concentrations of trace and major metals in acorn cup powder as determined by AAS and ICP-MS analyses.

Plasma Mass Spectrometry (ICP-MS) technique to determine additional metals, including Li, Cr, Se, Cd, Ba, and Pb. As shown in Table 3, the acorn cups exhibited very low concentrations of potentially toxic elements such as Cd, Co, Cu, Zn, Li, Se, Pb, Cr, and Ni, while maintaining appreciable levels of Fe, Mg, Mn, Ba and K^+, which are beneficial for microbial growth and denitrification processes. The relatively high iron content explains the yellowish-brown colouration of the solution after immersion of the acorn cups, indicating the presence of ferric iron (Fe^{3+}). Recent studies have shown that ferrous iron (Fe^{2+}) can act as an electron donor, enhancing nitrate removal efficiency under low C/N conditions[71]. Moreover, zero-valent iron (Fe^0) facilitates interspecies electron transfer among electroactive and denitrifying microorganisms, thereby improving denitrification rates[72]. These findings suggest that the naturally occurring Fe in acorn cups may enhance microbial electron transfer mechanisms involved in denitrification. Additionally, the significant presence of sodium and potassium suggests their importance as essential elements for bacterial activity, as reported by Wang, Feng and Deng[73], on the effect of potassium on nitrate removal from groundwater in agricultural waste-based heterotrophic denitrification systems.

The observed potassium ion (K^+) concentration remained below the 1000 mg / L threshold reported in a previous study[74]. Our findings confirm this, as the K^+ concentration in the acorn cups was also under 1000 mg / L, yet still sufficient to ensure effective denitrification. In contrast, when the threshold was exceeded in the cited experiment, nitrate removal efficiency declined, with only 85.73 ± 0.54 mg NO_3–N being removed over three consecutive operating cycles[73]. In our study, atomic absorption spectrometry revealed a K^+ concentration of 730.5 mg / L in 1 g of ash obtained after incinerating a larger quantity of acorn cup powder. This suggests that 1 g of acorn cup powder per litre in the denitrification system contributes less than 730.5 mg / L of K^+. According to Wang, Feng, and Deng[73], a concentration of 229.78 ± 25.80 mg–K / L can enhance the continuous

denitrification capacity using a similar solid-phase carbon source. Therefore, although our measured K^+ levels were higher, they remained within a range that could improve denitrification efficiency without the need for additional potassium. Trace elements are essential for denitrifying bacteria at the nano- or microgram levels. Therefore, these concentrations are sufficient for bacterial growth without resulting in excess metals in the water.

The results obtained by fluorescence X-ray spectrometry (XRF) are summarised in Table 4, which presents the percentage composition of trace elements in the acorn cups that could not be assessed via atomic absorption spectroscopy. As shown in the table, two compounds dominate: calcium oxide (CaO) at 1.98 % and silicate at 1.64 %, whereas all other compounds remain below 0.65 %. Calcium oxide, also known as quicklime, is recognised for its effectiveness in water treatment[75]. It plays key roles in pH regulation, metal hydroxide precipitation, flocculation, and decarbonation via calcium carbonate formation. Roset et al.[76] reported that CaO contributes to pipeline protection by forming stable carbonate films. Additionally, its dissolution releases hydroxide ions and carbonates that create alkaline conditions favourable to denitrifying bacteria[77].

In another study, Zhang et al.[78] utilised the precipitation of quicklime to form calcium carbonate, which provided a protective lining inside water distribution pipes. Their findings highlighted several beneficial effects of CaO, including water softening, pH adjustment, precipitation of metal hydroxides, flocculation, decarbonation via $CaCO_3$, and microbial inactivation. Regarding denitrification, the oxygen and carbonate ions released through CaO dissolution can enhance microbial activity, thereby promoting nitrate removal[79]. Although the concentrations found in our study are not particularly high, they may nonetheless contribute to improving water quality, particularly in systems such as aquaculture, where stable chemical conditions are essential. Based on our findings and supporting literature, we conclude that XRF analysis complements atomic absorption spectroscopy by revealing naturally occurring trace elements crucial to microbial processes. The presence of CaO in particular appears to offer additional benefits for effective nitrate reduction and water purification.

Scanning electron microscopy (SEM) images of acorn cup powder with particle sizes ranging from 0 µm to 355 µm are displayed in Fig. 4. The micrographs reveal a variety of pore shapes and sizes, with measured diameters of approximately 696 nm, 995 nm, 1.11 µm, 1.39 µm, 3.05 µm, 6.01 µm, and 6.62 µm predominantly within the micro- and mesoporous ranges. Previous studies have shown that such porosity facilitates strong bacterial adhesion to solid surfaces when immersed in aqueous environments[50,80]. In this context, the porous architecture of the acorn cup powder supports microbial colonisation, thereby enhancing the attachment and proliferation of denitrifying bacteria.

Result of heterotrophic biological denitrification analysis

As nitrates are frequent co-contaminants in water supplies, it was pertinent to investigate their removal under denitrifying conditions[81]. The control experiment, conducted without the addition of acorn cup biomass, provided valuable insights into the denitrification potential of the indigenous bacterial community under carbon-limited conditions. The results, presented in Fig. 5, illustrate the system's behaviour over the monitoring period. All experiments were carried out in triplicate, and the data represent mean values with their

Name	Acorn Cups %
Na_2O	0.0600
MgO	0.4300
Al_2O_3	0.6500
SiO_2	1.6400
P_2O_5	0.1100
SO_3	0.1500
K_2O	0.6200
CaO	1.9800
TiO2	0.0200
MnO	0.0100
Fe_2O_3	0.1500
Cr_2O_3	< 0.0010
NiO	0.0013
CuO	0.0042
ZnO	0.0020
SrO	< 0.0010
PbO	< 0.0010
BaO	0.0061
ZrO_2	< 0.0010
Cl	0.0200
PAF	94.2000
TOTAL	100.0300

Table 4. Chemical composition (%) of trace compounds in acorn cups as determined by fluorescence X-ray spectrometry (XRF).

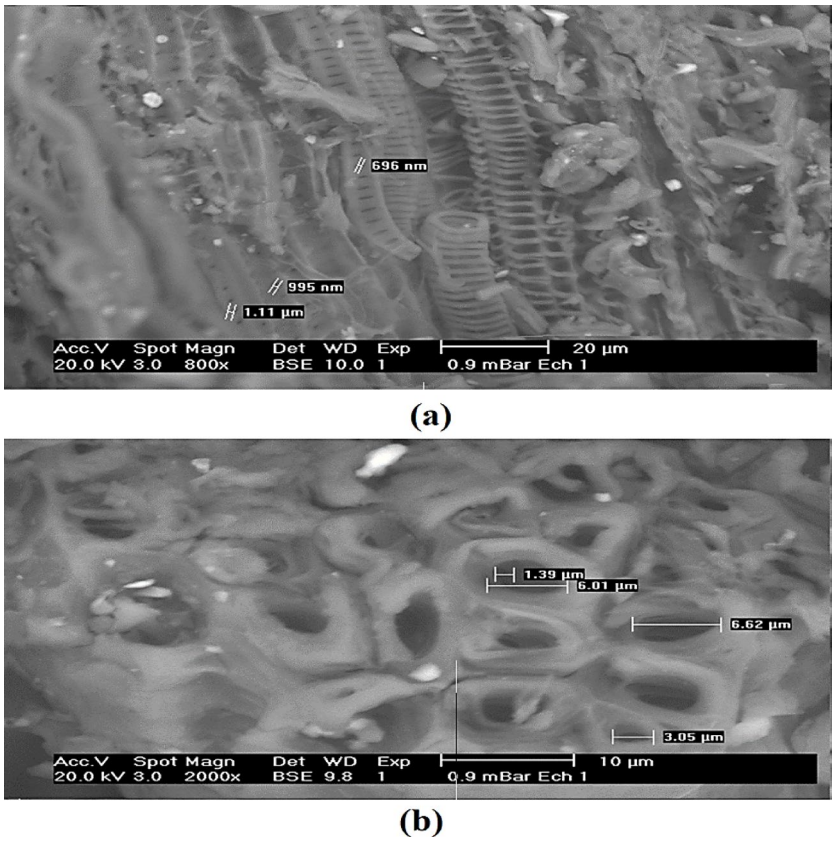
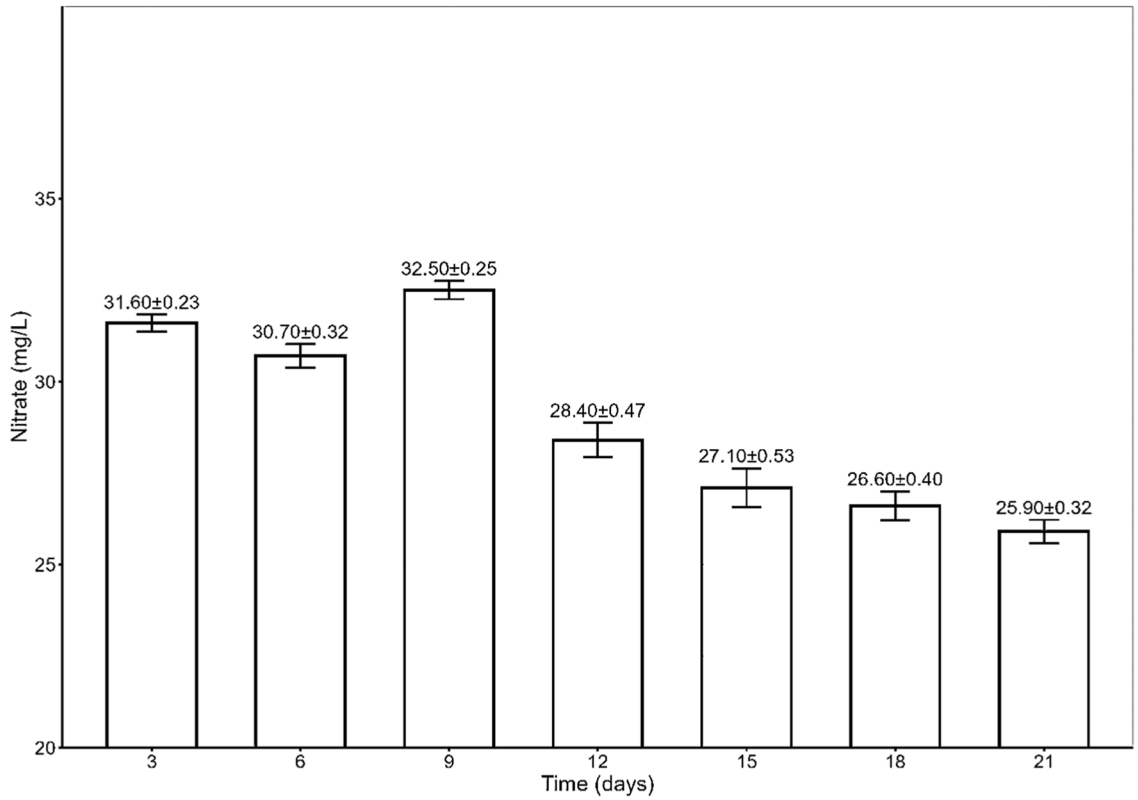


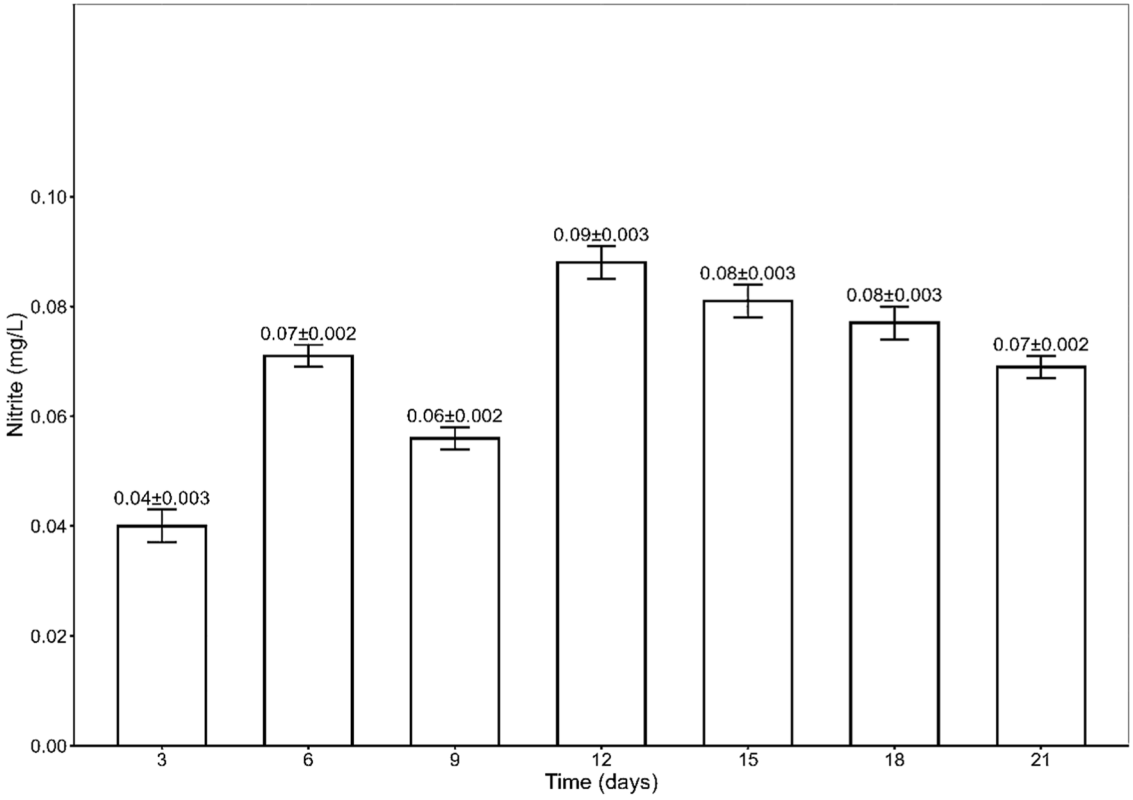
Fig. 4. SEM observation of acorn cup powder at two magnifications. (**a**) Structure with visible tubular and aligned micropores (800 × magnification) with pore diameters ranging from 696 nm to 1.11 µm. (**b**) More irregular mesoporous zones (2000 × magnification), with larger pore diameters between 1.39 and 6.62 µm.

corresponding standard deviations. Figure 5a shows the evolution of nitrate concentrations over 21 days. A gradual decline was observed, reaching approximately a 48 % reduction, which suggests partial nitrate removal through endogenous or residual carbon sources-though the process was limited in overall efficiency. As shown in Fig. 5b, the accumulation of nitrites is evident, peaking on day 9 and remaining elevated thereafter. This trend indicates incomplete denitrification, likely due to a lack of available electron donors. Figure 5c displays variations in pH across different initial nitrate concentrations. The pH remained relatively stable between 7.27 ± 0.07 and 7.05 ± 0.02, reflecting low microbial activity and limited acid production typically associated with active heterotrophic denitrification. Finally, Fig. 5d shows the nitrate removal efficiency as a function of initial nitrate concentration. A decline is observed from 48.2 ± 0.40 % at 50 mg / L to 35.10 ± 1.00 % at 150 mg / L, confirming the system's limited capacity in the absence of an external carbon source. Collectively, these results demonstrate that although some level of nitrate removal is achievable through endogenous activity, the lack of supplementary carbon significantly constrains both the rate and completeness of the denitrification process.

To investigate the temporal variation in nitrate and nitrite concentrations, a biomass concentration of 1 g / L with particle sizes ranging from 1 to 5 mm was added to a nitrate solution with an initial concentration of 50 mg / L and a pH of 7.10. The results, shown in Fig. 6a, illustrate the evolution of nitrate concentration during the denitrification process in the presence of acorn cups as a carbon source. A clear trend is observed: nitrate levels initially decrease, then temporarily increase around day 9, before gradually declining again until day 18. From day 18 to 21, the concentration stabilises, indicating the likely completion of denitrification. The transient increase in nitrate concentration after day 6, peaking on day 9, is attributed to the delayed release of nitrates from the acorn cup matrix. This observation is consistent with the findings of Gibert et al.[52], who reported nitrate release during the composting of organic materials. In the present study, the acorn cups were crushed rather than finely ground, potentially resulting in slower leaching kinetics due to larger particle size and reduced surface area. Incubation time had a pronounced effect on nitrate concentration. One-way ANOVA revealed $F(6,14) = 256.1$ ($p < 0.001$), and Tukey's HSD identified Day 9 (8.34 ± 0.32 mg / L) as significantly higher than all other time points (group "a"), with concentrations decreasing to 2.31 mg / L by Days 18–21 (group "e"). The trend shown in Fig. 6a rises from 7.05 mg / L on Day 3 to a peak on Day 9, followed by a smooth decline through to Day 21. Figure 6b presents the temporal variation in nitrite concentration. Nitrite accumulation is evident, with an initial peak on day 6, a decrease on day 9, and a second peak around day 12. This double-peak pattern likely reflects intermediate steps in the denitrification pathway, where the reduction of nitrate to nitrite occurs more rapidly than the subsequent reduction of nitrite to nitrogen gas. These observations highlight the transient accumulation of nitrites, a phenomenon commonly observed in systems where the availability of electron



(a)



(b)

donors and enzymatic activity fluctuate over time. Nitrite concentration also varied in a time-dependent manner (ANOVA $F(6,14) = 31.4$, $p < 0.001$). The highest accumulation was observed on Day 6 (0.034 ± 0.002 mg / L; group "a"), followed by a plateau between Days 9 and 12 (0.027–0.031 mg / L; group "ab"), and a return to minimal levels (0.010 mg / L; group "c") by Days 18–21 (Fig. 6b).

◀**Fig. 5**. Performance of the control system (without external carbon source). (**a**) Evolution of nitrate concentrations (mg / L) over 21 days under carbon-limited conditions; (**b**) Accumulation of nitrite concentrations (mg / L) over 21 days; (**c**) pH variation corresponding to increasing initial nitrate concentrations (50–170 mg / L); (**d**) Nitrate removal efficiency (%) at different initial nitrate concentrations, highlighting the system's limited denitrification capacity in the absence of added carbon.

It then gradually decreases until day 18, after which it stabilises and remains constant until day 21. The results indicate that the evolution of nitrite concentration follows the classic pattern of biological denitrification: an initial rise due to the reduction of nitrate to nitrite, followed by a gradual decline as nitrites are further reduced.

As explained by Tiedge et al.[82], nitrites represent an intermediate step in the conversion of nitrate to nitrogen gas, following the reaction:

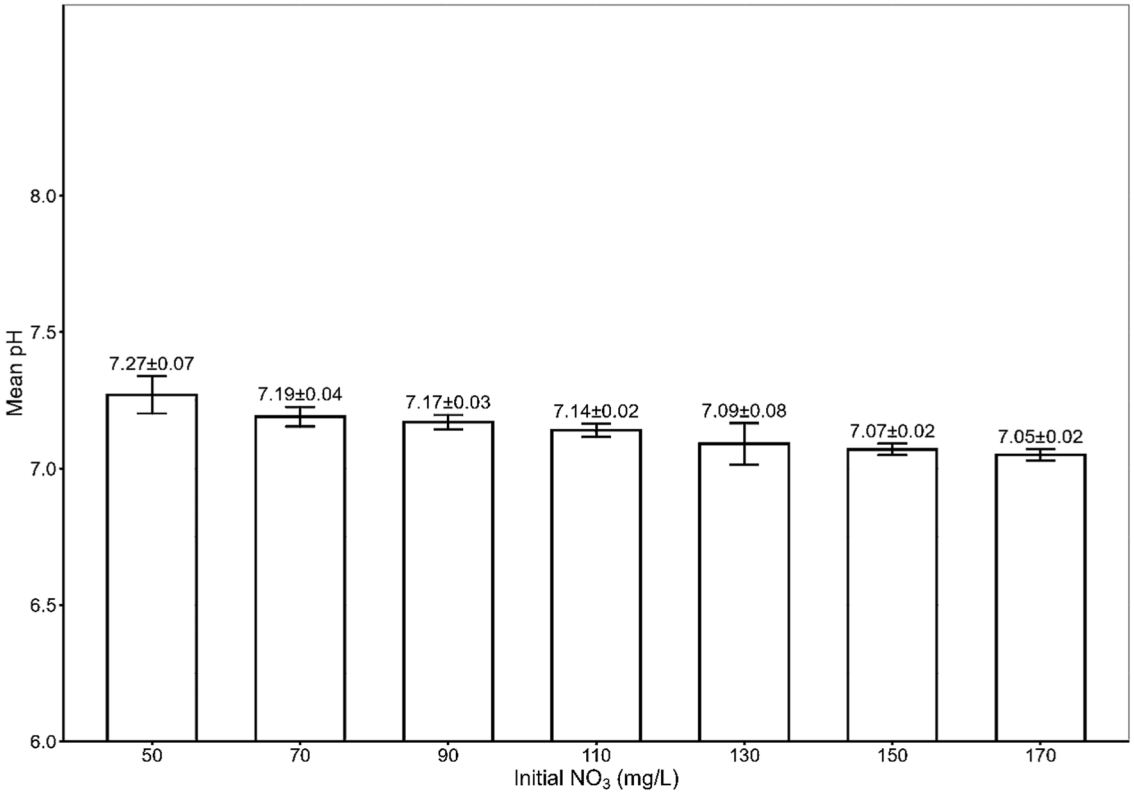
$$NO_3^- \rightarrow NO_2^- \rightarrow N_2$$

In this study, a two-phase pattern was observed, likely reflecting the sequential activity of denitrifying bacteria. Initially, a portion of nitrate is reduced to nitrite, resulting in the first concentration peak. As nitrite is subsequently converted to nitrogen gas, its concentration decreases. A second increase in nitrite concentration corresponds to the reduction of another fraction of nitrate, once again followed by a decrease due to further denitrification.

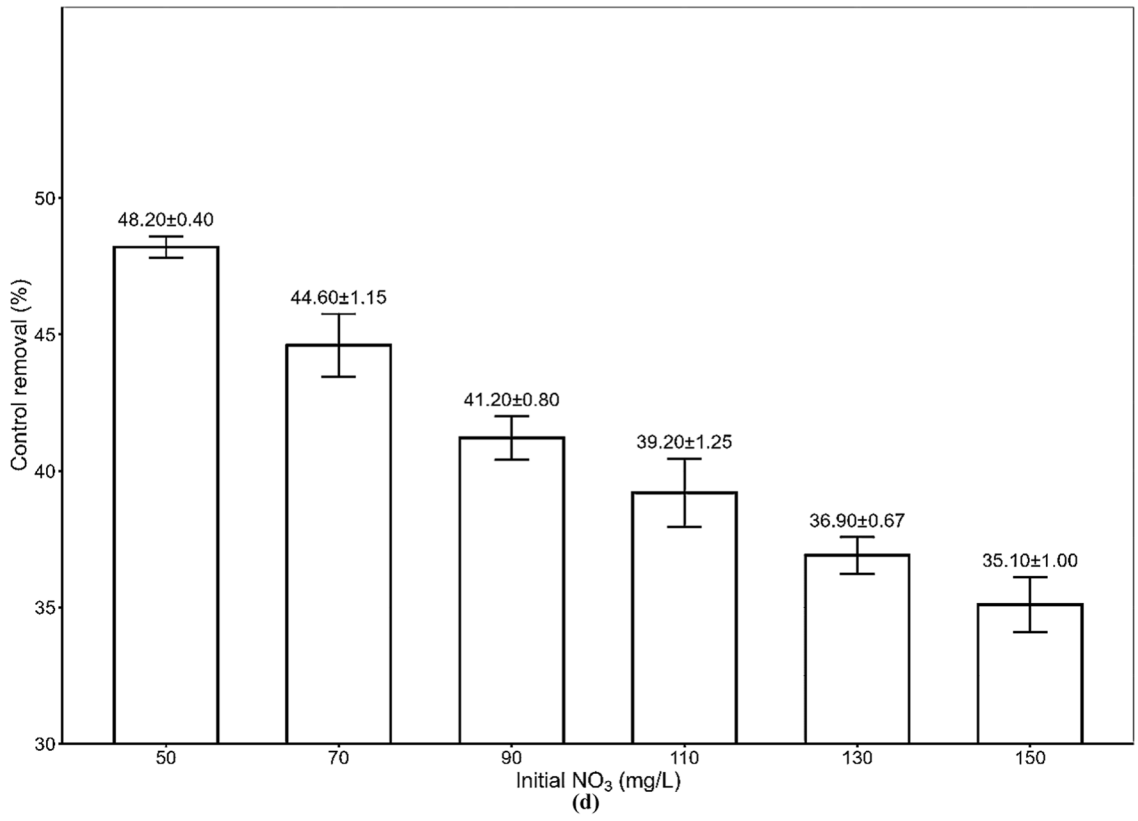
This progression aligns with the extended denitrification mechanism described by Alvarez et al.[83], summarised by the following reaction:

$$NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2.$$

Figure 7 presents the results obtained from the heterotrophic denitrification process using acorn cups as a solid carbon source under varying experimental conditions. Operational parameters were applied to assess the percentage of nitrate removal relative to the initial nitrate concentration in the solution. The tested concentrations were 50, 70, 90, 110, 130, and 150 mg / L, with the biomass dosage maintained at 1 g / L and an initial solution pH of 7.34. As shown in Fig. 7a, the nitrate removal percentage reached a maximum of 95.38 ± 0.13 % at the lowest tested concentration (50 mg / L). The efficiency slightly declined with increasing nitrate concentration, suggesting that although the carbon source remained effective, higher nitrate loads may require an increased supply of biodegradable organic matter. The percentage of nitrate removal displayed a strong inverse relationship with the initial NO_3^- concentration. ANOVA produced $F(5,12) = 190.6$ ($p < 0.001$), and Tukey's HSD revealed a stepwise decline from 95.38 % removal at 50 mg / L (group "a") to 88.37 % at 150 mg / L (group "e") (Fig. 7a). This trend was further supported by linear regression ($R^2 = 0.966$). A biomass concentration of 1 g / L and a pH of 7.31 were selected to monitor the variation in nitrite levels in relation to the initial nitrate concentrations. As shown in Fig. 7b, the concentration of nitrites increased with rising initial nitrate concentration. This trend is consistent with the expected behaviour of the denitrification process, where nitrite serves as an intermediate product. Progressive nitrite accumulation mirrored this pattern (ANOVA $F(6,14) = 216.0$, $p < 0.001$). Starting at a basal 0.010 mg / L (50–90 mg / L, group "e"), NO_2^- rose sharply to 0.082 mg / L at 170 mg / L (group "a"), with a positive correlation ($R^2 = 0.937$) that underscores intensified intermediate build-up at higher loads (Fig. 7b). To monitor the variation in pH in relation to the selected initial nitrate concentrations, the biomass was set at 1 g/L with an initial pH of 7.31. While pH is not a limiting factor for denitrification, it plays an important role in influencing the gaseous products formed during the process[50,84,85]. Denitrification was therefore assessed as a function of both pH and initial nitrate concentration. Figure 7c shows that pH increases as the initial nitrate concentration increases. Compared with Fig. 7a, it is clear that the denitrification rate is higher at lower initial nitrate concentrations. Specifically, for an initial nitrate concentration of 50 mg / L, the pH dropped to 7.02, and the denitrification percentage reached a maximum of 95.38 ± 0.13 %, confirming that denitrification tends to improve under near-neutral pH conditions. Similar findings have been reported in previous studies. Xiao-yu et al.[86] observed an optimum pH of 7.10, while Foglar and Briški[87] and Marinković et al.[88] reported optimum pH values of 7.40. These discrepancies can be attributed to differences in experimental models and conditions. In our case, the use of a slowly degradable solid-phase carbon source (acorn cups) contrasts with systems utilising soluble carbon compounds, such as methanol, or biological models such as cucumber seedlings. Such variations influence the buffering capacity, microbial community composition, and the production of acidic or basic metabolites during denitrification. In our study, the denitrification rate remained above 60 % across a pH range of 5.92 to 8.35. The variation in pH from 5.86 to 7.52 produced a significant change in denitrification efficiency, which is consistent with trends reported in the literature. These pH changes are mainly attributed to microbial metabolism during the denitrification process. However, it is also possible that residual compounds in the acorn cups, despite pre-washing, contributed slightly to initial pH variations. Final pH values declined modestly with increasing nitrate load (ANOVA $F(6,14) = 145.6$, $p < 0.001$). The highest pH (7.02 ± 0.04; group "a") occurred at 50 mg L^{-1}, while the nadir (6.53 ± 0.03; group "d") was observed at 70 mg / L, suggesting acidification through proton release during denitrification (Fig. 7c). The overall pH trend was weakly but significantly correlated ($R^2 = 0.313$). A nitrate concentration of 150 mg / L and an initial pH of 7.34 were used for this experiment. Biomass concentrations of acorn cups were varied from 1 to 10 g / L. The percentage of nitrate removal increased with the biomass concentration, reaching a maximum of 99.72 ± 0.17 % when the biomass exceeded 6 g / L (Fig. 7d). The role of biomass loading proved decisive. ANOVA $F(9,20) = 669.1$ ($p < 0.001$) was followed by Tukey's HSD, which classified removal at 1 g/L (87.54 ± 0.34 %, group "e") as significantly lower than at ≥ 6 g/L (> 99 %, group "a") (Fig. 7d). A linear model ($R^2 = 0.870$) confirms that each additional gram per liter of biomass



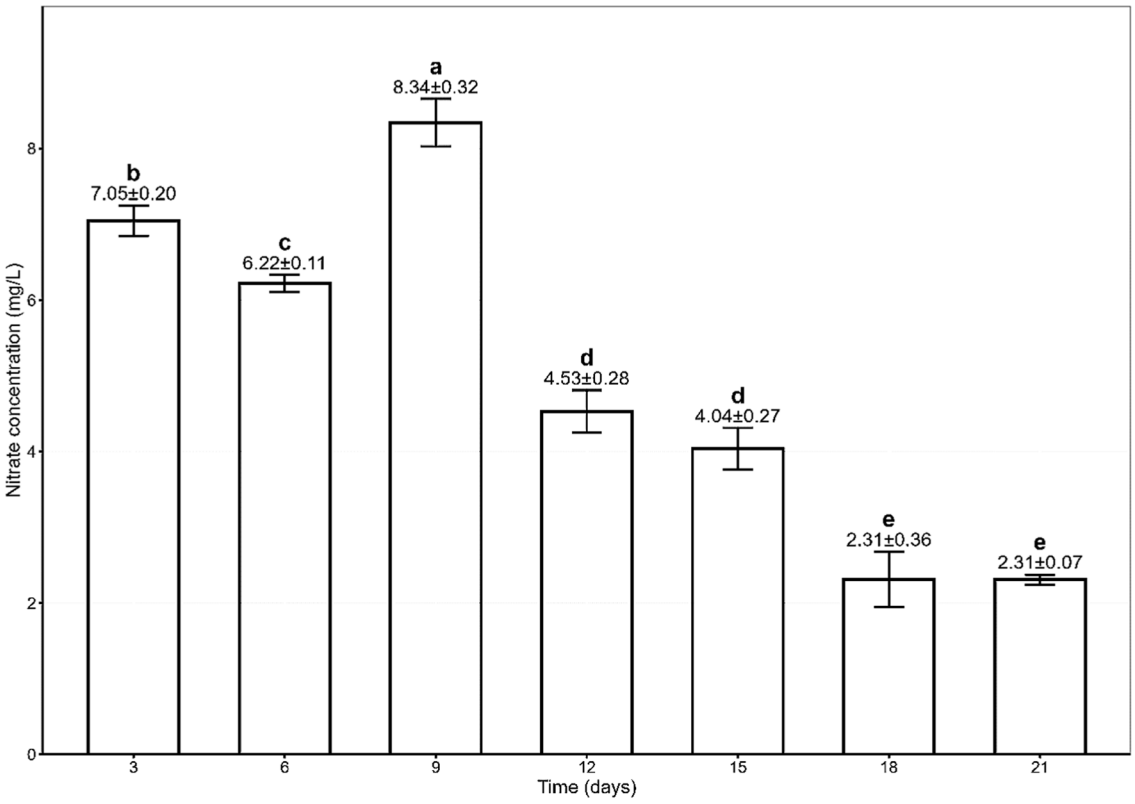
(c)



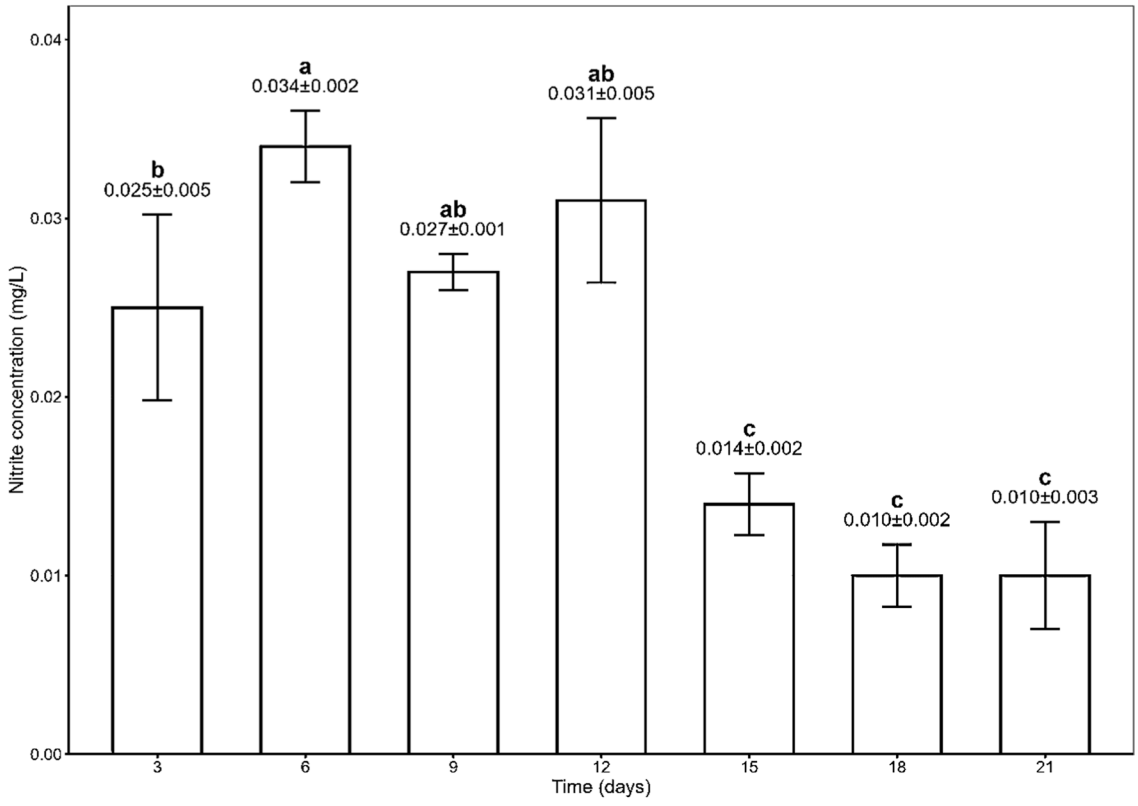
(d)

Fig. 5. (continued)

yields a marked uptick in denitrification efficiency. The improved nitrate removal observed with increasing

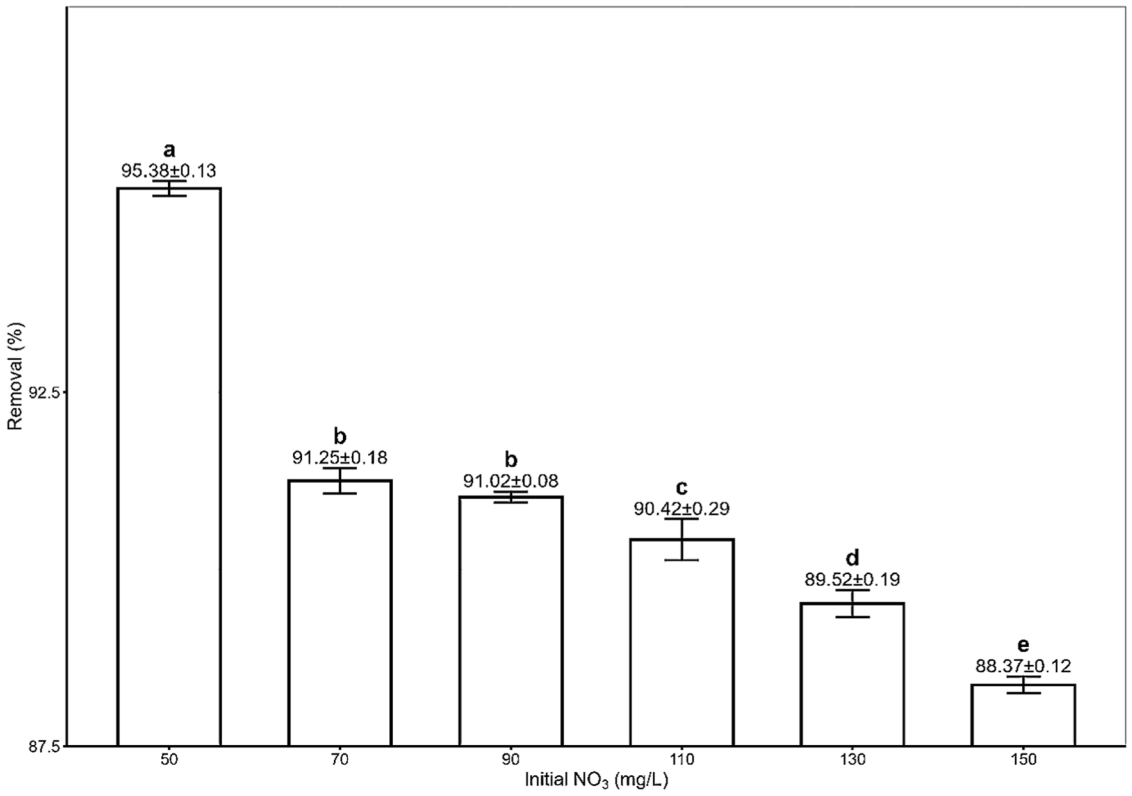


(a)

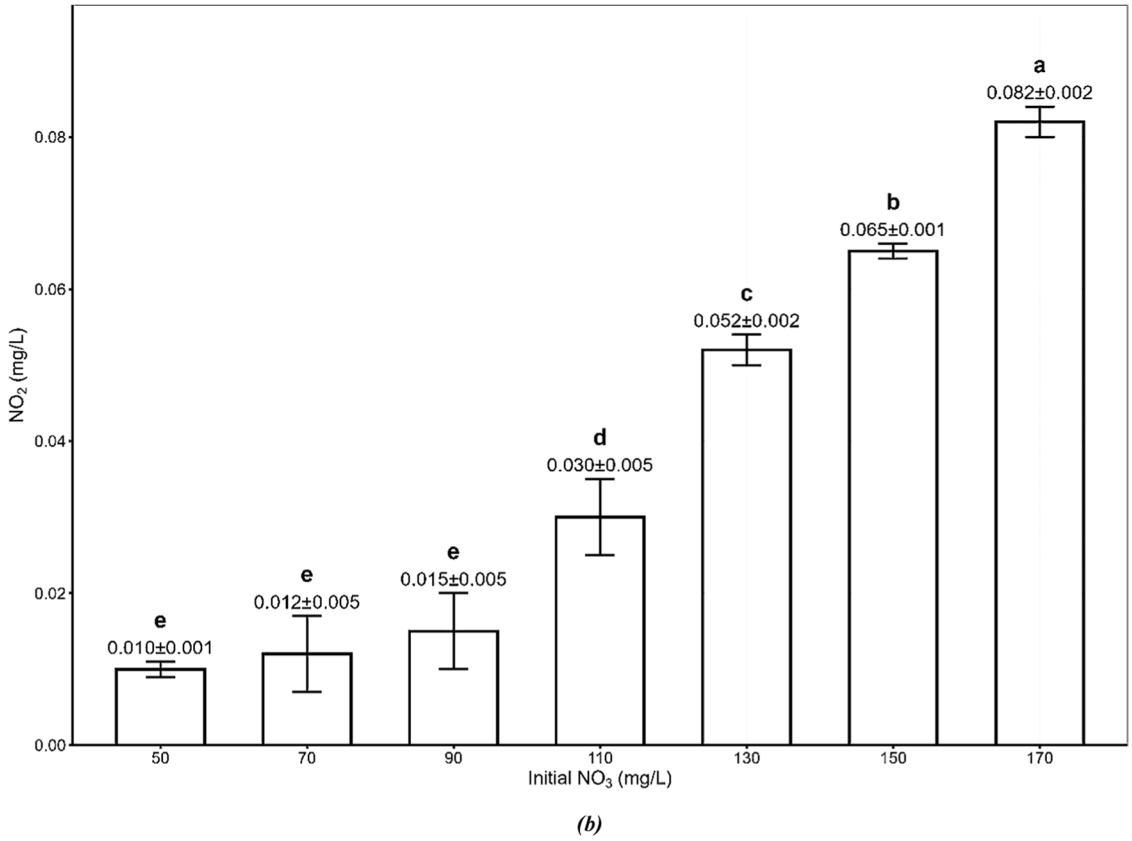


(b)

Fig. 6. Evolution of nitrogen species during batch-mode denitrification using acorn cups as carbon source. (**a**) Temporal variation in nitrate concentration (mg / L) over a 21-day period. (**b**) Temporal variation in nitrite concentration (mg / L) over a 21-day period.

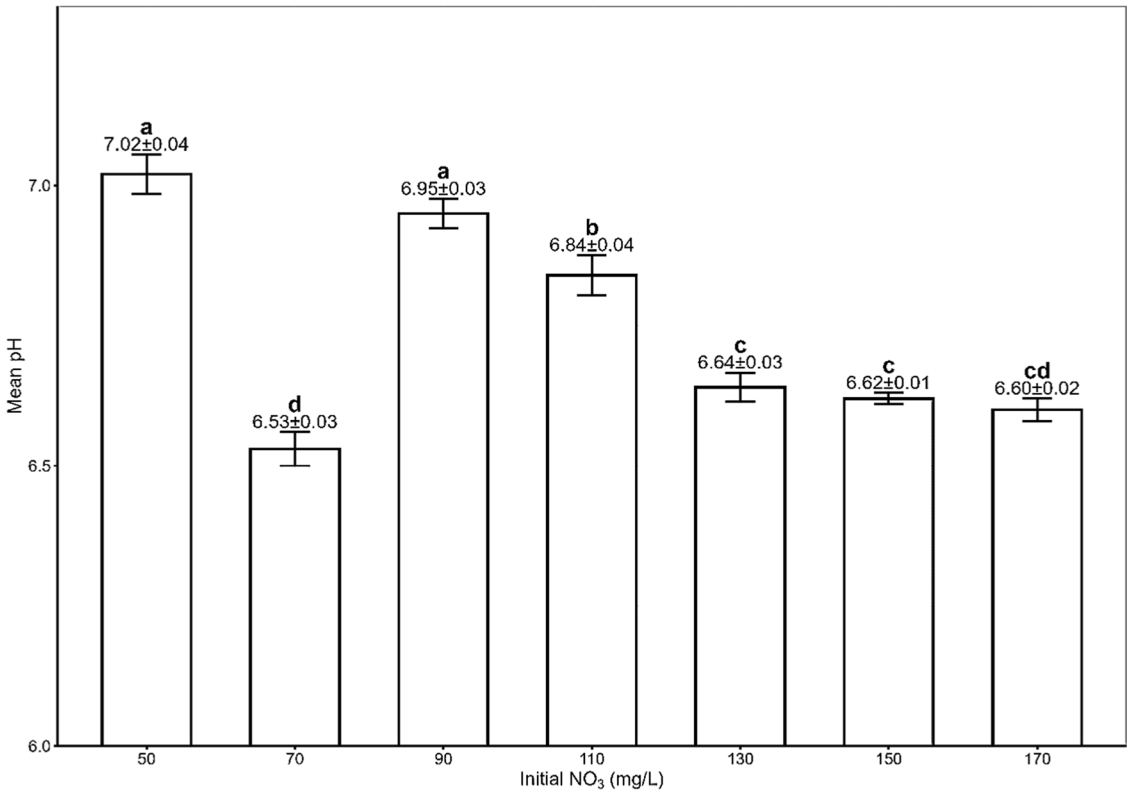


(a)

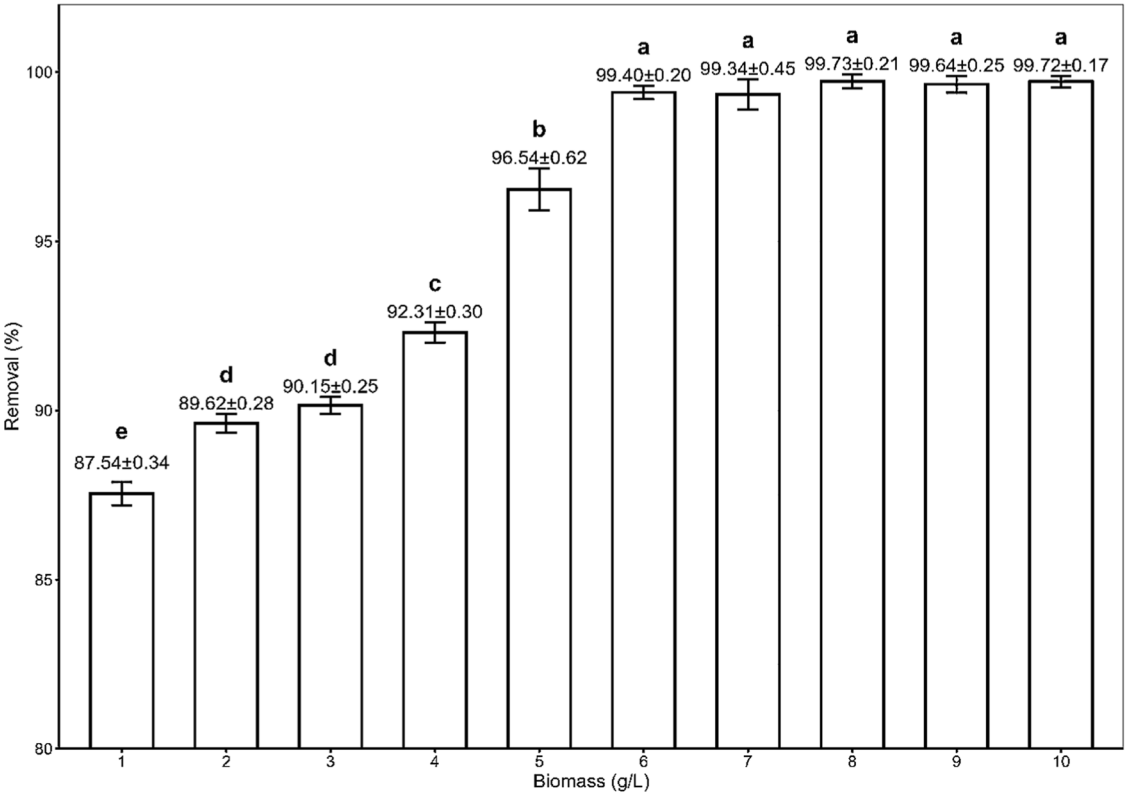


(b)

Fig. 7. Denitrification performance using acorn cups as a solid-phase carbon source: (**a**) Percentage of nitrate removal as a function of the initial nitrate concentration (mg / L). (**b**) Nitrite accumulation as a function of the initial nitrate concentration. (**c**) pH variation with respect to initial nitrate concentration; (**d**) Nitrate removal efficiency as a function of acorn cup biomass concentration (g / L).



(c)



(d)

Fig. 7. (continued)

acorn cup biomass indirectly suggests enhanced microbial activity, likely due to a larger surface area for bacterial attachment and increased availability of carbon. However, it should be noted that no direct measurements of bacterial growth (e.g., optical density, DNA quantification, or colony-forming units) were conducted in this study. Such assessments would provide a more comprehensive understanding of microbial dynamics and are recommended for future research efforts.

Direct comparisons with cupule-free controls using Welch's *t*-test underscored the marked impact of acorn-cup biomass. Nitrate concentrations in experimental conditions were significantly lower than in abiotic controls at all time points (Days 3–21, $p < 0.0001$ each), indicating an accelerated rate of nitrate consumption. Nitrite accumulation did not significantly differ from controls at Day 3 ($p = 0.0598$), but became markedly elevated from Day 6 onwards (p ranging from < 0.0001 to 0.0001). Nitrate removal efficiency under biomass-driven conditions significantly outperformed that of controls across all initial NO_3^- concentrations (50–150 mg / L; $p < 0.0001$ to 0.0001). Moreover, final pH values in experimental flasks were consistently and significantly lower than those in controls for all tested nitrate concentrations (50 mg / L $p = 0.0108$; 70 mg / L $p < 0.0001$; 90 mg / L $p = 0.0005$; 110 mg / L $p = 0.0005$; 130 mg / L $p = 0.0050$; 150 mg / L $p = 0.0001$; 170 mg/L $p < 0.0001$), reflecting pH-lowering effects mediated by biomass activity.

The denitrification rate increases with the availability of a carbon source. Indeed, an increase in carbon provides a larger surface area for microbial colonisation and supports the growth of denitrifying populations. As a result, a higher potential denitrification rate can be attributed to the presence of more metabolically active bacteria in the denitrification medium[89]. Compared to other carbon sources traditionally used in denitrification, this study highlights the key advantages of our selected material: biodegradability, non-toxicity, low cost, and ease of drying and grinding, making it a truly natural co-product.

These advantages are concentrated in a single carbon source, the acorn cup, setting it apart from alternatives such as methanol, which is only effective within a narrow dosage range due to its potential toxicity. In fact, excessive methanol concentrations may have severe adverse effects (the maximum permissible methanol concentration in water is 0.25 mg / L)[90]. Carbon sources derived from natural biomass provide both habitat and energy supply for microbial growth during denitrification[37,73]. However, some alternatives have notable limitations. While a few carbon sources offer advantages similar to those of acorn cups, many are either expensive or prioritised for other applications. Agricultural residues, such as maize, wheat, and rice straw, are commonly diverted to animal feed or energy production[91], which may limit their availability for denitrification. For example, date flour[36], though a potentially effective carbon source, is primarily reserved for food uses and is relatively costly. Likewise, banana peel powder[92] presents drying challenges, often requiring large-scale industrial dryers, leading to higher operational costs. In contrast, acorn cups, typically regarded as low-value agricultural or forest residues, may offer a promising avenue for valorising underutilised biomass. Their potential use as a carbon source in denitrification processes could help reduce reliance on conventional agricultural resources, pending further validation on a larger scale. In our experiments, denitrification yields reached up to 95.38 ± 0.13 % at neutral pH, with a maximum of 99.72 ± 0.17 %. These efficiencies can be attributed to the porous structure of the acorn cups, which promotes bacterial attachment, and to their nutrient-rich composition, providing trace elements essential for microbial metabolism. To better assess the role of acorn cups in the heterotrophic denitrification process, a control experiment was conducted under identical conditions but without the addition of any external carbon source. This setup allowed for a direct comparison and isolation of the effects of the acorn-based biomaterial. The results clearly showed that the presence of acorn cups significantly enhanced nitrate removal efficiency. While the test reactors achieved removal rates exceeding 90%, the control systems showed much lower performance, with nitrate elimination decreasing from 48% to approximately 35 % as the nitrate load increased from 50 to 150 mg / L. This difference confirms the carbon limitation in the control condition, consistent with findings in other studies in which denitrification is hindered by insufficient electron donors. Furthermore, nitrite accumulation patterns supported these observations. In the acorn-enriched systems, nitrite levels peaked early at low concentrations and were subsequently reduced, indicating a complete denitrification pathway. In contrast, the control showed continuous nitrite accumulation, peaking at levels more than double those recorded in the test reactors, suggesting a blockage at the intermediate stage due to the lack of available carbon. The pH profiles also differed. The pH in the control reactors remained slightly higher (7.05 ± 0.02–7.27 ± 0.07) than in the acorn-based systems (6.6 ± 0.02–7.02 ± 0.04), reflecting the lower microbial activity and limited acid production. This subtle shift in pH is in line with reduced heterotrophic metabolism and minimal nitrate reduction under carbon-deprived conditions. Table 5 presents a comparative summary of the key water quality parameters observed in this study including concentrations of nitrate and nitrite, pH variation, and biomass degradation alongside values reported in previous studies using various natural carbon sources. Denitrification performance varies notably depending on the type and origin of the plant-based carbon source. In this study, acorn cups demonstrated high nitrate removal efficiency (88.37 ± 0.12 %–95.38 ± 0.13 %), with a maximum NO_2^- concentration as low as ~ 0.03 mg / L and a final pH maintained within the neutral range (6.6–7.0), indicating a stable and efficient denitrification environment. Compared to other materials such as PCL composites[93,94], which also show good nitrate removal rates (> 87 %) but often accumulate more nitrite (up to 2 mg/L), acorn cups appear to offer more complete and balanced nitrogen removal with minimal NO_2^- accumulation. Notably, Qi et al.[95] using fermentation liquid from food waste also achieved high nitrate removal (~ 87 %) with near-zero NO_2^- detected, although under acidic conditions (final pH ~ 3.1), which could limit broader applicability due to potential microbial inhibition. In contrast, Shen et al.[96] and Wang et al.[73] reported variable performances depending on system configuration and carbon composition, with Wang's system showing extended operation time but moderate removal efficiency (80.7 %). Overall, acorn cups present a promising, sustainable alternative for biological denitrification, offering high performance within a short retention time (21 days), neutral pH stability, and minimal intermediate accumulation, rendering them attractive for practical applications in decentralised or low-maintenance systems.

Study/references	Carbon source	Initial NO_3^- (mg/L)	% NO_3^- removal	Max NO_2^- (mg/L)	Final pH	Duration (days)
This study	Acorn cups		88 ± 0.12 – 95.38 ± 0.13 99.73 ± 0.17 (exceeded biomass)	~ 0.03	6.6 – 7.0	21
Chen et al.[90]	PCL / PS (peanut shell); PCL / SB (sugarcane bagasse) PCL / TPS (thermal plastic starch)	20	87.60 ± 0.06 87.93 ± 0.05	2	Not specified	162
Li et al.[91]	PCL	25 – 35	80 – 90	More than 2	Not specified	249
Qi et al.[92]	Fermentation liquid produced from food waste	15	87.4 ± 7.2	Hardly detected ~ 0	3.1	35
Shen et al.[93]	PCL / Starch	25 15 – 50	90 47.50 – 98.42	1	6.47 – 7.48	60 25 – 184
Wang et al.[70]	banana peel + KCl	50	80.7 ± 0.9	0.69 ± 0.05	Not specified	~ 20

Table 5. Comparison of denitrification performance using various plant-based carbon sources reported in previous studies.

The meticulous statistical treatment presented herein confirms that acorn-cup biomass catalyses heterotrophic denitrification with remarkable efficiency. The pronounced temporal peak in NO_3^- on Day 9, followed by a sustained decline, is consistent with classical microbial nitrate reduction kinetics. The accompanying transient spike in NO_2^- highlights a rapid enzymatic progression through the denitrification pathway. Crucially, removal efficiency drops markedly under high nitrate loading, suggesting substrate saturation or inhibitory effects, whereas incremental additions of biomass lead to a linear increase in removal, underscoring the pivotal role of microbial density. The consistent acidification observed relative to controls supports proton release during reductive nitrogen metabolism. Collectively, these findings not only validate the efficacy of acorn-cup biomass in facilitating complete denitrification, but also elucidate the mechanistic interplay between substrate loading, microbial capacity, and reaction kinetics–insights that will inform scale-up and optimisation in wastewater treatment and bioremediation contexts.

This contextualisation positions the performance of acorn cups among alternative materials used in biological denitrification. Although the results obtained are promising, especially regarding nitrate removal efficiency and the stability of physicochemical conditions, it is important to acknowledge that this study was conducted at a laboratory scale and over a relatively short time frame. The current study did not investigate material regeneration, reuse, or long-term degradation. Nevertheless, the high physical stability of acorn cups observed during the experiment, along with their porous structure, suggests a potential for gradual biodegradation and extended use as a solid-phase carbon source. Further long-term research, including continuous-flow and real-world application trials, will be necessary to assess the sustained carbon release, durability, and life cycle of this biomaterial. Therefore, the scalability and industrial application potential of this technique should be considered as a potential rather than a confirmed outcome, pending future validation.

Conclusion

This study highlights the potential of acorn cups as a promising solid-phase carbon source for heterotrophic denitrification. Their porous structure facilitates microbial colonisation and activity, contributing to efficient nitrate removal from water. Additionally, acorn cups are biodegradable, easy to dry and grind, and contain low concentrations of trace elements, thereby reducing the risk of secondary contamination.

Unlike other biomaterials previously used for denitrification, such as date flour, banana peels, corn cobs, wheat and rice residues, or bagasse, acorn cups are generally regarded as low-value agricultural or forest residues. Many of the aforementioned alternatives are also prioritised for food, feed, or energy applications and may require substantial processing costs (e.g. drying and grinding) at an industrial scale.

Although the nitrate removal efficiency observed in this study is favourable, these findings were obtained under controlled laboratory conditions and over a relatively short experimental period. Therefore, the scalability and long-term performance of this method remain to be validated. Further research, particularly under pilot-scale and continuous-flow conditions, will be essential to assess the durability, carbon release dynamics, and cost efficiency of acorn cups in real-world water treatment applications.

Data availability

Should any raw data files be needed in another format they are available from the corresponding author upon reasonable request. Source data are provided with this paper. The authors declare that the data supporting the findings of this study are available within the paper.

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Author contributions

SI SMAIL Selma wrote the main manuscript text and conduct the experiment, BENBELKACEM Ouardia designed the research and prepared tables 1–5,Fauconnier Marie Laure and Lemlikchi Wahiba contributed equally to this work and prepared figures 1–7. All authors reviewed the manuscript.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

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