Industrial and agricultural wastes as a potential biofilter media for groundwater nitrate remediation

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ABSTRACT

Legislative measures like the Waste Framework Directive enforce the European Union member countries to increase the re-use of waste and/or the extraction of secondary raw materials. The Nitrate Directive requires the implementation of relevant measures to decrease groundwater nitrate concentration. This study evaluated the potential use of two dominant organic wastes in Turkey, tea factory waste and hazelnut husk, as potential carbon sources to stimulate nitrate removal in low cost permeable reactive barrier (PRB) systems. Leaching and batch experiments were carried out to determine the dissolution level and the degree of the heterotrophic denitrification process. Both organic substrates and inherent microbial communities supported the reduction of nitrate (NO_3^-) to nitrogen (N) gases. However, the percentage of organic substrate played an important role in the removal of nitrogenous compounds. The highest nitrate removal efficiencies were observed in flasks with 40% tea factory waste and 100% hazelnut husk, which were 64% and 97%, respectively. The corresponding zero-order reaction rates and half-lives were 3.03 mg N L^{-1} d^{-1} and 6 d^{-1} for tea factory waste and 5.17mg N L^{-1} d^{-1} and 4.4 d^{-1} for hazelnut husk. Of particular note, both wastes supported the denitrifying populations at such an excellent level that 99% of the nitrate was removed in the column study for a duration of 51 d under low and high flow rate conditions. Thus, the release of nitrate, ammonium and total organic carbon from wastes was not limiting its suitability in PRB systems.

Keywords: Industrial and agricultural wastes; Groundwater nitrate pollution; Remediation; Heterotrophic denitrification

1. Introduction

Waste volumes continue to increase, subsequently endangering human health and the environment and causing loss of resources. The total waste produced in the European Union (EU-28) in 2016 by all economic activities and households was about 2,533 million tonnes of which 8.5% were municipal solid wastes [1]. The Waste Framework Directive (WFD) (Directive 2008/98/EC) [2] provides the legislative framework across the EU by setting the basic concepts and principles related to waste management. The directive also requires member states to take appropriate measures to prevent or reduce waste production and to increase the recovery of waste using recycling, reuse or reclamation or any other process to extract secondary raw materials or the use of waste as sources of energy [3]. The directive also states that reuse or recycling takes preference over the use of waste as a source of energy [4]. The EU Landfill Directive, on the other hand, is supplementing the WFD by setting targets for the reduction of biodegradable wastes (bio-wastes) going to the landfill (Council Directive

99/31/EC) [5]. The total yearly production of bio-waste in the EU is estimated at 118 to 138 Mt of which around 88 Mt originate from municipal wastes and between 30 to 50 Mt from the food and drink industry [6,7]. The current and proposed revisions on the WFD and Landfill Directive include new targets on the recycling and landfilling of municipal wastes also containing specific bio-waste related elements for better bio-waste management [8,9].

Contamination of ground and surface water resources by nitrate has become a major problem worldwide [10–12]. Health concerns of high nitrate levels in drinking water are related to the higher risks of methemoglobinemia in infants and cancer [13]. The World Health Organization (WHO) and the EU have set a nitrate limit of 50 mg NO_3 L^{-1} in drinking water supplies (Drinking Water Directive 98/83/EC) [14,15]. For integrated protection and management of water resources, legislative instruments like the Water Framework Directive (Directive 2000/60/EC) [16] and the Nitrate Directive (Directive 91/676/EEC) [17] were developed by the EU. The cost of the implementation of EU directives and compliance of targets set by the EU was reported to be significant [18,19]. New microbial denitrification strategies have emerged for nitrate removal as more economical, practical and permanent solutions [20,21]. Numerous lab-scale and field-scale studies have shown that wood by-products (e.g. woodchip and plant-chip) [22,23], sawdust [24], sugarcane bagasse [25], mulch [26], and maize cobs [27] can be used as biofilter media in bioreactors like denitrification beds and walls/barriers treating nitrate-loaded effluents [28] and agricultural drainage water [29]. Recent studies focus on the selection of appropriate solid carbon sources as biofilter media presenting sustainability, long-term bioavailability and reactivity [30], lower pollution swapping potential [31], better hydrodynamic characteristics and consistent nitrate removal rates. Malá et al. [30] tested eight wood-based materials for in situ denitrifying bioreactors used for the treatment of high nitrate (43.1 mg N L^{-1}) agricultural runoff and found that beech woodchip, mulch and poplar woodchip represented the best results. Hou et al. [32] used rice bran as carbon and microbial sources for nitrate removal and determined that rice bran-gauze segmented columns presented the highest removal rate (262.20 g(m³d))$^{-1}$ with a 12 h hydraulic retention time. Hu et al. [33] treated woody sawdust with lime and peracetic acid to increase carbon availability and observed that the long-term denitrification rate of pretreated sawdust increased 4.5–4.8 times over that of untreated sawdust (29.3 mg N L^{-1} sawdust d^{-1}). Šereš et al. [34] studied birch and spruce woodchip and their mixture with gravel and found that denitrification bioreactors with mixed woodchip filling material yielded the highest load removal rates.

In this study tea factory waste and hazelnut husk, two dominant organic wastes in Turkey were investigated for their potential utility as organic carbon sources for denitrification bioreactors. Turkey is the largest hazelnut producer and fifth largest producer of tea in the world [35,36]. In-shell hazelnut varies between 420,000 and 800,791 tons per year (2008–2018), reaching a value of 515,000 tons in 2018 [37]. The husk is a major residue in nut production, accounting for about two-thirds of the wet weight of the hazelnut. Dede et al. [38] stated that about 300,000 tons of husk residues are produced per year based on a 1:3 ratio of dry matter of husk to total

dry matter of harvested hazelnut. Regarding tea production, the wet tea leave harvest increased from 1,100,257 tons in 2008 to 1,500,000 tons in 2018 [39]. The black tea produced at the end of the tea production process is approximately 20% of the fresh tea leaves entering the tea factory. Overgrown woody shoots are not graded as tea and are separated during the process as tea factory waste, which makes up 10%–20% of the dry tea produced. Malkoc and Nuhoglu [40] reported that the tea factories located in the Eastern Black Sea Region of Turkey produce about 30,000 tons of tea waste per year. As a candidate country, Turkey has enacted several EU directives, among them the WFD and Landfill Directive. Thus, Turkey is currently investigating the recovery of wastes as secondary raw materials or as an energy source. Turkey has also transposed the Water Framework Directive and Nitrate Directive. Turkey's goal is also to reduce nitrate concentrations in groundwater.

So far, residues of nut and tea production plants have not been used as organic media in bioreactors. The possibility of their use in denitrification bioreactors would similarly present low cost and sustainable solutions for other hazelnuts (e.g. Italy, Spain, USA, and Greece) and tea (e.g. China, India, Kenya, and Sri Lanka) producing countries. Within the scope of this study, (1) leaching tests were conducted to determine the dissolution level of nitrate, nitrite, ammonia and total organic carbon (TOC), (2) batch experiments were performed to assess the ability of organic carbon to stimulate denitrification and the nitrate removal rate at different organic waste doses, and (3) column experiments were carried out to investigate the long term carbon availability and nitrate removal efficiency.

2. Materials and methods

2.1. Industrial and agricultural wastes used as reactive materials

Hazelnut husk was obtained from local farms in Ordu (Middle Black Sea Region) after harvesting. The husk was cleaned of non-husk impurities, washed, dried at 105°C for 3 h. The tea-factory wastes were obtained from the Çaykur Tea Factory located in Çayeli Rize, the East Black Sea Region of Turkey. All wastes were air-dried and screened to obtain a fraction below 4 mm. In the leaching experiments, dry samples were used; whereas in the batch and column experiments saturated samples were utilized to avoid the presence of trapped oxygen. Saturation of wastes was carried out by filling 1,000 mL Duran bottles (DWK Life Sciences, Mainz, Germany) with hazelnut husk or tea factory waste and adding tap water until the bottles were full. These bottles were stored at temperatures below 4°C. Elemental analyses of wastes were carried out on homogeneous fine powder using Elementar Vario MACRO cube CHNS Elemental Analyser (Elementar Analysensysteme GmbH, Langenselbold, Germany). The elemental composition and some properties are presented in Table 1.

2.2. Leaching experiments

Leaching tests were performed in triplicate to determine the leachable nitrogen forms of reactive materials. Triplicate samples of 2.00 g each were placed into centrifuge tubes with

Table 1
Elemental composition and some properties of organic residues used in the study

	Elemental composition, % (Standard deviation)				Density	Porosity
	C	H	N	S	(g cm^{-3})	(%)
Hazelnut husk	42.10 (1.31)	5.66 (0.26)	1.21 (0.04)	0.24 (0.08)	0.56	84
Tea factory waste	48.17 (1.31)	6.57 (0.03)	2.72 (0.09)	0.22 (0.004)	0.70	74

50 mL deionized water. The tubes were placed in the dark on a rotary shaker for 66, 132, 198, and 264 h at 150 rpm. Following centrifugation, the supernatant was filtered using 0.45 μm MF-Millipore MCE Membrane syringe filters (Merck KGaA, Darmstadt, Germany) and then analyzed for nitrate (NO_3^-–N), nitrite (NO_2^-–N), ammonium (NH_4^+–N) and TOC.

2.3. Batch experiments

Batch tests were carried out in triplicate to determine the ability of wastes to provide dissolved organic carbon (DOC) for the activity of denitrifying bacteria. Ten 100 mL glass flasks were filled with an equal part in the volume of reactive material (approx. 20 cm^3). The reactive materials were an organic substrate and/or sand (3–4 mm). The percentage of organic substrate in reactive material was either 40%, 60%, or 100%. Mixtures consisting of organic residue and sand were carefully mixed to obtain homogeneous samples. Flasks with only sand or nitrate solution were also prepared as controls. Each flask was saturated with synthetic groundwater (32.4 mg L^{-1} NO_3–N) and sealed to create anoxic conditions. All flasks were covered with aluminum foil and were kept at room temperature (20°C) for 66, 132, 198, and 264 h. Previous results [41,42] suggested that microbial inoculation of the flasks was not needed; therefore, inoculation was similarly not carried out. During sampling, aqueous solutions in flasks were passed through 0.45 μm cellulose syringe filters and then analyzed for NO_3^-–N, NO_2^-–N, NH_4^+–N, TOC, pH, oxidation–reduction potential (ORP) and dissolved oxygen (DO).

2.4. Column experiments

Column tests were carried out for 51 d to investigate the ability of wastes to reduce nitrogen levels in the long term. For this purpose a lab-scale barrier system was set up with eight polyvinyl chloride columns, each having a length of 52.5 cm and an internal diameter of 5 cm. The barrier system was run with synthetic groundwater with two different flow rates in up-flow mode using two multichannel peristaltic pumps. The hydrodynamic character of columns was determined with a tracer test using 1,000 mg L^{-1} chloride solution. The flow rate and hydraulic retention time of columns of the slow rate barrier system were approximately 42 mL d^{-1} and 6.1 d, and of the high rate system were 84 mL d^{-1} and 3.25 d, respectively. The slow rate barrier system as consisting of five columns of which two were filled with 40% and 60% hazelnut husk or tea factory waste and one with only sand (100%). The high rate barrier system was consisting of three columns filled with 40% hazelnut husk or tea factory waste or only sand. All reactive materials were placed as saturated materials into columns full of water to avoid the presence of trapped oxygen. All columns were sealed to obtain anoxic conditions and were covered with an aluminum folio to avoid light penetration. Samples were taken four to five times a week and analyzed for nitrate, nitrite, ammonium, and TOC.

2.5. Analytical techniques

The aqueous solutions obtained from the leaching, batch and column experiments were sampled for nitrate, nitrite, ammonium and TOC analyses. pH, ORP and DO were measured only in samples of the batch and column experiments. pH and ORP were determined using a glass combination needle electrode (Intlab Redox-Pro pH) and a combination platinum Ag–Ag (3 M KCl) needle electrode (Intlab Redox-Pro V), respectively, interfaced with a Mettler Toledo Seven Multi multi-meter (Mettler Toledo International Inc., OH, USA). DO was measured using a portable DO meter equipped with a Hanna HI 9145 electrode (Hanna Instruments Ltd, Bedfordshire, UK). TOC of each sample was measured in triplicate with a high-temperature aqueous TOC analyzer (Tekmar Dohrmann Apollo 9000, Teledyne Tekmar, OH, USA). Thermo Scientific Helios Zeta UV/VIS spectrophotometer (Thermo Fisher Scientific Inc., MA, USA) analyzed nitrate, nitrite and ammonium concentrations in the solutes of leaching and batch experiments in accordance with methods# 8171, 8153 and 8038 of the HACH water analyses handbook, respectively (Hach Lange GmbH, Düsseldorf, Germany). Total inorganic nitrogen (TIN) concentrations for the leaching and batch experiments were calculated by summing the NO_3^-–N, NO_2^-–N, NH_4^+–N concentrations. Solutes of the column study were introduced to a Metrohm 850 Professional ion chromatography (IC) via an accompanying 858 Professional Sample Processor autosampler (Metrohm AG, Herisau, Switzerland). Among the IC data only results for the nitrogenous compounds and TIN were evaluated within this paper. Special care was given during all sampling and measurement processes to minimize aeration and assurance of homogenous sampling.

Microorganisms were harvested by passing 20–50 mL through sterile, syringe membrane-filters (0.20 μm pore size). Their deoxyribonucleic acid (DNA) was extracted and purified using PowerSoil DNA Isolation Kits (Mo Bio Laboratories Inc., Carlsbad, CA, USA) according to manufacturer's instructions, except a FastPrep-24 (MP Biomedicals, CA, USA) instrument was used to pulverize filters and disrupt cells. Genes representing denitrifying organisms (*nirS* and *nirK*) and total bacteria (16S ribosomal ribonucleic acid) were quantified by quantitative polymerase-chain-reaction

(qPCR) (Bio-Rad iCycler, Hemel Hempstead, UK) using SsoFast EvaGreen PCR reagents (Bio-Rad; 10 μL reactions) and previously designed primers (*nirS*: [43] and *nirK*: [44]). Reaction conditions involved initial denaturation at 94°C for 3 min.; 35–40 cycles of denaturation at 94°C (10s), primer annealing at 55°C (10s), and elongation at 60°C (10s). Post-analytical melt curves were conducted to verify product quality, and diluted plasmid controls, each containing the gene of interest, were used as standards [45].

3. Results

3.1. Leaching experiments

Concentrations of total nitrogen (TN) and organic carbon leached from the hazelnut husk and tea factory waste are presented as a function of time in Fig. 1. For both parameters, the leaching potential of tea factory waste rather seems at much higher degrees, when compared with the results of hazelnut husk. Hazelnut husk is a kind of raw material, whereas the waste obtained from a tea factory is a fermented material capable of readily evolving nitrogenous and organic compounds. The ultimate analyses of both substrates also show that the tea factory waste has relatively higher nitrogen and carbon in its elemental composition (Table 1). The highest concentrations of TIN and organic carbon were measured as 28.6 and 480.1 mg L^{-1} for husk and 104.9 and 2,155.1 mg L^{-1} for tea factory waste after 11 d, respectively. Additionally, the nitrogen released from the husk is mainly

in the form of nitrite, but ammonium and nitrate were also present in solutions. Nitrate was not measured in the leachates of tea factory waste, which mainly seems to release nitrogen in the form of ammonium. The leachable nitrogen forms of reactive materials are given in Table 2 as mg g^{-1}. Leaching of nitrate, ammonium, and TOC was also observed in other studies [27,31]. The findings of the current study show that nitrate leaching from hazelnut husk is generally lower than those reported by Gibert et al. [41] and Zhang et al. [46]. The amount of NO$_3^-$–N released by mulch, leaves, willow, and compost was recorded by Gibert et al. [41] as 0.11, 0.13, 0.18, and 0.28 mg g^{-1}, respectively. However, the amount of TOC leached from husk and tea factory waste was much higher than those reported by Gibert et al. [41], which was below 4.87 mg g^{-1}. McLaughlan and Al-Mashawbeh [47] studied the release of DOC from woody filtration materials and concluded that the leached DOC mass was varying significantly between the materials and to a lesser degree between different particle size ranges. Zhang et al. [46], on the other hand, stated that the initial high release of nitrogen and organic compounds might indicate a potential drawback to their use. Especially the high release of ammonium and TOC from tea factory waste might question its use for groundwater remediation.

3.2. Batch experiments

3.2.1. pH, ORP, and DO in solute

The pH recorded in the flasks with hazelnut husk remained neutral (between 6.7 and 7.3) over time (Fig. 2a). Flasks with tea factory waste, on the other hand, reflected slightly acidic pH conditions between 5 and 6.4 (Fig. 2b). For both substrates, all pH values increased slightly in the first 5–6 d, and decreased with increasing organic substrate percentages. Denitrifying microorganisms generally favor neutral pH conditions [12,46]. Thus, pH values of flasks with hazelnut husk would more likely stimulate the denitrification process. For both substrates, anoxic conditions were almost immediately established (Figs. 2c and d). Redox potential (Eh) values decreased with time and represented values below −100 mV for flasks with 40% and 60% tea factory waste, which was attributed to the consumption of DO resulting from the degradation of released organic carbon and the nitrification of ammonium. Values below −200 mV might cause excessive nitrite and sulfide formation [48]. The high redox potential (>110 mV) measured for the flasks with 100% tea factory waste may indicate possible inhibition due to elevated carbon, ammonium and/or nitrite levels. DO levels in the flasks with organic materials decreased rapidly from values between 38% to 46% saturation (100% sand) to percentages between 2%–5% for hazelnut husk and 2%–12% for tea factories waste (Figs. 2e and f), respectively. While a difference in DO values was not recorded for a flask with hazelnut husk, an apparent difference was observed for the flask with 100% tea factory waste when compared with the data of other flasks. Regarding the control flasks with only sand, DO values slightly decreased with time as was verified by the redox potential values. This was related to minor microbial activity in the flask with sand, which was only washed and not sterilized at the beginning of the experimental study.
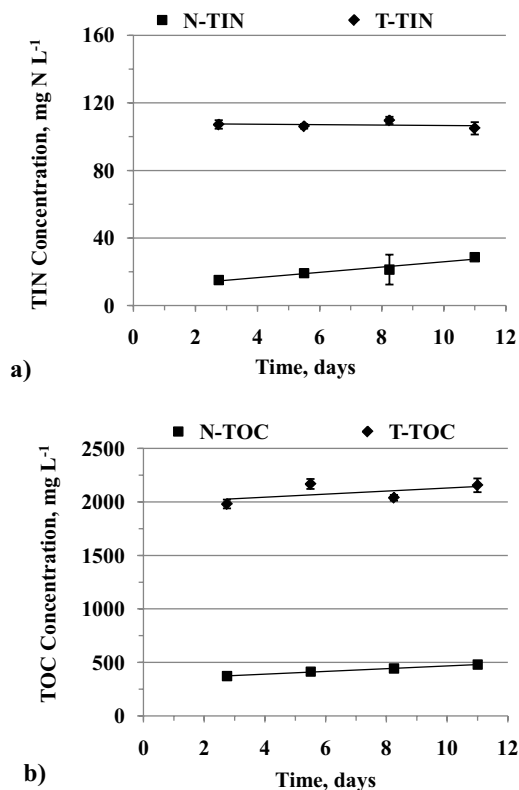

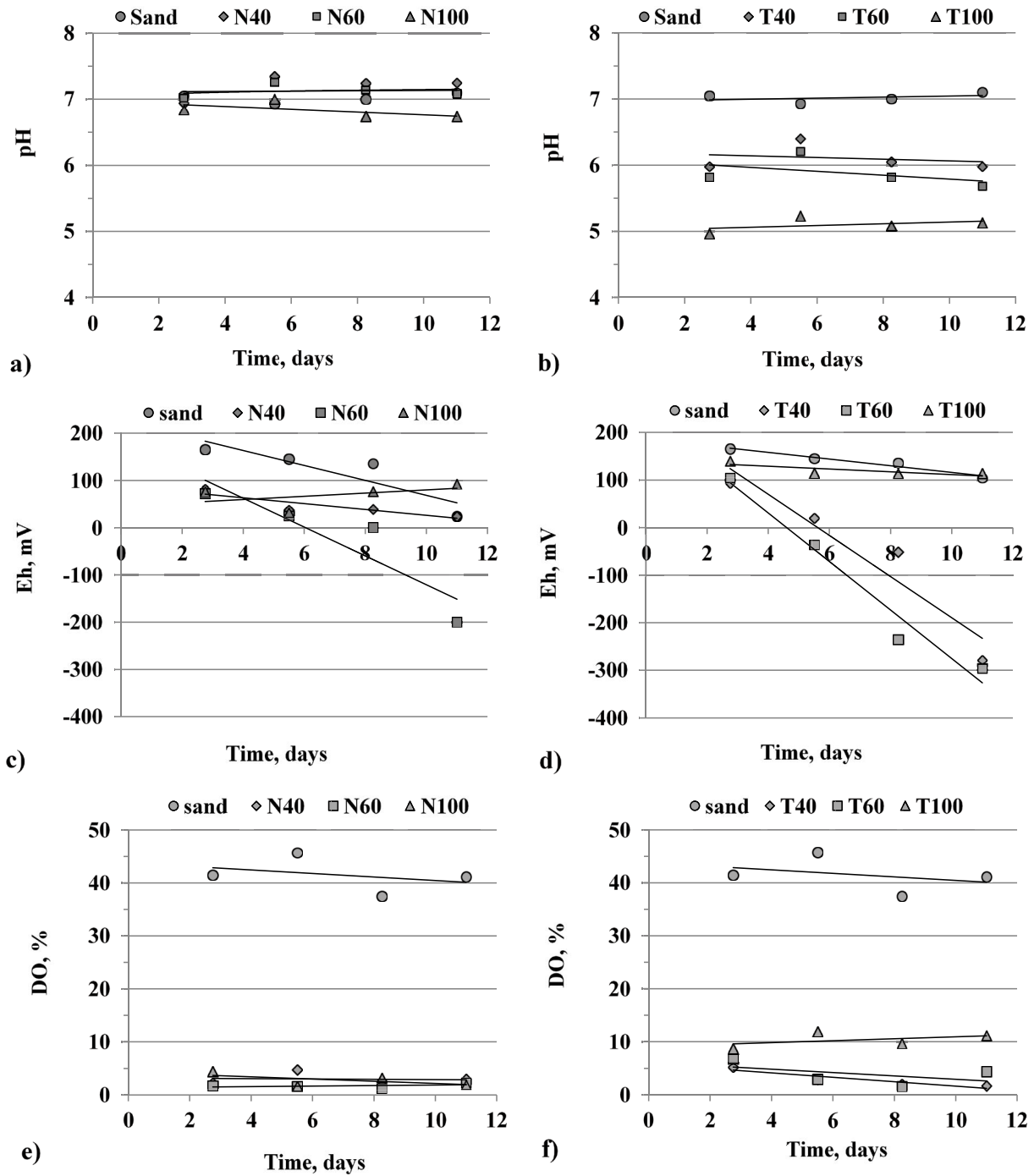Fig. 1. (a) Total nitrogen and (b) organic carbon concentrations in the solutes from leaching experiments.

Fig. 2. pH, Eh and DO in the solutes of batch experiments.

3.2.2. Nitrogen species in solute

Despite an initial increase in nitrate concentration, nitrate disappearance occurred in all flasks after 2–3 d (Fig. 3). The initial abrupt increase in nitrate was mainly related to the release of nitrate from the reactive material (e.g. hazelnut husk) and/or mineralization of released ammonium (e.g. tea factory waste). The nitrate reduction varied with the type and percentage of carbon sources. The hazelnut husk and tea factory wastes reflected different behaviors in the dynamics of nitrogenous compounds. An increase in the percentage of organic substrate in the flasks seems to have a positive effect on nitrate reduction with the hazelnut husk, whereas a negative influence was observed for the tea factory waste. The average nitrate concentrations decreased to approximately 11.7, 4.9, and 1.1 mg NO_3^-–N L^{-1} using hazelnut husk and 8.9, 13.7, and 22.1 mg NO_3^-–N L^{-1} using tea factory waste for 40%, 60%, and 100% organic substrate,
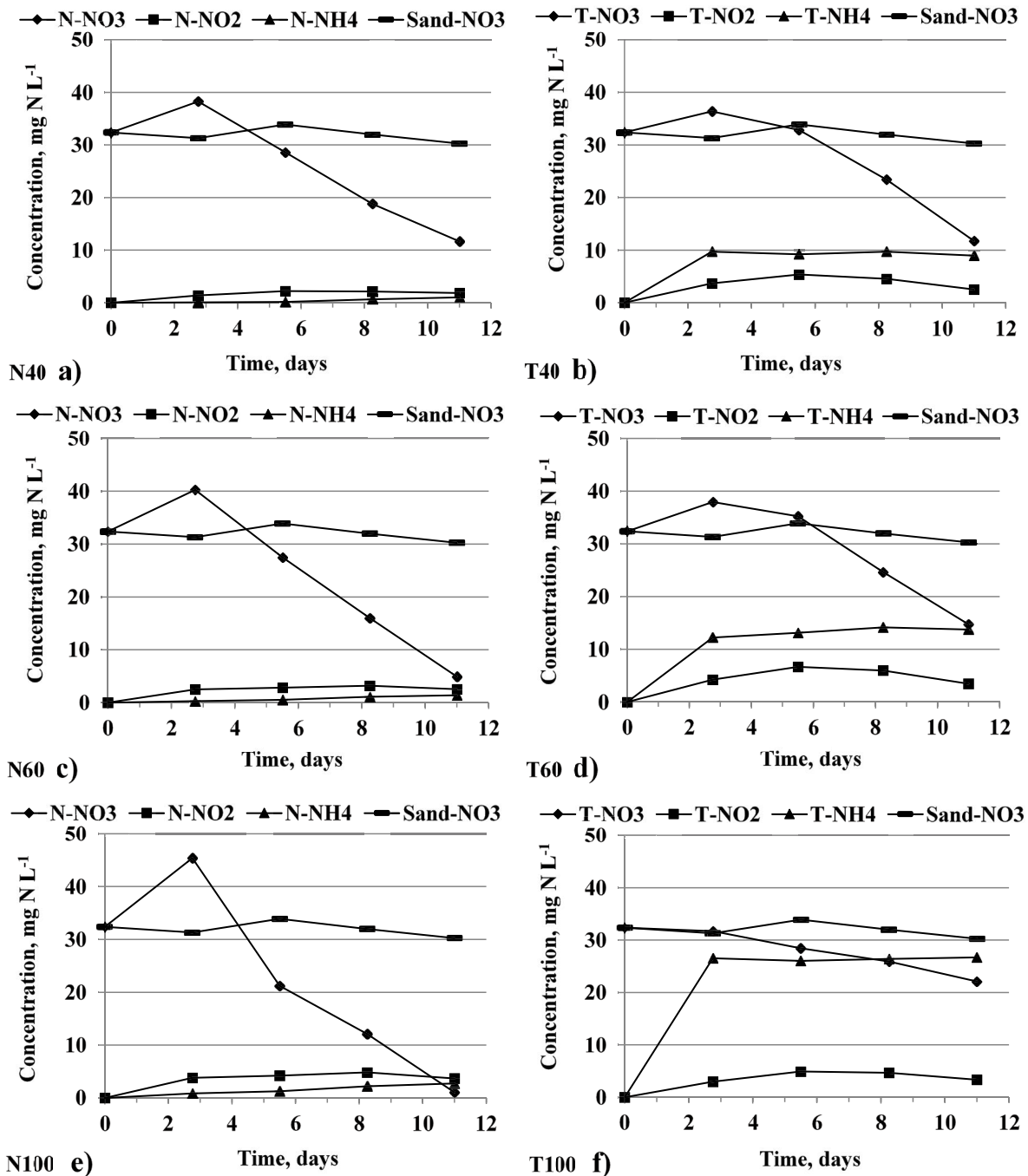
Fig. 3. Nitrogen species in the solutes of batch experiments. Boxes (a,c,e) represent results from hazelnut husks, whereas boxes (b,d,f) are from tea factory wastes at 40%, 60%, and 100% concentrations, respectively.

respectively. Little or no change in nitrate concentrations occurred in the control flasks (only sand or nitrate solution).

Nitrite accumulated in all flasks (Fig. 3), except in flasks with only sand and nitrate solution. Despite the higher nitrite leaching potential of hazelnut husk, nitrite concentrations observed for hazelnut husk were lower than those for tea factory waste (Table 1). The accumulation in both nitrate and especially nitrate was related to the mineralization of ammonium within the first 2–3 d which was also supported

by the decrease in DO concentrations (Figs. 2e and f). Tea factory waste represented much higher ammonium and TOC leaching potential than hazelnut husk that may have caused higher nitrate and nitrite concentrations increased within the first week but started to decline later on. Permanent enrichment of nitrite in the start phase was also reported by Krause Camilo et al. [26]. Generally, an increase in the percentage of organic substrate seems to increase the nitrate concentration. The highest nitrate concentrations were

observed as 4.8 mg NO_2^-–N L^{-1} for 100% hazelnut husk and 6.6 mg NO_2^-–N L^{-1} for 60% tea factory waste. These values decreased to approximately 3.7 and 3.5 mg NO_2^-–N L^{-1} on the 11th day, respectively. Nitrite accumulation was also reported by Zhang et al. [46] in tests with wheat straw (12.2 mg NO_2^-–N L^{-1}) and by Gibert et al. [41] in tests with hardwood and mulch (up to 21.0 mg NO_2^-–N L^{-1}). Gibert et al. [41] concluded that nitrite accumulation was probably due to the incomplete reduction of nitrate. Rivett et al. [12] stated that nitrate is preferentially used by denitrifiers to nitrite due to the difference in available energy from the reduction reaction. Lee et al. [49] related the high levels of nitrite to the relatively low nitrate reduction rate compared to nitrate reduction rate at the initial stage of the denitrifying process.

Accumulation of ammonium was especially observed for tea factory waste, which was attributed to its high leaching capacity and nitrogen content (Tables 1 and 2). The higher ammonium concentration observed by Grießmeier and Gescher [50] was attributed to the higher ammonium concentration in the wheat straw biomass. The ammonium concentration increased with the percentage of tea factory waste representing the highest concentration for 100% but remained stable for all percentages (Figs. 3e and f). The ammonium concentrations measured in flasks with 40%, 60%, and 100% tea factory waste were approximately 9.4, 13.3, and 26.4 mg NH_4^+–N L^{-1}, respectively. On the contrary, ammonium concentrations in flasks with hazelnut husk increased with time reaching values of 1, 1.4, and 2.7 m NH_4^+–N L^{-1} for 40%, 60%, and 100% organic substrate, respectively. Gibert et al. [41] determined ammonium concentrations up to 15.56 mg NH_4^+–N L^{-1} in similar batch experiments. It was also noted that the percentage increase in ammonium with respect to initial concentration was highest for 40% husk (between 2%–13%) and lowest for 100% husk (between 1.5%–3%). Since the calculated C/N ratios represent carbon limited conditions for 40% husk, the increase in ammonium might not be attributed to dissimilatory NO_3^- reduction to NH_4^+ dissimilatory nitrate reduction to ammonium (DNRA). DNRA is a further anaerobic reduction reaction that competes with denitrification and converts nitrate to ammonium rather than N_2 [46]. DNRA is generally known to occur at relatively higher C/N ratios [41,51]. Thus, for higher organic substrate percentages, where carbon abundance prevails, DNRA may be a counterproductive process in this denitrification study. It is interesting to note that carbon abundance was mostly observed in the tests with tea factory waste, where an increase in ammonium was not observed. On the other hand, very little ammonium formation (below 0.05 mg NH_4^+–N L^{-1}) was observed over time in flasks with only sand. Further studies on ammonium accumulation are needed.

3.2.3. Dynamics in TIN and organic carbon

The initial TIN concentration equaled the average concentration of prepared nitrate solutions (approximately 32.4 mg L^{-1}). This initial concentration increased to approximately 43–59 mg N L^{-1} and 58–70 mg N L^{-1} within 66 and 132 h for flasks with hazelnut husk and tea factory waste, respectively (Figs. 4a and b). In flask with only sand, an increase was not observed as expected. As presented above, the rise in TIN concentration as a result of NO_3^-, NO_2^-, NH_4^+ release and/or formation. Nitrate was generally the dominant inorganic nitrogen species in flasks with 40% and 60% organic substrate. More than 99% of TIN remained as nitrate in flasks with only sand. Ammonium, on the other hand, was the minor nitrogen (0%–24% of TIN) species in all flasks except 100% tea factory waste (39%–45% of TIN). The highest TIN concentrations were observed for flasks with 100% organic substrate. The TIN concentrations decreased as low as 16 and 29 mg N L^{-1} within 11 d. The observed TN removal efficiency was changing between 56%–73% and 15%–51% for hazelnut husk and tea factory waste, respectively.

Surprisingly the highest removal efficiencies were obtained for the highest hazelnut husk percentage and lowest tea factory waste percentage indicating the influence of TOC levels in solution. As can be seen from Figs. 4c and d, TOC concentrations continued to increase with time. For both substrates TOC concentrations were related to the substrate percentage, almost increasing systematically 3/2 and 3-fold with 60% and 100% substrate, when compared with 40% substrate. The lowest and highest TOC concentrations measured within 51 d were 27.7 and 81.0 mg L^{-1} for hazelnut husk and 175.2 and 537.7 mg L^{-1} for tea factory waste, respectively. TOC levels observed for hazelnut husk were in good agreement with the results of Gibert et al. [41]. Krause Camilo et al. [26] reported organic carbon consumption by aerobic dissipation processes and denitrification. The TOC removed per unit of nitrate reduced could not be assessed since a decrease in TOC was not observed. However, the electron donor to acceptor (C/N) ratio in denitrification was calculated for each substrate percentage [52]. The ratios were obtained by dividing the TOC concentration by either the nitrate concentration [TOC]/[NO_3^-] or TIN concentration [TOC]/[TIN]. It is interesting to observe that nitrate and TIN removal efficiencies and C/N ratios reflected a positive linear correlation for hazelnut husk ($r^2 \geq 0.83$) and tea factory waste ($r^2 \geq 0.97$) (Table 3). The C/N ratios indicated that the bacteria became carbon limited, especially in flasks with 40% hazelnut husk resulting in much lower removal efficiencies. Saliling et al. [48] stated that a deficiency of carbon can result in low nitrate removal and high nitrite production. On the other hand, in flasks with 60%–100% tea factory waste carbon abundance was observed. Carbon abundance might cause direct or indirect effects on denitrification [34]. Phenols, resin acids, tannins, lignins and volatile fatty acids that may be released as a result of organic matter degradation might have a direct toxic effect on the denitrification process. There might be other reasons that may have been induced by carbon abundance, influencing the activity of denitrifying bacteria. These might

Table 2
Average leachable amounts of nitrate, nitrite, ammonium, and TOC (mg g^{-1})

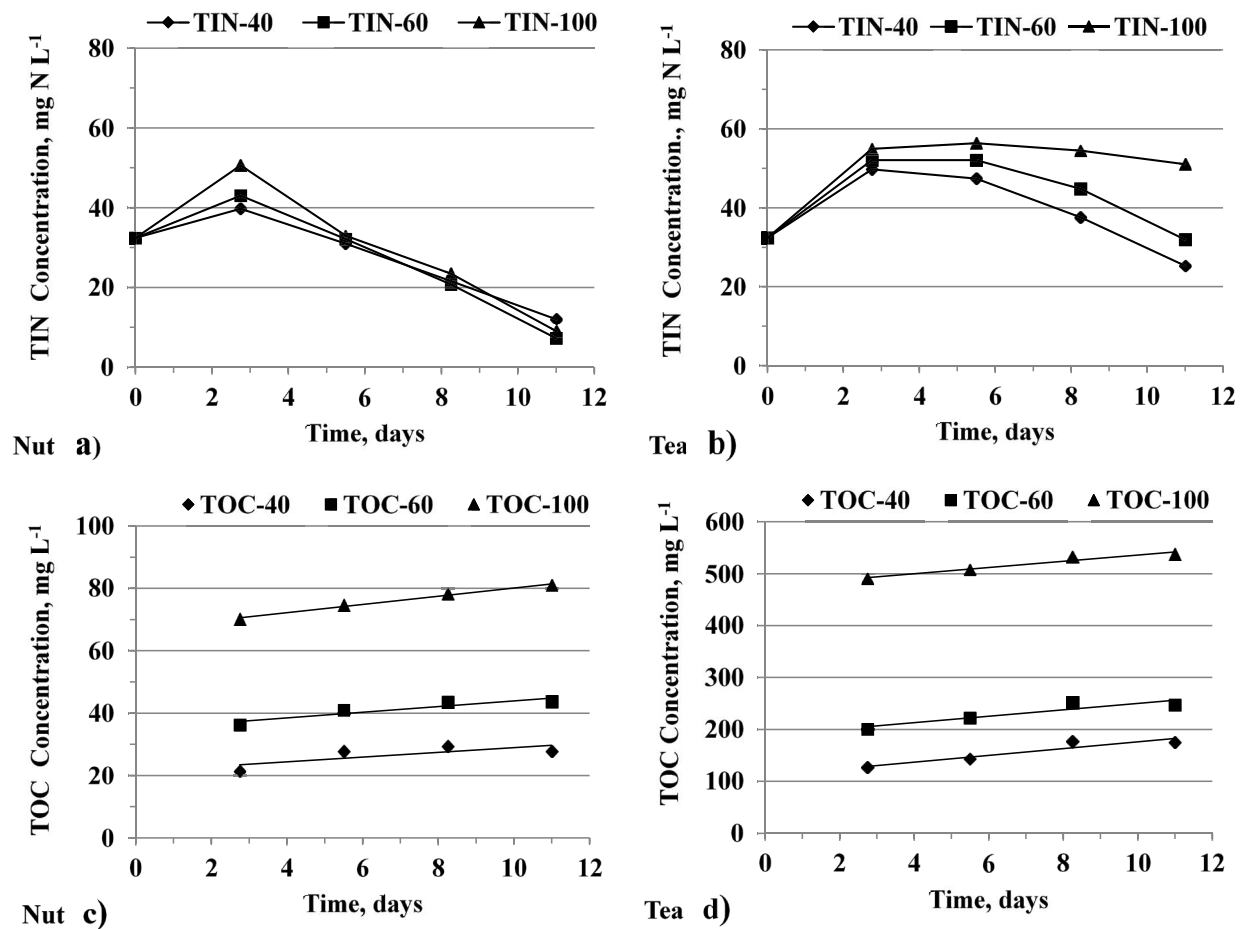| Reactive materials	NO_3^-–N	NO_2^-–N	NH_4^+–N	TN	TOC
Hazelnut husk	0.02	0.06	0.36	0.44	12.0
Tea factory waste	Bdl	0.03	2.61	2.64	53.9

Bdl: below detection limit.

Fig. 4. Dynamics in total inorganic nitrogen (a,b) and organic carbon (c,d) in batch experiments.

Table 3
Electron donor to acceptor (C/N) ratio in denitrification

Time (h)	[TOC]/[NO₃]			[TOC]/[TIN]		
	40%	60%	100%	40%	60%	100%
	Tea factory waste					
66	3.5	5.3	15.5	2.2	3.1	7.2
132	4.4	6.3	17.9	2.4	3.2	7.2
198	7.6	10.2	20.6	3.7	4.3	7.9
264	14.9	16.8	24.3	6.0	6.2	9.0
	Hazelnut husk					
66	0.6	0.9	1.6	0.5	0.7	1.2
132	1.0	1.5	3.5	0.8	1.1	2.1
198	1.6	2.7	6.5	1.1	1.6	2.6
264	2.4	8.9	76.5	1.5	3.0	5.1

be redox potential, pH and other environmental conditions that may inhibit the growth of denitrifying bacteria and result in the presence of other microorganisms [34]. Especially the stable redox potential values recorded for 100% tea factory waste indicate extreme inhibiting conditions in the flasks.

3.2.4. Presence of denitrifying bacteria

The presence of denitrifying bacteria was detected by targeting *nirS* and *nirK* genes in bacteria using quantitative PCR. The *nir* genes encode for nitrite-reductases, enzymes responsible for the conversion of nitrite to nitric oxide within the denitrification pathway. The enzyme *nirS* is a non-heme iron-containing enzyme, and *nirK* instead contains copper. Both genes exist in all batch systems (Table 4); however, higher concentrations exist in the hazelnut husk mixtures at almost equivalent concentrations ($10^{3.2}$ to $10^{3.5}$ genes per mL). The number of *nir* genes in the tea-leaf system was more greatly varied, with a wider range of community abundances, but gene abundances increased with higher levels of tea leaves. It suggests that the husks and tea-leaves either provided additional inoculation or supported bacteria growth (or both). While it is not possible to discern which process, we intended to verify whether biological denitrification is a possibility. It should be mentioned that the presence of the genes does not guarantee activity; however, it demonstrates that it could occur. Environmental conditions conducive for denitrification are still required for microorganisms to actively carry such biochemical reactions.

Table 4
NirK and _nirS_ concentrations in batch systems (log-transformed genes per mL)

Substrate		Log (_nirK_/mL)	Log (_nirS_/mL)
Hazelnut husk	40%	3.26 (0.62)	3.21 (0.01)
	60%	3.45 (0.03)	3.42 (0.38)
	100%	3.32 (0.15)	3.52 (0.05)
Tea factory waste	40%	1.86 (0.05)	0.77 (0.20)
	60%	2.07 (0.01)	1.62 (0.07)
	100%	2.52 (0.22)	3.47 (0.02)

Values represent means of duplicate samples and their ranges in parentheses.

3.3. Column experiments

3.3.1. Nitrogen species in solute

Despite the fact that all bioreactors were fed with a nitrate solution (approximately 32.2 mg L^{-1}) under continuous flow conditions an increase in nitrate concentrations in the outlet of columns was not recorded. Nitrate disappearance (<0.1 mg L^{-1}) occurred in all columns within 2 d irrespective of the source and percentage of organic materials and the flow rate (Fig. 5). Thus, both materials seem to provide organic carbon and abundant microbes into the bioreactor enabling a more effective denitrification process [32]. Nitrate concentrations rather remained high in the outlet of sand columns showing that lack of an organic carbon source is not supporting denitrification [34]. After approximately 20 d a very low increase in nitrite was recorded in the columns with hazelnut (<0.25 mg L^{-1}) and tea factory waste (<0.15 mg L^{-1}). A decrease in the percentage of organic matter did not reveal an important change in nitrate concentrations. However, the source and percentage of organic matter in columns seem to influence ammonium concentration, which was approximately 0.18 mg L^{-1} for hazelnut husk and 0.11 mg L^{-1} for tea factory waste for low flow rate columns containing 40% organic matter (Figs. 5a and c). For the same flow rate, columns with 60% organic matter revealed ammonium concentrations below 0.26 and 0.42 mg L^{-1} for hazelnut husk and tea factory waste, respectively (Figs. 5b and d). The ammonium concentrations observed for the high rate columns were not significantly different (Figs. 5e and f). The higher nitrate and ammonium concentrations recorded for hazelnut husk and tea factory waste, respectively, were related to the substrates leaching characteristics as presented in Table 2. Thus, columns with higher tea factory waste percentage reflected higher ammonium and columns with higher hazelnut husk percentage reflected higher nitrate concentrations. Zhao et al. [53] summarized the reason why nitrite was not further reduced to N$_2$, as a type of carbon source, carbon limitation, and microbes composition.

3.3.2. Dynamics in TIN and organic carbon

The TIN concentrations observed for the columns with organic material dropped almost immediately below 0.3 mg N L^{-1} within the first 2 d, while very little nitrate and ammonium concentrations were observed during the experiment. Regarding the TOC values in general, a continuous decline was observed for all columns with time. The TOC concentrations were different for the source and percentage of organic matter and the flow rate. As can be seen from Fig. 6a, TOC concentrations released from the slow flow rate columns filled with 40% and 60% hazelnut husk dropped from about 6.7 and 9.9 mg L^{-1} to about 3.7 and 6.3 mg L^{-1} within 51 d, respectively. TOC concentration recorded for the columns with tea factory waste was higher, dropping from about 86.9 and 120.1 mg L^{-1} to about 12.8 and 18.7 mg L^{-1} for columns with 40% and 60% substrate, respectively. Zhao et al. [53] similarly reported high TOC washout (approximately 165 mg L^{-1}) from woodchip bioreactors at the beginning of the experimental study (phase 1), which rapidly decreased later on. It was also concluded that the easily soluble fraction of carbon caused rapid microbial adaptation and thus high nitrate removal efficiency.

Increasing the flowrate of columns caused a general decline in TOC values (Fig. 6b). TOC values dropped from 30 mg L^{-1} to about 6.3 mg L^{-1} for columns with 40% tea factory waste, while TOC values recorded for columns with 40% hazelnut husk remained relatively stable within 51 d. Regarding the decline in TOC values, it can be stated that the undesired high TOC concentrations observed during the batch experiments were not of concern during continuous flow conditions. In general, TOC values were lower and more stable for columns with hazelnut than those filled with tea factory waste.

The reduction in TOC release from the organic materials did not seem to have an influence on nitrate removal as nitrate generally remained absent or very low (<0.1 mg L^{-1}) for the whole duration of the experiment. Thus, the calculation of [TOC]/[NO$_3^-$] was in general not possible due to the absence of nitrate, but [TOC]/[TIN] ratios that could have been calculated reveal that TOC was still abundant (>30) and thus supporting the denitrification process in the bioreactor for a duration of about 51 d under continuous flow conditions.

4. Discussion

4.1. Denitrification performance

Results of batch experiments revealed that the type and percentage of the organic substrate were influencing the nitrate removal efficiency. Thus, the denitrification potential was greatest for hazelnut husk, which was 64%, 85%, and 97% reduction for 40%, 60%, and 100% substrate, respectively. As the nitrate concentration decreased with time, the accumulation of both nitrate and ammonium was observed for flasks with hazelnut husk. Hazelnut husk reflected a higher leaching potential for nitrite (0.06 mg g^{-1}) than for ammonium (0.36 mg g^{-1}). However, the nitrite and ammonium concentrations observed in flasks were much lower than the concentrations calculated using the leaching data (Table 1). Additionally, the initial nitrate concentration (>38.3 mg NO$_3^-$–N L^{-1}) was higher than the nitrate solution prepared (32.4 mg NO$_3^-$–N L^{-1}) and the expected leaching levels (0.1–0.4 mg NO$_3^-$–N L^{-1}). Thus, the increase in nitrate might be related to the nitrification of ammonium and nitrite within the first 2–3 d, which was also supported by the decrease in DO concentrations (Fig. 2e). After the 3rd-day nitrate removal occurred in addition to nitrate,
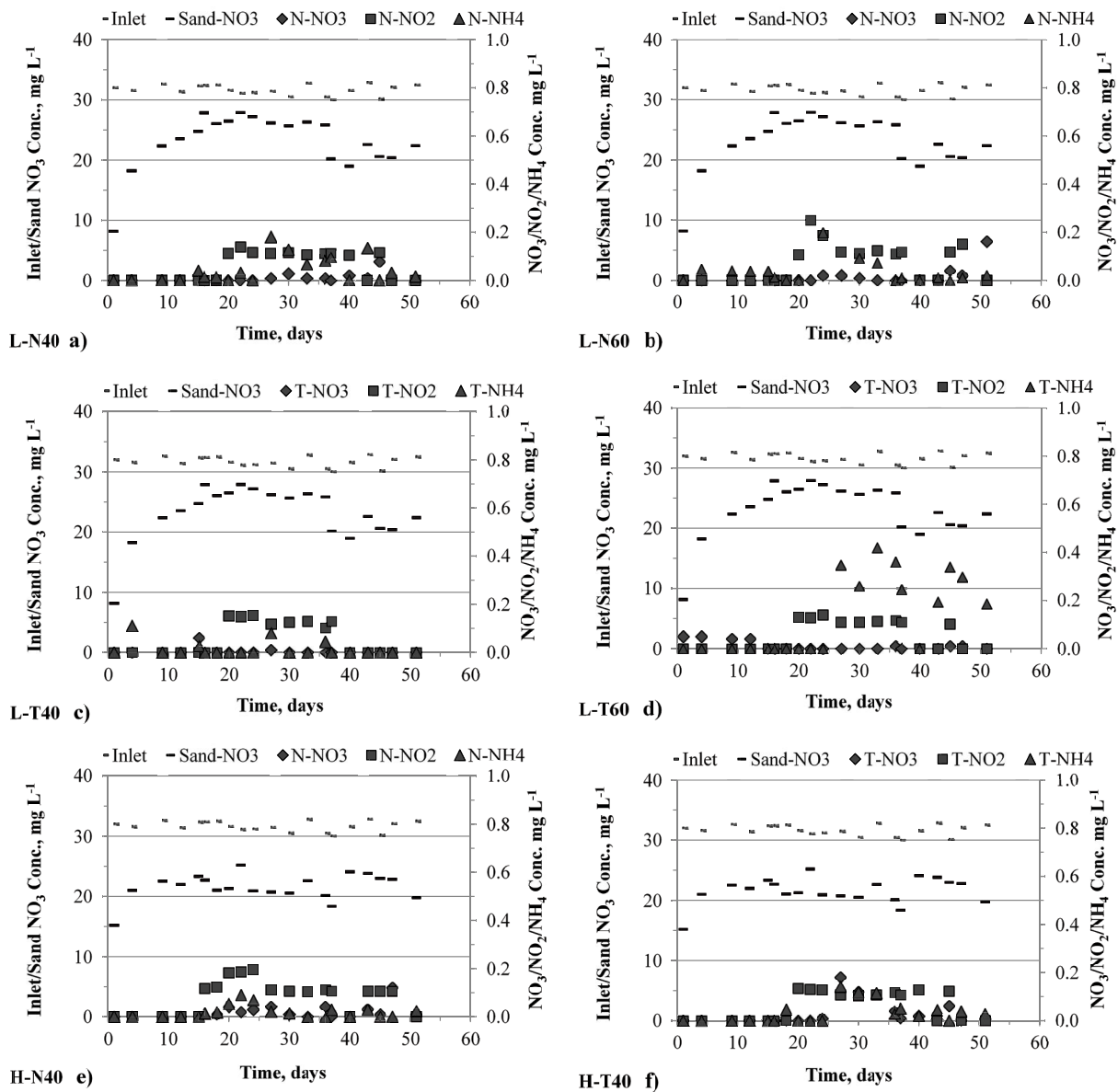
Fig. 5. Nitrogen species in the solutes of column experiments. Boxes (a,b,e) represent results from hazelnut husks, whereas boxes (c,d,f) are from tea factory wastes.

especially with an increase in hazelnut husk percentage. The ammonium concentration continued to increase, however, the fraction of final to initial ammonium concentration (C_t/C_0) decreased with increasing time. The nitrate concentrations decreased below 3.7 mg NO_2^-–N L^{-1}, however, ammonium concentrations reached 2.7 mg NH_4^+–N L^{-1}. The nitrate concentration decreased from 45.4 to 1.1 mg NO_3^-–N L^{-1} in flasks with 100% hazelnut husk, which is not concurrent with the ammonium formation. Thus, the removal of nitrate by DNRA could only be with minor importance (approximately 4.2%). Warneke et al. [54] and Long et al. [55] also suggested that DNRA is a minor process in nitrate removal. Greenan et al. [56] stated that the carbon to electron acceptor ratio was not sufficiently high enough to favor DNRA over denitrification, as DNRA accounted similarly for >4% of the

NO_3^-–N removed. There is no evidence that the increase in ammonium is a result of DNRA and not only leaching since ammonium leaching from hazelnut husk continued with time (Fig. 1).

The nitrate removal efficiencies observed for tea factory waste in the batch experiments were 64%, 55%, and 32% for 40%, 60%, and 100%, respectively. Similarly, as the nitrate concentration decreased with time, nitrite and ammonium accumulation occurred. Tea factory waste reflected a higher leaching potential for ammonium (2.61 mg g^{-1}) than for nitrate (0.03 mg g^{-1}). Nitrate leaching was not recorded. However, the nitrite concentrations observed in flasks were much higher and ammonium concentrations were much lower than the calculated leaching concentrations. Additionally, ammonium concentrations in solution

were not increasing with time. Thus, it can be accepted that ammonium formation was not the case. The initial nitrate concentrations (>36.4 mg NO_3^-–N L^{-1}) were higher than the nitrate solution prepared (32.4 mg NO_3^-–N L^{-1}). Thus, the increase in nitrate and nitrite might be similarly related to mineralization. After the 3rd day removal of both nitrate and nitrite occurred due to denitrification. Since no ammonium formation was observed, DNRA cannot be a counterproductive process in denitrification. Schipper et al. [57] similarly concluded that DNRA seems unlikely

to be a major sink for nitrate in the denitrification beds as no accumulation of ammonium was observed. The nitrate, nitrite and ammonium concentrations determined in flasks with 40% tea factory waste after 11 d was 11.7 mg NO_3^-–N L^{-1}, 2.5 mg NO_2^-–N L^{-1} and 9 mg NH_4^+–N L^{-1}.

Results of the column experiments revealed that nitrate removal was supported for the long term under continuous flow conditions. In general, both organic materials reduced the nitrate concentration below 0.1 mg NO_3^-–N L^{-1} throughout the duration. Formation of nitrate and ammonium occurred in all columns but was of no importance (<0.2 mg N L^{-1}), when compared with the results of batch experiments. Thus, environmental conditions generated during continuous flow conditions seemed promising for a real field-scale bioreactor.
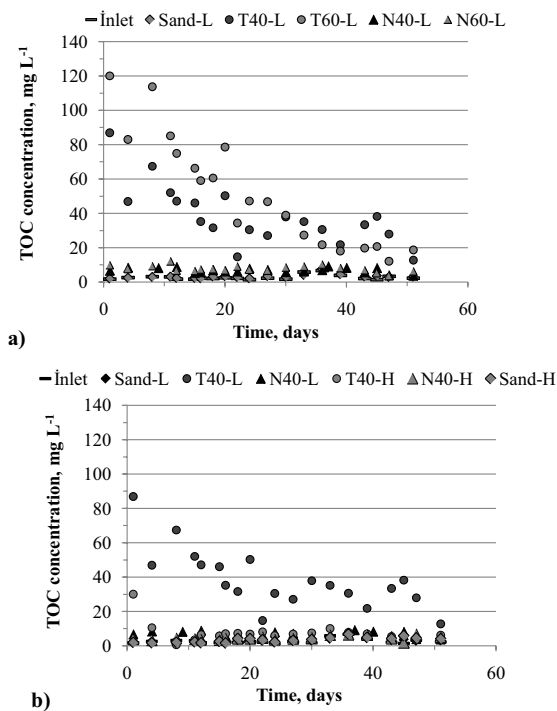
4.2. Denitrification rates

Regarding the differences in nitrogen dynamics observed for the batch experiments with hazelnut husk and tea factory waste, nitrogen removal rates were calculated applying both linear and non-linear regression analysis to nitrate concentrations measured after 66 h. For the column experiments, the regression analysis was avoided because of the lack of data, which was due to the absence of nitrate (below method detection limit (MDL)) in the outlet of most columns. However, it can be assumed that nitrate removal followed the zero-order model when nitrate is more abundant at the beginning but later changed to the first-order model when nitrate became limiting [53].

Considering the zero-order model, the decreasing slope of nitrate concentration (*C*) over time (*t*) was taken as the denitrification rate:

$$\frac{dC}{dt} = kC^0 \rightarrow C = C_0 - kt \rightarrow t_{1/2} = \frac{C_0}{2k} \tag{1}$$

Slopes obtained from the plot of $\ln(C/C_0)$ vs. (*t*) represented first-order reaction rates:

Fig. 6. Dynamics in total organic carbon in column experiments.

Table 5
Nitrate and TIN removal rates

NO_3 removal		Zero-order-model			First-order-model		
		k (mg N L^{-1} d^{-1})	r^2	$t_{1/2}$	k (d^{-1})	r^2	$t_{1/2}$
Hazelnut husk	40%	3.26	0.99	5.87	0.14	0.99	4.79
	60%	4.27	0.99	4.71	0.24	0.93	2.79
	100%	5.17	0.95	4.39	0.43	0.88	1.61
Tea factory waste	40%	3.03	0.95	6.00	0.14	0.89	5.11
	60%	2.92	0.95	6.50	0.12	0.91	5.97
	100%	1.14	0.99	13.90	0.04	0.99	16.20
TIN removal							
Hazelnut husk	40%	2.98	0.99	7.21	0.10	0.98	6.85
	60%	4.08	0.99	5.97	0.14	0.95	4.86
	100%	4.91	0.95	5.99	0.15	0.96	4.56
Tea factory waste	40%	3.61	0.82	8.06	0.08	0.79	8.26
	60%	3.07	0.69	10.42	0.06	0.69	11.83
	100%	1.01	0.58	33.73	0.02	0.58	44.15

$$\frac{dC}{dt} = kC \rightarrow \ln\frac{C}{C^0} = -kt \rightarrow t_{1/2} = \ln\frac{0,5}{k} \qquad (2)$$

where C_0 (mg L^{-1}) is the initial concentration, C (mg L^{-1}) is the concentration at time (t), k is the zero or first-order reaction rate constant, $t_{1/2}$ is the time needed to reduce half of the input concentration. Rates were representing nitrate removal after the initial increase in nitrate concentration. Additionally, a correction in nitrate concentrations for nitrate and ammonium were not made, but rates were also calculated for TIN. Table 5 summarises the rate parameters calculated for both zero and first-order kinetic models. Regarding the correlation coefficients, both kinetic models seem to describe the data well ($r^2 > 0.90$). However, slightly superior fits were achieved using the zero-order kinetic model, which indicates that the process was not depending on nitrate concentration but rather was controlled by independent parameters like the availability of organic carbon [58]. Correlation coefficients observed for TIN concentrations of tea factory waste were lower ($r^2 < 0.82$) implying that linear and exponential fit was not an excellent representation. It is generally known that the first-order kinetic model fits best at lower nitrate concentrations when nitrate becomes rate-limiting (≤ 1 mg NO$_3^-$–N L^{-1}), whereas zero-order kinetic model may apply over a wide range of nitrate concentrations [42,58]. A comparison of reaction rate constants represents, that nitrate and TIN removal rate was higher in flasks with hazelnut husk than with tea factory waste. Additionally, the rates calculated for hazelnut husk were increasing with organic substrate percentage indicating carbon limited conditions. On the contrary, the rates observed for higher amounts of tea factory wastes gradually decreased indicating inhibited microbial activity. Similarly, the lowest half-lives were determined for flasks with 100% hazelnut husk and 40% tea factory waste. The normalized values of removal rates (0.35–0.75 mg N L^{-1} d^{-1} g^{-1} organic substrate) were generally 10-fold higher than those observed by Gibert et al. [41] and consistent with rates reported by Greenan et al. [56].

5. Conclusions

Initial findings of batch experiments showed that both substrates developed conditions supporting the denitrification process without inoculation. Reaction rates observed by applying the zero-order kinetic model reflected that nitrate removal was depending on the availability of carbon and thus the organic substrate percentage. In studies with hazelnut husk carbon limited conditions and studies with tea factory waste, carbon abundance was observed. The highest nitrate removal rates were determined for 100% hazelnut husk and 40% tea factory waste. The corresponding half-lives calculated were about 4 and 6 d for hazelnut husk and tea factory waste, respectively. Gene enumeration suggests that denitrifying bacteria were present more in flasks with hazelnut husk than in tea factory waste. The high leaching potential of organic materials like tea factory waste may require control measures like pre- and post-treatment (e.g. pre-washing of tea factory waste or reuse of outlet water at DOC/nutrient-limited sites). However, long term column experiments reflected that the release of organic carbon and nitrogenous compounds is not negatively influencing the nitrate removal under continuous flow conditions. Results also showed that half-lives needed were much lower and that nitrate was almost absent throughout the experiment. Further studies on the potential use of tea factory waste as a fertilizer in alkaline soils also seem promising to comply with the Waste Framework and Landfill Directives.

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