

Article

Impact of Temperature and Residence Time on Nitrate Removal in Multimedia Denitrifying Bioreactors

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Abstract

Denitrifying bioreactors are edge-of-field best management practices used to reduce excess nutrients in agricultural drainage. Traditional systems rely on woodchips as both a carbon source and microbial habitat, but woodchip-only bioreactors often exhibit limited nitrate removal, particularly under low temperatures and high flow conditions. This study evaluated nitrogen removal in a multimedia bioreactor under varying environmental conditions. A non-ideal, continuous stirred-tank model was applied to estimate nitrogen removal rates and nitrate removal efficiencies in mesoscale reactors containing walnut-shell biochar, Brotex, and woodchips. Two configurations—woodchip–biochar with and without Brotex—were tested at 4 h and 12 h hydraulic residence times (HRTs) and temperatures from 6 °C to 14.5 °C. Nitrate removal was consistently higher in the non-Brotex treatments. At low temperature, average removal was 15.3% (3.21 g m^{−3} d^{−1}) and 50.5% (3.66 g m^{−3} d^{−1}) for 4 h and 12 h HRTs, respectively. Removal improved at higher temperatures, reaching 54.2% (4.77 g m^{−3} d^{−1}) and 79.7% (7.86 g m^{−3} d^{−1}). These results exceeded many nitrate removal values reported for woodchip-only systems in the literature under similar temperature and hydraulic residence time conditions, indicating that alternative media can enhance performance across a range of flow and temperature conditions, supporting broader application in diverse climates.

Keywords: denitrifying bioreactor; biochar; nitrate removal; nature-based solutions; temperature; hydraulic residence time



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1. Introduction

Denitrifying bioreactors are subsurface, media-filled trenches designed to intercept agricultural drainage water at the edge of field before it enters nearby waterways [1,2]. Traditional bioreactors use woodchips as both a carbon source and a physical substrate for microbial habitat [3,4]. This biodegradable carbon provides the electrons required for the microbial reduction of nitrate (NO₃[−]) via nitrite (NO₂[−]), nitric oxide (NO), and nitrous oxide (N₂O) ideally to nitrogen gas (N₂), a process that is favored under saturated, anoxic conditions [1]. In the absence of oxygen, microbes utilize nitrate as electron acceptor for anaerobic respiration, releasing nitrous oxide and/or nitrogen gas into the atmosphere and thereby achieving permanent nitrate removal from the water [5]. However, the performance of woodchip-based bioreactors is often diminished under cold temperatures and high flow

conditions—scenarios common during winter months in temperate climates or during storm-driven drainage events [2,5]. Low temperatures suppress microbial metabolic activity, while high flow rates reduce hydraulic residence times (HRTs), limiting the amount of time for nitrate reduction [6,7]. Enhancing bioreactor media composition presents an opportunity to improve nitrate removal efficiency across a broader range of environmental conditions [2].

The use of alternative media types may enhance nitrate removal by retaining microbial electron donor (carbon) and acceptor (nitrate/nitrite) and increasing surface area for microbial attachment, leading to higher cell numbers and metabolic activity of denitrifying microbes [8,9]. Promising alternatives include biochar and various non-carbon-based materials [6,10]. Biochar, a highly stable and mostly recalcitrant carbonaceous material, is produced through the pyrolysis of organic matter [10]. This process removes water and volatile compounds, resulting in a porous, chemically altered carbon material whose properties depend strongly on feedstock type and pyrolysis temperature [10,11]. Biochar made from different feedstocks has been shown to retain nitrate and organic matter through various surface sorption processes, potentially increasing nitrate and carbon availability for microbial respiration [10,11]. Non-carbon-based materials may serve as additional surfaces for microbial colonization, supporting the formation of structured communities of bacteria (biofilm) [9]. However, the performance of these alternative media types varies depending on their physical, chemical, and hydraulic characteristics [2]. Further research is needed to identify optimal media compositions and combinations that maximize nitrate removal efficiency under diverse environmental conditions.

Wood-based biochar (derived from hard or softwood) shows variable performance depending on several experimental factors. Generally, under long HRTs (>24 h), nitrate removal is moderate to high (~50–100%) [12,13]. When the HRT is reduced (8–12 h), nitrate removal efficiency declines to moderate levels (~40–50%) [13,14], likely due to a decreased contact time [10]. In wood-based biochar systems under conditions of low-influent nitrate loading (<5 mg L⁻¹), removal efficiency can drop dramatically (~10%) [15,16]. It has also been demonstrated that the highest-influent nitrate concentration and lowest-influent flow rate produced the highest nitrate removal capacity (0.11 mg g⁻¹) [17]. These studies demonstrate that HRT and influent nitrogen loading are key factors influencing the performance of wood-based biochar bioreactors, similar to woodchip-only systems.

Low temperatures can substantially reduce the removal of nitrate within denitrifying bioreactors. As of 2024, only four other published media-based, denitrifying bioreactor laboratory experiments used simulated agricultural drainage water and temperatures below 10 °C [5,18–20]. Some studies indicate a moderate negative correlation ($r \approx -0.5$) between temperature and nitrate removal efficiency in wood-based biochar systems [13]. In scenarios with low average temperatures (<10 °C), woodchip bioreactors have outperformed wood-based biochar bioreactors [21], likely due to a reduction in microbial metabolism [16]. These studies further demonstrate that low temperatures create challenges for nitrate removal in a variety of experimental situations and that more research in this area is greatly needed.

Limited studies have explored hybrid systems combining woodchips and wood-based biochar. However, even with high amendment rates (30–50% biochar by volume), nitrate removal remains low (<20%) [22,23]. This limited performance is likely due to the low carbon-to-nitrogen (C/N) ratio commonly associated with wood-based biochars, which can limit the amount of carbon available as an electron donor for the reduction process [23]. Other research has shown only marginal improvements in nitrate removal under high loading conditions when wood-based biochar is added to woodchip systems [24]. These findings suggest that biochar characteristics—particularly feedstock type—are critical to

bioreactor performance and that wood-based biochar may have limited effectiveness for nitrate removal.

The method of biochar production plays a critical role in determining its final characteristics, which can either enhance or inhibit microbial denitrification. Some studies on both wood-based and nutshell-derived biochars have shown that higher pyrolysis temperatures ($>750\text{ }^{\circ}\text{C}$) improve nitrate removal due to the development of a more crystalline structure and rougher surface texture [17], as well as the enhanced formation of stable aromatic structures [11]. Conversely, several studies indicate that wood-based biochars produced at lower pyrolysis temperatures ($300\text{--}500\text{ }^{\circ}\text{C}$) retain higher fractions of labile carbon, volatile matter, and oxygenated functional groups, which may enhance microbial activity and favor denitrification under suitable conditions [10,25,26]. Thus, pyrolysis temperature must be carefully selected to align with the intended function of the biochar in nitrate removal systems.

Some studies have investigated alternative, non-carbon-based forms of media for enhancing microbial denitrification. For example, Feyereisen et al. [27] tested a two-stage bioreactor system using corncobs followed by a chamber containing a plastic biofilm carrier. They observed a slight increase ($\sim 10\%$) in nitrate removal, which was statistically significant at moderate temperatures ($15\text{ }^{\circ}\text{C}$) but not at lower temperatures ($1.5\text{ }^{\circ}\text{C}$). In a green roof tray experiment, Beck et al. [28] evaluated a media blend consisting of 70% shell-based biochar and 30% tire-derived biochar, achieving a high level ($\sim 80\%$) reduction in nitrate leaching from rainfall runoff. While the tire-derived biochar may have contributed to improved structural properties, its effects were not tested independently of the shell-based component. Additionally, studies on the mineral zeolite have shown high nitrate removal efficiencies ($>90\%$) comparable to wood-based biochar and high-density polyethylene (HDPE) plastic after an initial acclimation period (~ 6 days), likely due to its high surface area, porosity, and surface roughness that support microbial colonization [29]. These findings suggest that non-carbon-based media and hybrid media combinations hold promise for enhancing nitrate removal in bioreactor systems.

Attempts have been made to improve the performance of both wood-based and biochar-based bioreactors to better accommodate low temperature and high flow conditions. Some studies have explored the use of electrostimulation to supply additional electrons to denitrifying bacteria [30,31]. Electrostimulation has been shown to moderately increase denitrification ($\sim +10\text{--}25\%$) in wood-based bioreactors [31] but is less effective when applied to walnut-shell biochar systems [30]. Moreover, the implementation of electrostimulation introduces additional costs and logistical challenges, limiting its feasibility for real-world applications. Another approach to enhance bioreactor performance is carbon supplementation. Roser et al. [20] showed an order of magnitude increase in denitrification due to continuous acetate dosing, as it provides an immediate and readily utilizable electron donor and carbon source for denitrifying microorganisms under low-temperature conditions. This supplementation has the effect of increasing microbial activity [20]. However, continuous dosing may pose logistical challenges and incur additional costs in field settings. Therefore, alternative strategies for improving bioreactor performance warrant further investigation.

This study investigates the effectiveness of a multimedia denitrifying bioreactor over a wide range of temperatures. Nitrogen load removed and nitrate removal rates were determined by coupling measured data with a mass balance model that represents non-ideal bioreactor hydraulics using a plug flow segment followed by continuous stirred-tank reactors in series (CSTRs), as developed by Han et al. [32]. In this previous study, the model was used to determine experimental bioreactor HRTs and other flow parameters using a conservative tracer (bromide) outflow curve. Furthermore, variation in microbial

abundance was incorporated into the modeling framework using total bacterial 16S rRNA gene copy numbers as a scaling factor to represent relative change in overall microbial biomass [33]. The model was used to calculate percent nitrate removal and nitrogen removal rates ($\text{g N m}^{-3} \text{d}^{-1}$) by fitting predicted effluent nitrate concentrations to measured data through optimization of the decay coefficient (κd) using Excel Solver™, minimizing the sum of squared errors (SSE). This approach accounts for hydraulic residence time lags and computes nitrate removal on a time-step basis under variable influent concentrations.

The media selected for this study were chosen to test whether combining complementary physical and chemical properties could improve nitrate removal under challenging environmental conditions. Hardwood woodchips served as the primary degradable carbon source and are representative of conventional denitrifying bioreactor media. Walnut-shell biochar was selected because previous studies have demonstrated that biochar can provide additional pore space, sorption capacity, and microbial attachment surfaces while potentially retaining nitrate and dissolved organic carbon within the treatment matrix. Brotex, a porous PET fiber matrix originally developed for floating treatment wetlands, was selected to provide additional microbial colonization surfaces without substantially reducing hydraulic conductivity. It was hypothesized that combining these materials would enhance microbial activity and nitrate removal relative to a woodchip–biochar mixture and woodchip-only bioreactors from the literature tested under similar HRTs and temperatures.

2. Materials and Methods

The experimental system consisted of the reduced-temperature mesoscale bioreactors previously described by Han et al. [32], Hackshaw [33], and Krider [34]. This testing apparatus used temperature control chambers to simulate springtime air and water temperatures in southern Minnesota (Figure 1). These chambers were used to test for denitrification within multimedia bioreactors under reduced temperature scenarios [32]. A 12-week laboratory experiment was conducted in the winter of 2016/2017 to test two different bioreactor media combinations under 4 h and 12 h HRTs (4 treatments $\times$ 3 replicates per treatment for a total of 12 troughs (i.e., containers housing the media)) at 6 °C for 4 weeks and 14.5 °C for 4 weeks, with a warming period of 4 weeks in between to mimic the gradual transition from cold to warm temperatures typically present in nature [32,34].

Troughs were surface-exposed (open-topped), horizontal bioreactors with half (6) containing one media combination (10% Brotex material, 10% walnut-shell biochar, and 80% hardwood woodchips by volume), while the other half (6) contained a second media combination (10% walnut-shell biochar and 90% hardwood woodchips by volume) (Figure 4 in [32]). Inflow was delivered from above the media surface through tubing into a gravel inlet zone, while effluent exited through a perforated vertical outlet pipe located near the downstream end of each trough, allowing for control of saturated conditions and external collection of effluent samples. Troughs were planted with wetland plant plugs (two of each fox sedge (*Carex vulpinoidea*), dark-green bulrush (*Scirpus atrovirens*), and rice cutgrass (*Leersia oryzoides*)) purchased from Cardno Native Plant Nursery in Indiana to mimic natural conditions likely to be found in the field [32,34]. Bioreactors were approximately $1.83 \text{ m} \times 0.31 \text{ m} \times 0.61 \text{ m}$, yielding a total volume of $\sim 0.35 \text{ m}^3$ and an aspect ratio of $\sim 6:1$ following guidelines presented by Christianson et al. [14].

Coarse grit black walnut shells (size 4/6, $\sim 4.76 \text{ mm}$) were purchased from Hammon's Products Company in Missouri and charred by slow pyrolysis in a mobile downdraft gasifier at 600 °C for 3 h by Char Energy, LLC. in Ada, MN, USA [32,34]. This temperature was selected because it falls within the midrange commonly used for producing stable, high-surface-area biochars, and it represents a typical operating temperature employed by the commercial charring service. The walnut-shell biochar was chemically characterized

by Eurofins (Hamburg, Germany) according to the guidelines introduced by the Biochar Science Network for obtaining the European Biochar Certificate (Appendix C). Brotex is a fibrous plastic matrix originally developed for BioHaven® floating treatment wetlands [35]. The material consists of a dense, porous network of recycled PET fibers designed to support microbial biofilm development [35]. For the purposes of mixing with the other substrate, large sheets of Brotex (3 m × 5 m) were cut into ~10 cm × 10 cm cubes. Media were arranged in a layered fashion, and all layers were inoculated with 80 mL of soil collected from an agricultural drainage ditch in southern Minnesota [32,34]. Additionally, each trough was topped with 0.025 m³ of the same agricultural soil [32,34].



Figure 1. Photo of experimental bioreactors inside a temperature control chamber (2.44 m × 1.22 m × 1.83 m) as part of the testing apparatus.

Throughout the course of the experiment, troughs received a water recipe designed to mimic the major chemical constituents and concentrations of agricultural drainage water

in southern Minnesota. This recipe was determined based on water-quality data collected at the Mullenbach Two-Stage Ditch in Mower County, Minnesota, as well as information provided by Zhang [12]. This recipe contained nitrate (NO_3^- , 30 mg L^{-1}), phosphate (PO_4^{3-} , 0.5 mg L^{-1}), calcium (Ca^{2+} , 55 mg L^{-1}), chloride (Cl^- , 150 mg L^{-1}), magnesium (Mg^{2+} , 20 mg L^{-1}), and potassium (K^+ , 5 mg L^{-1}) [34]. This nutrient-laden water was continuously mixed with filtered tap water (for removal of chlorine and chloramine) to produce the desired water recipe concentrations before delivery to the troughs. Flow to each bioreactor was individually controlled using variable area flow meters.

The experiment was conducted in temperature-controlled chambers with air-conditioning units equipped with a temperature-regulating device to further reduce air temperatures beyond the range inherent in the air-conditioning unit Figure 1 [34]. Setting up adequately reduced conditions took place over a span of 4 weeks, over which the temperature in the chambers was slowly reduced from 30 °C to 6 °C and nitrate measurements in the effluent were not yet taken [34]. Troughs were saturated in filtered tap water for the first week, and the second week employed a 24 h HRT of the drainage water nutrient recipe and 100 mg L^{-1} sodium acetate. Acetate was added during reactor startup to stimulate microbial recovery after air-drying the inoculum soil. This protocol was continued (minus the sodium acetate) for another 2 weeks while reducing the temperature by 2.8 °C every other day [34]. The HRTs were reduced to 4 h and 12 h for the corresponding treatments, and the temperature was reduced another 2.8 °C (to 6 °C) 1 week prior to the start of the experiment (5 December 2016).

Influent and effluent water samples were collected twice daily for each trough for 6 days per week and analyzed for nitrate using a Hach Nitratex Plus™ nitrate probe, flow rate by timing a volume collected, and DO, conductivity, pH, and ORP (oxidation reduction potential) using a YSI 6-Series Sonde™ [32,34]. Continuous influent nitrate measurements were collected every 15 min from 5:00 PM to 9:00 AM with the nitrate probe as well [32,34]. Air, water, and media temperatures were collected once daily for each trough for 6 days per week using Type K (chromel-alumel) thermocouples connected to a Campbell Scientific CR10X data logger [32,34]. Water samples were analyzed periodically at the University of Minnesota Research and Analytical Lab for QA/QC of nitrate and nitrite concentrations. All flow meters were cleaned as needed and reset daily at 5:00 PM [32,34].

Hydraulic parameters and effective HRT values were obtained from bromide tracer analyses previously reported by Han et al. (Figures 5 and 6 in [32]). Hydraulic parameters were optimized using the Excel Solver GRG Nonlinear Method to produce a universal curve of bromide concentrations over time (Figure 7 in [32]). The universal curve was used to determine in situ tracer detention time and employed to calculate actual HRT for the troughs (average of 3.80 h for the 4 h HRT and average 10.74 h for the 12 h HRT) [32]. The optimal configuration was produced using four reactors containing 98.1% of the total flow, with 3.7% short-circuiting and 1.1% dead space (Table 2 in [32]). A conceptual diagram of various flow components incorporated into the model is shown in Figure 1 in [32].

Individual nitrate measurements were removed if the trough flow rate was greater than 25% from the expected value and individual days were removed if more than 25% of the troughs met the previously stated criteria [32,34]. Nitrate data were determined to be non-normally distributed with unequal variances between treatments; thus, box-cox transformations were performed using the preferred form presented by Draper and Smith [36]. For calculating removal rates, the minimum sum of squared errors (SSE) was achieved using a lambda (λ) of 0.4, so the data were transformed prior to this analysis [32,34]. Nitrate removal was calculated for days in which the χ^2 (as $\sum(\text{O}_i - \text{P}_i)^2$) value was less than 30, where O_i and P_i are the observed and predicted values, respec-

tively [32,34]. This was chosen due to the natural split of the data and allowed for some variation between the model data and the actual data [32,34].

For statistical analysis, temperatures were grouped into three regimes: low (6.0 °C and 7.2 °C), mid (10.0 °C), and high (12.2 °C and 14.5 °C) [32,34]. Data were analyzed in SPSS (version 25) using a three-way mixed model ANOVA with the subjects of trough*replicate, the repeated measure of temperature regime, and the fixed factors of temperature regime, material, and HRT [32,34]. This analysis is based on a restricted maximum likelihood estimation, a type III sum of squares, and a diagonal repeated covariance type [32,34]. Significant differences in the exponent n in the microbial modification of the numerical approximation, as well as the nitrate removal efficiency and the nitrogen removal rate, all by temperature, are presented as results from the Fisher LSD test in ANOVA using XLSTAT™ (2016.1.1 version) [32,34]. Additionally, the Q10 value was calculated as the proportional change in the nitrogen removal rate with a 10 °C temperature change based on a linear regression model [32,34].

A combined plug–CSTR in a series bioreactor representing non-ideal flow hydraulics parameterized from bromide tracer tests to obtain effective HRT, short-circuiting, and dead space was modeled in Microsoft Excel™ [32]. The general formulations and analytical solutions, as well as the methodology for the sensitivity analysis, are given in [32]. A first-order formulation was selected to maintain consistency with the previously developed hydraulic model of Han et al. [32], which was developed for a combined plug flow–CSTR system operating under variable influent concentrations, flow rates, and effective hydraulic residence times. The specific mass-balance and kinetics equations used to calculate nitrate removal by denitrifying bacteria using first-order decay are provided in Appendix A.

To represent spatial and temporal variation in microbial biomass, we scaled the first-order nitrate decay coefficient (κ) with total bacterial abundance estimated from qPCR of 16S rRNA genes measured throughout the longitudinal profile of the media ([33]; Appendix B). Gene copy numbers were normalized to the run-specific mean and used to compute location-specific multipliers. The decay coefficient for each reactor segment was then adjusted by a power law function of the normalized abundance. Mass removal, efficiency, and volumetric removal rates were computed from the modeled time series.

Although the synthetic drainage water recipe was formulated to provide a target nitrate concentration of 30 mg L^{−1}, actual influent concentrations varied over time due to inconsistencies in mixing and nutrient delivery due to the gradual clogging of the nutrient line flow meter. Therefore, regression curves were developed to reconstruct influent nitrate concentrations at any given time for use in the nitrate removal model (Figure 2). For the first 20 days of the experiment, influent concentrations were estimated using linear regression equations based on 2–4 grab samples collected the following day. After day 20, polynomial regression equations were developed based on continuous overnight influent nitrate concentrations, recorded every 15 min from 5:00 PM to 9:00 AM using a nitrate probe, and used to estimate influent nitrate concentrations over each 24 h period starting from 5:00 PM [34]. When necessary, two separate polynomial equations were utilized: one for the initial rise in concentration following the reset and another for the subsequent decline caused by gradual clogging of the nutrient flow meter due to algal growth [34].

Nitrate removal was evaluated over 2–3 consecutive days, with the first day establishing initial influent flow and nitrate conditions. Nitrate removal was calculated after 6.75 h to allow stabilization of initial conditions [34]. The removal rate constant (κ_{d1}) was optimized using Excel Solver™ to fit the modeled effluent curve to observed grab sample data (typically two samples spaced 5 h apart) collected between 9:00 a.m. and 5:00 p.m. the following day [34].

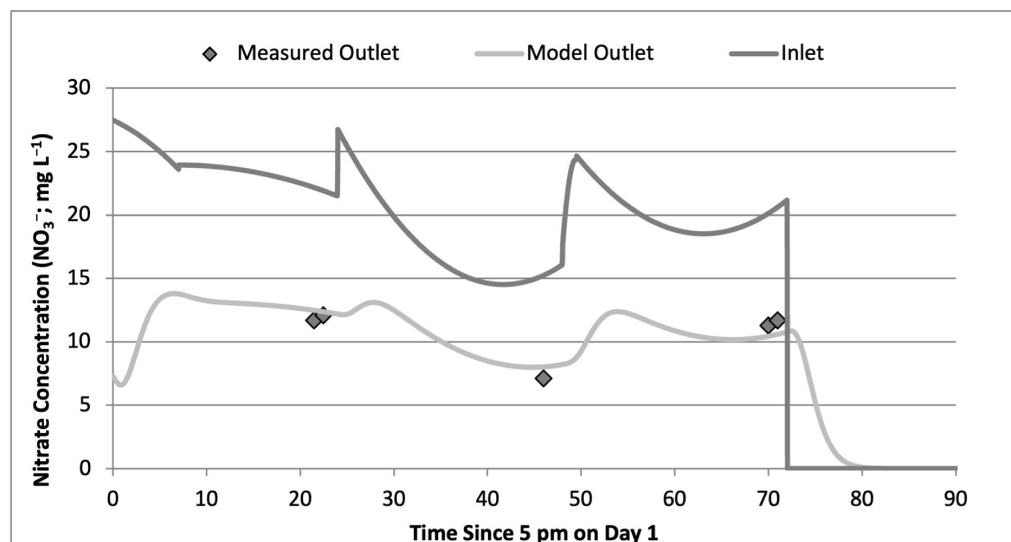


Figure 2. Graph of nitrate removal model results for 12–15 February 2017; 14.5 °C; 4 h HRT: treatment with Brotex, biochar, and woodchips; 46.97% nitrate removal; $\chi^2 = 3.49$.

Modeled effluent nitrate concentrations corresponding to measured values were graphed to determine the overall fit of the two datasets. A value of 0.98 was obtained for both a linear regression model coefficient (R^2) and a Nash–Sutcliffe Coefficient (NSE) on 461 data points. There was more scatter to the data for concentrations between 15 and 20 mg L^{−1} (visual estimate), so a three-part linear regression was applied to determine how the fit changes based on the effluent nitrate concentration. In the 0–15 mg L^{−1} range, the R^2 was 0.96, 0.41 in the 15–20 mg L^{−1} range, and 0.92 in the 20–28 mg L^{−1} range.

3. Results

3.1. Nitrate Removal

The range and average nitrate removal efficiencies, as well as nitrogen removal rates, varied among treatments and across temperatures (Figure 3). Across all observations included in the analysis, nitrate removal efficiencies ranged from 7.66% to 51.56% for the 4 h Brotex treatment, 11.27% to 63.32% for the 4 h non-Brotex treatment, 29.99% to 80.17% for the 12 h Brotex treatment, and 44.70% to 84.06% for the 12 h non-Brotex treatment. Corresponding nitrogen removal rates ranged from 1.07 to 7.50 g N m^{−3} d^{−1}, 2.07 to 9.66 g N m^{−3} d^{−1}, 2.22 to 6.12 g N m^{−3} d^{−1}, and 3.26 to 6.22 g N m^{−3} d^{−1}, respectively.

When treatment means were compared between the low-temperature and high-temperature regimes, nitrate removal increased with temperature for all treatments. Average nitrate removal efficiencies increased from 11.90% to 46.02% for the 4 h Brotex treatment, from 15.28% to 54.18% for the 4 h non-Brotex treatment, from 38.24% to 77.92% for the 12 h Brotex treatment, and from 50.49% to 79.67% for the 12 h non-Brotex treatment. Likewise, nitrogen removal rates increased from 2.38 to 7.03 g N m^{−3} d^{−1}, from 3.21 to 7.86 g N m^{−3} d^{−1}, from 2.97 to 4.86 g N m^{−3} d^{−1}, and from 3.66 to 4.77 g N m^{−3} d^{−1}, respectively. The temperature coefficient (Q10) across all treatments was 1.7.

There was no significant difference between replicates (days with the same temperature and troughs of the same treatment; $p = 0.673$) [34]. All three dependent variables (temperature regime, HRT, and material) had a significant effect on nitrate removal efficiency, both independently and combined, as well as the combined effect of temperature regime and HRT ($p < 0.001$) [34]. The combined effects of material with HRT and material with temperature regime did not have a significantly different effect on nitrate removal ($p = 0.587$ and 0.246, respectively) [34]. Across material and HRT, all three temperature

regimes were significantly different from one another ($p < 0.001$) with progressively more removal occurring as the temperature increased. Within both temperature and HRT, presence of the Brotex material had a significant negative effect on nitrate removed efficiency for the low-temperature regime ($p < 0.001$ and $=0.036$ for the 12 and 4 h HRT, respectively) and the high-temperature regime under the 4 h HRT ($p = 0.004$). Across material and temperature, HRT had a significant positive effect on nitrate removal efficiency ($p \leq 0.001$), with higher values produced under the 12 h HRT.

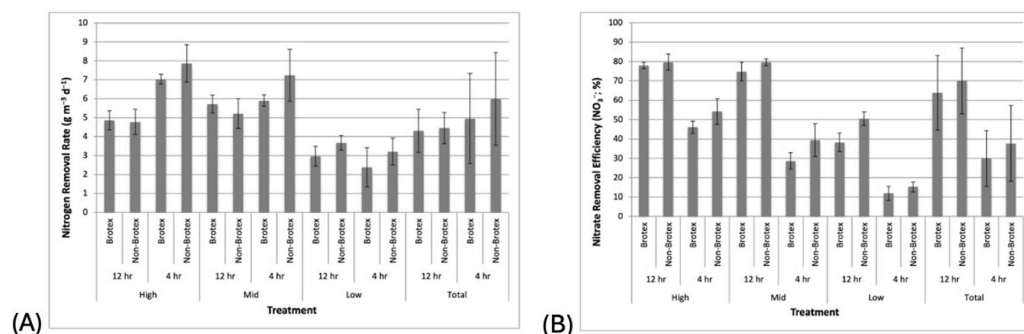


Figure 3. (A) Nitrogen removal rate ($\text{g N m}^{-3} \text{d}^{-1}$) as a function of bioreactor treatment with 1 SD error bar around the mean. Graph includes a breakdown by temperature regime (low (6.0°C and 7.2°C), mid (10.0°C), and high (12.2°C and 14.5°C)), as well as across temperature regimes (total). (B) Nitrate removal efficiency (%) as a function of bioreactor treatment with 1 SD error bar around the mean. Graph includes a breakdown by temperature regime (low (6.0°C and 7.2°C), mid (10.0°C), and high (12.2°C and 14.5°C)), as well as across temperature regimes (total).

Each of the three dependent variables (temperature regime, HRT, and material) had a significant effect on the nitrogen removal rate ($p < 0.001$, <0.001 , and $=0.023$ for each variable, respectively), but not the combined interaction ($p = 0.254$) [34]. Additionally, the combined effects of temperature regime and HRT, as well as material and HRT, had a significant effect on the nitrogen removal rate ($p < 0.001$ and $=0.032$, respectively) [34]. Across material and HRT, all three temperature regimes were significantly different than one another ($p < 0.001$), with progressively higher rates occurring as the temperature increased. Within both temperature and HRT, presence of the Brotex material had a significant negative effect on removal rates under the high-temperature regime for the 4 h HRT ($p = 0.024$) and under the low-temperature regime for the 12 h HRT ($p = 0.006$). Across material and temperature, HRT had a significant negative effect on the nitrogen removal rates ($p = 0.003$), as the rate was higher for the 4 h HRT due to associated differences in loading produced by higher flow rates [34].

Within both temperature and HRT, the presence of Brotex reduced nitrate removal efficiency under several conditions. Under the low-temperature regime, average nitrate removal efficiency decreased from 50.49% to 38.24% for the 12 h HRT treatment and from 15.28% to 11.90% for the 4 h HRT treatment when Brotex was added. Under the high-temperature regime and 4 h HRT, nitrate removal efficiency decreased from 54.18% to 46.02%. These reductions represented decreases of approximately 24%, 22%, and 15%, respectively, relative to the corresponding non-Brotex treatments.

3.2. Microbial Model

Insight into the microbial model was explored by considering the exponent n for different conditions [34]. This average value of the exponent is smaller for the high temperatures (0.039) than it is for the low temperatures (0.0515) [34]. There is a significant difference ($p < 0.001$) in the value of the exponent under the low- and high-temperature regimes [34]. There is not a significant difference due to the HRT ($p = 0.182$), but there is for

the combined effects of temperature regime and HRT ($p = 0.022$) [34]. The last biobag tubes (corresponding to the end of CSTR 3) had lower average total bacterial counts under low temperatures with high exponential adjustment factors (ns) and low decay coefficient ($\kappa d1$) values compared to high temperatures with low ns and high $\kappa d1$ values (8.43% less for the 12 h HRT and 53.89% less for the 4 h HRT) [34].

4. Discussion

To contextualize the performance of the multimedia bioreactors evaluated in this study, we compare our findings to results reported across laboratory, bench-scale/mesocosm, and field-scale denitrifying bioreactor systems. While laboratory comparisons are emphasized where possible, comparisons to field systems are also included to bracket the broader range of nitrate removal performance observed in real-world applications. Since relatively few laboratory studies report nitrate removal under temperatures below 10 °C and HRTs of 4 and 12 h, drawing on field benchmarks provides additional perspective. Differences in hydrologic complexity, thermal variability, and loading dynamics across scales are acknowledged. While field benchmarks strengthen context, factors dependent on scale and environmental conditions limit direct comparability. Presenting both laboratory and field comparisons provides a comprehensive view of potential performance while explicitly acknowledging contextual differences.

Nitrate removal efficiencies in these multimedia systems were consistently higher than values reported for laboratory and bench-scale woodchip-only bioreactors operated at similar temperatures and HRTs (e.g., [34,37]). For example, Chun et al. [38] achieved a 30–40% removal at a 12 h HRT and 16–17 °C, with performance dropping to 15% at 13 °C—considerably lower than the 75% removal observed here at ~14.5 °C under the same HRT. Wrightwood et al. [37] reported ~56% removal in a bench-scale continuous-flow woodchip bioreactor at ~22 °C, again lower than efficiencies observed here. It should be noted, however, that nitrate removal efficiency is influenced by influent nitrate concentration and loading rate, which differed among studies; therefore, these comparisons are intended to provide general context rather than direct performance equivalency. Overall, the literature consistently shows that woodchip-only bioreactors tend to exhibit significantly lower nitrate removal efficiencies, even under conditions of longer HRTs and higher temperatures.

The nitrogen removal rates achieved in this study are high relative to many reported laboratory and bench-scale systems, particularly under reduced temperature and short HRTs. Addy et al. [5] synthesized laboratory, mesocosm, and field studies and reported mean removal rates of 2.1 g N m⁻³ d⁻¹ at low temperatures (<6 °C) and 5.7 g N m⁻³ d⁻¹ at intermediate temperatures (6–16.9 °C). Lin and Volkenborn [39] reported laboratory woodchip bioreactor rates ranging from 0.4 to 4.3 g N m⁻³ d⁻¹ at 14 °C with a 24 h HRT. A more recent synthesis by Christianson et al. [2] reported median nitrate removal rates of 5.1 g N m⁻³ d⁻¹ (mean ± SD: 7.2 ± 9.6 g N m⁻³ d⁻¹; $n = 27$) across agricultural denitrifying bioreactors operating under a wide range of field conditions and temperatures. While these aggregated values overlap with those observed here, they largely reflect warmer seasonal operation and longer residence times than those evaluated in the present study. Taken together, these comparisons indicate that the removal rates observed here (4–6 g N m⁻³ d⁻¹ across 6–14.5 °C and 7–8 g N m⁻³ d⁻¹ for 4 h HRT treatments at 14.5 °C) fall toward the upper end of reported ranges for short-HRT, low-temperature systems without continuous soluble carbon addition. Some field-scale systems can achieve comparably high rates under longer HRTs, warmer conditions, or carbon supplementation, which helps contextualize the magnitude of these laboratory results while recognizing differences in scale and operating conditions.

First-order decay coefficients (k) observed here (0.04 – 0.30 h^{-1} ; mean 0.14 h^{-1}) were higher than those of many laboratory and field woodchip-only systems. Field values reported by Jaynes et al. [8] were 0.041 – 0.043 h^{-1} , and Chun et al. [40] reported $\sim 0.01\text{ h}^{-1}$ at the field scale. Laboratory estimates from Chun et al. [38] spanned <0.001 – 0.13 h^{-1} , and Lin and Volkenborn [39] reported 0.0008 – 0.0101 h^{-1} . Our maximum value (0.30 h^{-1}) approached the methanol-dosed field system of Moghaddam et al. [41] (0.369 h^{-1} , 10 h HRT). The elevated reaction rates observed in the current study may be attributed to the presence of biochar, which likely improved nitrate and biodegradable carbon availability for microbial denitrification through sorption and increased surface area for biofilm growth in the multimedia bioreactor system.

The experimental design did not include a woodchip-only control treatment operated concurrently with the biochar-amended reactors. As a result, the specific contribution of biochar to nitrate removal performance cannot be fully isolated from the effects of the mixed-media configuration under identical HRT and temperature conditions. Interpretations regarding the role of biochar, therefore, rely, in part, on comparisons to nitrate removal rates and denitrification performance reported for woodchip-only bioreactors in the published literature. While such comparisons provide useful context and suggest that the observed removal rates fall within or above previously reported ranges, they do not substitute for a direct internal control. Consequently, conclusions regarding biochar are framed as evidence of enhanced performance of the tested media mixtures relative to literature benchmarks rather than definitive attribution of causality to biochar alone.

Incorporation of the Brotex material did not result in enhanced nitrate removal and was associated with lower removal relative to woodchip-only systems reported in the literature. Previous studies examining porous plastic biocarriers (PBCs) have shown mixed results, with some reporting improved nitrate removal [42–44] and others observing variable or inconsistent performance [27]. In this study, Brotex was incorporated by replacing an equivalent volumetric fraction of hardwood woodchips, which ensured volumetric consistency between treatments but did not maintain functional equivalence in terms of electron donor mass or reactive capacity. Since woodchips provide the primary source of degradable organic carbon and electrons for denitrification, a reduction in nitrate removal would be expected based on mass balance considerations alone when a portion of the woodchip volume is replaced with an inert polymer matrix, independent of any material-specific limitations. Accordingly, the reduced performance observed in Brotex-amended treatments is interpreted primarily as a consequence of decreased reactive carbon availability, although differences in surface chemistry and electrochemical activity may still play a secondary role.

The influence of Brotex on reactor performance appeared to be primarily biological rather than hydraulic. Previous tracer analyses showed that the addition of Brotex increased drainable porosity from 53.6% to 55.4% but did not substantially alter overall hydraulic behavior because both treatment configurations exhibited minimal dead space ($\sim 1\%$) and short-circuiting ($\sim 4\%$) [32]. Thus, any treatment differences were more likely related to the additional microbial attachment area provided by the fibrous matrix than to changes in flow dynamics. Despite this potential advantage, Brotex did not consistently improve nitrate removal across temperature regimes, hydraulic residence times, or the duration of the experiment. This suggests that denitrification was more strongly constrained by environmental and operational factors, particularly temperature and hydraulic residence time, than by attachment surface area.

Several properties of the walnut-shell biochar may have contributed to the nitrate removal observed in this study. The material exhibited a high C:N ratio (199), indicating substantial carbon content relative to nitrogen within the biochar matrix (Appendix C).

In contrast, the measured surface area was relatively low compared with the European Biochar Certificate benchmark of $150 \text{ m}^2 \text{ g}^{-1}$ (Appendix C), suggesting that surface area alone was unlikely to explain the observed treatment performance. The biochar also contained positively charged ions and a range of trace metals that may have influenced denitrification processes, although the specific role of these constituents remains uncertain. Positively charged sites may have enhanced retention of negatively charged nitrate ions, potentially increasing nitrate availability within biofilms. It is also important to note that the characterization data represent fresh biochar; chemical properties may evolve during bioreactor operation as carbon and nitrogen compounds are adsorbed and transformed, as reported by Mukome et al. [45]. Finally, walnut-shell biochar produced at moderate pyrolysis temperatures ($<700^\circ\text{C}$) may retain greater quantities of aliphatic compounds and other labile functional groups than highly carbonized materials, which could provide conditions favorable for initial microbial colonization and activity (Christianson et al. [14]; Mukome et al. [45]; Zhang et al. [46]).

Influent nitrate concentrations fluctuated throughout the experiment, but most observations remained below approximately 12 mg N L^{-1} . As a result, some variation in nitrogen removal rate could be attributed to changes in nitrate loading, although the overall concentration range was relatively modest. Addy et al. [5] reported that denitrifying bioreactors receiving influent concentrations greater than 30 mg N L^{-1} generally exhibited higher nitrogen removal rates than systems receiving intermediate ($10\text{--}30 \text{ mg N L}^{-1}$) or low ($<10 \text{ mg N L}^{-1}$) nitrate concentrations. Since the majority of influent concentrations in the present study were well below this higher-loading threshold, changes in nitrate availability were unlikely to be the primary factor governing treatment performance. Temperature and hydraulic residence time, therefore, likely exerted a stronger influence on denitrification than the observed variation in influent nitrate concentration.

The modeling framework applied in Han et al. [32] was utilized to improve nitrate removal estimates provided by mass-balance equations under non-ideal and time-varying conditions. Although denitrifying bioreactors are physically static systems, tracer studies and prior work have shown that internal dispersion, entrance and exit effects, and media heterogeneity often lead to mixed-flow behavior. In Han et al. [32], bromide tracer tests demonstrated that actual HRTs differed from design values and that a portion of reactor volume behaved as mixed flow rather than ideal plug flow. Under these conditions, same-day influent–effluent comparisons can misattribute nitrate removal to incorrect loading periods. By explicitly accounting for hydraulic lag, short-circuiting, and effective treatment volume, the model enabled a more accurate estimation of nitrate removal rates and efficiencies. Additionally, since the experimental bioreactors also received variable inflow concentrations over time, a process-based model was employed to align influent loads with effluent response based on measured HRTs.

In this study, normalized 16S rRNA gene copy numbers were used to scale the decay coefficient in the nitrate removal model, allowing removal estimates to adjust for relative differences in microbial biomass among treatments. This measurement provides a reproducible metric of total bacterial abundance that can vary spatially and temporally. While other functional marker genes involved in various steps of the complete microbial nitrate reduction pathway—such as the nitrate reductase (napA/narG), nitrite reductase (nirK, nirS), nitric oxide reductase (norB), and nitrous oxide reductase (nosZ)—could have been used, previous studies have highlighted challenges in obtaining significantly different results with these functional marker genes under varying temperatures [19]. Additionally, at the time this study was initiated, limited research had incorporated nirK or nirS into bioreactor analyses, leaving their relevance in such systems largely unexplored [33]. Given

these limitations, 16S rRNA gene copy numbers were deemed the most appropriate and reliable variable for inclusion in this study.

An additional limitation of this study is that the experimental design was not intended to isolate startup effects or distinguish short-term and long-term treatment responses independently. Consequently, differences associated with reactor maturation, microbial establishment, and media aging could not be separated from the effects of temperature, hydraulic residence time, and media configuration. Future studies specifically designed to evaluate startup dynamics and long-term performance may provide additional insight into the temporal effects of the media on denitrification processes.

This work lays the foundation for a wide range of future experiments involving multimedia bioreactor systems. Laboratory studies on multimedia bioreactors could explore variations in media proportions, introduce wet-dry cycling, apply high-concentration nitrate pulses, inoculate systems with different soils or microbial communities, test broader-range HRTs, and evaluate alternative non-wood-based biochars. Additional analyses could include monitoring for the removal of other nutrients or contaminants, assessing the potential degradation of PBCs into plastic particulates within the media or effluent, and conducting gas and microbial sampling in suspected low-activity zones. Furthermore, the nitrogen removal model used in this study should be validated using field-scale data. Given the promising preliminary results, future work could aim to optimize system design for cost-effectiveness by using the most effective media combinations in minimal quantities.

5. Conclusions

The numerical solution to the continuous stirred-tank reactors in a series (CSTRs) of Han et al. [32] was a useful tool in this study. This model was shown to accurately predict the measured effluent concentrations by calibrating only the first-order decay coefficient. The model provided flexibility in analyzing nitrate removal for a relatively large experimental design using various media, temperatures, and residence times.

Optimized bioreactor systems have the potential to perform effectively under the reduced temperature and high-flow conditions typical of spring drainage in northern climates. In the multimedia bioreactors evaluated here, containing woodchips, walnut-shell biochar, and Brotex material, nitrogen removal rates and nitrate removal efficiencies exceeded many values reported for woodchip-only bioreactors in the literature under similar temperature and hydraulic residence time conditions. These findings demonstrate that the tested media combinations can achieve high nitrate removal performance under challenging low-temperature and short-HRT operating conditions. However, the addition of Brotex did not enhance reactor performance. The walnut-shell biochar may have contributed to treatment performance through a combination of physical and chemical properties, including sorption processes and provision of microbial attachment surfaces. Incorporating more direct measurements of denitrifying microbial activity into future bioreactor models could further improve understanding of the mechanisms controlling nitrate removal. Although the evaluated systems performed well, additional research is needed to optimize media composition and reactor design to further support nutrient reduction goals established by state and regional water-quality programs.

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Abbreviations

The following abbreviations are used in this manuscript:

DO	Dissolved oxygen
HRT	Hydraulic residence time
NO ₃ [−]	Nitrate
NO ₂ [−]	Nitrite
N ₂	Nitrogen gas
N ₂ O	Nitrous oxide
CSTR	Continuous stirred-tank reactor
qPCR	Quantitative polymerase chain reaction
DNA	Deoxyribonucleic acid
ORP	Oxidation-reduction potential
HDPE	High-density polyethylene
PBC	Polybutylene carbonate
PET	Polyethylene terephthalate
C/N	Carbon-to-nitrogen ratio

Appendix A. Equations for Theoretical Framework of Plug-CSTR in a Series Model

Nitrate mass balance for a reactor with dead space and short-circuiting is evaluated as

$$(f_s Q_{in} C_{in} + (1 - f_s) Q'_{in} C'_{in}) - Q_{out} C_{out} - \dot{R}_i = \frac{d((1 - f_d) V_r C_{out})}{dt} \quad (A1)$$

where Q_{in} and Q'_{in} are the volumetric flow rate into the reactor from the previous reactor and short-circuited flow that bypassed the previous reactor, respectively; C_{in} and C'_{in} are the corresponding concentrations (mass per volume); Q_{out} and C_{out} are the flow rate and concentration out of the reactor, respectively; V_r is the volume of the reactor; f_s is the fraction of the non-short-circuiting flow; f_d is the fraction of dead space; t is time; and $\dot{R}_i$ is the mass removal rate. For a continuous stirred-tank reactor, the concentration in the reactor is equal to that leaving. The right-sided term of Equation (A1) is the rate of change in mass in the reactor, the bracket term on the left-sided of Equation (A1) is entering mass rate, and $Q_{out} C_{out}$ is the mass rate leaving the reactor.

Nitrate removal by denitrifying bacteria was defined using a first-order reaction as

$$\dot{R}_i = -\frac{dC_{out}}{dt} = \kappa'_{b1}C_{out} = Q_{out}\kappa_{b1}C_{out} \quad (A2)$$

where κ'_{b1} is the first-order reaction coefficient reactor (time^{-1}) and κ_{b1} is a dimensionless first-order coefficient defined as $\kappa_{b1} = \kappa'_{b1}(V_r/Q_{out})$. The numerical solution to Equation (A1), as developed by Han et al. [32], computes the unknown concentration out of each CSTR (mg L^{-1}) for any given time step as

$$C_{out,2} = W_1(C_{in,2} + C_{in,1}) + W_2(C_{in',2} + C_{in',1}) + W_4C_{out,1} \quad (A3)$$

where subscripts 1 and 2 refer to values at the beginning and the end of the time step. The coefficients W_1 through W_4 are constant and known and defined as

$$W_1 = \frac{(\gamma_{in}f_s - (\gamma_{in} - 1))\frac{\Delta t}{2}}{t_d + (1 + \kappa_{b1})\left(\frac{\Delta t}{2}\right)} \quad (A4a)$$

$$W_2 = \frac{\gamma'_{in}(1 - f_s)\frac{\Delta t}{2}}{t_d + (1 + \kappa_{b1})\left(\frac{\Delta t}{2}\right)} \quad (A4b)$$

$$W_4 = \frac{t_d - \beta\left(\frac{\Delta t}{2}\right)}{t_d + (1 + \kappa_{b1})\left(\frac{\Delta t}{2}\right)} \quad (A4c)$$

where $\gamma_{in} = \bar{Q}_{in}/\bar{Q}_{out}$ and $\gamma_{in'} = \bar{Q}_{in'}/\bar{Q}_{out}$. The detention time (t_d) is defined as $\bar{V}_a/\bar{Q}_{out}$, where $\bar{V}_a = (1 - f_d)\bar{V}_r$ is the average total active reactor volume.

Appendix B. Equations to Process-Based Adjustments to Nitrate Removal

To account for changes in microbial abundance by treatment and over time, a microbial-based equation incorporating total bacteria cell numbers (approximated based on 16S rRNA gene quantification; Figure 19 in [33]) was integrated into the numerical model. This equation used empirical data from a quantitative real-time PCR analysis performed on DNA extracts from reactor media to determine the abundance of bacterial 16S rRNA genes per gram of treatment media [33]. For this analysis, 12 small mesh bags (AKA biobags) containing appropriately proportioned treatment media were placed in perforated vertical tubes along the horizontal profile corresponding to the outlets of tanks 1, 2, and 3. Each individual value of 16S rRNA gene copies was normalized by the average 16S rRNA gene copy number across all tubes for any given time using

$$R = \frac{16S_n}{16S_n} \quad (A5)$$

where the “n” subscript refers to tubes/tanks 1, 2, or 3.

Since there were no tubes corresponding to the reactor outlet, an exponential regression equation was used to interpolate the values for the outlet using the R values for tubes 1–3 plotted against reactor volume. Linear and polynomial regression equations estimated some outlet values as <0 , so these options were considered unreasonable and not used.

The decay coefficient was adjusted using a multiple (M) to reflect the ratio of the total number of bacteria (by volume of pore space) to the average [33]. This assumes a power relationship, with the constant exponent (n) and scaling coefficient (α), between decay

coefficients in each tube (κ_{d1i}) and bacteria 16S rRNA gene copy numbers (16S). The effect of the adjustments ($\dot{R}_i$) for reactor i is

$$\dot{R}_i = \frac{\kappa_{d1i}}{\kappa_{d1}} = \frac{\alpha(16S_i)^n}{\alpha(16S)^n} = \left(\frac{16S_i}{16S}\right)^n = M_i^n \quad (A6)$$

where the average first-order coefficient is determined directly from observed data. The adjusted first-order decay coefficient for reactor i (κ_{d1i}) is now defined as

$$\kappa_{d1i} = \kappa_{d1} M_i^n \quad (A7)$$

This adjustment is used in all of mass removal relationships, including the W terms of Equations (A4a)–(A4c).

For each time step j , the total mass of nitrate removed for all n tanks (R_R) is

$$R_R = \sum_{i=1}^n \dot{R}_i \Delta t \quad (A8)$$

The total mass of nitrate removed for all n tanks over a time interval (t_i to t_f) (R_T) is

$$R_T = \sum_{t_i}^{t_f} R_R \quad (A9)$$

Removal efficiency (R_e) (fraction), as the mass of nitrate removed per inflow nitrate mass (M_{in}) over the time interval, is now

$$R_e = \frac{R_T}{M_{in}} \quad (A10)$$

The nitrate removal rate (R_L) is found by

$$R_L = \frac{R_T}{T_f - T_i} \quad (A11)$$

where the difference in mass removed (R_T) is in g and the difference in time (T) is in days. To calculate the nitrogen removal rate, R_L is multiplied by 0.225 (the ratio of the mass of N in NO_3^-).

Appendix C. Biochar Characterization

Table A1. Table of biochar characterization properties as compared to standards determined by the International Biochar Initiative (IBI) and European Biochar Certificate (EBC). All measurements are taken from dry basis unless otherwise noted. Values presented are based on measurement using samples as received (*), and the maximum threshold range is determined by the soil tolerance level of application.

Property	Unit	Biochar	IBI	EBC
pH _{CaCl2} *	NA	6.6		≤10
C _{total}	%	87.6		>50
C _{org}	%	87.6	≥10	
C _{inorg}	%	<0.1		
C _{fixed}	%	83.5		
N _{total}	%	0.44		

Table A1. Cont.

Property	Unit	Biochar	IBI	EBC
C _{org} :N	ratio	199		
H	%	2.85		
O	%	7.5		
H:C _{org}	ratio	0.39	<0.7	<0.7
O:C	ratio	0.064		<0.4
Ash ₅₅₀	%	1.9		
Ash ₈₁₅	%	1.6		
EC *	µS/cm	116		
SA	m ² /g	0.6264		
Ca	mg/kg	1400		
Fe	mg/kg	1700		
K	mg/kg	5000		
Mg	mg/kg	360		
B	mg/kg	5		
Cd	mg/kg	<0.2	1.4–39	<1.5
Cr	mg/kg	2	64–1200	<90
Cu	mg/kg	11	63–1500	<100
Hg	mg/kg	<0.07	1–17	<1
Mn	mg/kg	27		
As	mg/kg	<0.8	12–100	<13
Na	mg/kg	65		
Ni	mg/kg	1	47–600	<50
P	mg/kg	480		
Pb	mg/kg	3	70–500	<150
S	mg/kg	110		
Si	mg/kg	270		
Zn	mg/kg	5	200–7000	<400
PAHs	mg/kg	162	6–300	<12

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