Effects of influent composition and substrate type on nitrate and *Escherichia coli* removal in laboratory-scale denitrifying bioreactors

By

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ABSTRACT

Escherichia coli (*E. coli*) and nitrate (NO_3^-) are common contaminants found in waters down gradient of wastewater sources such as septic tanks and agricultural operations. Carbon-rich subsurface denitrifying bioreactors are water treatment technologies that have the potential to decrease concentrations of NO_3^- and pathogenic pollutants released to water resources. Recent reports have indicated the potential for laboratory scale denitrifying woodchip bioreactors to significantly decrease *E. coli* concentrations at hydraulic retention times (HRTs) >10 hours. The goals of the current column study are to compare *E. coli* and NO_3^-+nitrite (NO_x-N) removal efficiency of various substrates (gravel, peanut shells, or woodchips) receiving influent water containing either NO_3^-+*E. coli*, *E. coli* only, or NO_3^- only, at a target HRT of 6 hours.

NO_x-N removal efficiency was significantly higher in woodchips (25%-30%) than both peanut shells (8%-18%) and gravel (-2%-2%), regardless of the presence of *E. coli*. Total nitrogen (TN) removal efficiency trends support that NO_x^- removal efficiency represent N removed from the system and not simply transformed to another species. *E. coli* removal efficiency was significantly higher ($p<0.05$) in gravel (medians of 88%-96%) than peanut shells (70%-86%) and woodchips (30%-67%), regardless of whether NO_3^- was added. When NO_3^-+*E.*

coli was treated, both woody substrates performed similarly. When influent water had *E. coli* only, peanut shells significantly outperformed woodchips. *E. coli* removal efficiency of woodchips and gravel significantly decreased when NO_3^- was not added.

Typically, effluent dissolved organic carbon (DOC) concentrations were significantly higher than influent concentrations; however, when influent water contained *E. coli* only, only woodchips significantly increased DOC concentrations. Effluent DOC concentrations never varied significantly by substrate type when receiving the same influent water.

In both scenarios where only one pollutant was added to influent water, peanut shells resulted in significantly higher ammonium (NH_4^+) concentrations than that of the influent. Peanut shells also released significantly more NH_4^+ when *E. coli* only was treated compared to when NO_3^-+*E. coli* was treated, whereas woodchips released significantly less when *E. coli* only was treated, and gravel remained unaffected by changes in influent composition.

In most scenarios, all substrates resulted in effective removal of NO_x and/or *E. coli*. Woodchips always removed the most NO_x and gravel always removed the most *E. coli* followed closely by peanut shells. Woodchips and gravel both achieved significantly better *E. coli* removal when NO_3^- was added to influent water, but peanut shells were not impacted by the addition. The findings reported here indicate that a paired-media approach may be best to treat for both contaminants and supports the need for further research into *E. coli* removal mechanisms and NO_x^- removal using novel substrates at varying HRTs and at the pilot- and field-scales.

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DEDICATION PAGE

I would like to dedicate this thesis to my husband, William Brink, and to my family for always believing in me and supporting me.

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1. INTRODUCTION

1.1 Pollutants

Contamination of ground and surface waters by inorganic nitrogen (nitrate (NO_3^-), nitrite (NO_2^-), and ammonium (NH_4^+)) and bacterial pollutants is a major environmental concern across the globe. As freshwater stores are depleted and human population continues to rise, responsible management of freshwater resources and aquatic ecosystems, which act as major food and revenue sources for countless civilizations, is becoming increasingly important.

1.1.1 Nitrate

Dissolved nitrogen in the form of NO_3^- is the most commonly identified groundwater contaminant globally (Bedessem et al., 2005). The U.S. Environmental Protection Agency (USEPA) identifies the maximum contaminant level (MCL) for nitrogen in the form of nitrate (NO_3^--N) in ground and surface waters at 10 mg/L (USEPA, 2002) due to increased risks of methemoglobinemia when concentrations are in excess (Humphrey et al., 2013). In addition, blackwater streams, typically low in nutrients, are common in the North Carolina coastal plain and are exceptionally sensitive to nutrient inputs, with increases of nitrogen (N) as low as 1 mg/L stimulating algal blooms (Humphrey et al., 2013) resulting in anoxic zones and an accumulation of toxins harmful to human and animal health (Zingone and Enevoldson, 2000). These events pose not only human health and ecosystem concerns but have also led to closure of water bodies for commercial fishing (Mallin and Cahoon, 2003) resulting in severe revenue loss. A vulnerability assessment of all US watersheds, based on risk factors such as soil percolation, runoff potential, and mass of animal nutrient application, ranked many North Carolina coastal plain watersheds, including the Albemarle-Pamlico and Cape Fear, among the most vulnerable in the country (Mallin and Cahoon, 2003).

1.1.2 E. coli

Escherichia coli (*E. coli*) is often concomitant with NO_3^- in ground and surface waters (Harter, 2003) and can cause human illness and/or death (Van Elsas et al., 2011). In addition, excessive concentrations of *E. coli* have also been associated with closures of economically valuable shell-fishing waters (Coulliette and Noble, 2008). Because of its intestinal origins (Odonker et al., 2013), *E. coli's* presence in water is interpreted as an indication that fecal contamination has occurred (Bain et al., 2014; Odonker and Ampofo, 2013). Although other fecal indicator bacteria such as total coliforms (TC) can be used to identify fecal contamination, *E. coli* is often chosen because it is more specific (i.e., there is a lower likelihood of *E. coli* introduction to water under scenarios other than fecal contamination) (Odonker et al., 2013). Fecal contamination is accepted as the biggest contributor to groundwater pathogen occurrence, as these pathogens pose the most widespread risk associated with drinking water (Morris et al., 2022) As such, *E. coli* is commonly used to determine efficacy of water treatment technologies aimed at removing pathogenic bacterial pollutants associated with fecal contamination (Al-Imara et al., 2021; Soupir et al., 2018; Stevik et al., 1999).

Groundwater serves 42.5 million people in the United States more than 2 million people in North Carolina (Mulhern et al., 2021). People dependent on groundwater are at higher at risk of gastrointestinal illness than those reliant on centralized water systems, as untreated groundwater has led to as many as 59.4 million cases of acute gastroenteritis globally (Morris et al., 2022). One large-scale analysis (n=32,839) of water collected from new North Carolina groundwater wells reported that 81 out of 90 counties collected *E. coli* positive samples with positive test rates ranging from 0.17%-12.7%. Samples collected from the outer coastal plains were more likely to report positive *E. coli* results than those farther inland (Morris et al., 2022).

As dependence on groundwater increases, demand for low-cost, low-maintenance and long-lasting approaches to groundwater remediation of bacterial and nutrient contaminants is required to ensure wide-spread access to safe drinking water supplies.

1.2 Pollutant sources

E. coli and N often come from many of the same sources and are often associated with human and other animal waste. Although many sources of N and *E. coli* exist, for the purposes of this paper, we will focus on those of particular concern in eastern North Carolina, which include septic systems and concentrated animal feeding operations (CAFOs).

1.2.1 Septic systems

Septic systems are designed to treat household wastewater on-site in rural and, less frequently, suburban locations (Robertson, 2021). In the United States (US), 22 million septic systems are in operation and, based on US Census Bureau data from 1999, release approximately four billion gallons of wastewater annually (Gerba and Smith, 2005). In North Carolina, 60% of residents utilize septic systems rather than municipal sewer systems (Humphrey et al., 2010). While the magnitude of effects on groundwater quality varies based on system type, maintenance, and installation factors, as well as soil characteristics, climate, and geology of the installation location (Humphrey et al., 2010; Yates, 1985), groundwater contamination associated with septic tanks is a common occurrence (Robertson, 2021). In fact, 60% of states, tribes, and territories reporting to the US Environmental Protection Agency (EPA) during the 2000 National Water Quality Inventory Report to Congress reported septic tanks as a major groundwater polluting source (Bedessem et al., 2005).

The nitrogenous waste entering septic systems primarily consists of organic nitrogen (ON) and NH_4^+. The systems are designed to mineralize ON to NH_4^+ within the septic tank, then

release effluent from the tank into a drain field where nitrification (unsaturated zone) and denitrification (saturated zone) can take place, converting NH_4^+ to NO_3^- and NO_3^- to N_2, respectively (Humphrey et al., 2015). However, due to improper installation and/or maintenance, as well as subsurface heterogeneities throughout installation sites, it is not uncommon for systems to underperform (Karathanasis et al., 2006). Because of this, NO_3^--N concentrations of >20 mg/L have been reported in groundwater adjacent to septic systems (Humphrey et al., 2015) and, in one North Carolina watershed, septic systems contribute an estimated 1.6 kg N/ha/year (Iverson et al., 2015).

Successful removal of bacteria from wastewater using septic systems has also proven problematic. After waste is treated in the septic tank, associated effluent has been shown to contain *E. coli* concentrations as high as 9.7×10^6 CFU/100 ml (Humphrey et al., 2011). Although installations of septic systems are required to adhere to specific guidelines meant to ensure proper bacterial treatment, natural heterogeneities in pore size and distribution within soils and variability in groundwater depth make ensuring proper treatment difficult and often result in inadequate *E. coli* treatment (Karathanasis et al., 2006). Successful pathogen filtration in septic drain fields relies on unsaturated flow conditions, relatively homogenous soil structures and an ideal soil size range (Karathanasis et al., 2006). When sediments are too coarse, water can drain through too quickly and without enough interaction with the sediment to result in effective filtration. When sediments are too fine, clogging may result in reduced soil permeability and system failure over the long term (Karathanasis et al., 2006). Results from an assessment of 10 soil samples from four groups (all of which fall within the guidelines set by the Kentucky Health Department) concluded that, even without visible macropores, only 20% of samples decreased fecal bacteria to the extent necessary to meet EPA criteria (Karathanasis et al., 2006).

1.2.2 Concentrated animal feeding operations (CAFOs)

CAFOs are facilities where animals are raised in high densities, predominantly indoors, for most of their lives, and are the primary manner by which animals are raised for human consumption today. In the US alone, these facilities create 133 million tons of manure annually (based on 1998 USEPA estimates) (Burkholder et al., 2007). The enormous amount of waste produced in swine CAFOs is typically held in waste storage lagoons which are constructed as basins extending into the subsurface and are designed for anaerobic treatment of animal waste. The base of these lagoons is often lined with clay bottoms, synthetic liners, or concrete; however, these liners frequently leak and allow waste to seep into the subsurface. In addition to subsurface seepage, when lagoons become overloaded, as may happen during large precipitation events, waste can spill over the side walls or cause the walls to fail entirely resulting in severe ground and surface water contamination (Burkholder et al., 2007).

CAFO-related coliforms have been tracked as far as 30 km downstream from spill sites, and when the bacteria settle down into the underlying sediment, they can exist at high concentrations for months (Burkholder et al., 2007). A study by Anderson and Sobsey (2006) reported significantly higher concentrations of *E. coli*, and a greater occurrence of antimicrobial resistant traits, at two swine farm sites as compared to two reference sites not associated with farming operations over the course of 1.5 years.

Unfortunately, closure of waste storage lagoons is not a guaranteed solution as the adjacent soils can be contaminated with NH_4^+ which, when exposed to air, is converted to NO_3^- (Yoon et al., 2014). As an example, a swine waste lagoon in operation for 25 years holds a buildup of 81,200 lb of NH_4^+-N in adjacent soils per acre of lagoon surface area and can impact soils at depths up to ~12 ft (Volland et al., 2003). North Carolina is not the only state facing

these high nutrient-load inputs from CAFOs; chemical and isotopic analysis of groundwater in Kansas revealed that animal waste contributed to NO_3^--N concentrations up to 22 mg/L at a depth of 161 ft (Volland et al., 2003).

When lagoons operate under ideal conditions and waste is successfully held for a full treatment cycle, the treated waste is then sprayed onto or injected into the subsurface of agricultural fields as fertilizer (Mallin and Cahoon, 2003). Although this practice does add nutrients necessary for plant growth, even at the recommended application rates, pollution of adjacent surface and groundwaters is often reported (Burkholder et al., 2007) For example, Evans et al. (1984) reported NO_3^--N concentrations ranging from 7-30 mg/L in a stream adjacent to a swine effluent sprayfield. In the early 1990s, one study found that twenty-two percent of domestic wells that were tested in eastern NC were found to have NO_3^--N concentrations above the MCL and reaching as high as 18 mg/L. Researchers identified agricultural operations as the most likely source of NO_3^- (Humphry et al., 2013). Although lagoon-related NO_3^- pollution is a nearly ubiquitous problem associated with agricultural fields amended with animal waste, bacterial pollution is more dependent on environmental conditions. Sunny, dry surface conditions typically inactivate *E. coli* within days; however, when rain events occur shortly after application, there are less unsaturated soils available to treat the bacteria and they may migrate into the subsurface or to nearby waterways more successfully (Mallin and Cahoon, 2003).

Consumption of water contaminated with hog waste can cause acute gastrointestinal distress (AGI), which manifests as nausea, vomiting, or diarrhea (Quist et al., 2022). In 2019, North Carolina was home to 9,500,000 swine, with the top producing counties (Duplin, Sampson, Bladen) located in the Coastal Plain (USDA, 2021). A recent study identified an increased occurrence of North Carolina residents who lived nearer to swine farms visiting the

emergency department (ED) due to AGI than by those who lived farther away. They reported an even higher increase in AGI related ED visits when the study was restricted to those residing in rural areas, after rain events, and in areas where poultry and swine CAFOs operate (Quist et al., 2022).

2. DENITRIFYING BIOREACTOR BACKGROUND

Subsurface denitrifying bioreactors are bed or wall type structures installed below the ground surface, and filled with carbon-rich, porous material. Historically, the purpose of these systems has been to intercept water containing high concentrations of NO_3^-; the carbon-rich substrate encourages aerobic respiration to decrease influent dissolved oxygen (DO) and supplies electrons and an attachment substrate to stimulate denitrification (Christianson et al., 2012). Denitrifying bioreactors have been extensively studied and have been categorized as a best management practice (BMP) by the United States Department of Agriculture (USDA). They offer a low-cost, low maintenance, long-lasting means of decreasing the mass of NO_3^- released into natural waterways from stormwater runoff (Lopez-Ponnada et al., 2017), on-site wastewater treatment systems (Humphrey et al., 2015; Lopez-Ponnada et al., 2017; Rambags et al., 2016; Rambags et al., 2019), animal waste (Aalto et al., 2022; Chambers, 2018; Lepine et al., 2016), and agricultural waters (Bell et al., 2015; Christianson et al., 2012; Soupir et al., 2018). More recently, they have shown promise in lab-, pilot-, and field- scale studies as effective pathogen removal mechanisms. The constraints and limitations on the ability of bioreactors to sequester bacterial pathogens such as *E. coli (*Rambags et al., 2016; Robertson et al., 2005; Soupir et al., 2018; Tanner et al., 2012) and *Salmonella* (Soupir et al., 2018), as well as plant pathogens such as *Phytophthora* spp. (Bell, 2019), are now being explored.

2.1 Denitrifying bioreactor nitrogen removal

Denitrification is the naturally occurring, microbially mediated process where NO_3^- and NO_2^- are reduced to inert dinitrogen gas (N_2). Denitrifying bacteria are facultative anaerobes which reduce NO_3^- in low oxygen conditions (ideally <0.02 mg/L but this varies by species of

denitrifying microbe) when carbon (C) and a viable attachment substrate are available (Christianson et al., 2012). In subsurface environments, limited available C is the most common hindrance to denitrification (Gibert et al., 2008). Denitrifying bioreactors act as a C source to stimulate efficient denitrification.

Denitrifying bioreactor N removal is typically reported as either mass removal rate (mass of N removed per bioreactor volume over time), removal efficiency (expressed as a percentage of N removed as compared to influent concentration), or load reduction (mass of N removed over time). The longer water remains in the bioreactor (higher HRT), and thus in contact with denitrifying microbes mediating the system's N-cycling processes, the more NO_3^- is removed from each pore volume of water (higher removal efficiency); however, mass removal rate and load reduction generally increase with flow rate, therefore decreasing with HRT (e.g. Hassanpour et al., 2017).

Denitrifying bioreactor exploration began in 1994 when Blowes et al. published results from pilot-scale mixed-media denitrifying bioreactors in Canada showing near 100% removal efficiency. Over the last several decades, the use of C-rich denitrifying bioreactors has proven useful in lab-, pilot- and field-scale experiments, removing up to 99% of influent NO_3^- (Christianson et al., 2012). The efficacy of these systems has proven to be highly dynamic and dependent on environmental conditions as well as system design and installation parameters. A few factors known to drive variations in denitrifying bioreactor performance include HRT, substrate type and influent characteristics.

The first denitrifying bioreactor review published was that of Schipper et al. (2010) which outlined the main types of denitrifying bioreactors as well as factors that limit performance and their efficacy. In 2012, Christianson et al. followed up with a review focused on

the practical use of denitrifying bioreactors for agricultural drainage; they lay out the requirements for denitrification which include influent NO_3^-+ NO_2^- (NO_x) as electron acceptors, denitrifying bacteria, a carbon source, and suitable DO conditions. They focused on the importance of carbon media choice due to its impact on N-removal performance and feasibility of installation. In 2016, Addy et al. performed the first quantitative analysis of published denitrifying bioreactor literature, analyzing 26 published studies of denitrifying beds, walls, and lab-scale columns focusing specifically on those using woodchips. They compared NO_3^- removal rates to design type, wood source, unit age, N limitation, and HRT. They found that lab-scale studies and bed designs often report similar removal rates which are significantly higher than those achieved by denitrifying walls but noted that only three studies reported wall bioreactor performance and that walls are often used in N limited systems, which may have influenced their findings. In addition, thorough comparisons were limited by the fact that only four of the 10 lab studies analyzed reported on more than one physical column unit; these limitations may have influenced the findings of lab-scale column comparisons.

2.1.1 Nitrogen removal mechanisms

Multiple, naturally occurring, microbially mediated N processing pathways exist; among them are denitrification, dissimilatory NO_3^- reduction to ammonia (DNRA), and anaerobic ammonium oxidation (ANAMMOX). The dominant pathway in any system depends on complex interactions between microbial community, substrate characteristics, and environmental factors (Carlson et al., 2020; Hartfiel et al., 2021; Lopez-Ponnada et al., 2017). Although denitrifying bioreactors are home to diverse microbial communities (Carlson et al., 2020; Hartfiel et al., 2021), they have been engineered in such a way that associated microbes tend to favor genes responsible for denitrification over other N processing pathways (Audet et al., 2021).

Denitrification occurs under low DO conditions (< 0.02 mg/L is ideal) and requires access to bioavailable C (Christianson et al., 2012).

2.1.2 Performance factors

Treatment efficiency of denitrifying bioreactors depends on a variety of factors including (but not limited to) HRT, influent composition, and substrate characteristics. As mentioned earlier, HRT is the amount of time that water spends within the system and increasing HRT typically increases treatment efficiency. Influent composition and substrate characteristics both affect removal efficiency by impacting the microbial community within the bioreactor. Below is a summary of studies exploring the impacts of HRT and substrate type, two of the main parameters of interest for this study.

How variations in HRT impact denitrifying bioreactor NO_3^- removal has been the focus of many denitrifying bioreactor studies at all scales. In 2015, Bell et al. assessed the effects of both temperature and HRT variations on the performance of field-scale mixed-species woodchip bioreactors receiving a simulated agricultural drainage water containing NO_3^--N concentrations ranging from < 0.1 mg/L-17 mg/L. NO_3^--N removal efficiency ranged from 20-98% and averaged 63%. Multiple regression models indicate that water temperature and HRT explain 43% of that variation. A more recent study focused on identifying differences in denitrification in lab-scale woodchip columns attributable to variations in HRT (ranging from 2-24 hours) and reported NO_3^- removal efficiency ranging from 8-55%, which tended to increase with HRT (Hoover et al., 2016). In 2017, Hassanpour et al. published a paper focused on the effects of seasonal variations in temperature and precipitation in six field-scale woodchip ± biochar bioreactors over the course of three years. They reported removal efficiencies from 20-98% resulting in an average of 50% which increased with HRT. Another team of researchers analyzed

NO_3^- removal using lab-scale woodchip bioreactor and reported changes in removal efficiency with both temperature and HRT but noted a more dramatic increase in NO_3^- removal efficiency with increased HRTs compared to those with increased temperatures. NO_3^- removal efficiency at a 12-hour HRT averaged 67% and 96% at an HRT of 24 hours at the same temperature (Soupir et al. 2018). Then in 2020, Abdi et al. reported that 99% of the NO_3^- in simulated nutrient-enriched agricultural runoff waters containing 18 mg NO_3^--N/L was removed when treated by a lab-scale denitrifying woodchip bioreactor paired with a phosphate filter operating at an HRT of 72 hours. However, when the same bioreactors were operated under a 21-minute HRT, the effluent released had NO_3^- concentrations similar or higher than those found in the influent.

Many published denitrifying bioreactor studies explore NO_3^- removal at longer HRTs (>10 hours) (e.g. Abdi et al., 2020; Soupir et al., 2018) and while they typically lead to higher removal efficiency, they can also lead to NO_3^- depletion resulting in increased pollutant swapping behavior (Hellman et al., 2021) (more on pollutant swapping in section 2.2 below). Because of this, as well as the need for in-field treatment at low HRTs (Christianson et al., 2012), understanding denitrifying bioreactor water treatment and impacts at low HRTs is and will continue to be an important focus moving forward.

As mentioned earlier, C availability is commonly the missing component which limits denitrification in natural subsurface systems (Xing et al., 2019). Denitrifying bioreactor substrates provide this C, and it is typically recommended that substrates be chosen with considerations to cost, porosity, carbon to nitrogen ratio (C:N), and potential for longevity (Christianson et al., 2012). In addition, the physical characteristics of bioreactor substrates influence microbial community composition (Carlson et al., 2020; Hellman et al., 2021). For example, Hellman et al. (2021) analyzed microbial communities developing in laboratory-scale

columns containing woodchips, straw, or sedge over the course of 270 days and reported that sedge and straw developed increasingly diverse communities over time, but woodchips did not. They also noted that differences in NO_3^- removal rate were reflected by differences in labile C content. Sedge and straw had significantly less total C, cellulose and lignin but significantly more hemicellulose and consistently removed more NO_3^- woodchips (when operated at similar HRTs). As we gain a better understanding of how substrate characteristics influence achieved water treatment, many researchers have taken to experimenting with innovative carbon sources aside from or in addition to woodchips.

Historically, substrates with lower C:N have been considered less desirable due to faster degradation and/or increased flushing losses, so while acceptable C:N of substrates varies widely, they are typically on the order of hundreds (Christianson et al., 2012). However, academic literature is rich with publications from researchers assessing raw and altered substrates with highly variable C:N in isolation and/or combined for use within denitrifying bioreactors. Among the substrates that have been tested are: various species of wood in the form of chips (Soupir et al., 2018; Abdi et al., 2020; Aalto et al., 2022), bark (Bell, 2019; Blowes et al., 1994; Camilo et al., 2023; Diaz-Garcia et al., 2003; Huang et al., 2015; Trois et al., 2010) and sawdust (Schipper and Vojvodić-Vuković, 2000) as well as mulch (Blowes et al., 2003; Camilo et al., 2013; Mala et al., 2017), hay, compost (Blowes et al., 1994), coconut husk (Rambags et al., 2019), corn cobs (Cameron and Schipper, 2010), walnut shells (Diaz-Garcia et al., 2003; Jing et al., 2010; Xu et al., 2020), peanut shells (Hu et al., 2022; Xing et al., 2019), almond shells (Diaz-Garcia et al., 2003; Diaz-Garcia et al., 2020), and flaxseed (Povilaitis and Matikiene, 2020); however, woodchips are ubiquitous throughout denitrifying bioreactor literature (Blowes

et al., 1994; Bell et al., 2015; Christianson et al., 2012; Rambags et al., 2019; Soupir et al., 2018).

2.2 Pathogen removal

Remediation of N pollution remains an important focus of denitrifying bioreactor research; however, attention is beginning to pivot toward the ability of these systems to decrease both bacterial and viral pathogens (Rambags et al., 2016; Rambags et al., 2019; Soupir et al. 2018). In 2005, Robertson et al. focused predominantly on their full-scale mixed wood-media bioreactor's ability to stimulate denitrification, but briefly reported on *E. coli* removal as well. They found that 79% of all effluent samples were completely devoid of *E. coli* regardless of the system receiving water with *E. coli* concentrations ranging from 20-200 CFU/100 mL. Then in 2012, Tanner et al. began exploring *E. coli* removal within various combinations of horizontal flow wetlands and carbonaceous bioreactors treating influent with *E. coli* concentrations averaging $3.5x10^6$ CFU/100 mL. They reported a range of mean fecal bacteria removal from 2.5-4.7log_{10}, with maximum removal achieved by the vertical flow sand media filter followed by woodchip and coconut husk denitrifying bioreactors. In 2016, Rambags et al. monitored a full-scale denitrifying woodchip bioreactor's ability to remove both *E. coli* and F-specific RNA bacteriophage (FRNA phage) viruses. They reported a consistent *E. coli* removal of 2.9log_{10} and 3.9log_{10} removal of FRNA phage regardless of the highly variable influent concentrations averaging $1.6x10^4$ MPN/100 mL. In 2018, Soupir et al. assessed the effects of HRT and temperature on bacterial removal and found that when comparing HRTs of 12 and 24 hours, temperature had a greater influence on performance than HRT did. *E. coli* concentration decreases ranged from 75-96%, with the lowest removal efficiency occurring at a 12-hour HRT and 10°C and the highest at 24-hour HRT and 21.5°C . Then in 2019, Rambags et al. compared removal of both *E. coli* and TC in fresh vs mature woodchip bioreactors to that of fresh and

mature coconut husk bioreactors operating at HRTs between 2-3.3 days and reported significant reductions in all cases. Fresh coconut husk was the only treatment that performed significantly better than the other substrates (1.8 $\log_{10}$ removal compared to other treatments which ranged from 1.2-1.7 $\log_{10}$ removal). However, the decrease in both *E. coli* and NO_3^--N removal efficiency of aged coconut husks vs that of fresh coconut husks was interpreted as a sign that coconut husks may not perform well over long periods of time, making them a less attractive denitrifying bioreactor substrate choice.

Total coliform and *E. coli* removal mechanisms in denitrifying bioreactors remain largely unexplored; however, they are likely similar to those being carried out in saturated slow sand filters and constructed wetlands containing various substrates including peat, aquatic plants, and gravel, both of which offer reportedly effective fecal coliform removal (usually 95 to >99%) (Vymazal, 2005). Many researchers have attributed *E. coli* removal in slow sand filters to the biological layer which often forms at the sand/air interface called the schmutzdecke (e.g. Haig et al., 2015) while others posit that removal is more dependent on sand physical characteristics, depending more on adsorption and microscopic surface structures (Chapetta, 2008; Weber-Shirk and Chan, 2007). *E. coli* removal in constructed treatment wetlands has been attributed to a combination of physical and chemical factors including inactivation, adsorption, settling (Boutilier et al., 2009), straining/mechanical filtration (Stevik et al., 2004), UV radiation, and adsorption to organic matter (Vymazal, 2005). Physical *E. coli* filtration using porous media is dependent on media grain size, bacterial cell size, hydraulic loading rate, and potential for clogging. Factors important for chemically driven pathogen removal such as adsorption or inactivation are organic matter content, biofilm development and electrostatic attraction, pH, temperature, and biotic interactions such as predation and inhibitory secretions from other

bacteria (Stevik et al., 2004). Particles with greater surface areas (i.e., smaller particles) provide more adsorption sites, additionally, rough surface textures decrease shear forces and decrease bacterial desorption rates. When macropores are present, media typically remove less bacteria due to decreased chance of bacteria contacting the surface, and adhesion to biofilms (Stevik et al., 2004).

2.3 Pollutant swapping

Pollutant swapping is a key issue which is becoming a more frequent topic of discussion in the assessment of denitrifying bioreactor performance. Pollutant swapping can generally be defined as "the increase in one pollutant as the result of a measure introduced to reduce another pollutant" (Healy et al., 2012). Pollutants of concern in this context include greenhouse gases (methane (CH_4), nitrous oxide (N_2O), and carbon dioxide (CO_2)), ammonia (NH_3), dissolved organic carbon (DOC), phosphorus (P), and dissolved metals (Healy et al., 2012) (including methylmercury derived from sulfate reduction) (Easton et al., 2015), which can all have adverse effects on aquatic ecosystems. Pollutant swapping potential can be determined by timing from start-up and/or flow dynamics (e.g., start-up period vs steady state, wet-dry cycles, etc.) and is influenced by operational factors, such as HRT and pH of water in the system, or by environmental factors such as influent NO_3^- concentrations as well as substrate characteristics (Carlson et al., 2020).

3. RESEARCH OBJECTIVES

Due to the diversity of pollutant sources and dynamic nature of the environments in which denitrifying bioreactors can be used, it is important to understand how NO_3^- and *E. coli* removal performance of various substrates compares and how those dynamics change with influent composition variability to allow for maximum water treatment efficiency with each installation. The goal of this research is to identify variations in woodchips, peanut shells and gravel's ability to remove *E. coli* and/or NO_3^- from each of three nutrient-amended and/or *E. coli* inoculated water (NO_3^-+ *E. coli*, *E. coli* only, or NO_3^- only) at a target HRT of 6 hours. We hypothesized that NO_3^- removal efficiencies would be greater among substrates with higher C:N and that *E. coli* removal would differ among substrates due to differences in surface properties.

4. MATERIALS AND METHODS

Three sets of triplicate up-flow bioreactor columns (Figure 1) (9 columns per set, 27 total columns), each filled with one of three substrates (woodchips, peanut shells, or gravel) were used to assess the NO_x^--N and *E. coli* removal efficiencies of each substrate under different influent compositions (NO_3^-+*E. coli*, *E. coli* only, or NO_3^- only) (Figure 2).

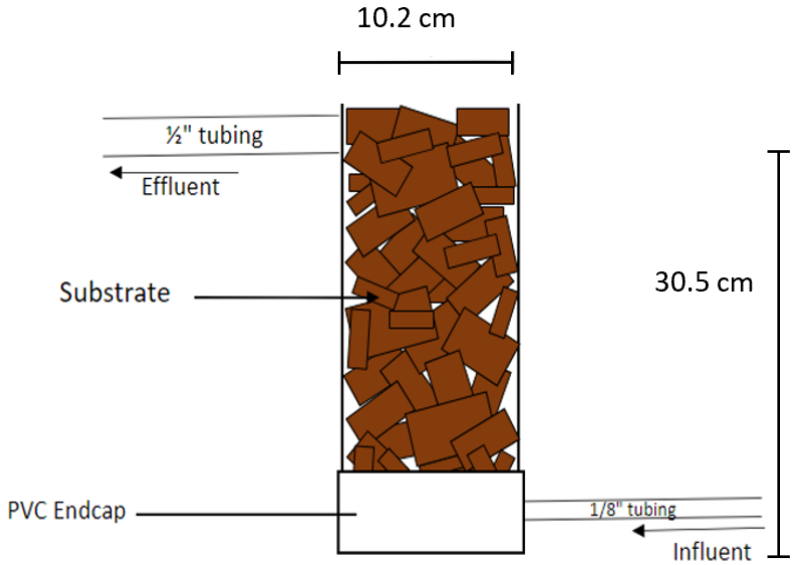


Figure 1. Column design replicated for 27 columns (9 with woodchips, 9 with peanut shells, 9 with gravel).

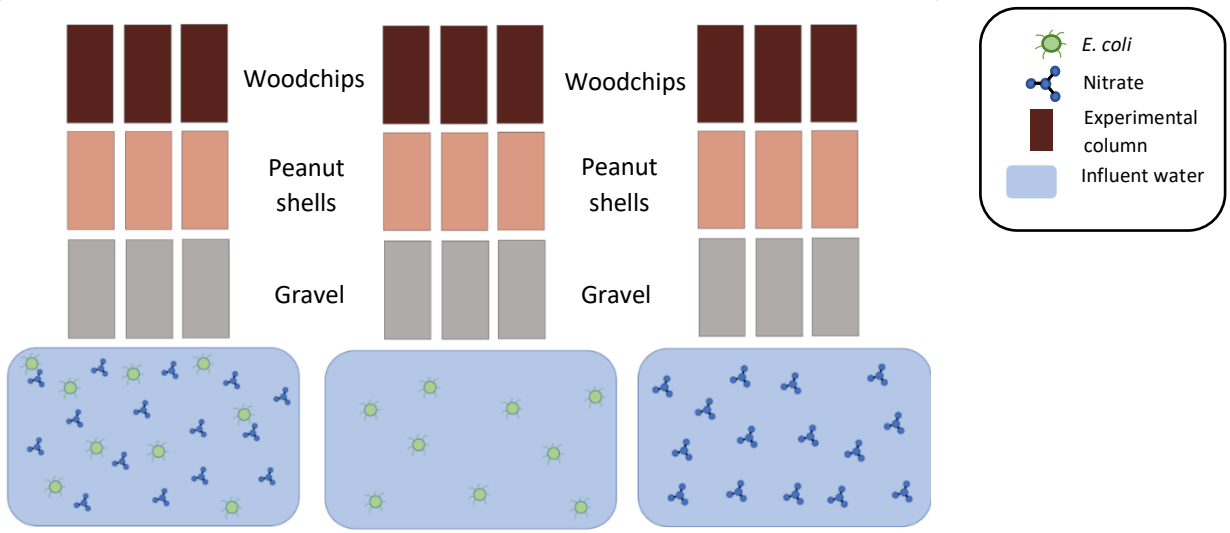


Figure 2. Schematic of experimental design including three physical replicates of each treatment consisting of a distinct substrate receiving influent water containing micronutrients (all) and either A) NO_3-N+*E. coli*; B) *E. coli* only; or C) NO_3-N only.

Although NO_3^- removal achieved using lab-scale bioreactors is often different than that of pilot- or field-scale (Addy et al., 2016), lab columns offer the opportunity to compare the impacts of different conditions (substrate type, temperature, influent composition etc.) on bioreactor performance (Pluer et al., 2016), which is the purpose of this experiment. Woodchips were chosen to represent the typical bioreactor substrate which have proven to perform well at NO_3^- and *E. coli* removal in some conditions, whereas peanut shells are a novel bioreactor substrate which are a readily available, inexpensive waste product (Adhikari et al., 2019). While NO_3^- removal using peanut shells as a bioreactor substrate has been explored in batch studies, no flow-through studies assessing NO_x nor *E. coli* removal abilities of peanut shells were found in the literature. Gravel was chosen as a non-carbonaceous control substrate to represent C limited conditions.

All columns were kept uncapped at room temperature and were operated at a theoretical total HRT of six hours. Flow rate to reach the theoretical total HRT was calculated as $Q = \frac{V*P}{HRT}$

where V represents column volume (2,492 cm^3), P represents total porosity (primary plus secondary porosity, see Tables 1 and 2) of the substrate, and HRT represents total HRT (360 minutes). Primary theoretical HRT was also calculated for each substrate using the same calculation but solving for HRT using Q (mL/min) that was calculated to reach six hours total HRT and replacing P with the primary porosity rather than total porosity.

One experimental run was completed each week over the course of five weeks. At the start of each run, all three influent tanks received identical amounts of both phosphate buffered saline (PBS) and micronutrients (described further in section 4.2) to support denitrifying microbes. Two of those influent stock tanks were inoculated with a green fluorescent protein (GFP) tagged *E. coli* strain and one of those two, as well as the third stock tank (which did not receive the *E. coli* inoculum), were amended with NO_3^-. When all stock tanks had been properly amended and/or inoculated, peristaltic pumps were started. After 15(± 1) hours, effluent grab samples were collected from each column and from the influent reservoirs; these samples were analyzed for NO_x-N, NH_4^+-N, and *E. coli*. Grab samples from triplicate replicate columns, packed with the same substrate and receiving the same influent, were combined to create individual bulk samples which were analyzed for DOC and total nitrogen (TN). After sample collection was complete, all columns that received influent inoculated with *E. coli* were flushed with water containing NO_3^- and micronutrients until no viable GFP *E. coli* was detected in the effluent. Pumps were turned off and columns remained saturated until the following week when another run was conducted.

Upon completion of experimental runs, a rhodamine dye tracer study (see Figure S1 in Appendix) was conducted using triplicate columns of each substrate receiving *E. coli* only and results indicate HRTs slightly lower than theoretical total HRT calculations. All theoretical and

measured HRTs can be found in Table 1. Mean (± SE) HRT of gravel columns was 4.8 (± 0.3) hours, 3.4 (± 0.2) for peanut shells, and 3.4 (± 0.4) for woodchips. These HRTs more closely align with the theoretical primary rather than total HRT calculation for each substrate.

Table 1. Calculated primary and total theoretical HRT as well as measured HRT from rhodamine dye tracer study results averaged over three replicate columns (± SE).

	Primary Theoretical HRT (hours)	Total theoretical HRT (hours)	Measured HRT (hours)
Woodchips	4.0	6	3.4 (± 0.4)
Peanut shells	4.5	6	3.4 (± 0.2)
Gravel	5.3	6	4.8 (± 0.3)

4.1 Column design and substrate characteristics

In total, 27 columns were constructed, each was packed with one of the three substrates and set to treat to one of three distinct influent water compositions. All columns were constructed of 32.2 cm tall, 10.2 cm diameter opaque white polyvinyl chloride (PVC) pipe fitted with an end cap on each base. 0.3 cm inner diameter PVC tubing was inserted into the side wall of each column near the base and received influent water and 0.6 cm inner diameter PVC tubing was inserted into the opposing side wall near the top of each column, through which effluent exited the column (Figure 1). The height of the saturated substrate column was 30.8 cm from the base of the PVC column to the base of the effluent tube.

The substrates assessed in this experiment were a waste woodchip mix predominantly consisting of hard wood species (East Carolina University, Greenville, NC, USA), raw peanut shells (Jimbo's Jumbo Peanuts, Edenton, NC, USA) and a granite gravel (E.R. Lewis Construction Company, Greenville, NC). Woodchips were chosen as they are the typical denitrifying bioreactor substrate, peanut shells are a readily available and inexpensive waste

product (Adhikari et al., 2019) and based on batch studies (e.g. Hu et al., 2022 and Xing et al., 2019), seem a plausible novel substrate capable of stimulating successful denitrification. All substrates were manually sieved to sizes between 0.6 cm-2.5 cm, a size-range similar to that which could be used in field-scale systems, and a sub-sample was sent to the NC State Agricultural lab (Raleigh, NC, USA) for C:N analysis (Table 2).

Table 2. Physical characteristics of each denitrifying bioreactor substrate used in the study.

	Primary porosity (%)	Secondary porosity (%)	Total porosity (%)	C:N ratio
Woodchips	56.6	28.1	84.7	114:1
Peanut shells	73.3	22.8	96.1	32:1
Gravel	45.0	5.0	50	N/A

Primary and secondary porosities of each substrate was measured (Table 2) following procedures outline by Mardani et al. (2020). Briefly, to determine primary porosity, sieved substrate and water were added to a 1-L container, and the volume of water it took to fill the pore spaces was recorded as the primary porosity. The containers were left overnight to allow the substrate to become saturated, and water was added as necessary to fill back up to one liter. After 24 hours, the mass of saturated material was recorded. Materials were then dried in oven at 105℃ for 24 hours and the mass recorded again; the difference between the saturated material mass and dried material mass was attributed to secondary porosity.

4.2 Influent solutions

Influent water was prepared using reverse osmosis (RO) filtered water amended with micronutrients to support denitrifying bacterial growth (Nadelhoffer, 1990; Soupir et al., 2018). Two source tanks, which each fed a distinct set of nine columns (three triplicate columns packed with each substrate), were inoculated with *Escherichia coli* GFP (ATCC 25922GFP), a strain that has been used in other environmentally focused bacteria transport studies such as Chiapponi

et al. (2020), where fluorescence was tracked through a constructed wetland system over the course of 19 days. One of those source tanks was not amended with NO_3^- (*E. coli* only), and the other, in addition to a third, was amended (NO_3^-+*E. coli* and NO_3^- only respectively). NO_3^- was added to a target concentration of 25 mg/L and influent water temperatures averaged 18 (± 0)º C.

A frozen (-80° C) culture of GFP tagged *E. coli* was purchased and used to grow isolated colonies on a tryptic soy broth (TSB) agar plate dosed with ampicillin to a final concentration of 100 µg/mL. A culture tube containing 10 mL of liquid TSB (ampicillin 100 µg/mL) was inoculated with bacteria from a single colony, collected with a disposable, sterile inoculating loop from the agar plate, and a liquid GFP culture was grown overnight (15 ± 1 hour). All cultures (broth and agar plates) were incubated at 37º C and liquid cultures were grown in automatic orbital shakers set at 180 RPM. Two µL of liquid culture/gallon water was suspended in 2.5 mL PBS/gallon of influent water prior to dispensing into *E. coli* only and NO_3^-+*E. coli* influent tanks. PBS suspensions were prepared immediately before inoculation to reduce time that cells were out of the ampicillin solution. Influent water containing *E. coli* was inoculated to a target concentration of $3.0x10^4$ CFU/100 mL. The NO_3^- only influent water received the addition of NO_3^- and PBS but no *E. coli* inoculum. All three tanks were constantly mixed using 80 gallon/hour submersible pumps (Simple Deluxe brand). The median *E. coli* concentration of influent water containing NO_3^- was $4.28x10^4$ CFU/100 mL (geometric mean of $4.58x10^4$ CFU/100 mL) and water without NO_3^- amendment was $3.14x10^4$ CFU/100 mL (geometric mean of $2.39x10^4$ CFU/100 mL) (Table 3).

4.3 Start-up

To simulate naturally occurring microbial communities in soils adjacent to a field-scale denitrifying bioreactor, soil from East Carolina University's West Research Campus was added to nutrient amended water which was pumped through all columns at the beginning of this experiment. The effluent water from all columns was combined and recycled for two weeks at a flow rate of ~2 mL/min (theoretical primary HRT of 10.4 hours for gravel, 17.6 hours for woodchips and 20.0 for peanut shells). At the end of the two-week period, the influent water was replaced with fresh, nutrient-amended RO water and effluent was no longer recycled.

4.4 Sample collection and analysis

Peristaltic pumps began distributing water to the columns immediately after the influent tanks were inoculated with GFP *E. coli*; effluent and influent grab samples were collected 15 (± 1) hours later to allow for stabilization of effluent NO_3^- concentrations (Pluer et al., 2016). *E. coli* only and NO_3^-+*E. coli* in influent tanks acted as a control for natural *E. coli* die off; by comparing the concentrations in the influent tank to those present in effluent at the same time point, the removal efficiency of each bioreactor column was quantified (Soupir et al., 2018).

Grab samples were collected in 125-mL acid washed, triple rinsed bottles from each column and from each influent tank. Triplicate influent samples were collected and analyzed in the same manner as effluent samples collected from triplicate columns. During each sampling event, NO_3^- only influent samples were collected, plated and incubated to verify the absence of GFP *E. coli*. All samples were held on ice during collection and transport then deposited into 4° C storage. *E. coli* samples were plated and incubated within six hours of collection, as were bulk samples for DOC and TN which were filtered (0.45 μm) prior to analysis (Pope et al., 2003; Rambages et al., 2005). Samples analyzed for NO_x^--N and NH_4^+-N were filtered (0.45 μm) and

frozen until analyzed. Influent and effluent water temperature, pH and dissolved oxygen (DO) were recorded using calibrated YSI ProDSS and Xylem EXO2 sondes, respectively. Influent water characteristics were recorded during grab sample collection. During each run, three EXO2 sondes were placed in flow through cells which collected effluent from three column replicates. Sondes were deployed when pumps were turned on and recorded data every two hours until sample collection was complete.

Effluent and influent samples were analyzed for NO_X-N, NH_4^+-N, DOC, TN and *E. coli* concentrations. NO_x and NH_4^+-N were analyzed using Smartchem 170/200 discrete analyzers (KPM analytics, Westborough, MA, USA) located in ECU's Environmental Research Laboratory using standard methods (APHA, 2017). Blanks consisting of deionized (DI) water were added to each run according to QA/QC protocol. TN and DOC were analyzed using the Hach (Loveland, CO, USA) DR6000 spectrophotometer and Hach test-n-tube kits. A DI water blank and a standard were included with each sample set. *E. coli* concentrations were quantified by plating and incubating samples collected from each column and the influent stock tank. 100 µL of each water sample was dispensed and spread evenly onto a tryptic soy agar plate containing 100 µg/mL ampicillin using an L-shaped cell spreader; each sample was plated in triplicate. Plates were incubated upside down at 37° C and resultant GFP *E. coli* colonies were counted in a dark room under a UV light.

4.5 Denitrification Enzyme Assay

After the last samples were collected and *E. coli* were flushed from the columns, the substrates rested under saturated conditions for ~8 months then denitrification potential was assessed using the acetylene inhibition method (Tiedje et al., 1994, Peralta et al., 2013). Denitrification enzyme assay (DEA) reportedly correlates with annual N loss in natural systems

and has been used in studies focused on comparing environmental effects on denitrification (Tiedje, 1994). Although the microbial population present during denitrification potential testing was presumably distinct from that existing during experimental runs, differences between substrate denitrification potential can be attributed to variations in experimental treatment differences (substrate type and/or influent composition); therefore, variations in DEA results can be interpreted as relative differences in denitrification potential based on substrate type.

To collect substrate samples for the DEA experiment, the columns were deconstructed and substrate from the entire column was homogenized, a sample was collected, weighed and held in an uncapped Wheaton bottle. A sample of each substrate was collected from each column that received influent treatments of NO_3^-+*E. coli* or NO_3^- only resulting in a total of 18 samples. Experimental (acetylene positive (A+)) and control groups (acetylene negative (A-)) were prepared for each sample. For this portion of the experiment, ~25 g of substrate was added to a 125-mL Wheaton bottle, then 75 mL of 1mM potassium nitrate (KNO_3) solution and 1.3 mL of a 100 mg/mL chloramphenicol solution were added to each bottle. Bottles were sealed with a septa-centered cap, shaken and purged with helium for five minutes while being vented with a needle. Gas samples were collected in helium evacuated exetainers once each hour. Between sample collection timepoints bottles were shaken on an orbital shaker set to ~180 RPM. Prior to collecting each sample, the bottle was shaken by hand to release N_2O within the substrate and accompanying liquid, then allowed to equilibrate, undisturbed on the bench, for five minutes. 15 mL of headspace gas was replaced with 15 mL of acetylene in A+ bottles and Helium (He) in A- bottles. 10 mL gas samples were collected from each Wheaton bottle and transferred by syringe to an exetainer vial at timepoints 0, 1, 2, and 3 hours during the assay. Gas samples were replaced with 10 mL 1:10 acetylene:He mix (A+) or 10 mL of He (A-).

The sample of gas collected in each exetainer was analyzed for N_2O using a gas chromatograph (GC) with electron capture detector (ECD) and single manual injector port (Shimadzu GC-2014). Using 0-100 ppm-v N_2O standards a calibration curve was created. Samples were then analyzed for N_2O concentrations (ppm), diluting with He as necessary to ensure measured concentrations fell within the range of the standard curve. Potential denitrification rates (ng N_2O/g DM/h) were obtained by regressing each sample N_2O concentration against elapsed time where the potential denitrification rates were interpreted as the slope of the sample regression line. As necessary, non-linearity of data was corrected by removing 1one of the four time point values; when more than one time-point sample was unusable, data from that experimental treatment replicate was discarded.

4.6 Data analysis

All data is reported as effluent concentration; *E. coli*, NO_x-N and TN concentrations are additionally expressed as removal efficiency calculated as $\varepsilon = \frac{C_{in}-C_{out}}{C_{in}}$, where ε represents removal efficiency, C_{in} represents the concentration in the influent solution and C_{out} represents the concentration in the effluent water. Statistical comparisons were made between treatments (substrate type vs influent composition) using R Studio Version 4.1.3. Q-Q plots and Shapiro-Wilke tests indicated that data sets were non-normally distributed, so non-parametric Kruskal-Wallis test was used to determine if significant ($\alpha = 0.05$) differences existed in a) NO_x-N removal efficiency compared by substrate type when all three receive identical influent water (NO_3^-+*E. coli* or NO_3^- influent treatments only); b) *E. coli* removal efficiency compared by substrate type when all three receive identical influent water (NO_3^- amendment or no NO_3^- amendment); c) *E. coli* removal efficiency of a single substrate when treating source water of distinct compositions (NO_3^- amendment or no NO_3^- amendment); and d) Effluent NH_4^+-N, TN,

and DOC concentrations between substrate type, and in the case of *E. coli* receiving columns, by influent treatment for each substrate. Pairwise Wilcoxon test with the Holm p-adjustment was used to determine which substrates or influent conditions resulted in significant differences between treatments. The Holm adjustment corrects errors associated with multiplicity intrinsic to performing multiple consecutive pairwise comparisons (Aickin and Gensler, 1996).

5. RESULTS AND DISCUSSION

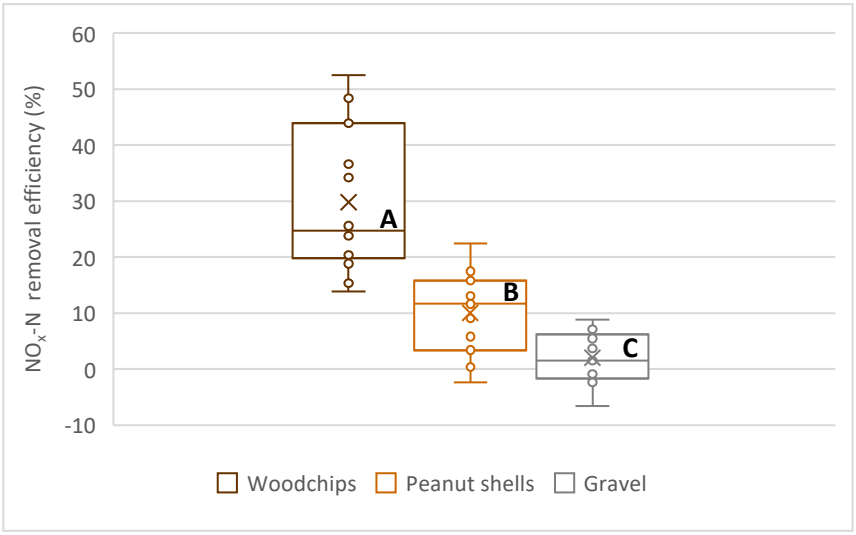
5.1 Nitrogen removal

N removal in denitrifying bioreactors is typically attributable primarily to denitrification (Audet et al., 2021). Multiple N processing pathways exist, however, and due to the diversity of denitrifying bioreactor microbes and their distinct N-cycling capabilities in different environments, results of the current study include analysis of N in the form of NO_x, NH_4^+, and TN to ensure a more holistic view of overall bioreactor performance.

5.1.1 Nitrate removal

Influent NO_3^- only and NO_3^-+*E. coli* treatments had an overall average NO_x-N concentration of 24 mg/L and *E. coli* only NO_x-N concentrations averaged 0 mg/L (Table 3). Removal efficiency of woodchips (25-30%) was significantly greater than peanut shells (8-18%) which significantly outperformed gravel (-2-2%) (Table 4, Figure 3). When NO_3^- only influent treatments were introduced, effluent concentrations and removal efficiency varied significantly between all substrate types. Gravel released water with the highest NO_x-N concentration (22.3 mg/L) followed by peanut shells (18.3 mg/L) then woodchips (14.9 mg/L). Because columns receiving *E. coli* inoculum were flushed between runs and columns receiving NO_3^- only were not, statistical comparisons were not made for NO_x-N treatment between bioreactors with identical substrates receiving flow with distinct microbiology.

3.1



3.2

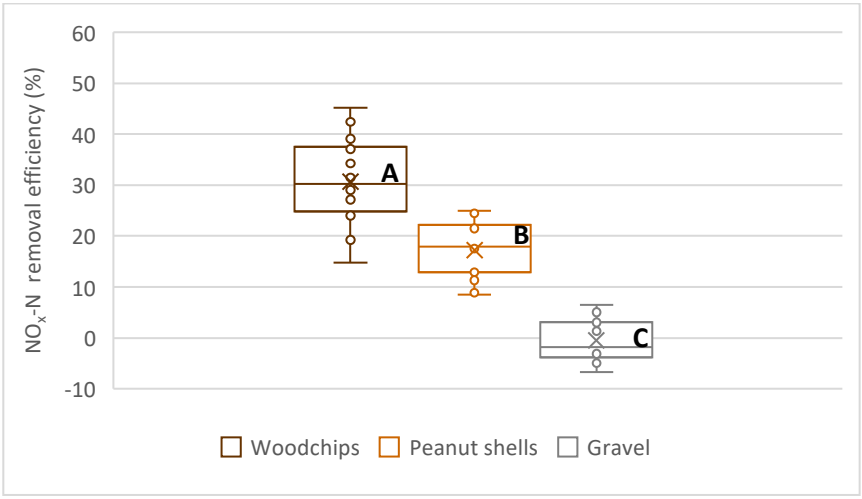


Figure 3. NO_x-N removal efficiency by substrate type receiving one of two distinct influent compositions 3.1) NO_3^- +*E. coli* and 3.2) NO_3^- only. Boxes outline the interquartile range where 50% of the data is found, the line through the middle represents median value, the 'x' represents the mean , hollow circles are individual data points and whiskers extend to minimum and maximum removal efficiency recorded during this experiment. Heterogeneous letters atop each medial line indicate significant difference ($\alpha = 0.05$) in removal efficiency. Note that gravel removal efficiency is not always > 0 – this net NO_x-N increase may be attributed to other N cycling processes that may be occurring in addition to denitrification.

Table 3. Median effluent concentration (interquartile range) calculated from three physical replicates over five runs for influent containing 3.1) NO_3^-+ *E.coli*, 3.2) *E. coli* only and 3.3) NO_3^- only. n=15 for *E. coli*, NO_x-N and NH_4^+-N analysis (apart from woodchips receiving NO_3^- only where n=14); n=5 for total nitrogen (TN); n=4 for dissolved organic carbon (DOC).

3.1	**NO_3^-+*E. coli***	**Woodchips**	**Peanut shells**	**Gravel**
E. coli				
Median concentration (CFU/100 mL)	$4.28x10^4$ ($1.19x10^4$)	$1.37x10^4$ ($1.33x10^4$)	$5.67x10^3$ ($9.67x10^3$)	$2.67x10^3$ ($3.00x10^3$)
Geometric mean (CFU/100 mL)	$4.58x10^4$	$9.51x10^3$	$6.11\ x10^3$	$8.84x10^2$
NO_x-N				
Median concentration (mg/L)	25.1 (5.1)	17.9 (5.3)	23.6 (5.8)	24.1 (3.5)
NH_4^+-N				
Median concentration (mg/L)	0.10 (0.25)	0.17 (0.17)	0.09 (0.21)	0.03 (0.01)
TN				
Median concentration (mg/L)	25.2 (3.1)	16.5 (2.6)	21.7 (3.4)	25.0 (2.8)
DOC				
Median concentration (mg/L)	1.95 (0.91)	5.89 (1.21)	5.46 (1.45)	4.94 (2.63)
3.2	***E. coli* only**	**Woodchips**	**Peanut shells**	**Gravel**
E. coli				
Median concentration (CFU/100 mL)	$3.14x10^4$ ($2.94x10^4$)	$1.17x10^4$ ($1.93x10^4$)	$7.83x10^3$ ($8.00x10^3$)	$3.33x10^3$ ($4.67x10^3$)
Geometric mean (CFU/100 mL)	$2.39\ x10^4$	$1.49\ x10^4$	$5.91x10^3$	$1.25x10^3$
NO_x-N				
Median concentration (mg/L)	0.1 (0.6)	0.0 (0.0)	0.3 (0.8)	1.6 (1.1)
NH_4^+-N				
Median concentration (mg/L)	0.10 (0.15)	0.05 (0.09)	0.46 (0.49)	0.03 (0.05)
TN				
Median concentration (mg/L)	1.0 (4.7)	2.1 (4.0)	2.8 (1.7)	4.8 (3.0)
DOC				
Median concentration (mg/L)	2.75 (3.20)	6.50 (4.3)	3.93 (2.54)	5.00 (1.67)
3.3	***NO_3^-* only**	**Woodchips**	**Peanut shells**	**Gravel**
E. coli				
Median concentration (CFU/100 mL)	0	0	0	0
NO_x-N				
Median concentration (mg/L)	21.6 (3.1)	14.9 (4.4)	18.3 (2.5)	22.3 (3.9)
NH_4^+-N				
Median concentration (mg/L)	0.10 (0.15)	0.17 (0.13)	0.40 (0.34)	0.04 (0.06)
TN				
Median concentration (mg/L)	22.2 (3.00)	15.6 (4.4)	18.1 (4.7)	20.9 (2.9)
DOC				
Median concentration (mg/L)	2.55 (2.30)	4.87 (0.61)	5.11 (0.28)	4.57 (0.46)

Table 4. Median total nitrogen (TN), NO_3^--N and/or *E. coli* removal efficiency (interquartile range) of each influent treatment and substrate type. n=15 for *E. coli* and NO_x-N analysis (apart from woodchips receiving NO_3^- only where n=14); n=5 for TN.

4.1 NO_3^-+*E. coli*	**Woodchips**	**Peanut shells**	**Gravel**
E. coli Median removal efficiency (%)	67.29 (16.21)	79.70 (16.18)	95.84 (5.28)
No_x-N Median removal efficiency (%)	24.67 (24.09)	7.68 (12.44)	1.51 (7.96)
TN Median removal efficiency (%)	31.53 (16.62)	9.52 (13.06)	2.90 (5.91)
4.2 *E. coli* only			
E. coli Median removal efficiency (%)	29.52 (32.83)	69.69 (12.37)	87.62 (18.21)
4.3 NO_3^- only			
No_x-N Median removal efficiency (%)	30.26 (12.73)	17.92 (9.28)	-1.86 (6.80)
TN Median removal efficiency (%)	29.07 (15.66)	20.16 (11.49)	8.11 (14.71)

Most lab-scale, flow through or batch denitrifying bioreactor studies operate systems at much higher HRTs (>6 hours and up to multiple days) than those assessed in the current study (e.g. Abdi et al., 2019; Cameron and Schipper 2009; Gibert et al., 2008; Greenan et al., 2006; Healy et al., 2011; Healy et al., 2015; Healy et al., 2006; Soupir et al., 2018). Because of the influence of both HRT and study scale on nitrate removal results, only laboratory scale woodchip bioreactor studies which operate at lower HRTs ranging from 2-6 hours were compared. For example, Chun et al. (2009) assessed the performance of a series of laboratory scale woodchip bioreactors reporting no impactful NO_3-N^- removal at an HRT of 2.7 hours; however, this was attributed to a lack of denitrifying bacteria inoculation prior to beginning the experiment. After the short HRT run, they increased HRT to 12.1 hours then subsequently decreased back down to 2.9 hours and reported an approximately 20% decrease in NO_3^- concentration. Pluer et al. (2016) performed a series of laboratory-scale experimental trials evaluating NO_3^- removal of both woodchip and woodchip-biochar amended denitrifying bioreactors at HRTs (2-12 hours) and reported that NO_x removal efficiency increased with HRT with approximately half of the influent N removed at an HRT of 8-10 hours. Refer to Figure 4 for a graphical comparison of NO_x removal efficiency vs HRT from the current study and other published studies similar in design to the current study.

To date, a few studies have also explored peanut shells as a carbon source for NO_3^- removal from various source waters. Although these experiments have been batch studies rather than flow through column or field-scale studies, due to the scarcity of documented flow-through studies, batch studies are discussed here. Xing et al. (2019) explored the use of peanut shells incorporated into double-layer carbon microspheres. Peanut shells were crushed to a very fine (<0.125 mm-0.3 mm) size range and were assessed for carbon release and denitrification

performance in batch studies. The researchers conclude that when grain size is <0.3 mm, chemical oxygen demand (COD) varies with grain size and that in all scenarios the COD released from the peanut shells peaked at 12-hours. They attributed this to a high specific surface area which initially releases large amounts of COD then gradually begins to reabsorb it. They reported that after 36 hours at 30 mesh size peanut shells removed 39.8% of initial NO_3^--N (which started at 25 mg/L); at 60 mesh size, they removed ~80% (leaving only ~4.9 mg NO_3^--N/L), and at 120 mesh size they removed nearly 75% of initial NO_3^--N. Better NO_3^- treatment obtained by Xing et al. (2019) as compared to the current study (10-17%) may be attributable to differences in HRT and particle size. The batch study reported results after 36 hours whereas the HRT in this study is closer to 3.4 hours. Additionally, peanut shells in this experiment were sieved to a much larger size range (> 0.6 cm) than in the Xing et al. (2019) study with the purpose of better representing field-scale particle sizes. Hu et al. (2022) compared NO_3^--N removal efficiency of various nut shells (5-10 mm particle size) in a batch study using a soaking solution containing 12-15.7 mg NO_3^--N. They sampled the solution every 24 hours over 20 days and reported that peanut shell NO_3^--N removal efficiency averaged 76.5%, the highest of the various nutshells.

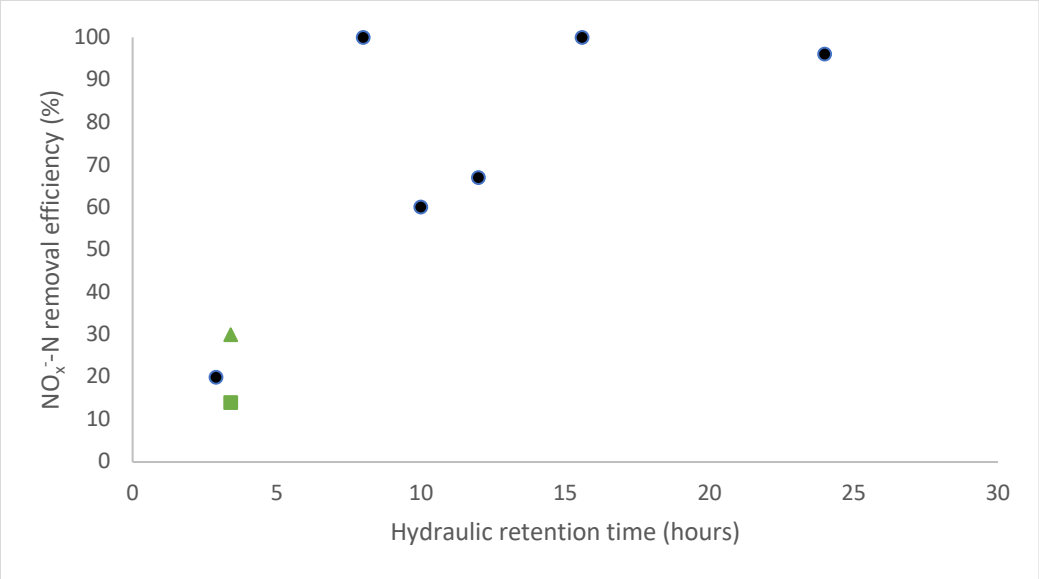


Figure 4. Comparison of HRT and NO_x-N removal efficiency reported by other lab-scale woodchip bioreactor flow-through studies (black circles) and the woodchips and peanut shells from the current study (green triangle and square, respectively). NO_x-N removals from this study are reported as averages across all experimental runs and column replicates (n=30 for peanut shells, n=29 for woodchips). Studies used for comparison include Chun et al., 2009; Cooke et al., 2001; Doheny et al., 2002 and Soupir et al., 2018.

5.1.2 Denitrification potential

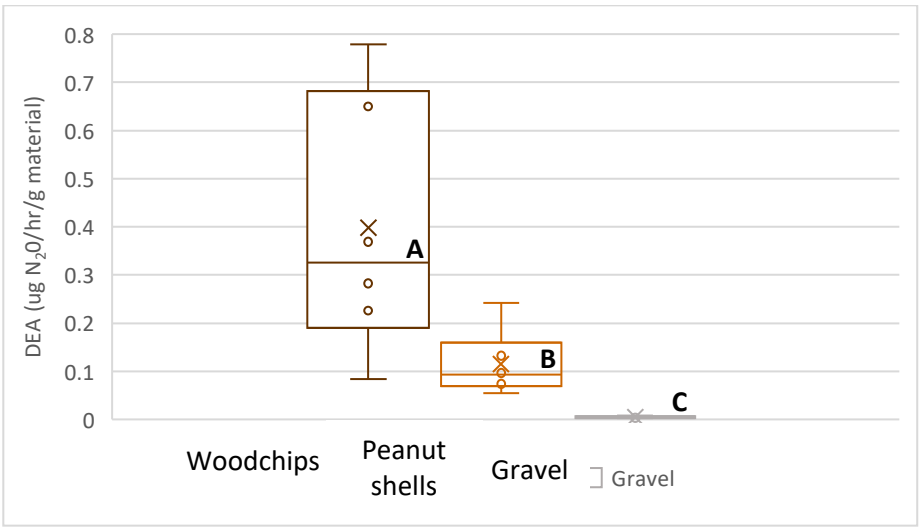
Trends from denitrification enzyme assays of each substrate typically followed NO_3^--N removal observed through weekly sampling conducted during experimental runs, with woodchips having removing significantly more NO_3^--N (0.23µgN_2O/hr*g and 0.57 µgN_2O/hr*g when influent had NO_3^-+ *E. coli* and NO_3^- only, respectively) followed by peanut shells (0.075 µgN_2O/hr*g and 0.015 µgN_2O/hr*g, respectively) then gravel (0.006 µgN_2O/hr*g and 0.004 µgN_2O/hr*g, respectively) (Table 5; Figure 5.1). Studies exploring the denitrification potential of peanut shell bioreactors are lacking, so comparisons are limited to published denitrification potential of woodchips bioreactors vs those observed in this study. Results reported here are slightly below or at the low end of other published DEA results including those reported by Long et al. (2011) who sampled a 14-year-old in-field woodchip denitrifying bioreactor at various depths over time and reported an average of 1.093 µgN_2O/hr*g bioreactor soil. Hathaway et al.

(2017) who used a similar method and reported N_2O production rates of moist woodchips ranging from 0.079-6.289 $\mu gN_2O/hr*g$. Differences in reported denitrification potential between the current study and other published literature may be a result of bioreactor scale, age, establishment and/or a lack of inflowing microbes which is usually present when treating natural/waste waters. Comparing denitrification potential to NO_3^--N removal efficiency resulted in a strong correlation for peanut shells and gravel (R^2 = 0.49 and 0.48, respectively) but almost non-existent for woodchips (R^2 = 0.01) (Figure 5.2). This could be the result, in part, of variations in microbial community interactions between the substrates. For example, NO_3^--N removal from peanut shell bioreactors may rely dominantly on denitrifying bacteria, so the denitrification potential results, which are a proxy for genes responsible for denitrification, represent the dominant NO_3^--N removal mechanism at work in peanut shell bioreactors. The incredibly weak correlation between the same two factors for woodchip columns may indicate another player in the denitrification process; one possibility is a fungal community component which may contribute to substrate decomposition in woodchips (Jeglot et al., 2021) but may not be able to grow in peanut shells due to their anti-fungal properties (Adikari et al., 2019).

Table 5. Median denitrification potential (interquartile range) of each substrate; n=6 for woodchips and peanut shells, n= 4 for gravel.

	$\mu g\ N_2O/h/g$ substrate
Woodchips	0.33 (0.49)
Peanut shells	0.09 (0.09)
Gravel	0.00 (0.00)

5.1



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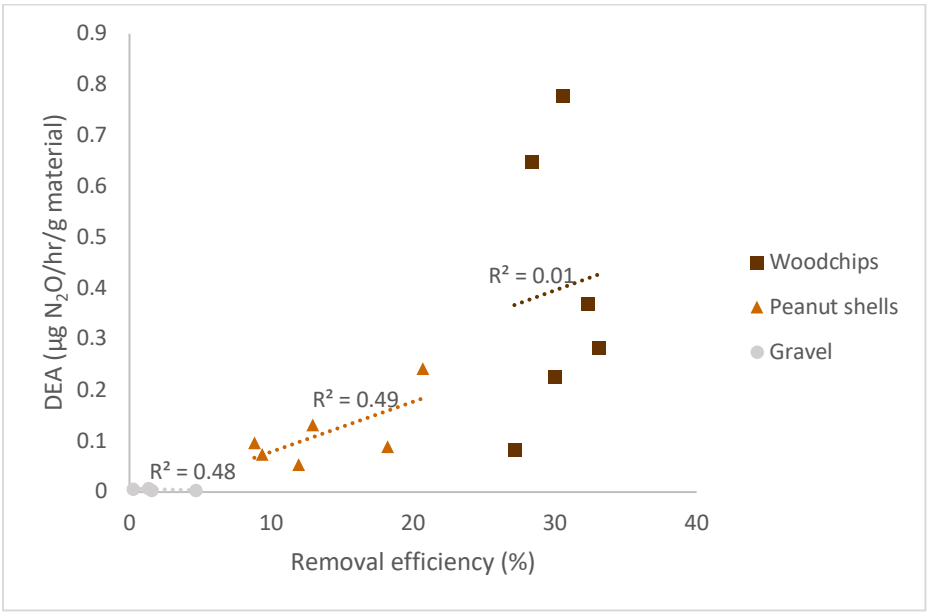


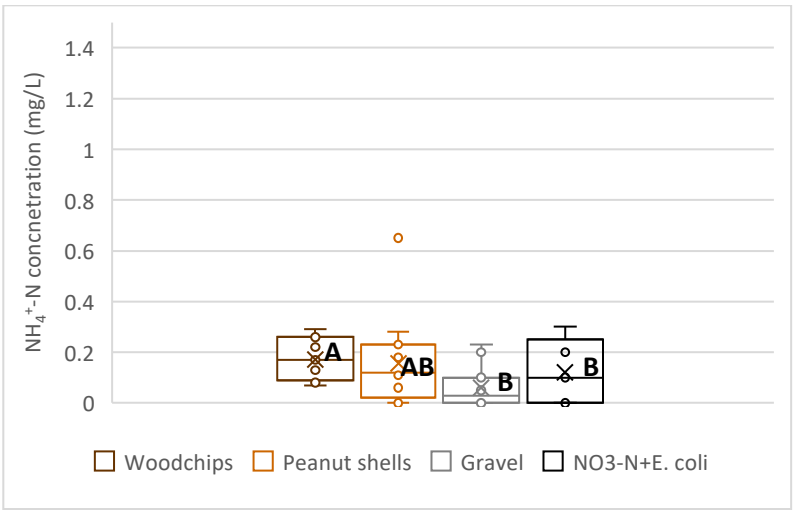
Figure 5. 5.1) Denitrification potential of each substrate type from columns that received NO_3^- with or without *E. coli.* Boxes outline the interquartile range where 50% of the data is found, the line through the middle represents median value and the 'x' represents the mean and whiskers extend to minimum and maximum removal efficiency recorded during this experiment. Heterogeneous letters atop each medial line indicate significant difference (α = 0.05) in removal efficiency. 5.2) Linear regression of denitrification potential and NO_x-N removal efficiency. Each point represents substrate and average removal efficiency of a single column over the experimental trial. n=6 for woodchips and peanut shells and n=4 for gravel due to two exetainer sample sets that had multiple sample losses.

5.1.3 Ammonium

Median concentrations of NH_4^+-N in influent waters across all treatments remained below 0.5 mg/L (Table 3). When NO_3^-+ *E. coli* influent was introduced, effluent NH_4^+-N concentrations from columns containing woodchips (0.17 mg/L) varied significantly from

influent concentrations (0.10 mg/L) but those containing peanut shells (0.09 mg/L) and gravel (0.03) did not (Table 3; Figure 6.1). When *E. coli* only influent was introduced, peanut shells (0.46 mg/L) were the only substrate to significantly increase NH_4^+-N concentrations from influent (0.1 mg/L) (Figure 6.2). Although DO concentrations (Table 6) were relatively consistent across all treatments, when NO_3^- only influent was introduced, NH_4^+-N concentrations of peanut shell effluent (0.40 mg/L) were significantly higher than that of influent water (0.1 mg/L) and gravel effluent (0.04 mg/L) had significantly lower NH_4^+-N concentrations than influent water (Figure 6.3). Woodchips released significantly more NH_4^+-N when treating NO_3^- +*E. coli* compared to that released when treating *E. coli* only (0.17 mg/L vs 0.05 mg/L respectively) (Figure 7.1) whereas peanut shells released significantly more when treating *E. coli* only (0.46 mg/L) compared to NO_3^-+*E. coli* (0.09 mg/L) (Figure 7.2). Gravel effluent NH_4^+-N concentrations remained unaffected regardless of NO_3^- presence or absence with *E. coli* (0.03 mg/L) (Figure 7.3).

6.1



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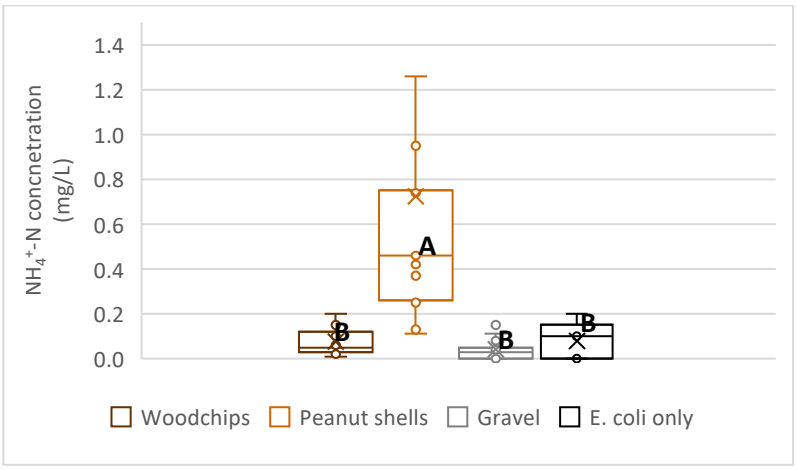


6.3

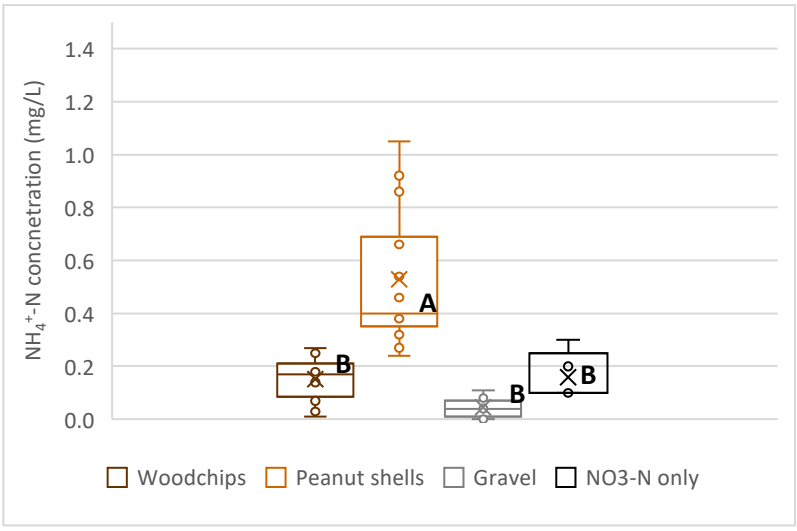
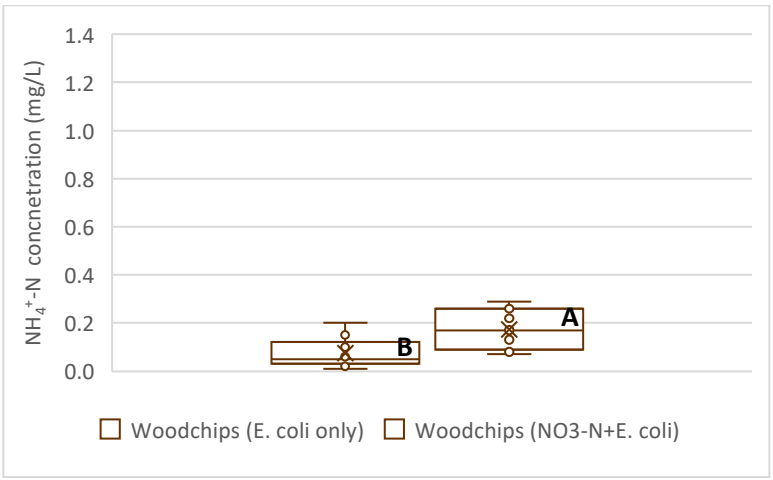


Figure 6. Median NH_4^+-N concentrations by influent treatment with 6.1) NO_3^-+ *E. coli;* 6.2) *E. coli* only*;* and 6.3) NO_3^- only. Boxes outline the interquartile range where 50% of the data is found, the line through the middle represents median value and the 'x' represents the mean and whiskers extend to minimum and maximum removal efficiency recorded during this experiment. Heterogeneous letters atop each medial line indicate significant difference ($\alpha = 0.05$) in removal efficiency.

7.1



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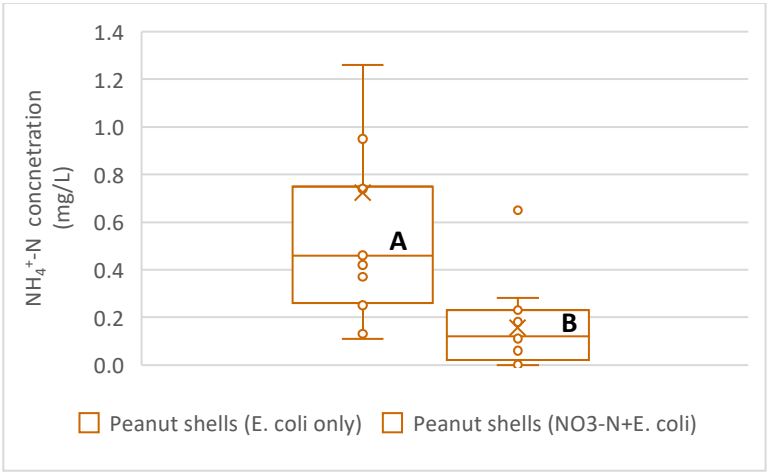

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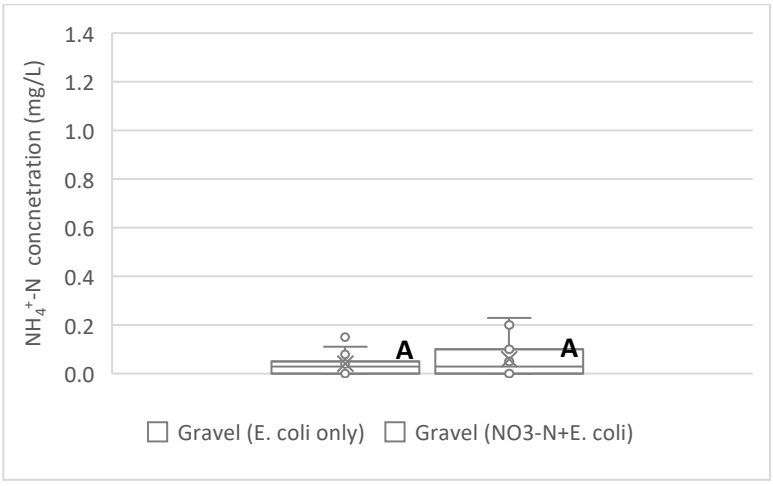


Figure 7. Median effluent NH_4^+-N concentrations compared by influent composition for 7.1) woodchips; 7.2) peanut shells; and 7.3) gravel. Boxes outline the interquartile range where 50% of the data is found, the line through the middle represents median value, the 'x' represents the mean and whiskers extend to minimum and maximum concentrations recorded for each treatment during this experiment. Heterogeneous letters atop each medial line indicate significant difference ($\alpha = 0.05$) in concentration.

Two pathways exist which result in the creation of NH_4^+-N; dissimilatory nitrate reduction to ammonium (DNRA) and ON mineralization. Our understanding of DNRA has improved dramatically over the last few decades (Tiedje, 1994). DNRA was only thought to occur in conditions similar to denitrification, but with the additional constraint of NO_3^- limitation, there is now much debate over whether the main driver is soil redox potential or $C:NO_3^-$ (Rutting et al., 2011). Regardless of the specific factors determining DNRA's contribution to N removal, it has been observed in systems similar to those being studied here (e. g., Hu et al., 2022). ON mineralization occurs through microbial decomposition of organic matter and has been observed to occur at C:N lower than 16:1 (Martin et al., 2019). Considering the low C:N, relatively high pH and reportedly fast degradation of peanut shells (Bernhard, 2010), ON mineralization may explain the significant increases in NH_4^+-N after peanut shell treatments; however, the lack of ON data hinders verification of this hypothesis. In the case of woodchip columns, where pH typically stayed below 7 and C:N is much higher (114:1), it seems more likely that DNRA played a role in increasing NH_4^+ concentrations (Hartfiel et al., 2022) which is supported by the greater NO_x-N removal efficiency of woodchips compared to peanut shells (Table 3 and Table 4).

Rambags et al. (2016) reported a lack of meaningful NH_4^+-N removal in woodchip bioreactors receiving wastewater with elevated *E. coli* concentrations; however, Martin et al., (2019) reported significant increases in NH_4^+-N in pilot-scale woodchip denitrifying bioreactors operating at 2-, 8-, and 16-hour HRTs. Healy et al. (2012) concluded that effluent NH_4^+-N concentrations typically increased with HRT in a lab-scale study assessing various carbon substrates. Xing et al., (2020) conducted batch experiments using microspheres created from peanut shells ranging from <0.125-0.3 mm and found no significant increase in NH_4^+-N over the

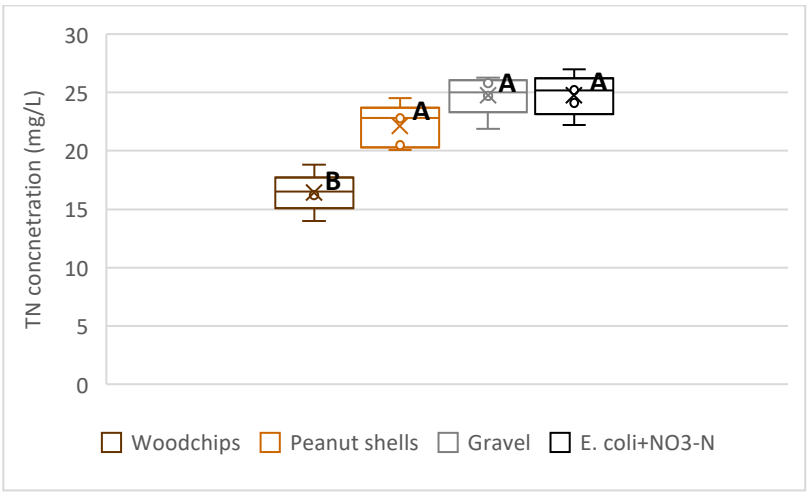
course of 36 hours; however, the influent water used in the current study was approximately 2-3 orders of magnitude lower than that used in batch experiments performed by Xing et al. which may have influenced N-cycling dynamics (Rutting et al., 2011). In contrast, Hu et al. (2022) conducted batch studies soaking various nut shells at a size range of 5-10 mm in a solution with initial NH_4^+-N concentrations ranging from 0.5-1.52 mg/L and reported a consistently high NH_4^+-N concentration from the peanut shell soaking solution compared to the other carbon sources (shells from pistachios, gingko nuts, walnuts, hazelnuts, pine seeds, chestnuts and macadamia nuts). This trend held true in the current research where peanut shells typically released effluent water with the highest NH_4^+-N concentrations.

5.1.4 Total nitrogen

Nitrogen speciation varies by both matrix and environmental factors (Christianson et al., 2021). Although most bioreactor studies don't report TN concentrations, in the current study bulk samples of each treatment (triplicate columns receiving the same influent water) were analyzed for TN to ensure that NO_x removal efficiencies represented net decreases in N within the system. Multiple studies have reported low TN removal (averaging 25%) which has been attributed to operation in unsaturated conditions where nitrification occurs (Lopez-Ponnada et al., 2017). Median TN concentrations from physical and temporal replicates from this study can be found in Table 3 and removal efficiencies in Table 4. The median TN concentration of influent water amended with NO_3^- across all experimental runs was 24.7 (3.5) mg/L (*E. coli* only influent water had a median TN concentration of 1.0 (4.7) mg/L). When NO_3^- and *E. coli* were both added to influent water, woodchips (16.5 mg/L) released water with significantly lower TN concentrations than that of peanut shell (21.7 mg/L) and gravel (25.0 mg/L) treatments (Figure 8.1). When influent water was inoculated with *E. coli* but not amended with NO_3^-, no effluent

TN values varied significantly from influent (Figure 8.2). When NO_3^- only influent was introduced, TN released from woodchip (15.6 mg/L) and peanut shell (18.10 mg/L) treatments were significantly lower than influent concentrations (22.2 mg/L) (Figure 8.3). In a series of batch studies, Gibert et al. (2008) reported that TN trends typically aligned with NO_3^- trends because NO_3^- was the dominant source of N, as is the case in this study; however, in the current study, TN removal efficiency is slightly greater than NO_x removal in most cases (Table 4).

8.1



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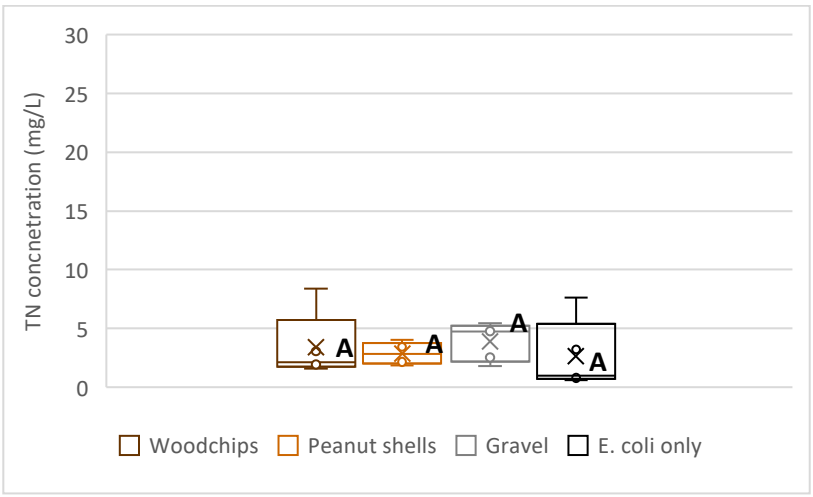

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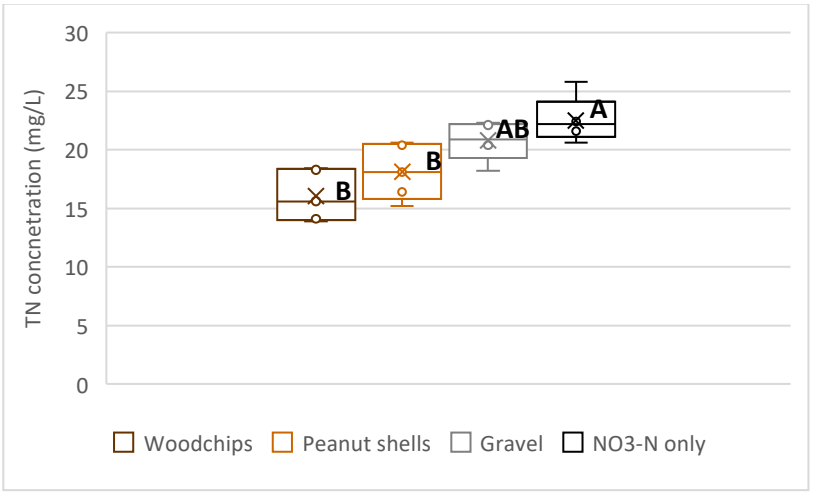
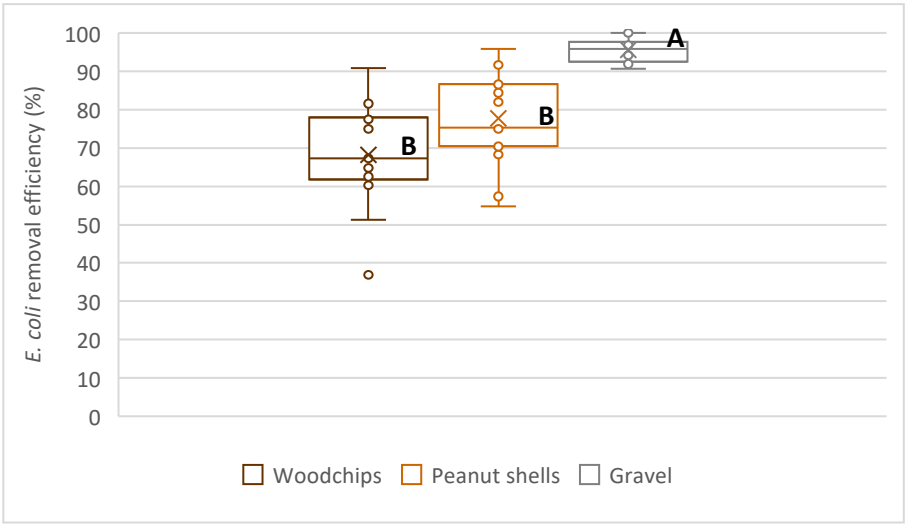


Figure 8. Total nitrogen (TN) concentrations of influent vs effluent from each substrate types by influent treatment with 8.1) NO_3^-+ *E. coli;* 8.2) *E. coli* only*;* and 8.3) NO_3^- only. Boxes outline the interquartile range where 50% of the data is found, the line through the middle represents median value, the 'x' represents the mean and whiskers extend to minimum and maximum concentrations recorded for each treatment during this experiment. Heterogeneous letters atop each medial line indicate significant difference ($\alpha = 0.05$) in concentration.

5.2 *E. coli* removal

Average influent *E. coli* concentration of both influent waters containing *E. coli* over the course of five weeks was $3.73x10^4 \pm 4.74x10^3$ CFU/100 mL (geometric mean of $3.31x10^4$ CFU/100mL). When NO_3^-+*E. coli* was treated, gravel (96%) removed significantly more *E. coli* than peanut shells (80%) and woodchips (67%) (Table 4, Figure 9.1). When *E. coli* only was treated, removal efficiency of gravel (88%) was significantly greater than that of peanut shells (70%) and woodchips (30%) removed significantly less than peanut shells (Table 4; Figure 9.2). Both woodchips and gravel had significantly lower removal efficiencies when *E. coli* only was introduced compared to when NO_3^-+*E.* coli was introduced but peanut shell removal efficiency was not significantly affected (Figure 10). Although both woodchips and gravel were both significantly impacted by influent composition, the magnitude of this effect was much greater for woodchips. Median removal efficiency of woodchips treating NO_3^-+*E. coli* was 38% greater than that of woodchips treating *E. coli* only whereas the same value for gravel only increased by 8% when NO_3^- was added. It is also important to note that while removal efficiency of gravel receiving *E. coli* only was lower than when gravel received the additional NO_3^- amendment, it was still higher than woodchip removal efficiency when NO_3^- was added. Removal efficiency of all substrates varied less when NO_3^- was added compared to when it was not, but overall, gravel performed most consistently.

9.1



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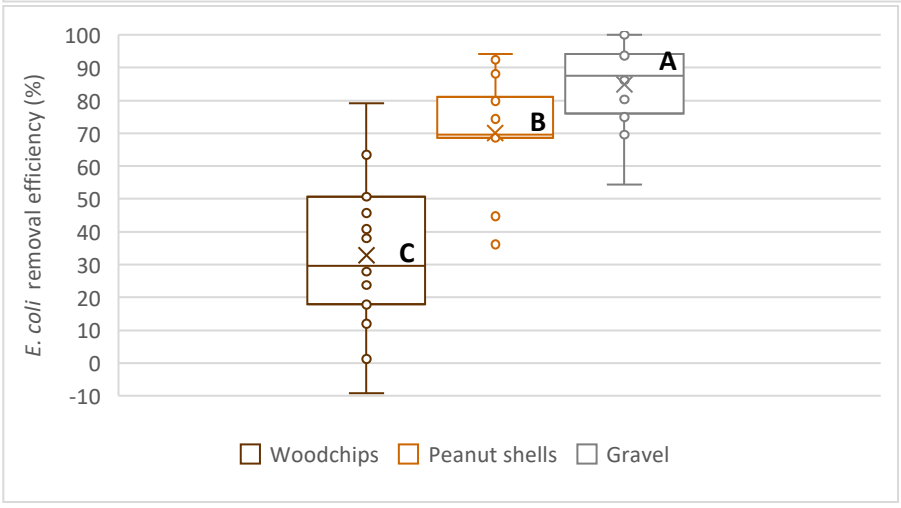
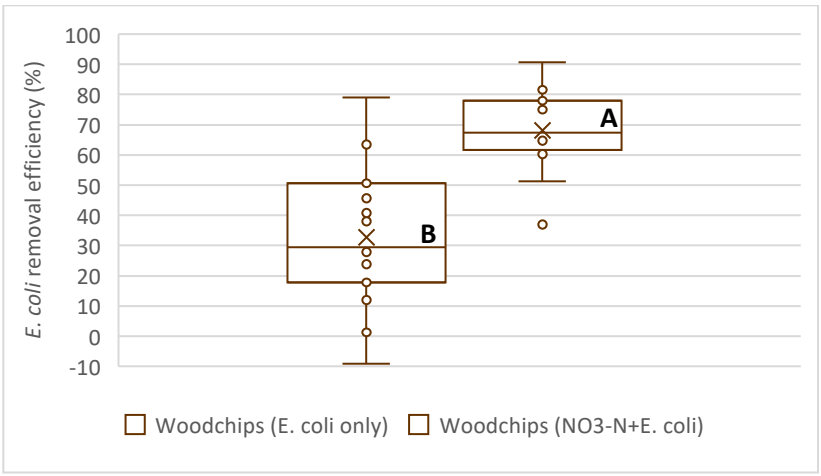


Figure 9. *E. coli* removal efficiency of 9.1) NO_3^-+*E. coli* and 9.2) *E. coli* only. Boxes outline the interquartile range where 50% of the data is found, the line through the middle represents median value and the 'x' represents the mean and whiskers extend to minimum and maximum removal efficiency recorded during this experiment. Heterogeneous letters atop each medial line indicate significant difference ($\alpha = 0.05$) in removal efficiency. Note the negative value for woodchips in 6.2, one sample from one column came back with a greater concentration of *E. coli* than was in the influent water. This may have been due to a release of previously filtered *E. coli*.

10.1



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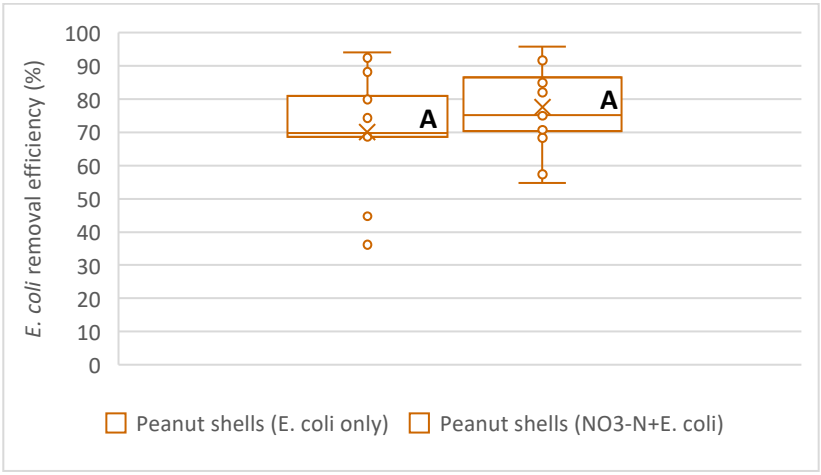

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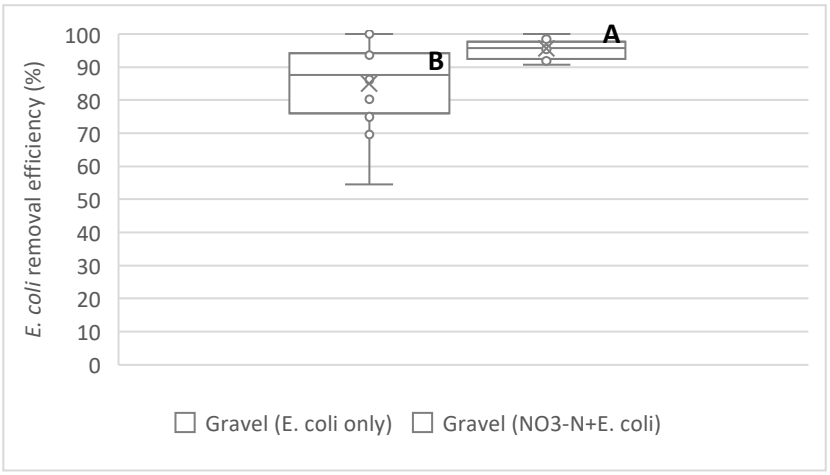


Figure 10. Median *E. coli* removal efficiency of 10.1) woodchips 10.2) peanut shells and 10.3) gravel compared when receiving influent with vs without additional NO_3^- amendment. Boxes outline the interquartile range where 50% of the data is found, the line through the middle represents median value and the 'x' represents the mean and whiskers extend to minimum and maximum removal efficiency recorded during this experiment. Heterogeneous letters atop each medial line indicate significant difference ($\alpha = 0.05$) in removal efficiency. Note the negative value for woodchips in 10.1 – in this case, one sample from one column contained a greater concentration of *E. coli* than was in the influent water. This may have been due to a release of previously filtered *E. coli*.

Studies similar to the current study have also found bioreactors containing either gravel or carbonaceous substrates as effective means of removing *E. coli* from either synthetic or real-world waste and agricultural waters (e.g., Robertson et al., 2005; Tanner et al., 2012; Rambags et al., 2016; Soupir et al., 2018). Direct comparison of these studies to the current study is challenging due to differences in experimental design (bioreactor design and scale, HRT, influent microbial concentration and chemistry, and substrate type); nevertheless, some comparisons can be made. Robertson et al. (2005) reported successful removal of all detectable *E. coli* (to achieve a concentration of <10 CFU/100mL) in 79% of samples when using a single-pass sand filter paired with Nitrex brand filter containing bark, sawdust and woodchip media. In the current study, no samples collected from woodchip or peanut shell columns contained such a low concentration. However, three samples out of 90 total samples collected (~3%) contained <1 CFU/mL: samples from two gravel columns receiving NO_3^- amended water (13% of this treatment) and samples from one gravel column receiving non NO_3^- amended water (7% of this treatment). As mentioned earlier, direct comparison of these results to the current study are difficult due to a difference in bioreactor scale (field vs lab), HRT (1-10 days vs ~4 hours), substrate type, and the use of a sand filter pre-treatment which, in some scenarios, decreased *E. coli* concentrations flowing into the bioreactor to a low range of $2x10\text{-}2x10^3$ CFU/100 mL. A few years later, Tanner et al. (2012) reported substantially improved *E. coli* removal when vertical flow sand media constructed wetlands treating primary wastewater were followed by carbonaceous denitrifying bioreactors containing either woodchips or coconut husks compared to those that were not when both systems were operated at an HRT of ~3.3 days. The increased efficacy of the systems accompanied by denitrifying bioreactors was speculatively attributed to nitrifier-induced deactivation of bacteria related to substantial pH change of the water. In the

previously mentioned study, water flowing into the bioreactors had an average pH ranging from 4.5-6.8 and average bioreactor effluent water pH ranged from 6.4-6.7. In the current study, average influent pH ranged from 5.9-6.2; gravel effluent had pH in the range of 6.6-7.1, woodchip effluent pH ranged from 6.9-7.3 and peanut shell effluent pH ranged from 6.8-7.2. Then in 2016, Rambags et al. reported that when receiving a solution with a mean *E. coli* concentration of $1.6x10^4$ CFU/100 mL the system averaged $2.9\log_{10}$ (>99%) reduction and most *E. coli* was removed from influent water after traveling the first 1m of their full-scale woodchip bioreactor which corresponded to an HRT ~6 hours. Most recently, Soupir et al (2018) reported on lab-scale bioreactors packed with woodchips operating at either 12-hour or 24-hour HRTs at either 10ºC or 21ºC and receiving influent with an average *E. coli* concentration of $4.5x10^4$ (± $2.6x10^4$) CFU/100 mL and 7.5 $x10^4$ (± 3.8 $x10^4$) CFU/100 mL, respectively. They found significant *E. coli* removal (75-96%) under all experimental temperatures and HRTs. At 12-hour HRT and 21°C, average *E. coli* removal was $3.8x10^4$ CFU/100mL or 96% of influent *E. coli* concentration. Results from this study as well as other similar studies are summarized graphically in Figure 11. Other studies have demonstrated high *E. coli* removal efficiencies (>90%), even when HRTs are quite different (12 vs. 24 hours). Results from the current study demonstrate that comparable *E. coli* removal efficiency can be achieved even when HRT is decreased to below 10 hours.

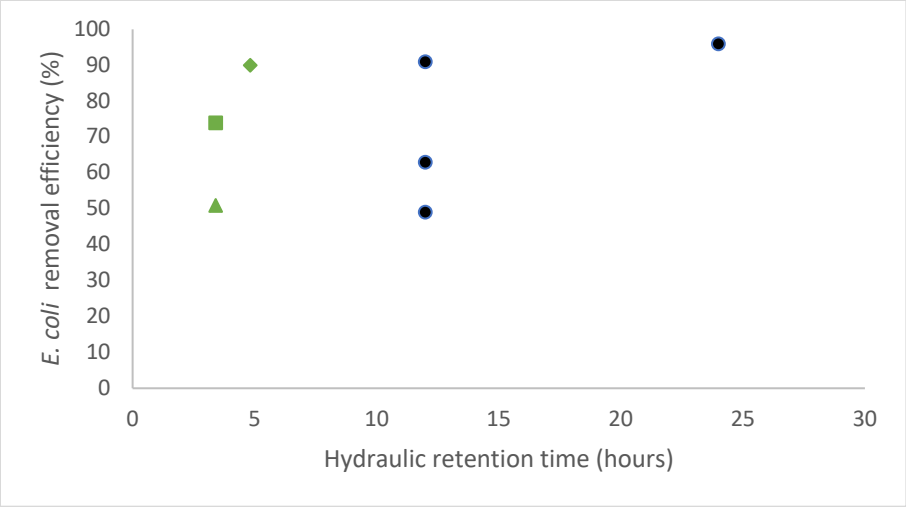


Figure 11. Graph comparing *E. coli* concentration removal efficiency vs hydraulic retention time from other laboratory-scale flow-through woodchip column studies (black circles) compared to woodchips (triangle), peanut shells (square), and gravel (diamond) from the current study (green symbols). Values for *E. coli* removal efficiency of each substrate type are averaged across all experimental runs and influent treatments (n=30 for each substrate type). Studies used for comparison are Soupir et al., 2018 and Mardani et al., 2020.

Organic matter content, pH, DO, HRT, grain size, porosity, and microorganism shape and size all affect microbe removal in water treatment technologies (Rambags et al., 2016). However, comparisons between distinct substrates which occupy columns of identical dimensions allows for a direct comparison of treatment efficacy not attributable to variations in HRT or physical microorganism characteristics (Rambags et al., 2016). *E. coli* removal efficiency of each substrate is likely driven by a combination of factors which overlap. Removal in gravel columns could be attributed to a combination of mechanical filtration related to a microscopically rough surface texture (Fig. 12) as has been explored in saturated slow sand filters (Weber-Shirk and Chan, 2007), adsorption related to electrostatic forces (Stevik et al., 2004), and adsorption or inactivation related to biotic interactions which have been pointed to in other systems meant to removal pathogens from water under saturated conditions such as constructed wetlands. Antimicrobial effects (Adhikari et al., 2019) and high surface area of peanut shells (Xing et al., 2022) may have affected *E. coli* removal dynamics as higher surface area increases the

likelihood of adsorption to particles (Rambags et al., 2016; Truluck, 2017) and provides greater space for potential biofilm formation (Zheng et al., 2021).

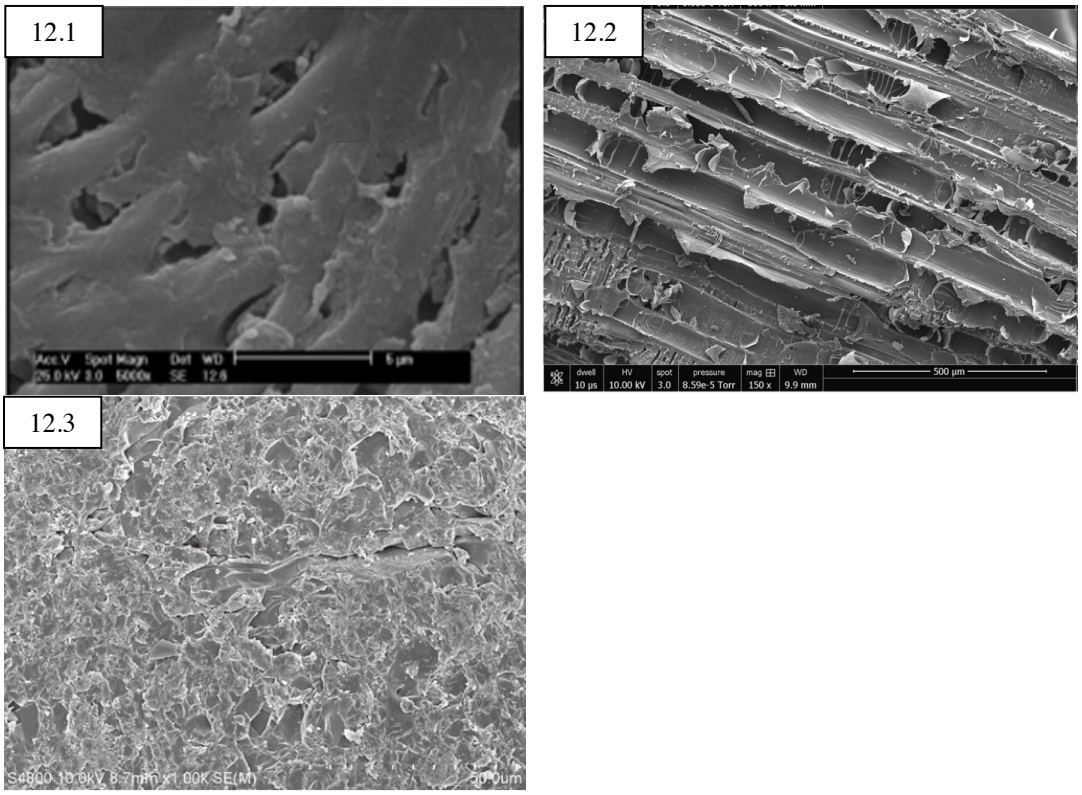


Figure 12. SEM images of 12.1) peanut shells; 12.2) woodchips; and 12.3) granite. (Adapted from: Bustamante-Bailon et al., 2021; Kasani et al., 2019; Kato et al., 2018, respectively).

The variation in magnitude of effect of the NO_3^- amendment added to influent water may point to a variation in *E. coli* removal mechanism between substrates. Microbial biofilm interactions have been proposed as a pathogen removal mechanism in other systems (Weber-Shirk and Chan, 2007). Most microbes rely on NO_3^- and NH_4^+ for growth (Kuypers et al., 2018); the increased *E. coli* removal performance of woodchips and gravel in the presence of NO_3^- may be due to bioreactor microbes growing more quickly and outcompeting the *E. coli* being introduced into the system by influent water (Natarajan, 2015). Because peanut shells' removal efficiency was not significantly impacted by presence of NO_3^- in the influent water, it is possible that *E. coli* removal is more dependent on mechanical filtration or changes to water chemistry (Weber and Legge, 2008) related to substrate characteristics rather than predation by or competition with the denitrifying bioreactor microbial community. Hu et al. (2022) found that

peanut shells also produced humic acid during batch studies, and humic acid is typically inversely related to *E. coli* survival in liquid cultures (Baker et al., 2020). This is just one example of many possible adverse effects that substrates can have on living contaminants.

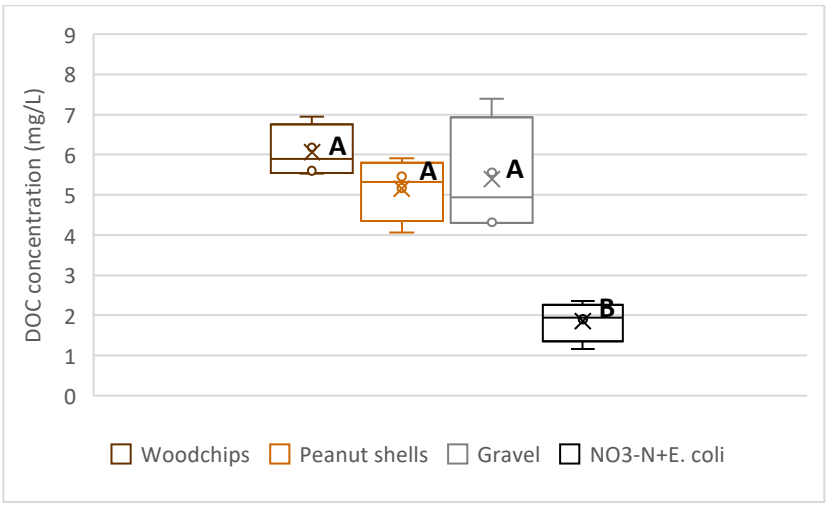
Although influent *E. coli* concentrations used in this experiment likely exceed those typically found in subsurface conditions, elevated concentrations of *E. coli* in North Carolina coastal plain waters have been documented during and after large precipitations events and lagoon overflows (e.g., Burkholder et al., 1997). In addition, high concentrations of *E. coli* transported from agricultural operations, peaking around $1x10^5$ CFU/100mL (Soupir et al., 2018), and septic systems, as high as 9.7×10^6 CFU/100 mL, have been documented (Humphrey et al., 2011). In scenarios such as those listed above, denitrifying bioreactor treatment using woodchips, peanut shells, or gravel would likely not decrease concentrations to a safe level for human contact; however, with strategic placement of subsurface denitrifying bioreactors containing a combination of gravel and/or peanut shells and woodchips (depending on expected NO_3^- concentrations), annual *E. coli* loads to natural waterways could be substantially reduced.

5.3 Water quality parameters

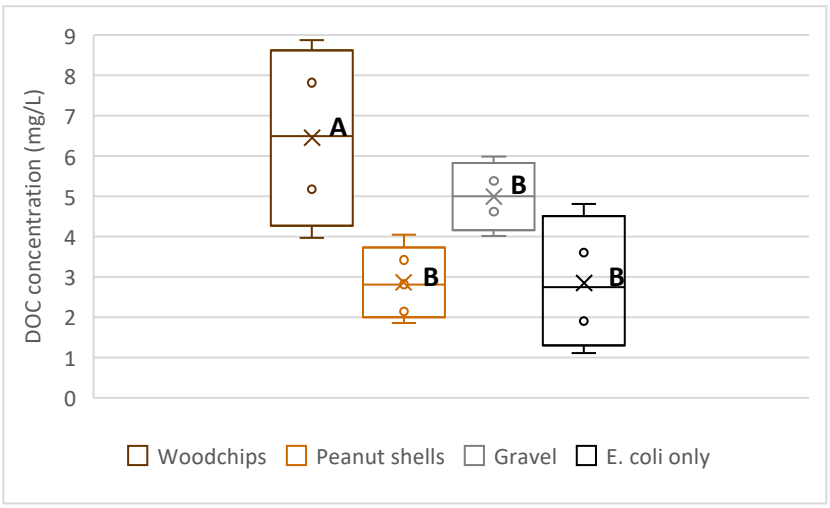
Median DOC values for all treatments explored in this study can be found in Table 3 and Figure 13. Whenever NO_3^- was present in influent water, effluent DOC of all three substrates was significantly higher than influent water (Fig. 13). The only significant difference between DOC released from bioreactor substrates treating the same influent water was in the case of woodchips treating *E. coli* only (Fig. 13.2). There were no significant differences found between effluent DOC concentrations when the same substrate treated water with NO_3^-+*E. coli* vs *E. coli* only (Fig. 14).

Elevated concentrations of DOC are typical in bioreactor effluent water and are likely associated with flushing of labile carbon and potentially release of other biological material (Bell et al., 2015; Hu et al., 2022). Concentrations reported here are at the low end of DOC concentrations reported in the literature which can reach 1-2 orders of magnitude greater (e.g., Abusallout and Hua, 2017; Bell et al., 2015; Hassnapour et al., 2017).

13.1



13.2



13.3

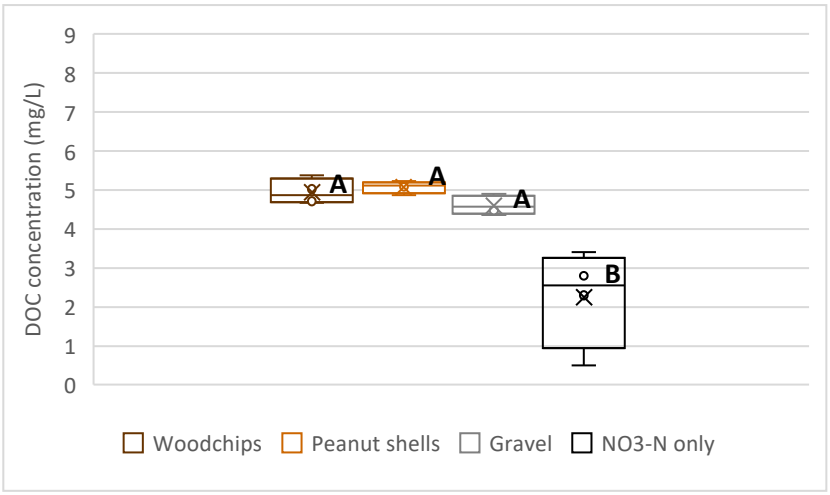
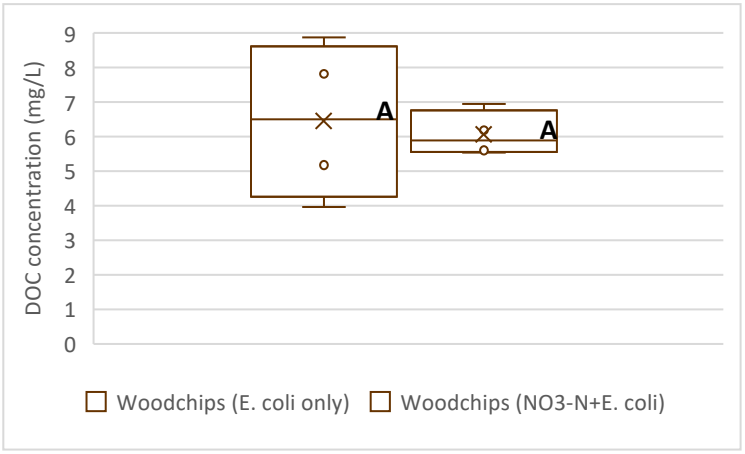
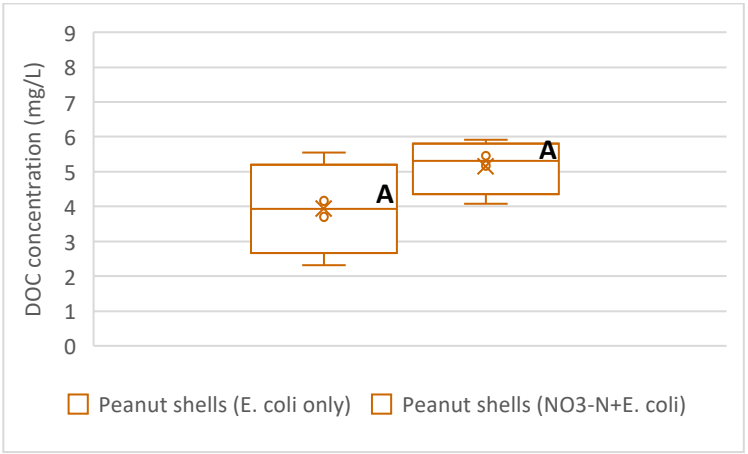


Figure 13. Dissolved organic carbon (DOC) by influent treatment with 13.1) NO_3^-+*E. coli;* 13.2) *E. coli* only and 13.3) NO_3^- only. Boxes outline the interquartile range where 50% of the data is found, the line through the middle represents median value, the 'x' represents the mean and whiskers extend to minimum and maximum concentrations recorded for each treatment during this experiment. Heterogeneous letters atop each medial line indicate significant difference ($\alpha = 0.05$) in concentration.

14.1



14.2



14.3

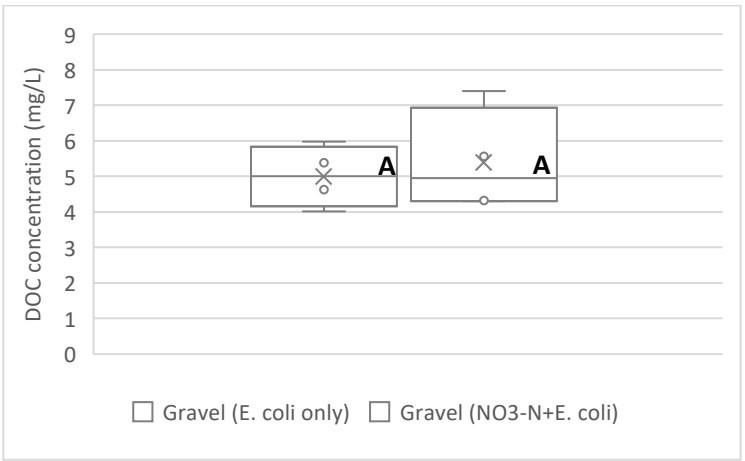


Figure 14. DOC concentration compared by influent composition for 14.1) woodchips; 14.2) peanut shells; and 14.3) gravel. Boxes outline the interquartile range where 50% of the data is found, the line through the middle represents median value, the 'x' represents the mean and whiskers extend to minimum and maximum concentrations recorded for each treatment during this experiment. Heterogeneous letters atop each medial line indicate significant difference ($\alpha = 0.05$) in concentration.

Mean DO and pH concentrations of influent and effluent water can be found in Table 6. Average influent DO concentration was 8.4 mg/L, which is similar to concentrations recorded in bioreactor field studies (e.g., Martin et al., 2019; Bell et al., 2015). When influent water contained NO_3^-+*E. coli*, effluent DO concentrations from all treatments was lower, on average, than that of influent water (9.3 mg/L). For the other two influent treatments (*E. coli* only and NO_3^- only) both woodchips (5.3 mg/L and 6.8 mg/L, respectively) and peanut shells (7.3 mg/L and 6.7 mg/L, respectively) released water with lower DO concentrations than that of influent water (8.8 mg/L and 7.1 mg/L, respectively), and DO concentrations of effluent water was consistently highest from gravel columns (9.2 mg/L and 8.6 mg/L, respectively). When NO_3^-N was present in influent water, DO was higher in woodchip effluent than that that of peanut shells. However, when no NO_3^- was added to influent water, DO was lower in woodchips than it was in peanut shell effluent. Gibert et al. (2008) conducted column studies and reported a much larger drop in DO after bioreactor treatment than was observed in this study. NO_3^- removal rates achieved in this study may have been dictated, in part, by elevated DO levels throughout the column allowing denitrifying microbes to use DO rather than NO_3^- as the electron receptor (Christianson et al., 2011). Although lab-scale columns, due to their limited size, do not necessarily reflect DO dynamics found in field-scale systems (Christianson et al., 2011), the results of this study provide a useful comparison of NO_3^- removal performance between substrate types under variable influent conditions.

Denitrification is believed to be inhibited when DO is above 0.5 mg/L (Christianson et al., 2012). While DO concentrations in this study never reached 0.5 mg/L or below, significant differences in effluent NO_x-N concentrations compared to influent were observed. Considering these reductions in NO_x-N, as well as DEA results, denitrification appears to have occurred.

Additionally, even when bulk conditions are not anoxic, bacteria can form microcosms of anoxic space where denitrification can occur (Smriga et al., 2021). This observation is supported by Martin et al. (2019) who used leave-one-out cross validation to determine the effect of various parameters (temperature, influent NO_3^-N concentration, HRT and DO) on NO_3^- removal efficiency and found that DO contributed to only 0.19%. Environmental and hydrologic conditions like soil composition and wet-dry cycles can also result in denitrification "hot-spots" or "hot moments" within systems (Weitzman et al., 2021).

Typically, the process of denitrification increases pH levels (Christianson et al., 2012). Regardless of influent or substrate type, all treatments resulted in an effluent pH greater than that of the respective influent pH. All effluent pH values, regardless of substrate type or influent composition, fall within the temporarily acceptable range for aquatic ecosystems set by the USEPA (6.5-9) (USEPA, 1976). The results reported here fall in line with other work assessing denitrifying bioreactor substrates in lab-scale columns studies such as Gibert et al. (2008).

Table 6. Mean (±standard error) influent and effluent dissolved oxygen and pH from columns receiving water A) with NO_3^-+*E. coli* B) *E. coli* only and C) NO_3^- only.

	NO_3^-+*E. coli*	Woodchips	Peanut shells	Gravel
Dissolved oxygen (mg/L)	9.3 ± 0.4	7.9 ± 0.1	6.9 ± 0.4	8.8 ± 0.1
pH	5.9 ± 0.1	7.3 ± 0.0	7.2 ± 0.0	6.9 ± 0.1

	E. coli only	Woodchips	Peanut shells	Gravel
Dissolved oxygen (mg/L)	8.8 ± 0.4	5.3 ± 0.1	7.3 ± 0.1	9.2 ± 0.0
pH	6.1 ± 0.1	6.9 ± 0.0	7.2 ± 0.03	7.1 ± 0.1

	NO_3^- only	Woodchips	Peanut shells	Gravel
Dissolved oxygen (mg/L)	7.1 ± 0.9	6.8 ± 0.1	6.7 ± 0.1	8.6 ± 0.0
pH	5.9 ± 0.1	6.7 ± 0.0	6.8 ± 0.0	6.6 ± 0.02

6. CONCLUSIONS

This study demonstrates potential for woodchips, peanut shells, and gravel to be effective means of sequestering *E. coli* and/or NO_3^- from shallow groundwater as denitrifying bioreactor substrates at low HRTs (<6 hours). NO_3^--N removal efficiency of woodchips (29%-30%) consistently outperformed that of peanut shells (17%-10%) and the gravel control (1%-2%) regardless of the presence of *E. coli*. TN removal efficiency trends typically align with NO_3^- removal efficiency trends but, in most scenarios, are slightly greater, indicating that reported NO_3^--N removal wasn't simply theproduct of N transformations. Lab-scale denitrifying bioreactor studies have reported NO_3^--N removal > 99% at HRTs of 8 hours (Cooke et al., 2001), however, few lab-scale studies have explored NO_3^--N removal at HRTs as low as those explored here. Although NO_3^--N removal achieved with peanut shells in the current study was relatively low, removal efficiency achieved with woodchips compares favorably to other lab- and field-scale studies which have reported NO_3^--N removal rates near 20% at HRTs of 2-hours (Bell et al., 2015; Chun et al., 2009).

E. coli removal efficiency of gravel continuously outperformed both carbonaceous substrates. Removal efficiency of peanut shells (70%-78%) remained unaltered by variations in influent chemistry whereas removal efficiency of woodchips (33%-69%) and gravel (85%-96%) were both significantly improved by the presence of NO_3^- in influent water. Effluent NH_4^+-N concentrations were typically low (< 0.7 mg/L). Although the range of *E. coli* removal efficiencies achieved in the current study is quite variable depending on substrate used, removal efficiencies observed here are similar to those achieved in other lab-scale denitrifying bioreactor

studies which have reported upwards of 90% removal efficiency at HRTs ranging from 12 hours to multiple days (Rambags et al., 2016; Rambags et al., 2019; Soupir et al. 2018).

Improving our understanding of NO_3^--N and *E. coli* removal at low HRTs, like those explored in this study, will allow for identification of optimal HRTs for each substrate in various conditions so denitrifying bioreactors can be effectively exploited. In environments where water is typically high in both NO_3^- and *E. coli*, a combination of substrates, like woodchips and gravel, would likely result in the highest remediation of both contaminants. In environments with variable NO_3^- concentrations where *E. coli* concentrations tend to be elevated, peanut shells may offer a better solution to remediation of both contaminants than woodchips or gravel. In locations where NO_3^- is typically more problematic than *E. coli*, woodchips could offer a better option than peanut shells or gravel.

This study warrants further investigation into denitrifying bioreactor dynamics with variations in influent chemistry as well as into bacterial filtration mechanisms in these systems. Furthermore, assessment of interactions between substrate types on *E. coli* and NO_3^- may uncover novel ways to combine substrates to result in more effective site-specific remediation efforts. Moving forward, further exploration into peanut shell bioreactor microbial communities, dynamics, N end products, and their potential for pollutant swapping will be necessary. Once operation of peanut shell bioreactors is more thoroughly understood through column studies and an optimal HRT has been identified, analysis of performance at the pilot- and field-scale will be required before peanut shell bioreactors become a viable, widespread option.

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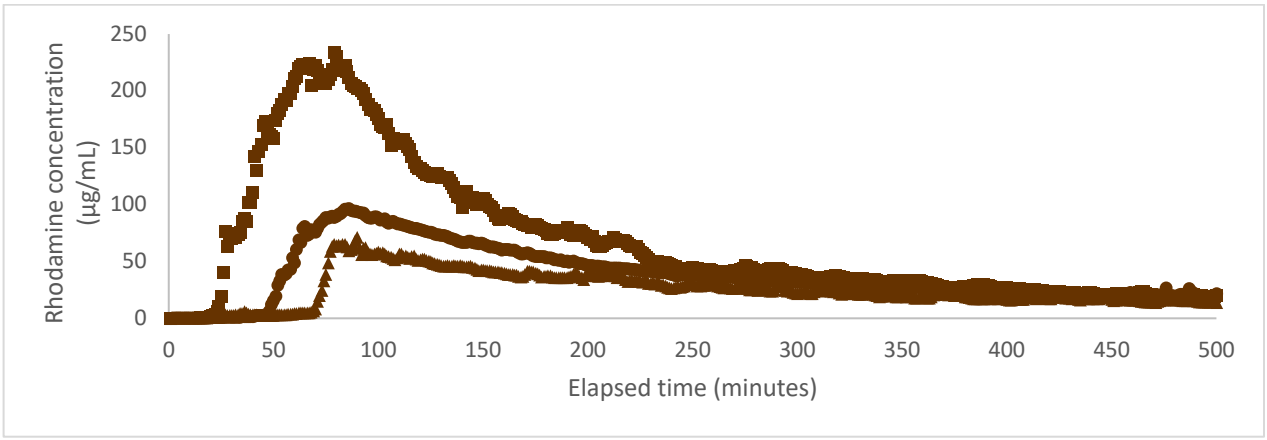
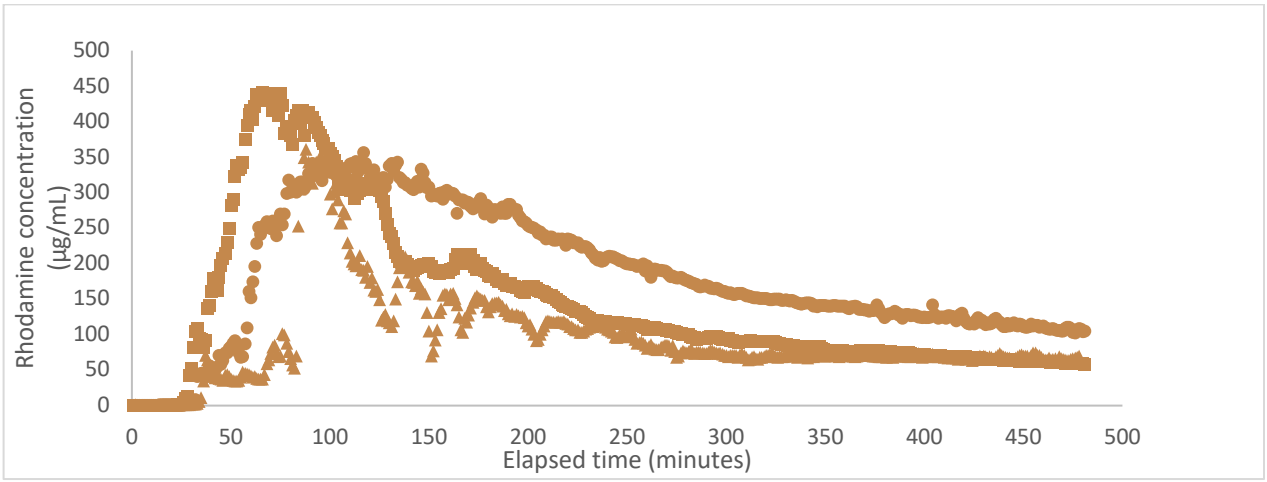
APPENDIX

1.1



1.2



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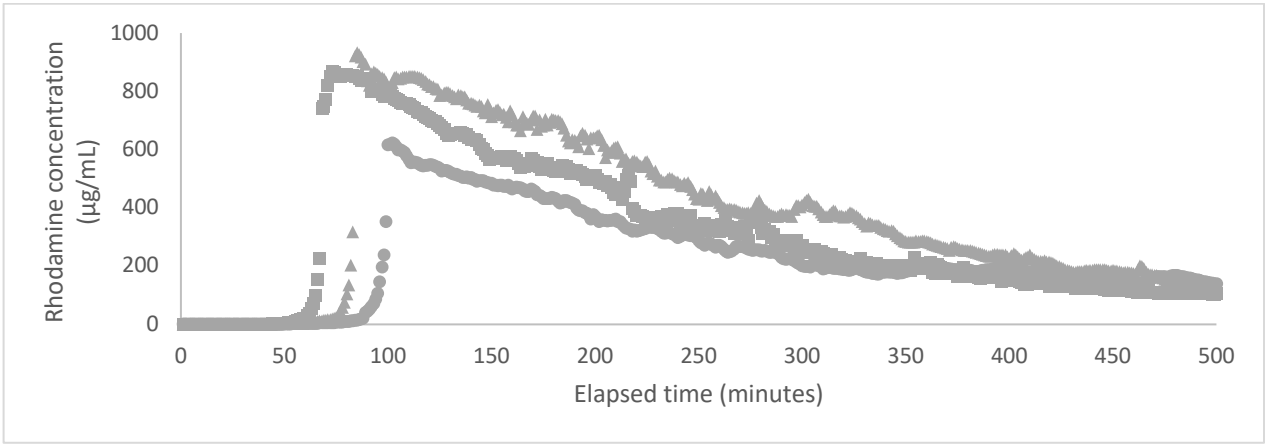

Figure S1. Graphs show dye concentration over time. Each graph shows results from three physical replicates of each substrate type 1.1) woodchips; 1.2) peanut shells; and 1.3) gravel. Distinct symbols (square, circle and triangle) within each graph represent results from a distinct physical column. Exo2 sondes were placed in one of 3 low-volume flow-through cells and collected water from a single column; a rhodamine dye (Bright Dyes, Kingscote Chemicals, Miamisburg, OH, USA) plug was introduced to each column preceded and followed by fresh water.