Universiteit Utrecht

Deltares

Investigating the potential of dissolved organic matter (DOM) induced denitrification in Dutch groundwater

Gemma Spaak
April 2014

Deltares

Title
Investigating the potential of dissolved organic matter (DOM) induced denitrification in Dutch groundwater

Pages
69

Keywords
Dissolved organic matter, denitrification, organic matter characterization

Summary
Denitrification is the dominant nitrate attenuation process in the subsurface and is mostly governed by the presence of a suitable electron donor. Dissolved organic matter (DOM) is a complex mixture of compounds originating from different sources and its potential to act as an electron donor for denitrification is not well understood.
This research aimed to investigate the potential of DOM induced denitrification in groundwater. Groundwater was collected from various locations across the Netherlands and laboratory experiments assessed groundwater denitrification potential by measuring NO_3, NO_2, NH_4 and DOC during a 60 day incubation. Organic matter characteristics were evaluated using spectroscopic and fractionation methods. Results show potential for DOM induced denitrification in Dutch groundwater but it remains unclear how DOM is utilized in this process. Partial oxidation and HS moieties with electron donating capacities could be supplying electrons but other NO_3 removal processes such as assimilation into biomass can also play a role. It was attempted to identify relationships between organic matter characteristics and the observed NO_3 removal but no clear correlation was found. It has been suggested that the presence of hydrophilic compounds such as carbohydrates, organic acids and proteins enhance the biodegradability of DOM. This relationship was not found between the hydrophilic fractions of DOM and the observed NO_3 removal. Analytical techniques used were also unable to identify variations in groundwater samples that correlated to land use or depth. Analytical techniques characterizing the molecular structure of DOM are possibly more adequate to link DOM to its origin.

Contents

1 Introduction

Nitrate (NO_3) is a common groundwater pollutant in the Netherlands and other parts of the world, especially in regions of intensive agriculture (Zwart et al. 2004). Main sources of NO_3 in groundwater originate from agricultural activity and uncontrolled land discharges of raw and treated wastewater (Shrimali and Singh, 2001). In 1991, the European Commission set the limit for NO_3 in groundwater at 50 mg L^{-1} (Boumans et al. 2005). The Dutch national institute for public health and environment (RIVM) reported in 2012 that NO_3 concentrations have decreased in the upper 30 meters of Dutch groundwater since 1992, but in the sandy regions of the Netherlands NO_3 concentrations still exceed the environmental threshold value.
Upon ingestion NO_3 can be converted to much more poisonous nitrite (NO_2), which causes health problems by diminishing oxygen transfer in the human body (methaemoglobinaemia, WHO, 2004) and can be converted to carcinogenic nitrosamine (Swann, 1977, Shrimali and Singh, 2001).
Due to its high solubility, conventional water treatment processes (e.g. coagulation, filtration) cannot be used to remove NO_3 from drinking water. Therefore other techniques need to be applied to eliminate NO_3, which are relatively expensive and merely displace NO_3 to a concentrated waste brine that may pose a disposal problem (Shrimali and Singh, 2001). Thus, alternative removal options such as natural attenuation of NO_3 in soils and aquifers are desirable. The most significant process for removing NO_3 from the subsurface is considered to be denitrification (Peterson et al. 2013, Rivett et al. 2008) which is the focus of this study. Denitrification is a process in which microorganisms use organic matter or inorganic compounds as electron donors to reduce NO_3 to nitrogen gas (N_2).

In the case of heterotrophic denitrification, carbon supply is the most important factor limiting NO_3 removal in groundwater environments (Jahangir et al. 2012, Rivett et al. 2008, Baker et al. 2000). There are indications that leached dissolved organic matter (DOM) and sediment derived organic matter can play an important role as source for heterotrophic denitrification. For instance, studies on water extractable organic carbon and soil derived DOM support the hypothesis that DOM amends denitrification (Siemens et al. 2003, Peterson et al. 2013, Castadelli et al. 2013). Moreover, Neff (2001) stated that DOM fluxes to deeper soil horizons might support up to 30% of the subsoil microbial activity.

In contrast to evaluations in soils, not many denitrification studies focused on natural DOM present in shallow and deeper groundwater and the results vary greatly. For example, Well et al. (2005) observed substantial denitrification rates in the saturated zone of sandy, loamy and peat soil material (0.13 – 26.6 mg N kg^{-1} d^{-1}) and found close relationships between denitrification capacity and electron donor concentration (i.a. organic carbon, sulphide). In contrast, Weymann et al. (2010) found low denitrification activity (0.2 – 13 µg N kg^{-1} d^{-1}) in shallow groundwater systems. Siemens et al. (2003) observed little changes in dissolved organic carbon (DOC) and NO_3 concentrations during incubations with groundwater collected under agricultural fields and accounted denitrification inactivity to the low bioavailability of organic matter. Both Weymann et al. (2010) and Siemens et al. (2003) found no correlations between DOC and denitrification activity, even though DOC concentrations were high (5 – 40 mg C L^{-1}). This indicates that denitrification rates are controlled by DOM quality (i.e. the chemical composition of DOM) rather than the total amount of organic carbon.

In short, the (redox) reactivity of groundwater DOM varies and more research is needed to understand its spatial and temporal variability. Both the soil zone and organic matter present in aquifer sediments contribute to groundwater DOM. Land use, land management, biological activity, environmental factors and groundwater age are all factors that influence DOM properties. As groundwater DOM is a complex mixture of organic materials originating from different sources, insight into changes in its bioavailability and chemical properties will provide a better understanding of differences in denitrification (denitrifier) activity. Various methods are

available for DOM characterization but it is unclear which chemical property controls bioavailability. Bioavailability is often determined under aerobic conditions and expressed as oxygen consumption, but not much research has focused on bioavailability under anaerobic conditions. It has been suggested that the aromaticity, the degree of complexity and condensation of the molecules, the hydrophobicity and the content of carbohydrates affect the microbial stability of DOM (Kalbitz et al. 2003).

This study focuses on examining groundwater DOM as a potential electron donor (source) for denitrification by (i) conducting a laboratory denitrification experiment and (ii) investigating the changes in chemical composition of DOM during the denitrification process. Groundwater samples used for the laboratory experiment originated from locations with different land use and different depths. Changes in DOM chemical composition were investigated using the following analytical techniques:

1 UV-Vis absorbance
2 Fluorescence spectroscopy
3 Resin fractionation
4 Pyrolysis gas chromatography mass spectrometry (Py/GC/MS)

Figure 1.1 summarizes the workflow of this study.

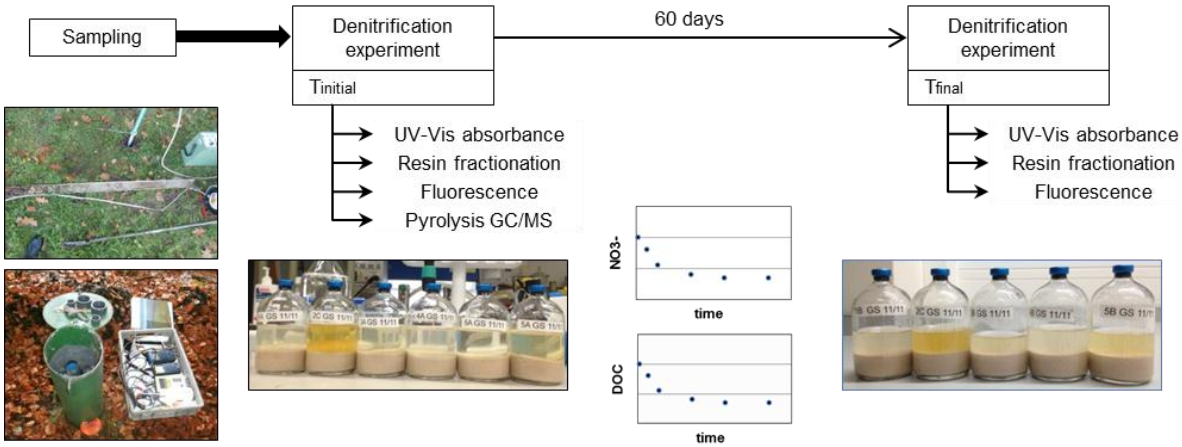


Figure 1.1 Schematic representation of the experimental procedures

Specific research questions addressed are:

- Is there potential for natural DOM amended nitrate removal in Dutch groundwater?
- Is there a link between nitrate removal rates under laboratory conditions and DOM characteristics and which of the characteristics is a suitable predictor for biodegradability?
- Does the chemical composition of DOM change during denitrification?
- Can the chemical composition of DOM be explained by changes in depth, land use and geology (i.e. environmental constraints)?

2 Background information

In order to investigate whether DOM can be used as electron donor for groundwater denitrification, it is important to understand (1) DOM composition, (2) behaviour and source of groundwater DOM and (3) the main factors and/or processes that inhibit or facilitate groundwater denitrification. This section provides a brief overview of these three topics.

2.1 DOM classification

DOM is known to include a broad spectrum of organic constituents with molecular weights ranging from several hundreds to more than 300,000 Da (Piccolo et al. 2002) which vary in composition, chemical and physical properties. DOM can be seen as a complex mixture of aromatic and aliphatic hydrocarbon structures that have attached amide, carboxyl, hydroxyl, ketone, and various minor functional groups (Leenheer, 2003).
Two major types of compounds can be distinguished (Stevenson, 1994, Piccolo, 2002):

- *Non-humic substances:* compounds belonging to the well-defined classes of organic compounds such as amino acids, carbohydrates, lipids, polysaccharides, proteins, waxes and resins.
- *Humic substances (HS):* supramolecular, yellow to black substances of self-assembling heterogeneous and relatively small molecules derived from the degradation and decomposition of biological material. HS are predominantly stabilized by weak dispersive forces. Hydrophobic and hydrogen bonds are responsible for the apparent large molecular size.
  HS can be generally characterized as being rich in oxygen-containing functional groups, notably COOH but also phenolic and/or enolic OH, alcoholic OH and C=O of quinones.

HS can be further separated into humic acid (HA), fulvic acid (FA) and humin. Fulvic acids have a lower average molecular weight and higher acidity (Figure 2.1) than humic acids and represent the main fraction of HS (fulvic acid/humic acid mass ratio is generally around 9:1; Rodriguez et al. 2011). Humic acids are often colloidal in form due to their large size and exhibit more aromaticity, UV absorbance and have more colour than fulvic acids.

It is important to note that HA and FA are both operationally defined classes based on their pH dependent character, which can be accounted to the presence of functional groups subject to acid-base equilibria. Next to the subdivision between non humic substances and HS, DOM can also be classified based on its hydrophobic and hydrophilic character (Figure 2.2).

In summary, HS are a mixture of substances originating from:
- the decay of plant tissues, and
- microbial metabolism,

which vary with different general properties of the ecosystem (e.g. vegetation, climate and topography).

When referring to the dissolved fraction of organic matter, the terms DOM and DOC are used interchangeably. DOM refers to the entire organic molecule and includes other elements such as oxygen and hydrogen, whereas DOC only represents the carbon fraction. Organic matter is therefore difficult to quantify and measurements of organic carbon are preferred (Thurman, 1985). As such, general discussions about dissolved organic matter incorporates the acronym DOM while reference to specific results or the carbon content refers to DOC. Conversions

between the two measurements are conducted by assuming that DOM is 45–50% organic carbon by mass (McDonald et al. 2004).
DOC is the organic carbon passing through a 0.45 µm filter (Thurman, 1985). The use of the 0.45 µm pore size is one of convenience, and it has recently come under criticism as being inadequate for the removal of colloidal species (McDonald et al. 2004). Nevertheless, the operational definition of DOC has remained.

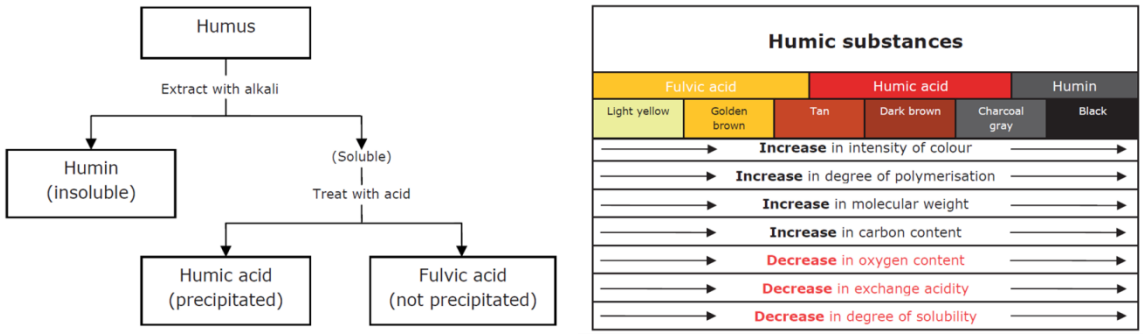


Figure 2.1 Classification and general properties of humic substances (van Zomeren, 2007).

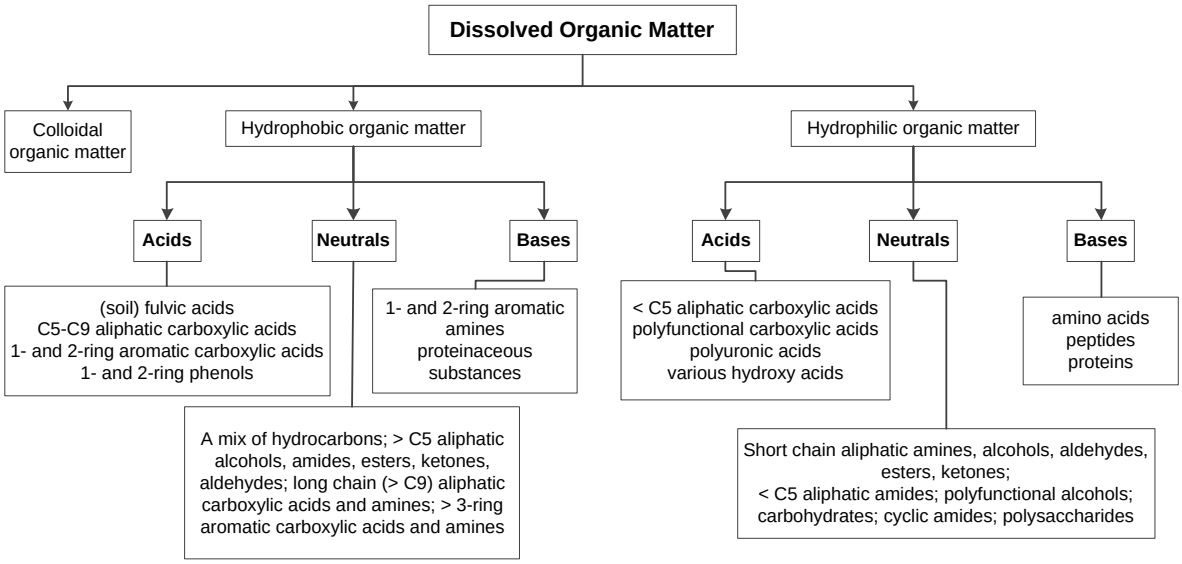


Figure 2.2 DOM classification (modified from Leenheer (2003) and Swietlik et al. 2004).

2.2 Behaviour and role of DOM in aquatic systems

DOM is present in all natural waters and DOC concentrations of aquatic systems range from <1 mg L^{-1} in most groundwaters to several tens of mg L^{-1} in the brown water of swamps. Rivers typically show DOC concentrations of 1 – 10 mg L^{-1} (Frimmel, 2005, Thurman, 1985).
Although DOM is a minor component of the groundwater carbon cycle, it plays an important role for groundwater quality as it impacts the transport and availability of metals, radionuclides and organic pollutants (Aravena et al. 2004, Wong et al. 2010).
The main sources of groundwater DOM are: (1) the soil zone, (2) organic matter present in aquifer sediments (allochthonous substances) and (3) material resulting from biological activities (autochthonous substances). In addition, water from waste water treatment plants can contribute significantly to the load of refractory organic substances (ROS) in aquatic systems (Frimmel, 2005).
The amount of soluble terrestrial organic matter that reaches the groundwater table depends on environmental and biochemical factors including soil type, climate, hydrologic flow system, vadose zone thickness, oxidation, microbial mineralization, adsorption and precipitation (Wassenaar et al. 1990). Soil types can be distinguished based on their humic acid/fulvic acid ratio (Stevenson, 1994). Forest soils generally have a lower humic acid/fulvic acid ratio than peat or grassland soils. Forest soils are also known to be less aromatic in nature, have a lower optical density in the visible region, have a lower C but higher H content and more closely resemble fulvic acids than grassland soils (Stevenson, 1994).
DOM inputs into the mineral soil generally greatly exceed DOM outputs with seepage (Marschner et al. 2003). Mechanisms and controls of DOM degradation in soils are however still poorly understood (Kalbitz et al. 2000).

In situ soil organic matter (originating from peat layers or organic materials in aquifer sediment) can contribute to groundwater DOM by either:

- biogenic or abiotic decomposition of organic matter, or
- polymerization of low molecular weight DOM components produced by bacterial action on soil organic matter.

Humic substances make up a significant percentage of groundwater DOM and are characterized by low oxygen (36%) and high carbon (53%) content, which is mainly a result of biochemical processes that occur in the vadose zone (Wassenaar et al. 1990).
The degree to which DOM affects groundwater quality depends on its availability to support microbial metabolism (Chapelle, 2012), i.e. its bioavailability and biodegradability. Bioavailability describes the potential of microorganisms to interact with DOM whereas biodegradability refers to the actual utilisation of organic compounds by microorganisms. DOM bioavailability can be reduced by physical restrictions, such as inaccessibility of DOM in very small pores or chemical restrictions, such as sorption of DOM to mineral surfaces.

DOM biodegradation is controlled by numerous factors and can be divided into three categories (Marschner et al. 2003):

1. Intrinsic DOM characteristics (e.g. molecular size and structure, functional group content, elemental composition),
2. Soil or aquifer sediment properties (metal or nutrient availability, microbial community structure, soil texture, presence of toxic substances),
3. External factors (temperature, rainfall regime, vegetational cycles, seasonal changes).

In the case of intrinsic DOM characteristics, studies on DOM in soils and surface water indicate that molecular size is only a secondary attribute and the primary factor controlling DOM biodegradability is structural characteristics (Marschner et al. 2003).

Compounds with alkyl or aromatic structural units generally accumulate during decomposition of soil organic matter and have been associated with a low biodegradability, whereas carbohydrates and amino acids are highly decomposable in soils (Kalbitz et al. 2003).

Easily utilisable substances such as carbohydrates are mostly found in the hydrophilic neutral fraction of DOM, which is enriched with carbohydrates from cellulose and hemicellulose breakdown and from microbial origin (Guggenberger et al. 1994). The DOM acidic fractions (hydrophilic and hydrophobic) are more recalcitrant and consist of highly degraded plant-derived compounds. Hydrophilic acids can be differentiated from hydrophobic acids by their higher degree of oxidative biodegradation. A laboratory incubation experiment of soil carbon fractions conducted by Jandl et al. (1997) confirmed that mineralisation of acidic carbon fractions was significantly lower than mineralisation of the hydrophilic neutral fraction.
Hydrophobic organic neutrals show the closest relationships to the refractory soil humin (Guggenberger et al. 1994) with a relatively high content of non-carbohydrate aliphatics.

That being said, there are no systematic studies relating properties of DOM to its biodegradability. Carbon fractions are operationally defined and are not necessarily a good indication of biodegradation. In the case of specific compounds (e.g. sugars, proteins, tannins) that are known for their different biodegradability, variation in biodegradation can be accounted to the presence of these substances. DOM however mainly comprises of decomposed organic material (HS) and biodegradability of these substances is controlled by the presence of structural components. Enzymes are needed for the breakdown of structural components and little is known about the diversity and efficiency of these enzymes (Marschner et al. 2003). Moreover, studies investigating the effect of other factors (e.g. metal or nutrient concentrations) often show conflicting or ambiguous results. Research on DOM biodegradation would benefit from the development of a standardized protocol so that inter-laboratory comparisons can be made.

Humic substances are also known to be redox-active and can act as terminal electron acceptors in anaerobic microbial respiration (Klüpfel et al. 2014). Quinones are considered to be one of the principal moieties responsible for reversible electron transfer when HS are first reduced and subsequently reoxidized. Under oxic conditions, HS can also act as electron donors. Upon oxidation a wide variety of HS moieties release protons and undergo irreversible follow up reactions (Aeshbacher et al. 2012).

2.3 Denitrification in groundwater

Denitrification involves the reduction of nitrate to nitrous oxide, nitrite and eventually nitrogen gas by microbially mediated or abiotic reactions. The denitrification half reaction can be written as follows:

$$2NO_3^- + 12H^+ + 10e^- \rightarrow N_2 + 6H_2O \quad (1)$$

The microbially mediated conversion of NO_3 can be performed by either heterotrophic bacteria or autotrophic bacteria. Heterotrophic denitrification involves organic matter as an electron source whereas for autotrophic denitrification, electrons can originate from the oxidation of iron(II) or reduced sulphur. Denitrifying bacteria tend to be ubiquitous in surface water, soil and groundwater (Beauchamp, 1989) and are mostly facultative anaerobic heterotrophs, obtaining both their energy and carbon from the oxidation of organic compounds. In general, the absence of oxygen and the presence of an appropriate electron donor, e.g. organic carbon, reduced sulphur or iron(II) facilitates denitrification. Such conditions are often spatially and temporally variable. Moreover, denitrification occurrence is also strongly related to nitrate formation by nitrifiers. As such, denitrification is mostly found at oxic/anoxic interfaces in aquifers and in near river environments, including riparian and hyporheic zones (Figure 2.3). Note that riparian zones are characterized by high organic matter contents in the soil and denitrification is limited by NO_3 input rather than the presence of an appropriate electron donor (organic carbon). Denitrification also occurs under the presence of pyritic or high organic carbon sedimentary layers in aquifers (Zhang et al. 2012). Rivett et al. (2008) provided an extensive review on denitrification in groundwater, addressing the different electron donors and effects of environmental conditions, which is beyond the scope of this report. In summary, the critical limiting factors are oxygen, electron donor concentration and electron donor availability. Variability in other environmental conditions such as nitrate concentration, nutrient availability, pH, temperature and the presence of toxins appear to be less important.

Heterotrophic denitrification using DOC

If organic carbon acts as the electron donor, denitrification rates are often related to the amount of DOC in porewater or groundwater rather than the total amount of solid organic carbon present since all microbial uptake mechanisms require a water environment (Rivett et al. 2008, Marschner et al. 2003). The reaction of nitrate with organic matter can be written as follows (Jorgensen et al. 2004):

$$5CH_2O + 4NO_3^- \rightarrow 2N_2 + 4HCO_3^- + CO_2 + 3H_2O \quad (2)$$

The stoichiometry indicates that 1 mg C/L of DOC is capable of converting 0.93 mg N/L of nitrate to nitrogen gas. The actual availability of DOC will vary and is controlled by the nature and quantity of the carbon source.

Denitrification in the Dutch subsurface

Denitrification is a topic of great interest in the Netherlands due to the intensive Dutch agriculture and high input rates of fertilizer nitrogen (N). Many agricultural soils are characterized by a surplus of N; the annual input of N is higher than the amount that is removed by agricultural harvest products as many agronomic experiments have suggested that crops typically use only half of the applied N (Boumans et al. 2005, OECD, 2001). Consequently, there is a high potential for N leaching to ground- and surface water. Nitrogen is mainly leached as NO_3 as this is the most soluble N species, but ammonium and organic N are also susceptible to leaching (Boumans et al. 2001).

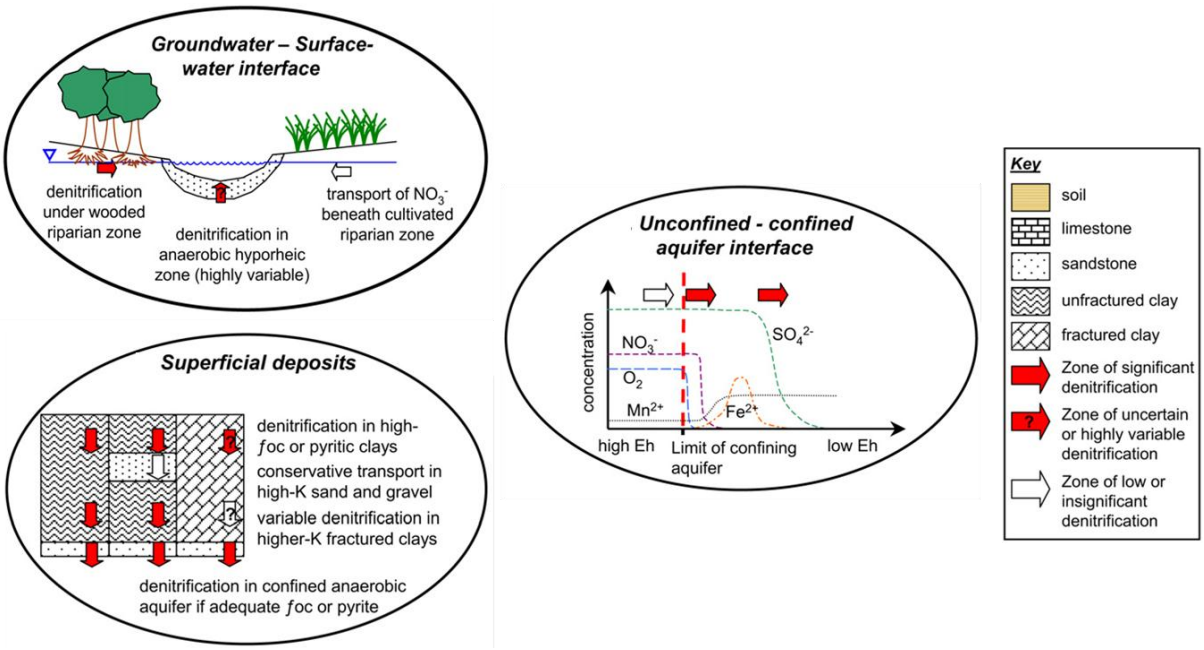


Figure 2.3 Conceptual model of denitrification occurrence in the subsurface environment (modified from Rivett et al. 2008)

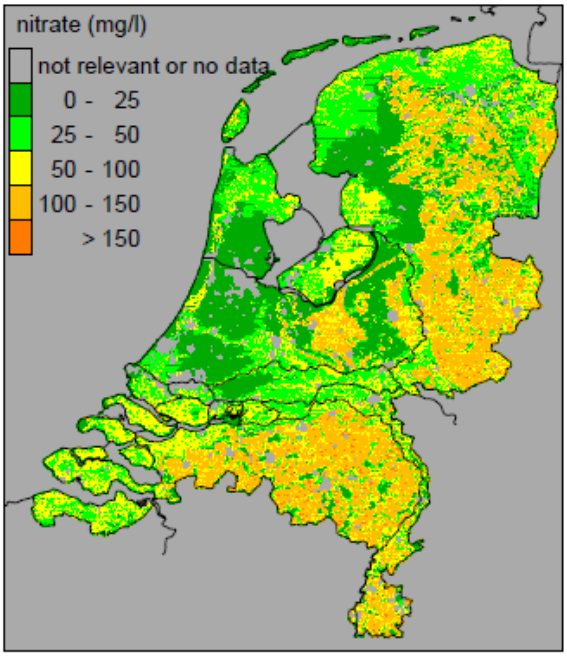


Figure 2.4 Nitrate concentrations in upper groundwater in the Netherlands (Zwart et al. 2004)

Nitrate concentrations in the upper groundwater are mainly regulated by the following four factors:

1 *Groundwater table*. In The Netherlands groundwater levels are very shallow; mostly within 5 meters below the surface. In winter, the groundwater table can reside within 0.5 - 1 meter below the soil surface. As the groundwater table approaches the root zone, anaerobic conditions are more likely to occur, causing a decrease in nitrate by denitrification (Boumans et al. 2001).
2 *Precipitation and evapotransporation*. Changes in precipitation and evaporation result in varying groundwater recharge, causing either dilution or evapoconcentration of nitrate in soils and groundwater.
3 *Farm management*. Nitrate application rates including manure and ammonia/urea application (and amounts), crop type and tillage operations influence nitrate leaching.
4 *Soil texture*. Sandy soils are more susceptible to nitrate leaching than clay or peat soils, influencing the amount of nitrate recharging the groundwater. The same holds for well-drained soils versus poorly drained soils.

The southern and eastern parts of The Netherlands have more agricultural activity and predominantly comprise of permeable sandy soils (Pleistocene deposits, fluviatile sediments medium to coarse sand), resulting in higher groundwater NO_3 concentrations in these areas (Figure 2.4) than the western part of the Netherlands, which is characterized by clayey, peat and fine to medium sand deposits. Most of the southern and eastern parts are characterized by NO_3 concentrations well above the environmental threshold value of 50 mg NO_3 L^{-1}.

Velthof (2003) conducted a study on 467 Dutch agricultural soil samples identifying relations between potential denitrification and crop type. The samples were subdivided into either grassland, maize or other arable land. Potential denitrification was determined by an anaerobic incubation of nitrate amended soils. Results showed that potential denitrification were distinctly higher in grassland than maize or other arable land, whereas no significant differences were identified between maize and other arable land. Possible explanations for the higher potential denitrification in grasslands are the accumulation of organic matter due to the continuous presence of the grass (with high root biomass), in comparison to tillage in the other crop types. Note that tillage also increases the oxygen concentrations in the soils which are unfavourable with regards to denitrification.

2.4 Other nitrate removal pathways

Although heterotrophic (respiratory) denitrification is considered as the primary nitrate attenuation process in groundwater (Rivett et al. 2008, Peterson et al. 2013), alternative nitrate removal pathways can also play important roles in aquatic ecosystems. These include: dissimilatory nitrate reduction to ammonium (DNRA), anaerobic ammonium oxidation (anammox), reduction of nitrate coupled to abiotic or biotically mediated oxidation of iron and denitrification coupled to the oxidation of reduced sulphur (H_2S, S^0, FeS_2) (Figure 2.5).
Burgin (2007) reviewed nitrate removal processes in aquatic ecosystems and discussed the potential prevalence of alternative pathways under different conditions. DNRA and nitrate reduction coupled to iron oxidation are thought to be more important in nitrate limited-environments, whereas heterotrophic denitrification is favourable under carbon-limited conditions (Figure 2.6). Under certain aquifer conditions (Pyrite organic rich clay lenses and the presence of thiobaccillus denitrificans), Pyrite (FeS_2) can be the favourable electron donor (Zhang et al. 2012, Korom et al. 1992) Sulphur driven nitrate reduction is inhibited by free sulphide and annamox is inhibited by many simple organic compounds. It is suggested that the relative availability of labile carbon, reduced sulphur and reduced iron are the key determinants of nitrate removal pathways.

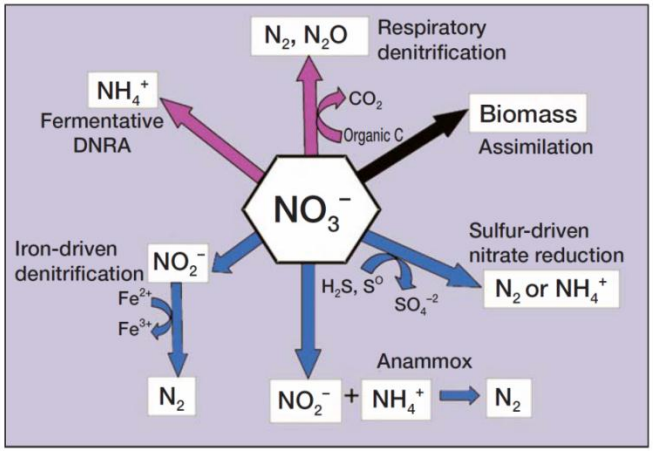


Figure 2.5 Illustration of the different possible pathways and fates of nitrate removal (Burgin, 2007)

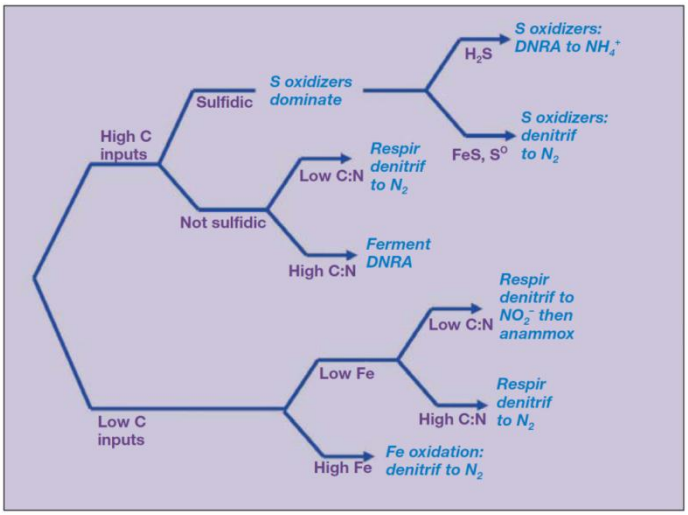


Figure 2.6 Hypothesized controls on predominant dissimilatory pathways of nitrate removal (Burgin, 2007)

3 Materials and Methods

3.1 Sampling locations and procedures

Groundwater samples originated from 12 different locations across The Netherlands (Figure 3.1) which vary in depth, land use and geology. Natural DOC concentrations range between 3.3 and 77.4 mg C L^{-1}.

Details of the different sampling locations are provided in Table 3.1 and Figure 3.2. Sample 13 and deeper groundwater samples were obtained from accessible wells. Shallow groundwater samples (sample 1-5) were collected with the open borehole technique. A borehole of c.a. 2 meter depth was drilled using an Edelman hand auger. In the borehole a pipe was placed with at its end a perforated section (c.a. 0.5 meters), which was covered with a filter gauze. In all cases a PVC sampling tube was inserted in the borehole or well and groundwater was extracted with an Eijkelkamp peristaltic pump (type 12.25) and passed through an Eijkelkamp 0.45 µm disposable filter. In situ measurements of pH, Eh, EC, T and O_2 were obtained using a flow through cell (to avoid air contact) and samples were taken when water quality parameters stabilized (Figure 3.1). pH and EC were measured with a WTW multi 340i meter, O_2 was measured with a WTW 330i oxi meter and Eh and T were measured with an Eijkelkamp meter (type 18.28). Groundwater samples were stored at 4°C until further analysis. Borehole photographs from samples 1-5 are found in Appendix A.

Table 3.1 Sample locations

Sample code	Original ID	X (RD)	Y (RD)	Depth well (m)	Groundwater level (m)	Land use
1	IP09C	159297	409125	1.98	0.63	agriculture grass
2	IP09A	159412	410730	1.97	1.2	agriculture grass
3	IP09D	158186	408831	1.99	0.85	agriculture (crop)
4	IP09B	160177	410484	1.98	1.09	agriculture maize
5	BD03A	183863	395168	1.99	0.73	forest
6	B45B0121	158975	413725	10.6	0.94	agriculture
7	B32E0085	162588	473642	9.63	1.03	grass
8	B32A0466	146100	469850	10.9	1.39	forest
9	B32A0467	145010	465299	10.68	1.26	forest
10	19GP0207	123009	506755	25	1.08	grass
11	25BP0908	111176	495429	11	1.48	grass/peat
12	25BP0908	111176	495429	30	1.48	grass/peat
13	NA	136734	463655	0.96	0.95	natural - peat

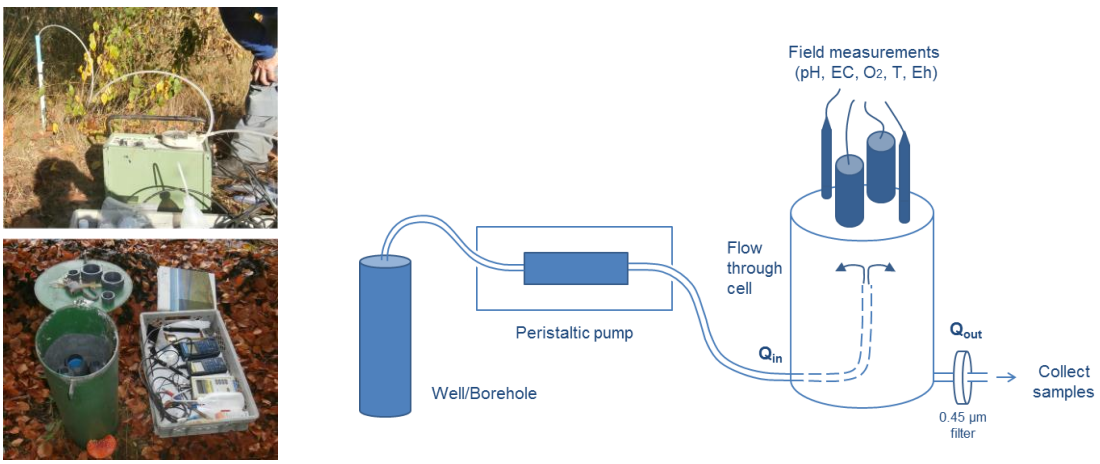


Figure 3.1 Schematic representation of the sampling procedure

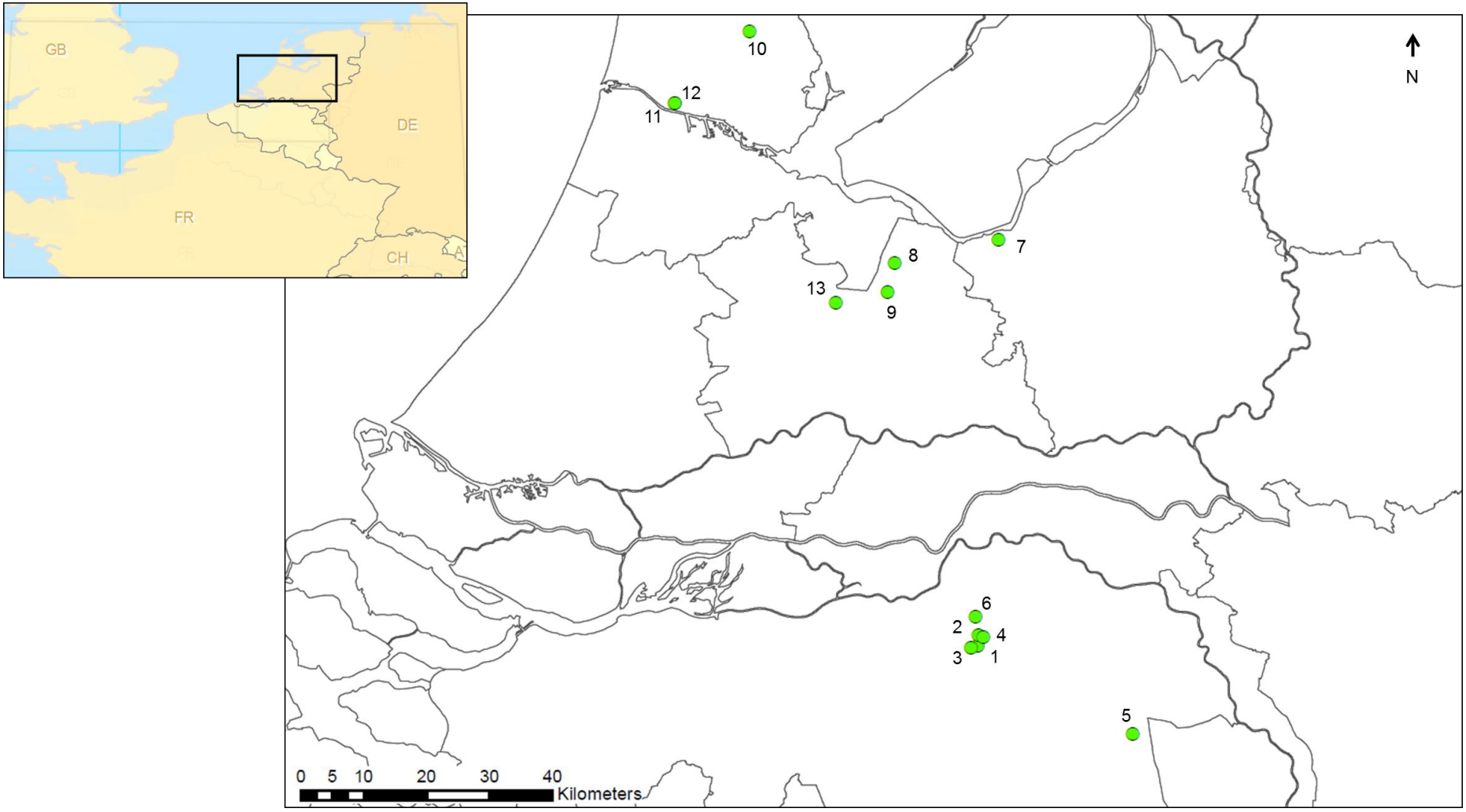


Figure 3.2 Sampling locations

3.2 Analytical procedures

Fluoride, chloride, bromide, nitrite, nitrate, sulphate and phosphate were measured using a dionex ion chromatograph.
Ion chromatography was used (1) to determine natural concentrations in groundwater and (2) to determine concentrations after the denitrification experiment. Note that anions were not measured at the onset of the denitrification experiment.
During the denitrification experiment NO_3 and NO_2 were measured using colorimetric methods developed in cooperation with the faculty Science Ecology and Biodiversity group at Utrecht University. The method is based on the reduction of NO_3 to NO_2 by hydrazine sulphate using copper sulphate and zinc sulphate as catalysts (Shand et al. 2008). A mixture of sulphanilamide and N-(1-naphthyl)ethylenediamine dihydrochloride (NEDD) was used as colour reagent (Griess diazotization reaction) to obtain a pink colour. Nitrite was measured by the same procedure but omitting the hydrazine solution and substituting an equal amount of demi water. Absorbance was measured at 540 nm at 37°C with a SPECTROstar nano microplatereader. Details on reaction time and reagent concentrations are in Appendix B.

Ammonium (NH_4) determination involved the reaction of NH_4 as monochloramine with salicylate and dichloroisocyanurate which gives indophenol blue (Jüttner, 1999). Nitroprusside was used as catalyst and after heating at 37°C absorbance was measured at 660 nm. Absorbance was measured with a SPECTROstar nano microplatereader. Details on reagent concentrations are in Appendix B.

Iron, Fe(II) and Fe(tot), was also determined using a colorimetric method. Ferrozine reacts with divalent iron to form a stable magenta complex species. To measure Fe(tot) hydroxylamine hydrochloride was used as reducing agent. The maximum absorbance was recorded at 562 nm. Details on reagent concentrations are in Appendix B.

DOC was determined with a Shimadzu 5000 TOC analyser after acidification and purging to remove inorganic carbon (NPOC).

3.3 Denitrification experiments

The laboratory experiment involved testing the biodegradability of DOM by quantifying the decrease in DOC and NO_3 during 60-day batch incubations of field filtered (refer to section 3.1) groundwater with nitrate additions. In this case NO_3 removal is used as a measure for denitrification and terms are used interchangeably. Groundwater samples were stored for three weeks before the experiment, and therefore were sparged with N_2 (tube with a large needle was placed in the sampling bottles, connected to N_2 gas and left to sparge for 10 minutes) to remove oxygen from the water prior to preparing the incubation flasks.

250 mL incubation flasks were filled with 100 g of purified silver sand (Merck, washed with acid), 135 mL filtered groundwater and 15 mL of unfiltered mixed groundwater as inoculant. A mixture of unfiltered groundwater was added to inoculate the groundwater samples with a comparable microbial community. Three incubation flasks were prepared for each groundwater type with varying NO_3 concentrations (1200, 120 and 0 µM NO_3).
Based on the stoichiometry of equation 2, 11.52 mg C L^{-1} could be oxidized by 1200 µM NO_3. Natural DOC concentrations of the groundwater samples range between 3.3 and 77.4 mg C L^{-1}, with an average of 31.5 mg C L^{-1}. Injection of 1200 µM NO_3 was chosen so that changes within DOC concentrations were detectable with the NPOC method.
The flasks were sealed with neoprene stoppers and the headspace was flushed with a mixture of N_2 and CO_2 (80% N_2, 20% CO_2) to an end pressure of 0.2 bar. All samples were continuously shaken in the dark at 20°C over a period of 60 days. During the incubation, groundwater samples were extracted from the incubation flasks using a needle and a syringe. A total of seven time measurements were obtained.

3.4 DOM characterization methods

3.4.1 UV-Vis absorbance

Individual UV-Vis absorption coefficients were recorded at 365 nm, 465 nm and 665 nm using a Shimadzu UVmini 1240 UV-Vis spectrophotometer. Samples were analysed in a 1 cm optical glass cuvette and were blanked against Milli-Q water. The E4:E6 absorption ratio was calculated as a_{465}/a_{665} (E4:E6). The E4:E6 ratio indicates a reverse relationship with aromaticity (progressive humification and increased concensation, large content of polycondensated aromatic-ring structures, Piccolo et al. 2002). E4:E6 ratios greater than 5 are considered to indicate a dominance of fulvic acids while values below 5 are indicative of humic acids (Osborne et al. 2007).

3.4.2 Fluorescence

Fluorescence spectroscopy is a sensitive and selective technique used for analysis of organic substances. DOM molecules are excited by irradiation at a certain wavelength and the emitted radiation is measured at a different wavelength. Fluorescence peaks at specific excitation (λ_{Ex}) and emission (λ_{Em}) wavelengths can distinguish protein like and humic like substances and are helpful in describing structural compositions of the humic materials. There are many different methods to interpret the data; from peak picking to numerical modelling schemes like parallel factor analysis (PARAFAC). For this study PARAFAC as well as fluorescence intensity ratios were used to analyse the data.

Excitation Emission Matrices
Fluorescence spectra were measured with an Olis DM 45 spectrofluorimeter using a xenon lamp. Excitation-emission matrices (EEMs), a result of merging a series of emission scans from excitations over a range of wavelengths, were created by scanning excitation wavelengths between 240 and 400 nm with 5 nm intervals and emission wavelengths between 350 and 550 nm with 5 nm intervals.
The fluorescence intensities were blank corrected with intensities measured for Milli-Q water.
Inner filter effects (IFE), sometimes referred to as self-absorption, are caused by the absorption of the exciting as well as the fluorescent light by the fluorophore itself or by another component of the sample. Corrections for inner filter effects are necessary at absorbance values greater than 0.04 cm^{-1} at 254 nm (Ohno et al. 2002, Stedmon et al. 2009). For this study, IFE were disregarded as absorbance values were lower than 0.02 cm^{-1} at 254 nm. These absorbance values are unreliable as measurements were performed in Hellma optical glass cuvettes. Hellma optical glass cuvettes cannot be used at shorter wavelengths, as the transmission through these cuvettes is below 10% at wavelengths shorter than 300 nm. Unfortunately the use of wrong cuvettes was realized after all measurements were completed, and therefore IFE could not be taken into account.

To ensure data is spectrally inter-comparable between instruments and over time, fluorescence intensities were calibrated by normalizing the fluorescence signal to the integrated area of the Raman peak of water (Stedmon et al. 2009). The Raman peak is a result of non-elastic scatter and because it is a fixed property of water, it can be used to calibrate measurements made on different instruments. The integral of the Raman peak was determined by daily measurement of the fluorescence spectra of Milli-Q water. Normalized fluorescence data is shown in so-called Raman Units (R.U.).

The resulting fluorescence EEMs were analysed in MATLAB by PARAFAC, using the DOMFluor toolbox (available at http://www.models.life.ku.dk/). PARAFAC can decompose a fluorescence signal of a data set into the underlying individual fluorescent phenomena or components

(Stedmon et al. 2008). These components can be broadly characterized (e.g. into humic like fluorophores or protein like fluorophores) but can also be linked to specific compounds.
The PARAFAC components of this study were compared to OpenFluor, an on-line database of published fluorescence spectra (http://www.openfluor.org), which contains excitation and emission spectra of known fluorophores as well as EEMs of PARAFAC components extracted from mixtures. The advantage of OpenFluor is that matches between components have high statistical similarity in both excitation and emission spectra, in comparison to the conventional method of comparing component excitation and emission peaks.

Fluorescence indices

The fluorescence index (FI) was calculated as the ratio of emission intensity for λ_{Em} 450 nm to λ_{Em} 500 nm at excitation wavelength λ_{Ex} 370 nm:

$$FI = \frac{fluorescence\ intensity\ (\lambda_{Ex}(370)\ \lambda_{Em}(450))}{fluorescence\ intensity\ (\lambda_{Ex}(370)\ \lambda_{Em}(500))} \quad (3)$$

FI provides a metric for distinguishing terrestrial sources from microbial sources (McKnight et al. 2001). Terrestrial DOM refers to terrestrial plant material and soil organic matter and microbial DOM refers to autochthonous microbial material (e.g. extracellular release or leachate of algea or bacteria). A fluorescence index smaller than 1.4 (FI < 1.4) indicates a terrestrial origin (and high aromaticity) of DOM and FI > 1.9 corresponds to a microbial DOM source (and lower aromaticity).
The biological/autochthonous index (BIX) assesses the relative contribution of autochthonous DOM (Huguet et al. 2009, Birdwell et al. 2010) and is determined as:

$$BIX = \frac{fluorescence\ intensity\ (\lambda_{Ex}(310)\ \lambda_{Em}(380))}{fluorescence\ intensity\ (\lambda_{Ex}(310)\ \lambda_{Em}(430))} \quad (4)$$

Values of BIX between 0.8 and 1.0 correspond to freshly produced DOM, whereas values below c.a. 0.6 are considered to contain little autochthonous OM (Birdwell et al. 2010).

3.4.3 Fractionation of DOM

DOM was fractionated according to the technique of Van Zomeren (2008) (taken up in the ISO 2012: ISO 12782-5), isolating DOM into four fractions based on defined operational conditions using organic resin (DAX-8) (Figure 3.3).

In short, humic acids were precipitated at pH 1 and fulvic acids together with the hydrophobic organic neutral fraction were adsorbed onto DAX-8 resin. The organics remaining in solution after resin addition are classified as hydrophilic organic substances. Fulvic acids were subsequently desorbed from DAX-8 resin and DOC concentrations of the humic acids (HA), fulvic acids (FA) and hydrophilic organic substances (Hy) were measured. Hydrophobic organic neutrals (HON) were determined from a mass balance calculation between measured $TOC_{FA+Hy+HON}$ TOC_{FA} and TOC_{Hy}. If TOC_{FA} + TOC_{Hy} < $TOC_{FA+Hy+HON}$, then HON equals $TOC_{FA+Hy+HON} - (TOC_{FA} + TOC_{Hy})$.

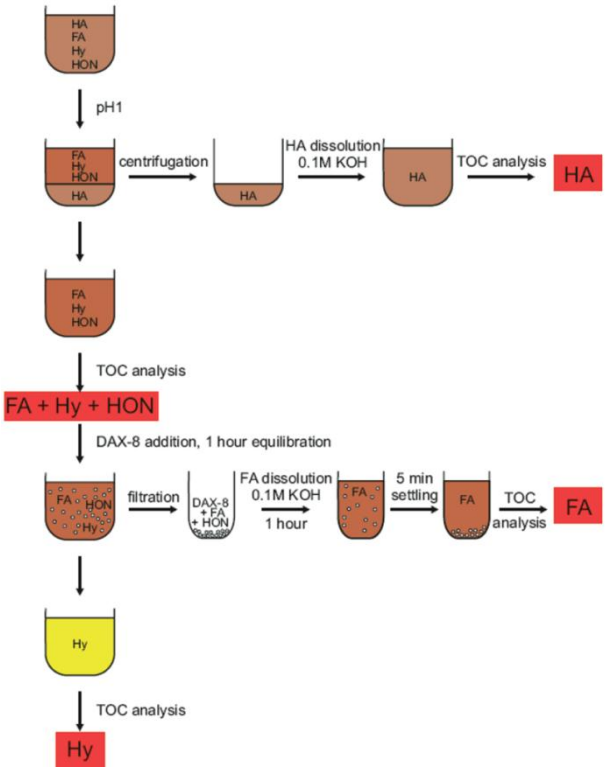


Figure 3.3 Schematic representation of fractionation procedure (van Zomeren, 2007)

3.4.4 Pyrolysis gas chromatography mass spectrometry

A selection of groundwater samples were freeze dried and investigated using Curie-point pyrolysis mass spectrometry (Py/GC/MS).

Pyrolysis was carried out on a Horizon Instruments Curie-Point pyrolyser. Samples were heated for 5 s at 600°C. The pyrolysis unit was connected to a Carlo Erba GC8060 gas chromatograph and the products were separated by a fused silica column (Varian, 25 m, 0.32 mm i.d.) coated with CP-Sil5 (film thickness 0.40 µm). Helium was used as carrier gas. The oven was initially kept at 40°C for 1 min; then heated at a rate of 7°C/min to 320°C and maintained at that temperature for 15 min. The column was coupled to a Fisons MD800 mass spectrometer (mass range m/z 45-650, ionization energy 70 eV, cycle time 0.7 s). Identification of the compounds was carried out by comparing their mass spectra using a NIST library (in Masslab).

4 Results

4.1 Characterization of groundwater samples

A total of 13 different groundwater samples were analysed in this study, comprising of 6 shallow (phreatic) groundwater samples (between 1 and 2 meters) and 7 deeper groundwater samples (between 10 and 30 meters). DOC concentration ranged between 3.3 and 77.4 mg C L^{-1} and NO_3 concentration ranged between 0 and 97.9 mg L^{-1} (Table 4.1). Phreatic groundwater originated from agricultural grass areas, agricultural crop areas, a forest and a natural peat landscape. Oxygen concentrations varied between 0.47 and 5.40 mg L^{-1} being lowest in groundwater from agricultural grass soils. Oxygen concentrations in deeper groundwater were below 1.2 mg L^{-1}. Field pH ranged between 4 and 7.8, with low pH found in groundwater from forest areas and high pH measured in deeper groundwater. All phreatic groundwater was fresh with EC values below 634 µS/cm. Electrical conductivity of deeper groundwater ranged between 123 and 11130 µS/cm (fresh to brackish).

Field measurements of pH and EC were in the same order of magnitude as historic measurements. Nitrate concentrations were generally higher over the historic measurement period (1991 – 2007) than in the fall of 2013, when groundwater samples for this study were obtained.

The geography of the Netherlands can be divided into a Pleistocene and Holocene area at the surface (Figure 4.1). The Pleistocene is characterized by permeable fluvial and glacial or periglacial deposits. During the Holocene, sea level rose and the rivers changed to more meandering systems leaving finer sediments than in the Pleistocene. As such, Holocene deposits are mostly clays, peat and fine to medium sands whereas Pleistocene deposits comprise of fine to coarse sand and gravels. Phreatic groundwater samples originate from the Pleistocene area. Deeper groundwater was collected from both the Pleistocene and the Holocene area.

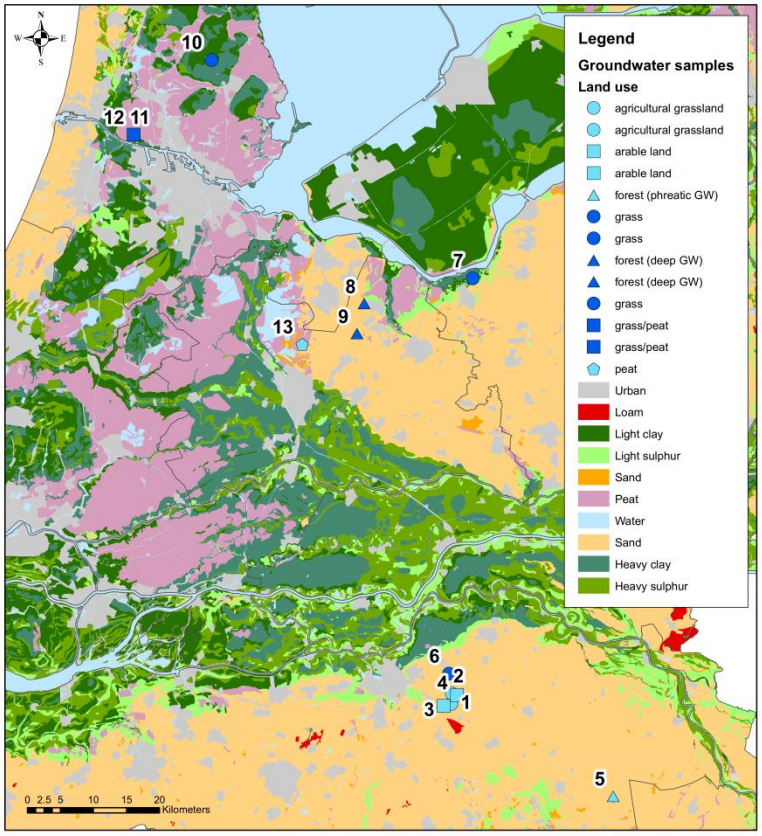


Figure 4.1 Sample distribution

Table 4.1 Field measurements, DOC and NO_3 concentrations of groundwater samples

						Field measurements						
Sample code	**depth well (m)**	**groundwater level (m)**	**land use**	**DOC (mg L^{-1})**	**NO_3 (mg L^{-1})**	**water colour**	**water clarity**	**EC (µS/cm)**	**pH**	**O_2 (mg L^{-1})**	**Eh (mV)**	**T (°C)**
1	1.98	0.63	agriculture grass	11.6	n.a.	light brown	turbid	634	6.7	0.47	-72	13.2
2	1.97	1.2	agriculture grass	77.4	2.4	dark brown	turbid	460	6	0.76	17	14.8
3	1.99	0.85	agriculture (crop)	16.9	20.5	light brown	turbid	139	6.1	5.40	129	13.2
4	1.98	1.09	agriculture maize	9.1	n.a.	brown	turbid	305	5.1	2.90	119	15
5	1.99	0.73	forest	63.0	1.0	yellow	turbid	467	4	7.95*	288	17.5
6	10.6	0.94	agriculture	22.0	0.2	light yellow	clear	773	6.7	0.20	-25	12.6
7	9.63	1.03	grass	52.8	n.a.	yellow	clear	7260	6.6	0.69	-85	13.8
8	10.9	1.39	forest	3.3	2.2	colourless	clear	248	6.1	1.19	90	11.9
9	10.68	1.26	forest	15.5	0.4	light yellow	clear	123	5.7	0.37	72	12.7
10	25	1.08	grass	42.5	n.a.	dark orange	clear	4360	7.8	0.74	NA	11.9
11	11	1.48	grass/peat	33.5	n.a.	light yellow	clear	4740	7.3	0.55	NA	11.4
12	30	1.48	grass/peat	20.8	n.a.	light yellow	clear	11130	7.1	0.6	NA	11.4
13	0.96	0.95	natural - peat	40.5	97.9	light yellow	turbid	605	7.0	NA	NA	9.0

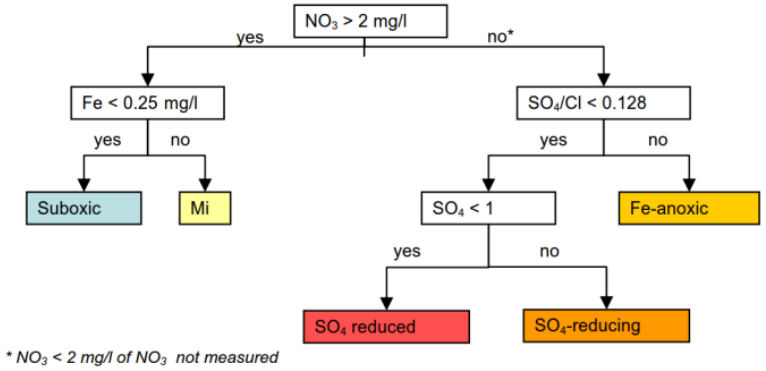
Table 4.2 Historic groundwater quality obtained from DINO, data and information of the Dutch subsurface (average concentrations are reported)

	Historic measurements (DINO)										
Sample code	**pH**	**EC (µS/cm)**	**NO_3 (mg L^{-1})**	**NH_4 (mg L^{-1})**	**SO_4 (mg L^{-1})**	**Cl (mg L^{-1})**	**Ca (mg L^{-1})**	**K (mg L^{-1})**	**Fe (mg L^{-1})**	**DOC (mg L^{-1})**	**measurement period**
1	6.2	591	43.0	2.3	47.6	48.1	43.3	60.6	1.5	98.6	2002-2007
2	6.2	353	24.2	0.9	44.8	13.8	36.5	27.3	0.5	65.1	2002-2007
3	6.4	609	67.0	0.9	45.9	38.1	57.6	39.8	3.8	31.7	2002-2007
4	6.5	453	30.0	1.1	36.0	36.2	39.7	17.5	1.9	42.7	2002-2007
5	4.7	222	4.9	0.3	33.3	29	9.2	1	0.7	15.3	2002-2007
6	NA	97	0.2	0.7	281.5	49.9	118	3.043	50.8	11.1	1992-1994
7	NA	760.7	1.8	32.4	13.1	2009	387	16.0	12.6	49.8	1985-1993
8	7	21.4	0.3	0.6	19.6	19.6	22.6	1.9	4.3	3.5	1991-1994
9	6.6	57.3	138.0	5.7	74.8	30.5	57.6	10.8	0.8	8.6	1991-1994
10	7.6	4200	0.2	29.5	<0.5	559	40	50.3	0.5	55	2001
12	7.1	7310	0.4	16.8	211	1770	321	22.5	9.3	33.5	2001

Samples were classified into 5 redox classes using NO_3, Fe, SO_4 and Cl concentrations (Table 4.3), as the presence (or absence) of these substances are indicators for the groundwater redox state. Detailed explanation of this classification can be found in Groenendijk et al. 2009.
Groundwater samples collected in the west of the Netherlands (Holocene) are characterized by sulphate reducing redox conditions while all other samples are suboxic to iron reducing.

Table 4.3 Redox classification of different groundwater samples

Sample	Redox classification
1	Fe anoxic
2	mix
3	suboxic
4	Fe anoxic
5	Fe anoxic
6	Fe anoxic
7	SO_4 reduced
8	mix
9	Fe anoxic
10	SO_4 reduced
11	SO_4 reduced
12	SO_4 reducing
13	mix



4.2 Denitrification experiments

Changes in NO_3 and DOC in the incubation flasks were quantified by seven time measurements during a 60 day batch incubation experiment. This section includes the time series of NO_3, NO_2, NH_4, DOC and pH as well as concentrations of anions and iron (Fe) (i) initially measured on the groundwater samples and (ii) measured after the experiment.
Note that even though all samples were injected with a fixed amount of NO_3, initial concentrations differ due to contributions from the originating groundwater and the unfiltered groundwater mixture that was added to represent the microbial community. Initial NO_3 concentrations (at the start of the denitrification experiment) were calculated based on NO_3 additions and NO_3 measured before the experiment (Appendix C).

Time evolution of solution composition
Solution composition for each of the samples is listed in Table 4.5. Except for chloride (Cl), fluoride (F) Fe and N-species, concentrations measured before and after the denitrification experiment remained constant. A slight pH increase was observed in all samples.
Increasing Cl concentrations can be explained by the addition of unfiltered groundwater (*Table 4.3*), which is a mix of groundwater from all sample locations to avoid activity differences due to differences in microbial community. With the exception of sample 3, 4 and 8, Fe(II) concentrations decreased during the experiment. Removal of Fe(II) can be accounted to denitrification coupled to Fe(II) oxidation, converting Fe(II) to less soluble Fe(III). Fe(III) can then precipitate as Fe(III) hydroxides and/or oxyhydroxides or can attach to the quartz grains, removing it from the aqueous phase.
Decreasing Fe(III) concentrations (sample 4) could be a result of pH increase as Fe(III) solubility is pH dependent, being more soluble at low pH and alkaline pH.

N – species

A decrease in NO_3 concentrations was observed in all samples and varied between 30 and 80% of the initial concentration (T_0). For the 1200 µM NO_3 treatment, removal rates were higher during the initial 11 days of the incubation. After 11 days NO_3 removal rates decreased, but NO_3 removal was still significant ranging between 229 and 683 µM NO_3.
The bottles injected with 120 µM NO_3 also showed decreasing NO_3 concentrations. After 37 days NO_3 was completely removed from all samples except for sample 13, which had a very high natural NO_3 concentration.
NO_2 concentrations increased in all samples during the 60 day experiment and final NO_2 concentrations ranged between 10 and 656 µM. Note that sample 13 (peat sample) had the lowest NO_2 production, but the highest NO_3 removal. NO_2 production rates seem to follow the same trend as NO_3 removal rates, showing high production in the first 10-20 days of the incubation period followed by a slower increase. But NO_2 formation was not completely explaining the observed NO_3 removal.

Trends in NO_3 and NO_2 concentrations were approximated by four different models:

1 Single zero-order model (zero order kinetics)
$$NO_3(t) = NO_3(0) - vt$$
$$NO_2(t) = NO_2(0) + vt$$
where v: rate [µM day^{-1}].

2 Double zero-order model; describing the initial rate (first 11 days) and the final rate (two zero order rate laws). Rate equations are identical to model 1.

3 Single exponential model (first order kinetics with one rate constant)
$$\frac{NO_3(t)}{NO_3(0)} = \exp(-kt)$$
$$\frac{NO_2(t)}{NO_3(0)} = (1 - \exp(-kt))$$
where k: rate constant [day^{-1}].

4 Double exponential model (first order kinetics with two rate constants)
$$\frac{NO_3(t)}{NO_3(0)} = (1-\alpha)\exp(-k_1 t) + \alpha \exp(-k_2 t)$$
$$\frac{NO_2(t)}{NO_3(0)} = (1-\alpha)(1 - \exp(-k_1 t)) + \alpha\,(1 - \exp(-k_2 t))$$
Where k_1: initial rate constant [day^{-1}] and k_2: final rate constant [day^{-1}]. Alpha is a measure of DOM properties rather than NO_3 or NO_2 properties. (1-α) refers to labile DOM and α is the more recalcitrant part of the DOM pool.

For the two linear models, linear regressions were used to fit the curves and calculate standard errors of the associated rates. The single exponential model was fitted by plotting concentrations on a logarithmic scale and determining the k values by linear regression. In the case of the double exponential model, a least squares optimization method was used to fit the curves.
The double linear model and the double exponential model were both able to successfully describe trends in the measured data. From the double linear model reaction rates can directly be assessed from the slope whereas the double exponential model is more complex and there is not a unique solution for the three parameters (α, k_1, k_2) that gives a good fit. The double exponential model also fails to incorporate decreasing trends of NO_2 (Figure 4.4). On the other hand, the double exponential model uses all seven time measurements to fit the curve whereas the double linear model fits a linear regression to the first three measurements and the last four measurements. As such the double linear model shows an abrupt rate change after 10 days. Results of both models are presented (Figure 4.2, Figure 4.3, Figure 4.4, Figure 4.5). Rates and k-values of the double linear and double exponential model are found in Appendix C.

It is evident from the zero-order removal rates and the double exponential model that there is a high level of variation in NO_3 removal across the 13 samples. Deeper groundwater samples have a faster initial NO_3 removal phase that levels off after approximately 10 days ($k_1 > k_2$). Phreatic groundwater samples show a more gradual removal ($k_1 \approx k_2$). Nitrite inhibition is probably not

influencing the removal trend as this takes place at NO_2 concentrations of 3-4 mmol/L (Bollag and Henninger, 1978, Glass et al. 1997) and maximum NO_2 in the incubation bottles was 0.7 mmol/L. The variation in NO_3 removal could however be influenced by microorganisms present in the filtered groundwater.

To investigate relations between NO_2 production and NO_3 removal, NO_2 production rates were divided by NO_3 removal rates (v_{NO2}/v_{NO3}), for the initial rate and final rate of the double linear model (Table 4.4). If v_{NO2}/v_{NO3} is equal to 1, all loss of NO_3 can be accounted for by production of NO_2. If the ratio is less than 1, loss of NO_3 is greater than production of NO_2 indicating that NO_3 is being reduced to either N_2O or N_2. Ratios of v_{NO2}/v_{NO3} were less than 1 for all samples, implying a greater loss of NO_3 than production of NO_2. This can be explained by the reduction to either N_2O or N_2 or assimilation in microbial biomass. Some samples showed an increase in the v_{NO2}/v_{NO3} ratio, indicating that the rate change between $v_{NO3(initial)}$ and $v_{NO3(final)}$ was larger than the rate change between $v_{NO2(initial)}$ and $v_{NO2(final)}$.

A linear regression was also fitted to the NH_4 time series (Appendix C). No distinct concentration changes were observed and rates varied between 0.8 and 8.3 µM day^{-1}. Decreasing concentrations could be a result of nitrification (conversion NH_4 to NO_3 with residual oxygen left in the incubation bottles) or perhaps assimilation into biomass.

DOC

In contrast to the N species, no significant changes in DOC concentration were observed (Figure 4.5).

A statistical F-test was performed on time series (T_0 – T_7) of each sample between bottles injected with NO_3 and bottles where no NO_3 was added (to test if the variance of DOC concentrations between bottles with and without added NO_3^- is the same). In all cases $F < F_{crit}$ at a 95% confidence interval, indicating that there is no statistical difference between the time series (no rejection of the null hypothesis) and variations are most likely due to measurement errors.

A linear regression was fitted through the DOC results (Figure 4.5). Rates were mostly negative and ranged between 0.6 and 9.9 µM day^{-1} (Appendix C).

Table 4.4 Rate comparison between NO_2 and NO_3.

Sample	$v_{NO2(initial)}/v_{NO3(initial)}$	$v_{NO2(final)}/v_{NO3(final)}$
1	0.38	0.59
2	0.19	0.23
3	0.14	0.12
4	0.17	0.10
5	0.003	0.71
6	0.32	0.08
7	0.12	1.04
8	0.10	0.29
9	0.57	0.78
10	0.28	0.20
11	0.19	0.06
12	0.12	2.33
13	0.02	0.44

Table 4.5 General chemistry before and after the laboratory experiment (concentrations at the start of the experiment were calculated).

	Before the experiment										At the start of the experiment					After the experiment							
Sample	**F**	**Cl**	**Br**	**SO_4**	**PO_4**	**$Fe_{(tot)}$**	**Fe(II)**	**HCO_3**	**NH_4**	**pH**	**F**	**Cl**	**Br**	**SO_4**	**PO_4**	**F**	**Cl**	**Br**	**SO_4**	**PO_4**	**$Fe_{(tot)}$**	**NH_4**	**pH**
	µM	**µM**	**µM**	**µM**	**µM**	**µM**	**µM**	**µM**	**µM**		**µM**	**µM**	**µM**	**µM**	**µM**	**µM**	**µM**	**µM**	**µM**	**µM**	**µM**	**µM**	
1	n.a.	1896	n.a.	452	n.a.	5	5	2896	104	6.7	1	3241	2	430	2	51	3481	n.a.	420	n.a.	1	50	7.1
2	21	471	2	502	n.a.	32	30	2512	85	6	20	1966	4	475	2	2	1993	n.a.	446	n.a.	6	51	7.0
3	2	87	n.a.	72	n.a.	2	0	604	54	6.1	3	1681	3	93	2	45	1967	n.a.	96	n.a.	2	33	7.0
4	n.a.	891	n.a.	643	n.a.	138	0	248	83	5.1	1	2468	3	597	2	n.a.	2778	2	610	n.a.	1	47	6.3
5	27	2487	n.a.	402	n.a.	12	10	0	35	4	25	3779	3	386	2	19	4114	3	410	n.a.	8	42	5.7
6	n.a.	935	n.a.	523	n.a.	93	73	5452	47	6.7	1	2425	3	493	2	49	2746	n.a.	510	n.a.	0	36	7.5
7	n.a.	50077	78	n.a.	6	326	310	21127	1614	6.6	1	46013	72	29	7	n.a.	47117	73	22	n.a.	0	1246	7.7
8	n.a.	484	n.a.	504	n.a.	6	0	747	26	6.1	1	1990	2	476	2	42	2265	n.a.	489	n.a.	1	45	7.0
9	n.a.	258	n.a.	19	n.a.	45	42	670	197	5.7	1	1733	2	45	2	40	2029	n.a.	47	n.a.	4	162	6.7
10	35	17214	39	n.a.	194	14	13	28634	1630	7.8	32	16850	37	29	174	71	17199	33	19	182	3	1112	7.8
11	n.a.	32424	56	275	60	81	55	12279	389	7.3	1	30226	52	273	55	13	31088	48	278	68	2	302	7.5
12	23	95011	154	n.a.	n.a.	282	270	14208	1286	7.1	21	85576	139	30	2	50	86769	105	22	n.a.	0	1024	7.3
13	21	1054	n.a.	357	n.a.	7	5	361	71	7.0	20	2501	3	346	2	2	2751	n.a.	349	n.a.	1	43	7.0

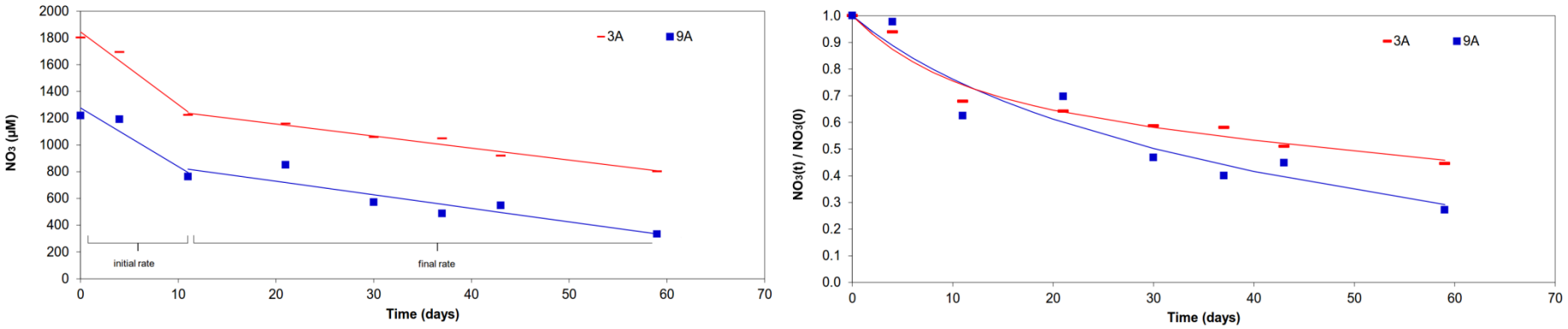


Figure 4.2 Double linear fit and double exponential fit of NO_3 for sample 3 and 9.

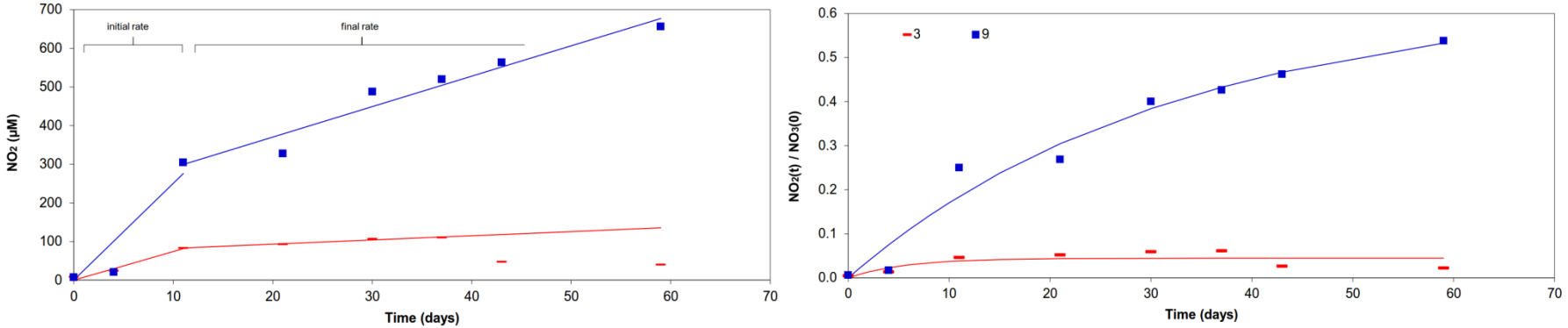


Figure 4.3 Double linear fit and double exponential fit of NO_2 for sample 3 and 9.

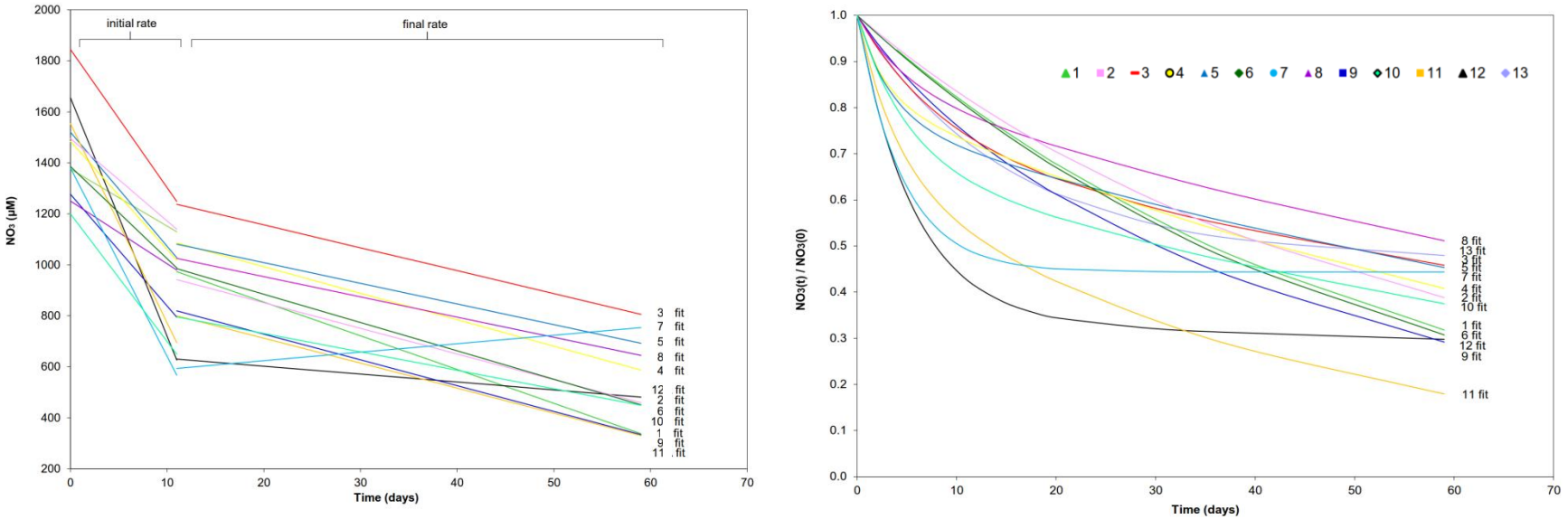


Figure 4.4 Double linear fit and double exponential fit of NO_3 for all 13 groundwater samples

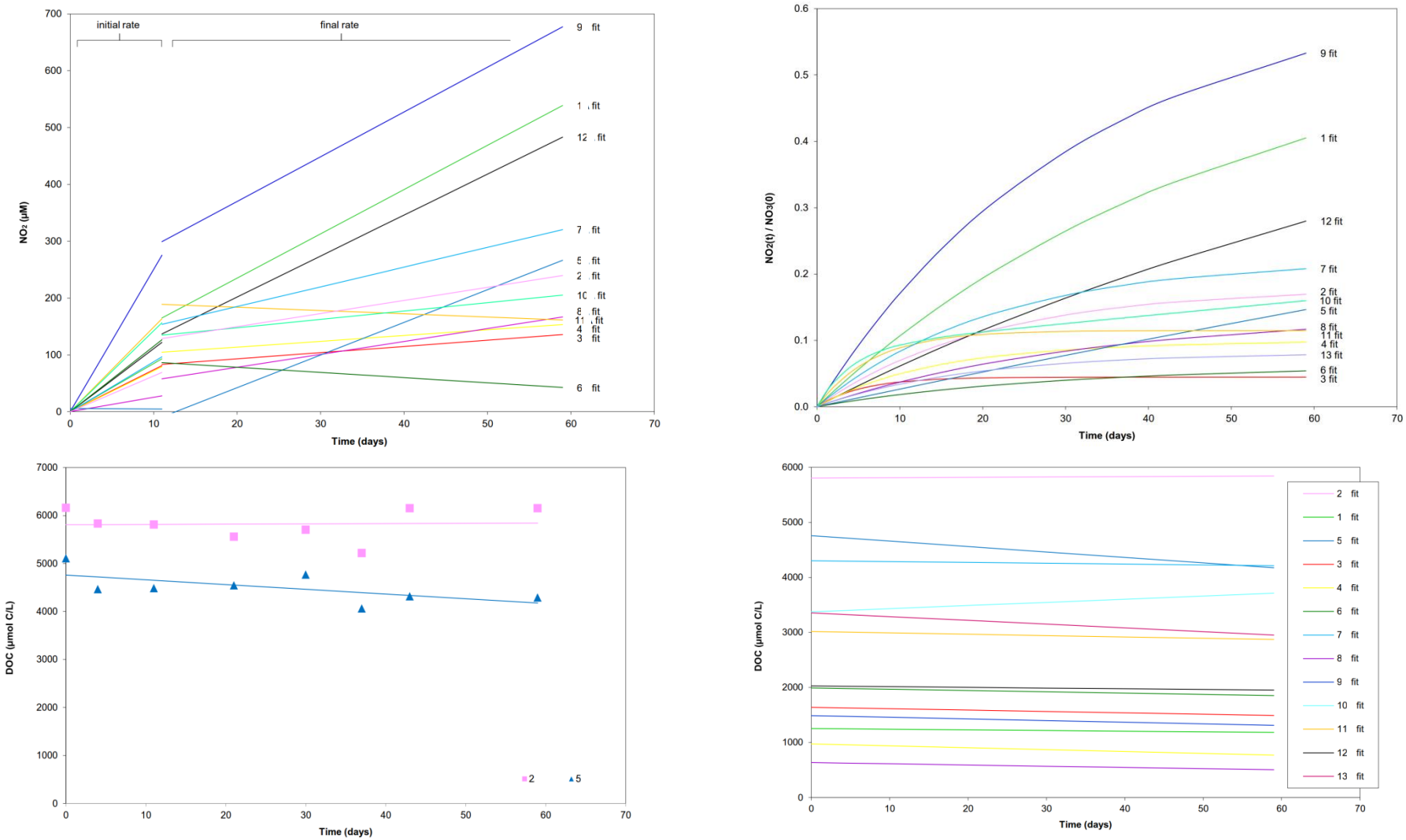


Figure 4.5 Double linear fit and double exponential fit of NO_2 for all 13 groundwater samples (top graphs). Linear regression for DOC concentrations (bottom graphs)

4.3 DOM characterization

4.3.1 UV-Vis absorbance

Single wavelength UV-Vis absorbance and absorption ratios were determined for all 13 samples prior to and after the incubation. The absorbance of humic substances and chromophoric dissolved organic matter (CDOM; component of DOM that absorbs light) is known to increase with decreasing wavelength in an exponential fashion (Boyle et al. 2009), due to the overlapping absorption spectra of the many functional groups in DOM (Dobbs et al. 1972). The single absorption values recorded for all samples are in accordance with this trend (Appendix D).

CDOM is known to have a yellowish colour which can be quantified by the absorption of blue light (Boyle et al. 2007). Blue light has a wavelength of approximately 440 - 490 nm and therefore the absorption values of 465 nm were used as colour classification which, in turn, can reveal information on DOM composition (Figure 2.1). Absorbance measurements at 465 nm (a_{465}) correlated well with colour variations determined by visual analysis (Figure 4.6), but there is no correlation between a_{465} and DOC concentration. Chemical differences of soil types (section 2.2) were not reflected in single absorbance values. Phreatic groundwater originating from a forest soil (sample 5) did not exhibit lower absorbance at 465 nm than groundwater originating from grassland or peat soils. In contrast, the E4:E6 ratio does specifically show lower aromaticity for phreatic groundwater originating from a forest soil compared to E4:E6 ratios from grassland or peat soil (Table 4.5, refer to section 2.2). The E4:E6 ratios also changed due to the denitrification experiment, resulting in both increasing and decreasing E4:E6 ratios. Note that despite its popularity and continued application, E4:E6 ratios have been shown not to hold to its relationship with aromaticity (Piccolo et al. 2002, Chin et al. 1994).

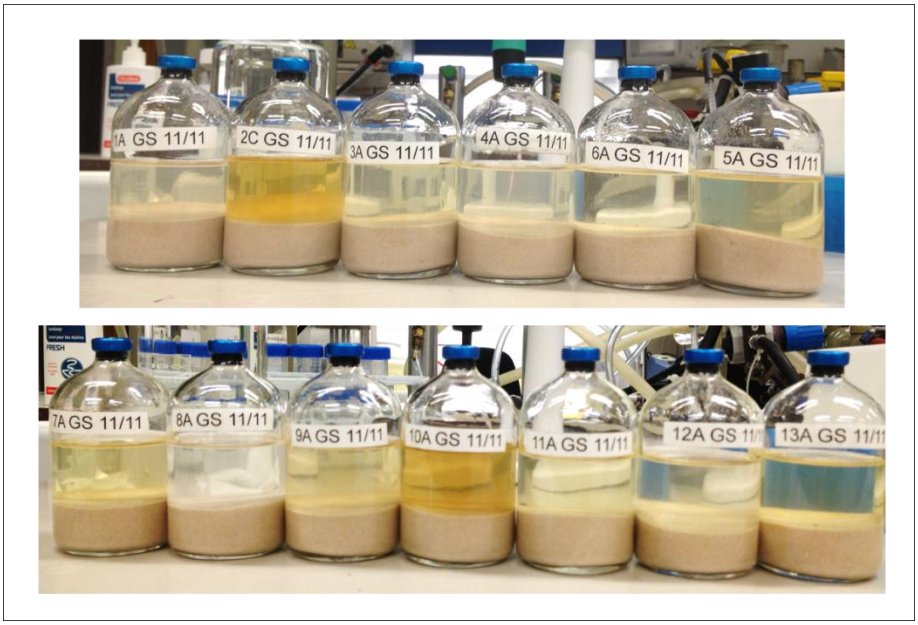


Figure 4.6 Incubation flasks with groundwater from the 13 different sample locations at the onset of the experiment

4.3.2 Fluorescence

Fluorescence EEMs were collected for samples 1 and 3-13 before the incubation (1) and after the incubation (2), using initially sampled groundwater and groundwater from the incubation bottles amended with 1200 µmol/L NO_3. Sample 2-2 (post incubation) could not be measured as

there was not enough groundwater left after the fractionation procedure. Measured EEMs of the 25 samples shown on the same scale are in Appendix D.

As mentioned in section 3.4.2, IFE were not taken into account during the fluorescence measurements. Correction for IFE is most often done by diluting the sample based on absorbance values, and is therefore not possible after the fluorescence measurement. There is a recent study that proposes to use a mathematical correction procedure based on the intensity of the Raman scatter from water (Larsson et al. 2007), however due to time constraints this procedure could not be applied here. Inner filter effects are caused by self-absorption of the exciting as well as the fluorescent light by the fluorophore itself and therefore reducing the fluorescence signal. The mechanism of IFE is different from quenching, but the effect is similar (Larsson et al. 2007). DOC concentration effects should also be taken into account. The fluorescence intensity is thought to be greater at higher DOC concentrations as more fluorescing molecules are present, but Mobed et al. (1996) found little variation in the fluorescence spectra when humic substance concentration changed from 5 to 100 mg/L. In contrast, higher DOC concentrations have shown to cause a shift from shorter to longer excitation and emission wavelengths (Kalbitz et al. 2003). Because of these issues with regards to IFE and DOC concentration, interpretation of the results, especially between different samples, is done with caution.

Differences in magnitude and type of fluorescence were initially distinguished by visual analysis of the EEMs. All samples had a similar fluorescence pattern, comprising of two distinct peaks with excitation maxima at 350 nm and 390 nm (Figure 4.10). The fluorescence peak at 350 nm seems to be uniformly present, whereas the peak at 390 nm is not present in all samples. Fluorescence intensities ranged between 4.4 and 0.2 R.U. Differences in fluorescence intensities were more pronounced between different samples than before and after the denitrification experiment.
Fluorescence intensities can be correlated to DOC concentrations with higher fluorescence intensities found in samples containing higher DOC concentrations (Figure 4.7). This correlation is clearer for initially sampled groundwater than for groundwater obtained from the denitrification experiment (Figure 4.8), which could be a result of the addition of the unfiltered groundwater or due to differences in microbial growth during the experiment. Fluorescence intensity is not well correlated with UV absorbance at 465 nm. Interesting to note is that maximum fluorescence changed due to the denitrification experiment, however there was no uniform increase or decrease observed. Some samples increased in fluorescence (1, 3, 4, 8, 9, 10, 11 and 12) and some decreased in fluorescence (5, 6, 7 and 13). Especially sample 5 showed a large decrease in fluorescence compared to other samples, from 4.0 to 2.7 R.U.

PARAFAC analysis of the 25 measured EEMs separated the dataset into four underlying components. Figure 4.9 shows the spectral properties of each component as contour plot (EEM) and as surface plot. These components do not represent single fluorophores but rather groups of fluorophores with similar fluorescence properties. Based on comparison with other fluorescence studies (OpenFluor) the components can be identified as humic like fluorophores and fulvic like fluorophores (Table 4.6). Investigation of measured, modelled and residual EEMs showed that the 4 component model reproduces the main features of the measured EEMs, but fails to incorporate a protein like fluorescent signal found in sample 1-2, 4-2, 6-2, 8-2 and 12-2 (Figure 4.11). Five, six and seven component models were evaluated to investigate whether the protein like signal could successfully be added as an additional component. This was not the case and therefore the four component model was accepted. The protein like fluorescence is probably not present in enough samples to be added as a separate component. It should also be noted that when dealing with DOM fluorescence, both the number and characteristics of the underlying fluorescent signals are unknown and using datasets with more than 100 samples greatly simplifies the model validation process (Stedmon et al. 2008).

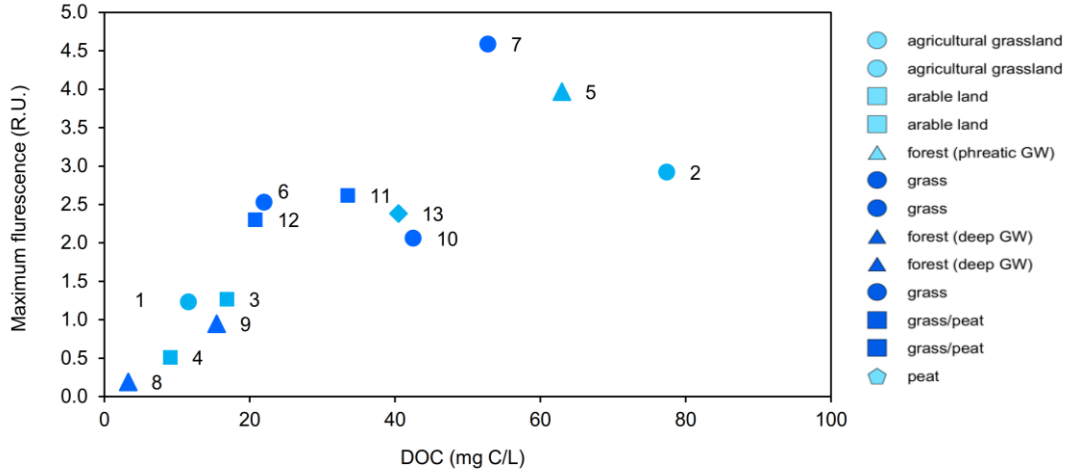


Figure 4.7 Maximum fluorescence versus DOC concentration of groundwater samples before the denitrification experiment

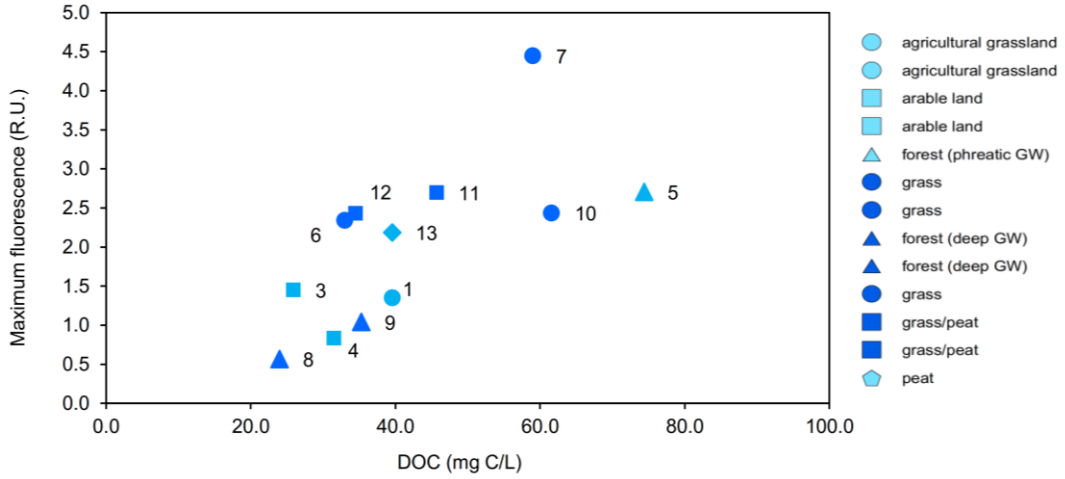


Figure 4.8 Maximum fluorescence versus DOC concentration of groundwater samples after the denitrification experiment

The results of the PARAFAC decomposition can be used to quantify the contribution of each component, and examine the fluorescence signal in more detail. For each component, relative fluorescence intensities obtained before and after the denitrification experiment were compared amongst the 13 groundwater samples (Figure 4.13). Component contribution and distribution between all samples were surprisingly similar. Component 1 and 2 make up the majority of the fluorescence signal, both exhibiting excitation fluorescence at longer wavelengths, suggesting that these fluorophores are more aromatic in nature or contain several functional groups (Coble et al. 1998). Component 2 shows much more variation in contribution to different groundwater samples than component 1. Variation in component 2 seems to be correlated to DOC concentrations. Note that higher DOC concentrations can shift the fluorescence signal to longer wavelengths and because component 2 resides at longer wavelengths than component 1, the variation of component 2 could be an artefact of this. Component 3, a fluorophore group found in agricultural environments, was dominantly present in shallow agricultural groundwater but was also found in deeper groundwater sampled in forest areas. No clear trend could be identified in changes observed in component contribution before and after the denitrification experiment. For all samples the difference in relative fluorescence of each component (as shown in Figure 4.13) was plotted against i.a. DOC concentrations, decrease in NO_3 and increase in NO_2 which did not result in any correlation.

Table 4.6 Fluorescence component identification

This study			
Component number	Excitation max (nm)	Emission max (nm)	Description (match found in OpenFluor)
1	355-365	430-450	Similar to component 1 from Murphy et al. (2011). Terrestrial humic like fluorescence (**H-terr**) in high nutrient and wastewater impacted environments
2	395-405	450-490	No match found in OpenFluor. Closest resemblance to component 2 from Stedmon et al. (2005): fulvic acid fluorophore group present in all environments. Can have a terrestrial or autochthonous origin.
3	305-325	425-445	Similar to component 5 from Stedmon et al. (2005). UVA humic like fluorescence, common to a wide range of freshwater environments. Belongs to a humic fluorophore group exported from agricultural environments which has a terrestrial/anthropogenic origin (**H-UVagr**).
4	325-345	390-400	Similar to component 5 from Søndergaard et al. (2003) and component 4 from Stedmon et al. (2003). UVA or visible humic like fluorophores (**H-UVvis**)
Previously identified (Stedmon et al. 2005)			
Label	Excitation max (nm)	Emission max (nm)	Description
A	260	380-460	UV humic-like
C	320-360	420-460	Visible humic-like
D	390	509	Soil fulvic acid
E	455	521	Soil fulvic acid
M	290-310	370-410	Marine humic-like
T	275	340	Protein-like (tryptophan)

Fluorescence indices range between 1.7 and 2.4, indicating a predominant microbial DOM source (Table 4.7). This is in contradiction to the EEM results, which indicate a dominant humic like DOM source. Moreover the FI determined in this study do not correspond with previously determined FI on aquatic humics and soil DOM (Birdwell et al. 2010). Variance of FI between different samples decreased during to the laboratory experiment, which can be accounted to the addition of unfiltered groundwater (which is equal for each sample). In contrast, BIX values do not exceed 0.8 which suggests that there is little freshly produced DOM of biological or microbial origin. On the other hand there is a correlation between FI and BIX, where higher FI values correspond to higher BIX values (Figure 4.9)

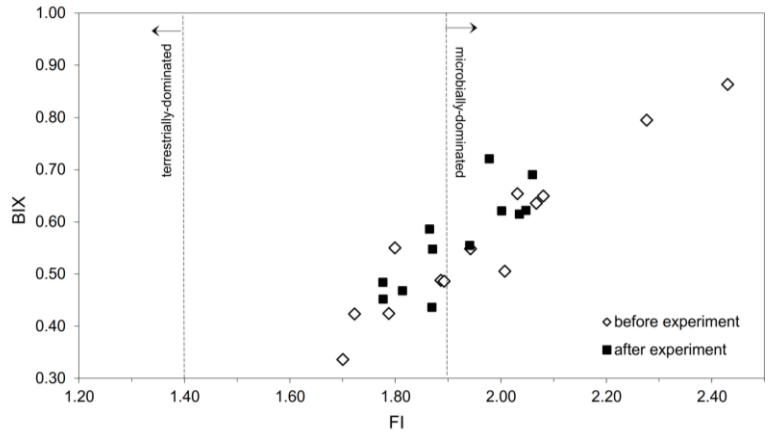


Figure 4.9 FI versus BIX for groundwater samples before the experiment and after the experiment

Table 4.7 Results of spectrophotometric characterization methods

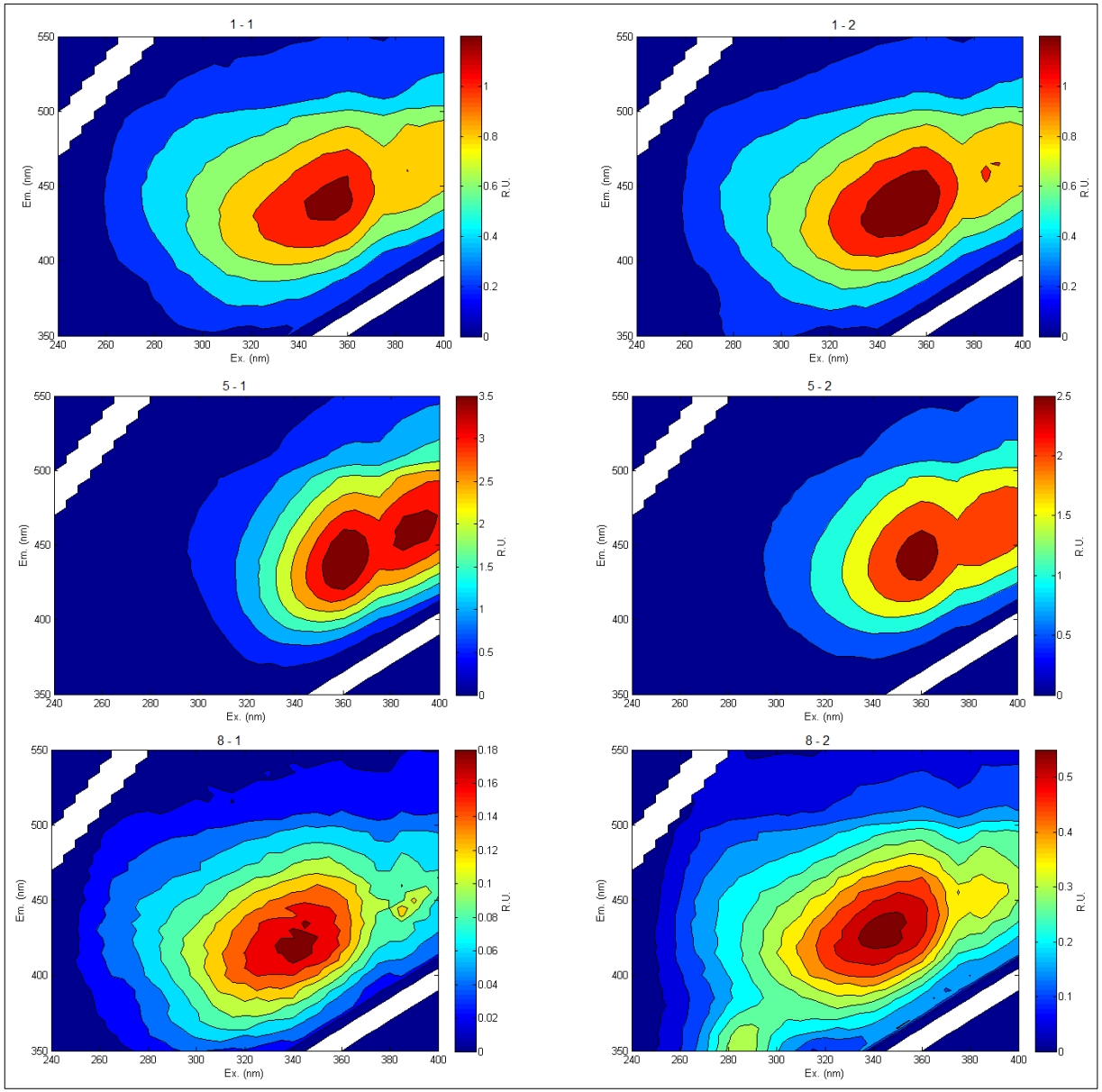
This study												
	Before incubation						After incubation					
Sample	DOC (mg L^{-1})	a_{465} (cm^{-1})	E4:E6[a]	Maximum fluorescence (R.U.)	FI	BIX	DOC[b] (mg L^{-1})	a_{465} (cm^{-1})	E4:E6[a]	Maximum fluorescence (R.U.)	FI	BIX
1	11.6	0.022	2.4	1.2	1.80	0.55	39.6	0.02	5.0	1.3	1.87	0.59
2	77.4	0.138	8.6	2.9	1.70	0.34	76.2	0.142	4.6			
3	16.9	0.019		1.3	1.89	0.49	25.9	0.03	10.0	1.5	1.87	0.55
4	9.1	0.019	1.7	0.5	2.28	0.79	31.5	0.015	1.9	0.8	2.06	0.69
5	63.0	0.038	38.0	4.0	2.01	0.50	74.4	0.055	3.7	2.7	1.87	0.44
6	22.0	0.179	3.1	2.5	2.07	0.64	33.0	0.015		2.3	2.05	0.62
7	52.8	0.033	3.3	4.6	2.08	0.65	59.0	0.023		4.4	2.04	0.61
8	3.3	0.013	1.0	0.2	2.43	0.86	24.0	0.002		0.6	1.98	0.72
9	15.5	0.043	7.2	0.9	1.72	0.42	35.3	0.051	3.0	1.0	1.78	0.45
10	42.5	0.147	4.1	2.1	1.79	0.42	61.6	0.115	5.8	2.4	1.78	0.48
11	33.5	0.021	21.0	2.6	1.94	0.55	45.7	0.021		2.7	1.94	0.55
12	20.8	0.007	7.0	2.3	2.03	0.65	34.5	0.014	14.0	2.4	2.00	0.62
13	40.5	0.028		2.4	1.89	0.49	39.6	0.032		2.2	1.81	0.47

Previously identified (Birdwell et al. 2010)

Category	FI	BIX
Sediment OM	1.2 – 1.6	0.6 – 1.2
Aquatic humics	1.0 – 1.3	0.3 – 0.4
Soil porewater	1.2 – 1.5	ND
Sediment porewater	1.5 – 1.8	0.6 – 1.1
Microbial mats, aqueous extracts	>2	>2
Protein	>2 (2.5)	>>2 (5.2)

[a]: *In some cases a_{665} was zero and cells are left blank*

[b]: *DOC concentrations are higher than initially measured due to the addition of unfiltered groundwater (approx. 5 mg C L^{-1}) and DOC release of the rubber septa used on the incubation bottles. DOC results in section 4.2 have been corrected for this.*



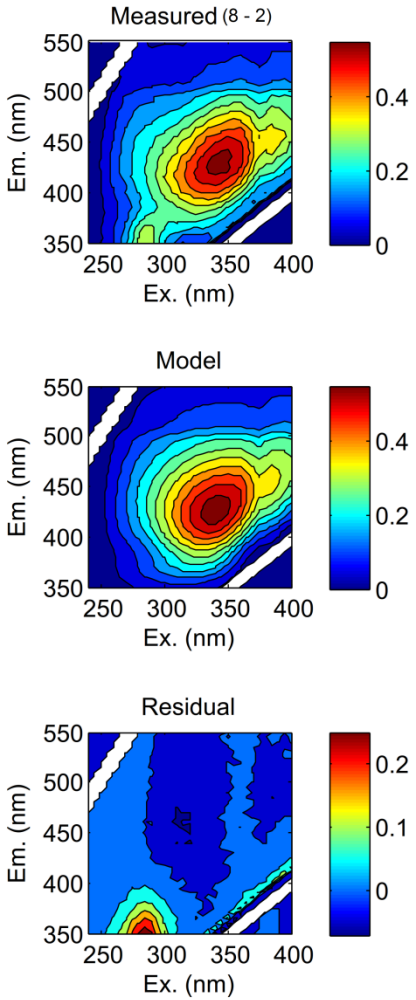


Figure 4.10 (left) EEMs of sample 1, 5 and 8 prior to and after the denitrification experiment
Figure 4.11 (above) Example of the measured, modelled and residual EEMs from the PARAFAC modelling.

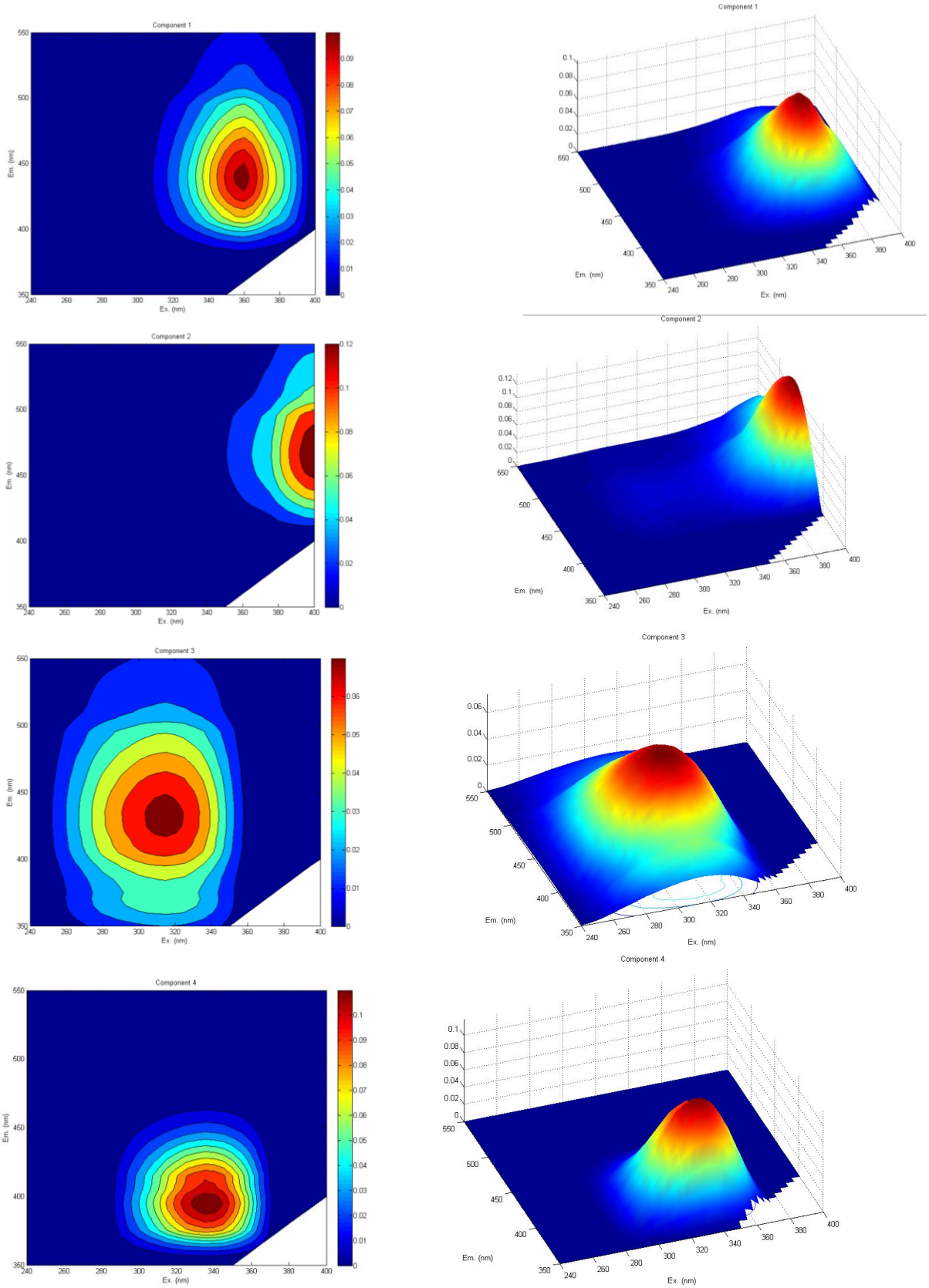


Figure 4.12 EEMs and surface plots of the 4 components identified by PARAFAC modeling

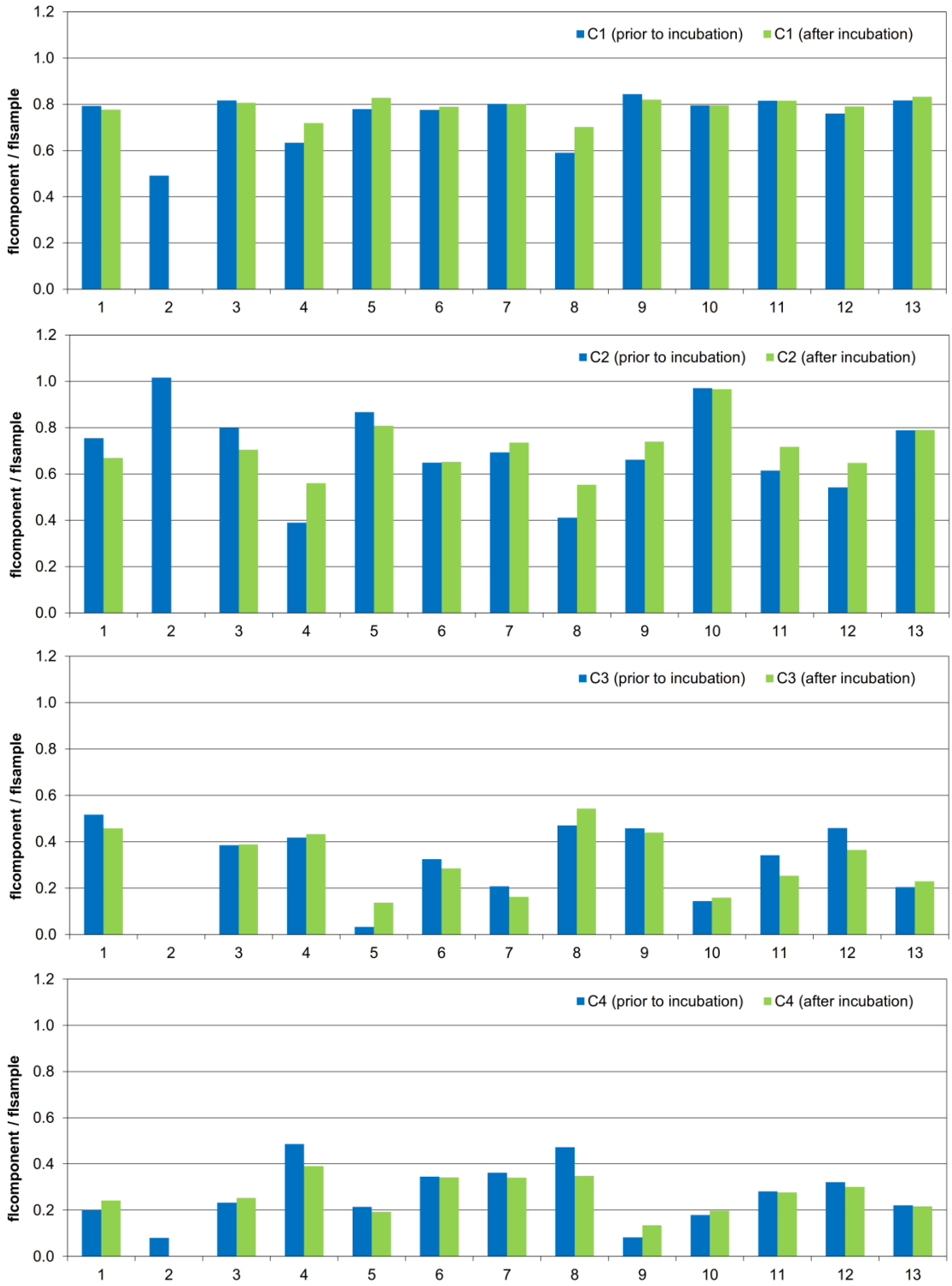


Figure 4.13 Relative contribution of the four components for each groundwater sample

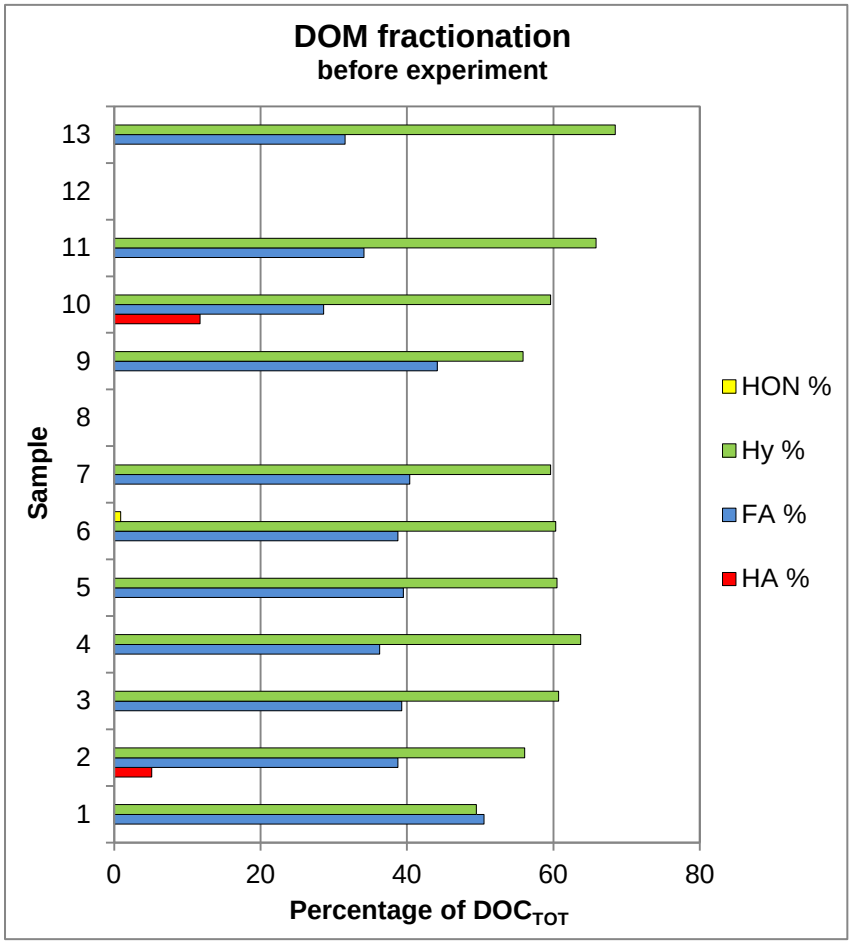
4.3.3 Fractionation

HA, FA, Hy and HON fractions are in Figure 4.14 (as percentage of DOC) and were measured before and after the batch incubation. The difference between initially measured DOC and DOC obtained from the fractionation mass balance was between 0.3 and 20%. Results show a general trend of HON being produced at the expense of y. n some cases (sample 4, 9) Hy increased and HON was produced at the expense of FA, however the amount of Hy and HON produced in these samples was very low. HA did not show significant changes. Minor differences were found amongst samples, which is in accordance with the fluorescence results. Dissolved organic carbon concentrations changed only marginally during the denitrification experiment (Figure 4.5), which would indicate that the change in fractions is due to internal shifts. However these DOC concentrations are corrected values using a blanc measurement (only demi water) and an inoculum blanc (15 mL unfiltered groundwater and 135 mL demi water). True measured DOC concentrations are in fact 5 – 20 mg/L higher than initially measured (Table 4.7).

As the concentration of HON is determined from a mass balance calculation (HON is the fraction retained onto the resin after washing with 0.1 M NaOH), this fraction is very susceptible to variations due to measurement errors, both from the fractionation procedure and from the TOC measurements. HON production could also be originating from the rubber septa used for the incubation flasks (butyl rubber) instead of from the natural DOM pool. For this reason changes in the hydrophilic fractions (acids and neutrals; Hy) and hydrophobic acids (FA) are more reliable.

4.3.4 Pyrolysis gas chromatography mass spectrometry

The Py/GC/MS results were inconclusive due to a contamination (unnatural C_8 and C_{10} fatty acids either free or bound to glycerol) dominating the pyrogram signal, which was present in all samples. The source of the contamination is currently unresolved and therefore the results are excluded from the main report. The Py/GC/MS results are in Appendix C.



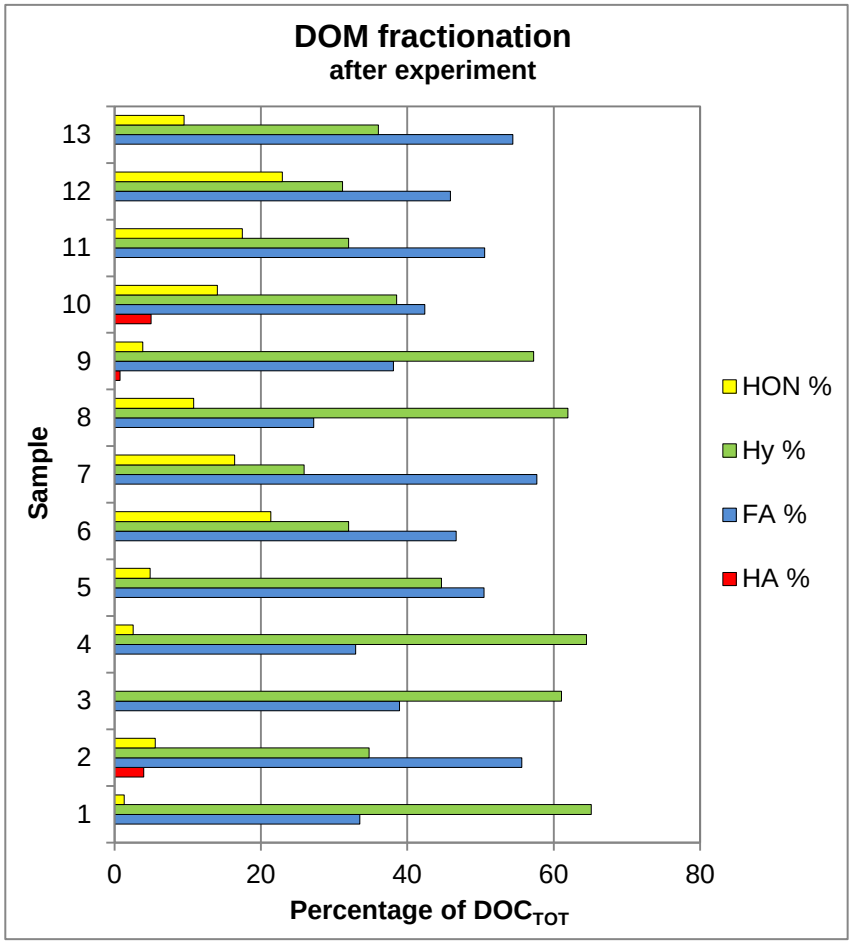


Figure 4.14 Resin fractionation results

5 General discussion

5.1 Linking NO_3 removal to DOC

Electron donor context
Previous studies on soil DOM amended denitrification resulted in DOM mineralization and increasing CO_2 concentrations (Kalbitz et al. 2003, Qualls, 2005). The results presented here show that there is potential for groundwater denitrification, as NO_2 was produced and NO_3 decrease was between 49 and 82 % of initially determined NO_3. However, NO_3 removal is not fully accounted for by mineralization of DOM, as DOC concentrations decreased only marginally. Note that even though DOC concentrations remained unchanged it does not necessarily mean that no mineralization took place, if microbial growth is occurring these two processes can counterbalance one another in the NPOC measurement. On the other hand the amount of assimilable organic carbon generally does not exceed 40-50% of the DOM pool, and therefore all of the NO_3 removal cannot be accounted to microbial growth. As such it is thought that DOM is either partly oxidized or there is another electron source that facilitates the reduction of NO_3.

To investigate potential electron donors that can explain the observed losses of NO_3, electron consumption and production were calculated from concentration changes measured during the denitrification experiment.
Electron consumption was quantified by: (1) the production of NO_2 by reduction of NO_3 to NO_2 and (2) further reduction of the remaining NO_3 removed (remaining NO_3 = $TOT\Delta NO_3$ – $TOT\Delta NO_2$) to either N_2O or N_2.
Electron production was quantified by: (1) the loss of DOC due to mineralization (2) Fe(II) oxidation and (3) anammox.
Sulphur driven nitrate reduction was also considered but as sulphate concentrations remained unchanged, this process was disregarded. Details on calculation methods and assumptions used for the electron balance are in Appendix F.
Electron production was subtracted from electron consumption (Table 5.1) and the remaining amount of consumed electrons were compared to the electron donating capacity (EDC) of humic substances (mmol e^- (g HS)$^{-1}$ (Aeshbacher et al. 2012). Results are shown in Table 5.1 and it can be concluded that the above mentioned oxidation reactions cannot account for the observed deceases of NO_3. The electron balance required an electron donor that was consumed at the expense of NO_3 reduction. DOC only makes up approximately 50% of DOM and if comparing EDC of DOM to literature derived EDC of HS, EDC of DOM needs to be multiplied by two.

Partial oxidation of organic matter is the most probable explanation for the surplus of electron consumption in the laboratory experiment. The fractionation results do indicate that the DOM pool changed in composition during the incubation period, but the results are in part contradictory to the idea of partial oxidation of DOM. Hydrophobic acids are less accessible to microbial degradation than hydrophilic compounds, (Kalbitz et al. 2003) which could explain the dominant decreasing trend of Hy in the fractionation results.
A transition of hydrophilic compounds to hydrophobic neutrals suggests that compounds rich in polymeric carbohydrates as well as carboxylic and ketonic carbons are being transformed to non-carbohydrate aliphatics (Guggenberger et al. 1994). When considering the transformation of carboxylic carbon to aliphatics, the oxidation state of carbon would change from +III to 0, indicating that electrons are taken up instead of released. On the other hand, conversion of carbohydrates to aliphatic compounds could potentially release electrons depending on the oxidation state of carbon (varies between – I and +I for most carbohydrates). Note that this is a very simplistic way of looking at the molecular make up of DOM and the underlying reactions are

probably more complex. Perhaps the increase in HON could be accounted for by the 'stripping' of humic substances from labile functional groups or moieties.
Fluorescence results also fail to identify specific compound changes during the incubation experiment that can be correlated to partial oxidation. Fluorescence intensities are very similar before and after the incubation and changes in fluorescence can be accounted to changes in DOC concentrations. On the other hand fluorescence measurements did show an increase in protein like fluorescence indicating microbial activity. Growth was also observed in the incubation bottles when comparing cell count of incubation flasks with and without added NO_3 using flow cytometry (pers. comm. M. Hefting).

If it is assumed that partial oxidation is indeed taking place it is surprising that DOC is acting as electron donor for denitrification as groundwater DOM is generally thought to be recalcitrant. However note that most groundwaters are known to contain DOC concentrations < 5 mg L^{-1} and bioavailability restrictions could well play a role. In this case groundwater samples were chosen based on their historically high DOC concentrations. However more surprising is the uniform chemical composition of DOM across a wide range of groundwater origins.

Which electron donor was used remains enigmatic and it can only be speculated. Initial provision of H_2 by flushing the bottles is unlikely as a N_2/CO_2 mixture was used. It cannot be excluded that NH_4 release from organic matter drove nitrate consumption via anammox as DON was not measured.
Photosynthetic nitrate assimilation could be playing a role however this is very unlikely as no algal growth was observed in the bottles and samples were kept in the dark during the majority of the incubation experiment.
Manganese is known to play a role in N cycles of marine sediments (Hulth et al. 1999) and can act as an electron donor during NO_3 reduction. Manganese was not measured during this study and its contribution cannot be quantified. Again Mn oxidation is very unlikely as it would involve the production of (black) insoluble Mn oxides, which was not observed in the incubation bottles. Historic Mn measurements of sample 6, 7, 8 and 9 were also very low and ranged between 0.3 and 1.3 mg/L (DINO).

As there is no evidence for complete reduction of NO_3 to N_2, the question arises whether NO_2 is actually being reduced or whether removal mechanisms other than reduction are playing a role. Perhaps NO_2 can be covalently bonded to humic substances or can attach to the sand grains. This is difficult to address as there are no studies on the sorption capacity of NO_2 to HS or sand particles.

Further research would be required to verify these observations and to identify underlying mechanisms of nitrate removal.

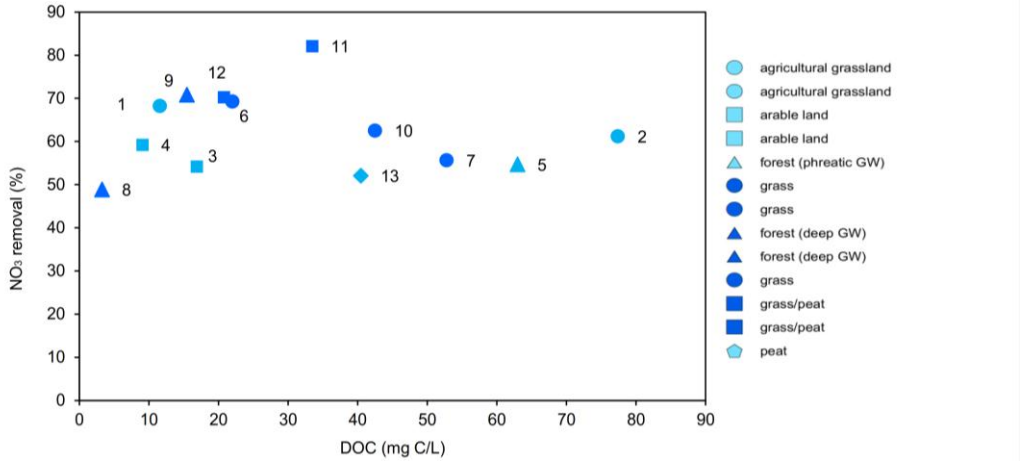


Figure 5.1: Nitrate removal (based on double exponential model) versus initially measured DOC concentration

Table 5.1: Electron mass balance for the denitrification experiment

Sample	electron consumption[a] (µmol e^- L^{-1}) $NO_3 \rightarrow N_2O$	electron consumption[b] (µmol e^- L^{-1}) $NO_3 \rightarrow N_2$	electron production (µmol e^- L^{-1})	remaining electrons (mmol e^- (g DOC)$^{-1}$ $NO_3 \rightarrow N_2O$	remaining electrons (mmol e^- (g DOC)$^{-1}$ $NO_3 \rightarrow N_2$	EDC range (mmol e^- (g HS)$^{-1}$
1	3073	3596	292	177	211	1 -5
2	3415	4177	131	46	56	1 -5
3	3756	4675	613	149	193	1 -5
4	3442	4234	868	188	246	1 -5
5	2981	3609	2335	11	21	1 -5
6	3329	4102	636	109	141	1 -5
7	2064	2429	1037	20	28	1 -5
8	2398	2927	522	238	305	1 -5
9	2230	2460	909	68	80	1 -5
10	2923	3543	1471	68	82	1 -5
11	4681	5776	1006	105	136	1 -5
12	3917	4656	1212	114	145	1 -5
13	5568	6955	1622	96	130	1 -5

[a]: *electron consumption if excess NO_3 removal (excess NO_3 = $TOT\Delta NO_3$ – $TOT\Delta NO_2$) is converted to N_2O*

[b]: *electron consumption if excess NO_3 removal (excess NO_3 = $TOT\Delta NO_3$ – $TOT\Delta NO_2$) is converted to N_2*

Prediction of denitrification using DOM properties

Identifying relationships between the observed NO_3 reductions and compositional changes in the DOM pool could reveal more information about the processes occurring during the experiment and potentially justify the idea of partial oxidation of DOM.

Figure 5.1 shows that the groundwater sample with the lowest DOC concentration had the least amount of NO_3 removal. For DOC concentrations lower than 35 mg L^{-1} it seems like there is a positive correlation between NO_3 removal and DOC concentration, but this correlation does not hold for 5 of the 13 samples, which is a substantial amount when dealing with such small data set. The two samples with smallest (3.3 mg C L^{-1}) and largest (77.4 mg C L^{-1}) natural DOC concentration only showed a 10% difference in NO_3 removal. Whether there is a link between DOC concentration and NO_3 removal should be examined with a larger data set.

A poor correlation between NO_3 removal and DOC concentration suggests that NO_3 reduction is dependent on the quality of DOM rather than concentration.

The denitrification experiment only showed evidence for the reduction of NO_3 to NO_2, which often occurs in carbon limiting environments. This could be indicative of the bioavailability of groundwater DOM.

Confirmation on the link between DOM quality and changes in NO_3 could not be extracted from the measurements performed in this study. Relationships between NO_3 removal and (1) the change in hydrophilic compounds, (2) the change in fulvic acids, (3) absorbance at 465 nm and (4) the E4:E6 ratio were investigated and no correlations were found. Groundwater depth also does not seem to influence NO_3 removal. There was no relationship between the reduction of NO_3 and shallow or deeper groundwater (Figure 5.1).

Most DOM becomes mineralized or is retained in soils, with only a part reaching aquatic systems (Kalbitz et al. 2012). Because of this it is often thought that groundwater DOM is generally more recalcitrant than soil DOM. However, the results presented here show highest NO_3 removal in deeper groundwater samples rather than phreatic groundwater. This may suggest that groundwater transported DOM or DOM originating from aquifer sediments play a larger role than soil leached DOM.

NO_3 removal was higher in samples originating from grasslands than from arable crops. The quality of DOM is suggested to decrease when grassland soils are converted to arable crops since there are less water extractable carbohydrates and amino N-compounds in arable crop soils (Chantigny et al. 2003). The two phreatic groundwater samples originating from grasslands (1 and 2) both showed higher NO_3 removal than samples originating from arable land (about 10%). Note that details on crop rotation and land management are not known for the sampling locations. The net effect of management practices can be poorly predictable under field conditions because various soil properties are modified at the same time, resulting in confounding and counteracting effects on DOM (Chantigny et al. 2003).

Even though there are no systematic studies relating properties of DOM to its biodegradability, literature suggests that DOM is more readily available for use by microorganisms if it contains relatively high proportions of hydrophilic materials, such as carbohydrates, organic acids and proteins. High contents of hydrophobic materials (e.g. lignin) decrease biodegradability (Bourbonniere et al. 2006, Marschner et al. 2003). The distribution of hydrophilic and hydrophobic compounds (fulvic acids) of groundwater analysed in this study do not show a correlation with NO_3 removal, and therefore it cannot be concluded which DOM property is a suitable predictor for DOM biodegradability. Bioavailability of DOM is even more difficult to quantify as this mostly depends on in situ conditions such as pore size, or sorption of DOM to solid surfaces.

By using a highly variable data set of DOM origin and concentration, it was expected to see more differences in DOM characteristics between samples. Surprisingly, especially for the DOM fractionation, DOM composition was very uniform across a diverse sample set. As fluorescence spectroscopy was the most sensitive tool out of all the characterization methods applied (with the exception of Py/GC/MS), it could be useful to redo fluorescence measurements on diluted groundwater samples. Moreover other techniques should be considered in order to predict DOM bioavailability for denitrification.

5.2 Correlations between DOC characterization techniques

No relationships were found between the different DOM characterization techniques. There are however some similarities which should be pointed out:

- The two samples with a humic acid fraction at the onset of the experiment (2 and 10) exhibited the highest a_{465} values and had the largest contribution of component 2 in the fluorescence results.
- The sample with the lowest E4:E6 ratio (8) also resulted in the lowest absorption at 465 nm and had the least amount of NO_3 removal.
- Both fluorescence and fractionation showed surprisingly similar results amongst different samples despite the large variation in sample origin.

On the other hand the trend proposed by Osborne et al. 2007 linking E4:E6 ratios smaller than 5 to a dominance of fulvic acids is not in accordance with the fractionation results. Moreover the fluorescence indices FI and BIX are contradictory and do not provide substantial information about DOM quality.

These findings show that there are limitations to the application of the different characterization methods and there is no clear evidence which method is more accurate to predict changes in DOM that can be linked to biodegradation.

5.3 Linking DOM composition to its origin

Groundwater samples used in this study showed a large variation in colour distribution amongst the samples with no distinct pattern between phreatic or deeper groundwater, forest or agricultural origin. In contrast DOM fluorescence and fractions were very similar for these samples. As such, detailed molecular analysis of DOM using Py/GC/MS or nuclear magnetic resonance spectroscopy (NMR) could provide more insight in the variation in DOM composition.

The uniformness of DOM fluorescence across different samples originating from different geological settings, land use and depth gives rise to the questions whether the chemical composition of DOM actually varies within the studies environments and which factors govern the chemical composition of DOM groundwater. One would expect a much more land use influenced DOM pool in phreatic groundwater (horizontal flow dependent on percolation) than groundwater originating from 10-30 meter depths, where vertical groundwater flow is much more dominant and DOM can be transported from other locations. Similarities within the fluorescence signal could be an artefact of high DOC concentrations (see section 4.3.2) but it could also imply that fluorescence is incapable of identifying differences between soil derived DOM and sedimentary derived DOM.

6 Conclusions and Recommendations

The results and discussion have shown the following:

- There is potential for DOM induced denitrification in Dutch groundwater but it remains unclear how DOM is utilized in this process. There is no evidence for DOM mineralization and it is thought that partial oxidation as well as HS moieties with electron donating capacities could be supplying electrons. NO_3 removal by incorporation into microbial biomass could also play a role.
- Spectrophotometric DOM characterization (UV-Vis absorbance and fluorescence measurements) did not show a large variation amongst samples, nor between measurements performed before and after the denitrification experiment. Fluorescence EEMs did show an increase in protein-like fluorescence, indicative of microbial activity.
- Resin fractionation subdivided DOM into four fractions and the contribution of each fraction was very similar in all groundwater samples. The fractionation results did show a change in chemical composition of DOM, as hydrophilic compounds decreased and hydrophobic neutrals increased. This trend is in contradiction to the idea of partial oxidation of DOM, which adds to the complexity of DOM acting as an electron donor.
- There is no correlation between denitrification rates and DOM characteristics and therefore it is not possible to identify underlying processes of NO_3 reduction. It remains unknown whether this is due to the inadequacy of the characterization techniques or because of measurement errors.
- DOM characteristics cannot be clearly linked to sample origin (land use and/or depth). Again it remains unknown whether this is due to the inadequacy of the characterization techniques or due to measurement errors. In the first option, methods identifying molecular structure (Py/GC/MS or Nuclear Magnetic Resonance spectroscopy, NMR) could provide a better correlation to sample origin.

Recommendations

This research used a data set of samples originating from extremely different environments, expecting to obtain a high level of variance in DOM characteristics. In contrast to the expectations DOM characteristics were surprisingly similar, and no clear relationship can be found between either reductions in NO_3 and DOM composition or DOM origin and DOM composition.

The denitrification experiment showed that NO_3 removal occurred in all samples and more importantly NO_2 production took place, which is a good indicator for (the first step) of denitrification. As such, it could be attempted to redo the fluorescence characterization as this was the most sensitive and selective characterization technique used in this study (Maitilianen et al. 2010) and because there was a relationship with DOC concentration. Perhaps this technique would reveal more differences in DOM composition if concentrations are below c.a. 10 mg L^{-1} and kept constant throughout the data set.

To further investigate the potential of DOM as electron donor for denitrification, a data set comprising of a smaller variety of groundwater samples should be analysed in triplicate. Measurements of chlorofyll, C:N ratios, gas phases (N_2O, N_2, CO_2) and identification of the microbial community would give more insight in the NO_3 reduction process. To explore the possibilities of partial DOM oxidation, compound analysis techniques (Py/GC/MS or NMR) can be used to determine compositional changes and carbon oxidation states prior to and after the experiment.

7 Acknowledgements

This thesis was accomplished through collaboration between the faculty of Earth Sciences at Utrecht University and Deltares. I would like to thank several people who have contributed to this research:

Prof. Dr. Jasper Griffioen, Dr. Mariet Hefting and Dr. Thilo Behrends for arranging this project and for all the useful discussions concerning measurement techniques, data acquisition and data interpretation.

Dr Mariet Hefting and Gerrit Rouwenhorst, for arranging the use of equipment at the Faculty of Science Ecology and Biodiversity and helping me solve numerous measurement issues.

Dr. Klaas Nierop, without whom I could not have completed the pyrolysis gas chromatography mass spectrometry measurements.

Prof. Dr. Karsten Kalbitz and Dr. Joris Haftka for their great support on spectrophotometric characterization methods.

Remco Koper, Dr. André van Zomeren and Dr. Jos Vink for all their help and advice on DOM fractionation.

Dineke van de Meent and Rob van Galen, for providing me with help and support with regards to TOC measurements.

Fredericke Hannes, Piet Peereboom, André Cinjee and Erik van Vilsteren for their support at the Deltares laboratory.

8 References

Addy, K., Kellog, D.Q., Gold, A.J., Groffman, P.M., Ferendo, G., Sawyer, C. (2002). In situ push-pull method to determine ground water denitrification in riparian zones. J. Environ. Qual. 31: 1017-1024.

Aeschbacher, M., Graf, C., Schwarzenbach, P., Sander, M. (2012). Antioxidant properties of humic substances. Environmental Science and Technology 46: 4916-4925.

Aravena, R., Wassenaar,L. I., Spiker, E. C. (2004). Chemical and carbon isotopic composition of dissolved organic carbon in a regional confined methanogenic aquifer. Isotopes Environ. Health Stud. 40: 103-114.

Baker, M. A., m Valett, M.H., Dahm, C.N. (2000). Organic carbon supply and metabolism in a shallow groundwater ecosystem. Ecology 81: 3133-3148.

Baker, M. A., Vernier, P. (2004). Hydrological variability, organic matter supply and denitrification in the Garonne River ecosystem. Freshwater Biology 49: 181-190.

Barnes, R. T., Smith, R.L., Aiken, G.R. (2012). Linkages between denitrification and dissolved organic matter quality, Boulder Creek watershed, Colorado. Journal of Geophysical research 117.

Birdwell, J. E., Summers Engel, A. (2010). Characterization of dissolved organic matter in cave and spring waters using UV-Vis absorbance and fluorescence spectroscopy. Organic Geochemistry 41: 270-280.

Boumans, L. J. M., Fraters, B., van Brecht, G. (2001). Nitrate in the upper groundwater of 'De Marke' and other farms. Netherlands Journal of Agricultural Science 49: 163-177.

Boumans, L. J. M., Fraters, B., van Brecht, G. (2005). Nitrate leaching in agriculture to upper groundwater in the sandy regions of The Netherlands during the 1992-1995 period. Environmental Monitoring and Assessment 102: 225-241.

Bourbonniere, R.A., Creed, I.F. (2006). Biodegradability of dissolved organic matter extracted from a chronosequence of forest-floor materials. J.Nutr. Soil Sci. 169: 101-107.

Boyle, E. S., Guerriero, N., Thiallet, A., Del Vecchio, R., Blough, N.V. (2009). Optical properties of humic substances and CDOM: relation to structure. Environ. Sci. Technol. 43: 2262-2268.

Bradley, P. M., Fernandez, Jr, M., Chapelle, F.H. (1992). Carbon limitation of denitrification rates in an anaerobic groundwater system. Environ. Sci. Technol. 26.

Burgin, A. J., Hamilton, S.K. (2007). Have we overemphasized the role of denitrification in aquatic ecosystems? A review of nitrate removal pathways. Front Ecol Environ 5: 89-96.

Cannavo, P., Richaume, A., Lafolie, F. (2004). Fate of nitrogen and carbon in the vadose zone: in situ and laboratory measurements of seasonal variation in aerobic respiratory and denitrifying activities Soil Biology & Biochemistry 36: 463-478.

Castaldelli, G., Colombani, N., Vincenzi, F., Mastrocicco M. (2013). Linking dissolved organic carbon, acetate and denitrification in agricultural soils. Environ Earth Sci 68: 939-945.

Chantigny, M. H. (2003). Dissolved and water-extractable organic matter in soils: a review on the influence of land use and management practices. Geoderma 113: 357-380.

Chapelle, F. H., Bradley, P.M., Journey,C.A., McMahon, P.B. Assessing the relative bioavailability of DOC in regional groundwater systems. Groundwater 51: 363-372.

Chin, Y., Aiken, G., O'Loughlin, E. (1994). Molecular weight, polydispersity, and spectroscopic properties of aquatic humic substance. Environmental Science and Technology 28: 1853-1858.

Craig, L., Bahr, J.M., Roden, E.E. (2010). Localized zones of denitrification in a floodplain aquifer in southern Wisconsin, USA. Hydrogeology Journal 18: 1867-1879.

De Klein, C. A. M., van Logtestijn, R.S.P (1994). Denitrification in the top soil of managed grasslands in The Netherlands in relation to soil type and fertilizer level. Plant and Soil 163: 33-44.

Eschenbach, W., Well, R. (2013). Predicting the denitrification capacity of sandy aquifers from shorter-term incubation experiments and sediment properties. Biogeosciences 10: 1013-1035.

Fennel, K., Brady, D., DiToro, D., Fulweiler, R.W., Gardner, W.S., Giblin, A., McCarthy, M.J., Rao, A., Seitzinger, S., Thouvenot-Korppoo, M., Tobias, C. (2009). Modeling denitrification in aquatic sediments. Biogeochemistry 93: 159-178.

Fraters, B., Kovarm K., Willems, W.J., Stockmarr, J., Grant, R. (2003). Monitoring the effectiveness of the EU Nitrates Directive Action Programmes. D. Fraters, RIVM.

Fraters, D., Boumans, L.J.M., van Drecht, G., de Haan, T., de Hoop, W.D. (1998). Nitrogen monitoring in groundwater in the sandy regions of The Netherlands. Environmental Pollution 102.

Groenendijk, P., Renaud, L.V., Janssen, G.M.C.M., Jansen, S., Heerdink, R., Griffioen, J., van der Grift, B. (2009). A new compliance checking level for nitrate in groundwater. Wageningen, Alterra.

Groffman, P. M., Altabet, M.A., Bohlke, J.K., Butterbach-Bahl, K., David, M.B., Firestone, M.K., Giblin, A.E., Kana, T.M., Nielsen, L.P., Voytek, M.A. (2006). Methods for measuring denitrification: diverse approaches to a difficult problem. Ecological Applications 16: 2091-2122.

Guggenberger, G., Zech, W., Schulten, H. (1994). Formation and mobilization pathways of dissolved organic matter: evidence from chemical structural studies of organic matter fractions in acid forest floor solutions. Organic Geochemistry 21: 51-66.

Hartog, N. (2003). Reactivity of organic matter and other reductants in aquifer sediments. Geosciences, Utrecht University.

Hood, E., Fellman, J., Edwards, R.T. (2007). Salmon influences on dissolved organic matter in a coastal temperate brown-water stream: an application of fluorescence spectroscopy. Liminology and Oceanography 52: 1580-1587.

Hooijboer, A.E.J., Kleine de, A. (2012). Water quality on farms. RIVM report 680123001/2012.

Huguet, A., Vacher, L., Relexans, S., Saubusse, S.,Froidefond, J. M., Parlanti, E. (2009). Properties of fluorescent dissolved organic matter in the Gironde Estuary. Organic Geochemistry 40: 706-719.

Hulth, S., Aller, R. C., Gilbert, F. (1999). Coupled anoxic nitrification/manganese reduction in marine sediments. Geochimica et Cosmochimica Acta 63: 49-66.

Israel, S., Engelbrecht, P., Tredoux, G., Fey, M.V. (2009). In situ batch denitrification of nitrate-rich groundwater using sawdust as a carbon source-Marydale, South Africa. Water Air Soil Pollut 204: 177-194.

Jahangir, M. M. R., Johnston, P., Khalil, M.I., Richards, K.G. (2012). Linking hydrogeochemistry to nitrate abundance in groundwater in agricultural settings in Ireland. Journal of Hydrology: 212-222.

Jandl, R., Sollins, P. (1997). Water-extractable soil carbon in relation to the belowground carbon cycle. Biol Fertil Soils 25: 196-201.

Jüttner, F. (1999). Interference with ammonium determination by the indophenol-type reaction of salicylate and dichloroisocyanurate. Fresenius J Anal Chem 363: 128-129.

Kalbitz, K., Geyer, S., Geyer, W. (2000). A comparative characterization of dissolved organic matter by means of original aqueous samples and isolated humic substances. Chemosphere 40: 1305-1312.

Kalbitz, K., Schmerwitz, J., Schwesig, D., Matzner, E. (2003). Biodegradation of soil-derived dissolved organic matter as related to its properties. Geoderma 113: 273-291.

Klupfel, L., Piepenbrock, A., Kappler, A., Sander, M. (2014). Humic substances as fully regenerable electron acceptors in recurrently anoxic environments. Nature geoscience 7: 195-200.

Knowles, R. (1982). Denitrification. Microbiological reviews 46: 43-70.

Korom, S. F. (1992). Natural denitrification in the saturated zone: a review. Water resources research 28: 1657-1668.

Kraus, T. E. C., Bergamaschi, B.A., Hernes, P.J., Spencer, R.G.M., Stephanauskas, R., Kendall, C., Losee, R.F., Fuijii, R. (2008). Asessing the contribution of wetlands and subsided islands to dissolved organic matter and disinfection byproduct precursors in the Sacramento-San Joaquin River Delta: A geochemical approach. Organic Geochemistry 39: 1302-1318.

Larsson, T., Wedborg, M., Turner, D. (2007). Correction of inner-filter effect in fluorescence excitation-emission matrix spectrometry using Raman scatter. Analytica Chimica Acta 583: 357-363.

Laverman, A. M., van Cappellen, P., van Rotterdam-Los, D., Pallud, C., Abell, J. (2006). Potential rates and pathways of microbial nitrate reduction in coastal sediments. FEMS Microbiol Ecol 58: 179-192.

Lawaetz, A. J., Stedmon, C.A. (2009). Fluorescence intensity calibration using the raman scatter peak of water. Applied spectroscopy 63.

Leenheer, J. A., Croue, J. (2003). Characterizing dissolved aquatic organic matter. Environmental Science and Technology 19.

Marschner, B., Kalbitz K. (2003). Controls of bioavailability and biodegradability of dissolved organic matter in soils. Geoderma 113: 211-235.

Matilainen, A., Gjessing, E. T., Lahtinen, T., Hed, L., Bhatnagar, A., Sillanpaa, M. (2011). An overview of the methods used in the characterisation of natural organic matter (NOM) in relation to drinking water treatment. Chemosphere 83: 1431-1442.

McDonald, S., Bishop, A.G., Prenzler, P.D., Robards, K. (2004). Analytical chemistry of freshwater humic substances. Analytica Chimica Acta 527: 105-124.

McDowell, W. H., Zsolnay, A., Aitkenhead-Peterson, J.A., Gregorich, E.G., Jones, D.L., Jodemann, D., Kalbitz, K., Marschner, B., Schwesig, D. (2006). A comparison of methods to determine the biodegradable dissolved organic carbon from different terrestrial sources. Soil Biology & Biochemistry 38: 1933-1942.

McKnight, D. M., Boyer, E.W., Westerhoff, P.K., Doran, P.T. (2001). Spectrofluorometric characterization of dissolved organic matter for indication of precursor organic material and aromaticity. Liminology and Oceanography 46: 38-48.

Mobed, J. J., Hemmingsen, S.L., Autry, J.L., McGown, L.B. (1996). Fluorescence characterization of IHSS humic substances: total luminescence spectra with absorbance correction. Environmental Science and Technology 30: 3061-3065.

Murphy, K. R., Hambly, A., Singh, S., Henderson, R. K., Baker, A., Stuetz, R., Khan, S. J. (2011). Organic matter fluorescence in municipal water recycling schemes: toward a unified PARAFAC model. Environmental Science and Technology 45: 2909-2916.

Neff, J. C., Asner, G.P. (2001). Dissolved organic carbon in terrestrial ecosystems: a synthesis and a model. Ecosystems 4: 29-48.

OECD (2001). Environmental indicators for agriculture, Organisation for economic co-operation and development (OECD).

Peterson, M. E., Curtin, D., Thomas, S., Clough, T. J. , Meenken, E.D. (2013). Denitrification in vadose zone material amended with dissolved organic matter from topsoil and subsoil. Soil Biology & Biochemistry 61: 96-104.

Piccolo, A. The supramolecular structure of humic susbtances: a novel understanding of humus chemistry and implications in soil science. Advnces in Agronomy 75.

Postma, D., Boesen, C. (1991). Nitrate reduction in an unconfined sandy aquifer: water chemistry, reduction processes and geochemical modeling. Water resources research 27: 2027-2045.

Qualls, R. G., Richardson, C.J. (2003). Factors controlling concentration, export, and decomposition of dissolved organic nutrients in the Everglades of Florida. Biogeochemistry 62: 197-229.

Qualls, R. G. (2005). Biodegradability of fractions of dissolved organic carbon leached from decomposing leaf litter. Environ. Sci. Technol. 39: 1616-1622.

Ringuet, S., Sassano, L., Johnson, Z.I. (2011). A suite of microplate reader-based colorimetric methods to quantify ammonium, nitrate, orthophosphate and silicate concentrations for aquatic nutrient monitoring. Journal of Environmental Monitoring 13: 370-376.

Rivett, M. O., Buss, S.R., Morgan, P., Smith, J.W.N., Bemment C.D. (2008). Nitrate attenuation in groundwater: a review of biogeochemical controlling processes. Water research 42: 4215-4232.

Rutting, T., Boeckx, P., Muller, C., Klemedtsson, L. (2011). Assessment of the importance of dissimilatory nitrate reduction to ammonium for the terrestrial nitrogen cycle. Biogeosciences 8: 1779-1791.

Shand, C. A., Berwyn, L.W., Coutts, G. (2008). Determination of N-species in soil extracts using microplate techniques. Talanta 74: 648-654.

Shrimali, M., Singh, K. P. (2001). New methods for nitrate removal from water. Environmental Pollution 112: 351-359.

Siemens, J., Haas,M., Kaupenjohann, M. (2003). Dissolved organic matter induced denitrification in subsoils and aquifers? Geoderma 113: 253-271.

Sondergaard, M., Stedmon, C. A., Borch, N. (2003). Fate of terrigenous dissolved organic matter (DOM) in estuaries: Aggregation and bioavailability. Ophelia 3: 161-176.

Stedmon, C. A., Markager,S., Bro, R. (2003). Tracing dissolved organic matter in aquatic environments using a new approach to fluorescence spectroscopy. Marine Chemistry 82: 239-254.

Stedmon, C. A., Markager, S. (2005). Resolving the variability in dissolved organic matter fluorescence in a temperate estuary and its catchment using PARAFAC analysis. Liminology and Oceanography 50: 686-697.

Stedmon, C. A., Bro, R. (2008). Characterizing dissolved organic matter fluorescence with parallel factor analysis: a tutorial. Liminology and Oceanography: Methods 6: 572-579.

Stevenson, F. J. (1994). Humus Chemistry Genesis, Composition, Reactions, John Wiley & Sons, Inc.

Swietlik, J., Sikorska, E. (2004). Application of fluorescence spectroscopy in the studies of natural organic matter fractions reactivity with chlorine dioxide and ozone. Water research 38: 3791-3799.

Torrento, C., Urmeneta, J., Otero, N., Soler, A., Vinas, M., Cama, J. (2011). Enhanced denitrification in groundwater and sediments from a nitrate-contaminated aquifer after addition of pyrite. Chemical Geology 287: 90-101.

Van Zomeren, A. (2008). On the nature of organic matter from natural and contaminated materials; isolation methods, characterisation and application to geochemical modeling. Soil Science, Wageningen Univeristy.

Velthof, G. L. (2003). Relations between mineralization, denitrification and indications for soil quality in agricultural soils. Wageningen, Alterra.

Wassenaar, L., Aravena, R., Fritz P., Barker, J. (1990). Isotopic composition (13C, 14C, 2H) and geochemistry of aquatic humic substances from groundwater. Organic Geochemistry 15: 383-396.

Weymann, D., Geistlinger, H., Well, R., von der Heide, C., Flessa, H. (2010). Kinetics of N2O production and reduction in a nitrate-contaminated aquifer inferred from laboratory incubation experiments. Biogeosciences 7: 1953-1972.

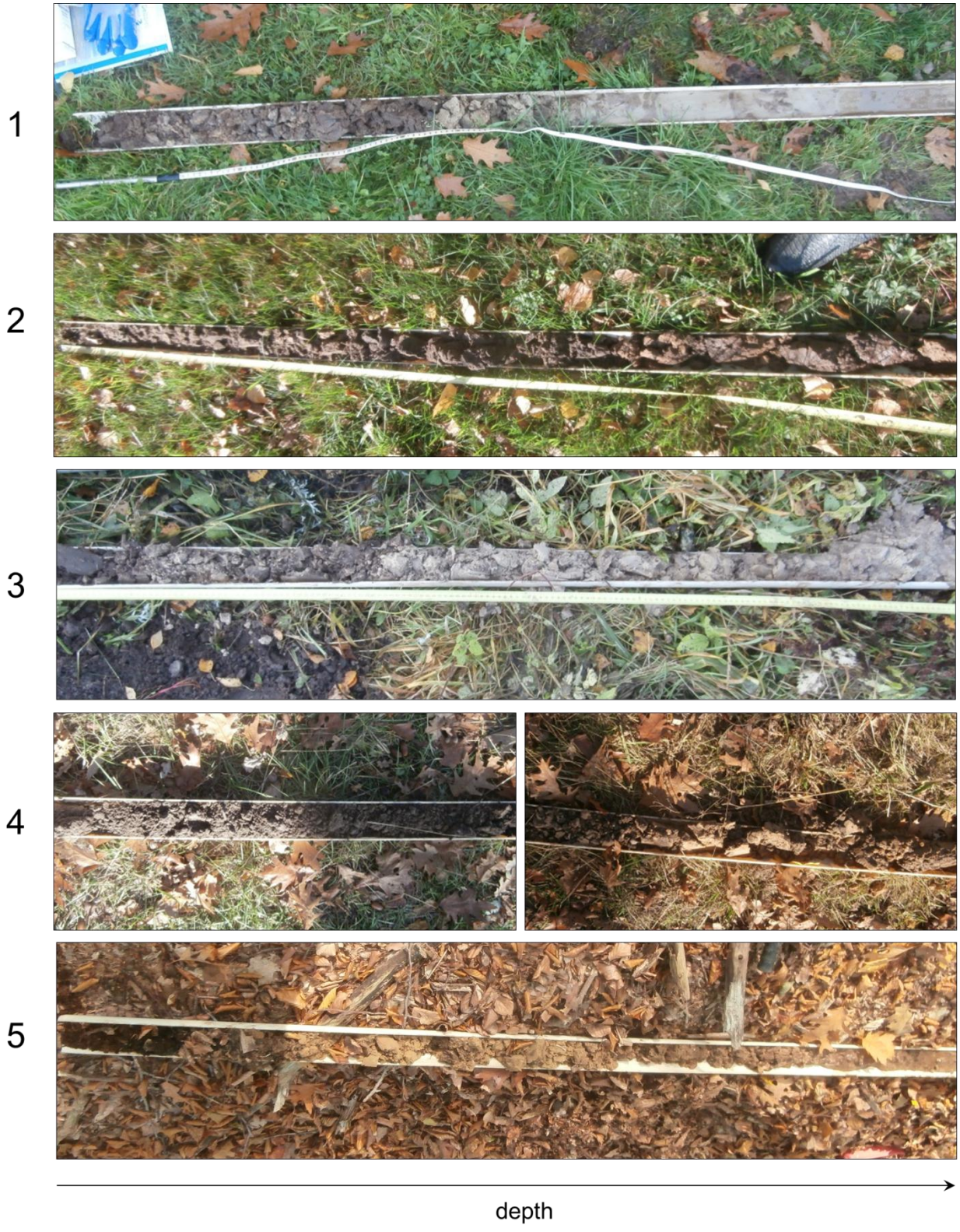
Wong, J. C. Y., Williams, D.D. (2010). Sources and seasonal patterns of dissolved organic matter in the hyporheic zone. Hydrobiologia 647: 99-111.

Zhang, Y., Slomp, C.P., Broers, H.P., Bostick, B., Passier, H.F., Bottcher, M.E., Omoregie, E.O., Lloyd, J.R., Polya, D.A., van Cappellen, P. (2012). Isotopic and microbiological signatures of pyrite driven denitrification in a sandy aquifer. Chemical Geology 300-301: 123-132.

Zwart, K. B., Velthof, G.L., van Groenigen, J., Heinen, M., van der Grift, B., Boumans, L. (2004). Denitrification in agricultural soils in the Netherlands, Alterra, TNO, RIVM.

Appendix A: Borehole photographs

Cores from shallow groundwater samples (1-5). Wells were drilled using an Edelman hand auger. Depth of all wells was c.a. 2 meters.



Appendix B: Determination of N-components and iron

Colorimetric measurements were performed using a SPECTROstar nano microplate spectrophotometer to determine NO_3^-, NO_2^- and NH_4^+. 96 well microlon 200, PS F-Bottom microplates were used.

Nitrate
The method used to measure NO_3^- is based on the reduction of NO_3^- to NO_2^- using hydrazine sulphate. The reagents used are listed in Table B.1

Table B.1

Solution	Contents	Amount	Conc.
A. NaOH solution			0.5 M
B. Reduction solution	D	1 L	
	E	2 mL	
	F	20 mL	
C. Color reagent	G	Equal parts of G and H	
	H		
D. Hydrazine sulphate			2.59 mM
E. Copper sulphate			16.2 mM
F. Zinc sulphate			35.8 mM
G. Sulphanilamide		20 g L^{-1}	116 mM
H. N-(1-naphthyl)ethylenediamine dihydrochloride (NEDD)		1 g L^{-1}	3.85 mM

20 µL of sodium hydroxide solution was pipetted into the microplate followed by 100 µL demineralized water, 6 µL sample and 50 µL reduction solution. The mixture was shaken for 20 minutes after which 50 µL of color reagent was pipetted in each well. The optical density was measured at 540 nm at 37°C directly after addition of the color reagent and the concentrations were determined against a range of standards (0-2 mmol NO_3^- L^{-1}) prepared from KNO_3.
The method was tested by changing the ratio between sample volume and demineralized water. Using a relatively small sample volume of 6 µL still resulted in reliable calibration curves with minimal variation between different measurements. Therefore dilution of samples was not necessary.

Nitrite
Nitrite measurements were performed using the same procedure used for NO_3 but omitting the hydrazine solution and substituting an equal amount of demi water. Concentrations were determined against a range of standards (0-1 mmol NO_2 L^{-1}) prepared from $NaNO_2$.

Ammonium
Ammonium was measured using the salicylate-dichloroisocyanurate method adapted from the Scalar Autoanalyzer procedure. The reagents used are listed in Table B.2
. 38 µL of sample was pipetted into each well followed by 150 µL complex buffer, 50 µL sodium salicylate solution, 20 µL sodium nitroprusside solution and 50 µL sodium dichloroisocyanurate solution. The microplate was shaken for 60 minutes at 37°C and optical density was measured at 660nm at the same temperature. Concentrations were determined against a range of standards (0-4 mmol NH_4 L^{-1}) prepared from NH_4Cl.

Table B.2

Solution	Contents	Concentration
A. Complex buffer	$C_4H_4KNaO_6 \cdot 4H_2O$	33 g/L
	$C_6H_5Na_3O_7$	24 g/L
B. Sodium salicylate	$C_7H_5Na_3O_7$	80 g/L
C. Sodium nitroprusside	$Na_2Fe(CN)_5NO \cdot 2H_2O$	0.25 g/L
D. Sodium dichloroisocyanurate	$C_3Cl_2N_3NaOH$	0.08 g/L

Iron (Fe(II) and Fe(tot))

Ferrozine (monosodium salt hydrate of 3-(2-pyridyl)-5,6-bis (4-phenylsulfonic acid)-1,2,4 triazine p-p'-disulfonic acid) reacts with divalent iron to form a stable magenta complex species.

Reagents

A. Ferrozine
   0.493 g ferrozine and 0.771 g ammonia acetate were combined and diluted to 100 mL with demi water.
B. Reducing agent
   Hydroxylamine hydrochloride ($H_2NOH.HCl$, 99%, Merck)
   1.4 mol L^{-1} prepared in a solution of hydrochloric acid 2 mol L^{-1}. 9.729 g of $H_2NOH.HCl$ diluted in 10 mL HCL 2M.
C. Buffer
   Ammonium acetate: a 5 mol L^{-1} solution adjusted at pH 9.5 with a solution of ammonium hydroxide (28-30%, NH_4OH, Merck). 5 mol L^{-1} ammonium acetate, 77.08 g of ammonium acetate diluted in 200 mL demi water. Then pH is adjusted with NH_4OH.

To determine Fe(tot) 1.2 mL of the sample or standard was pipetted in a disposable cuvette. 200 µL ferrozine solution (A) and 300 µL of the reducing agent (B) was added. After 20 minutes 200 µL of the buffer solution (C) was added and the solution was stirred with a plastic stick. The maximum absorbance was recorded at 562 nm. For Fe(II) the reducing agent was replaced with water. Concentrations were determined against a range of standards (0-100 µmol Fe^{2+} L^{-1}) prepared from $Fe(NO_3)_3$.

Appendix C: Additional results denitrification experiment

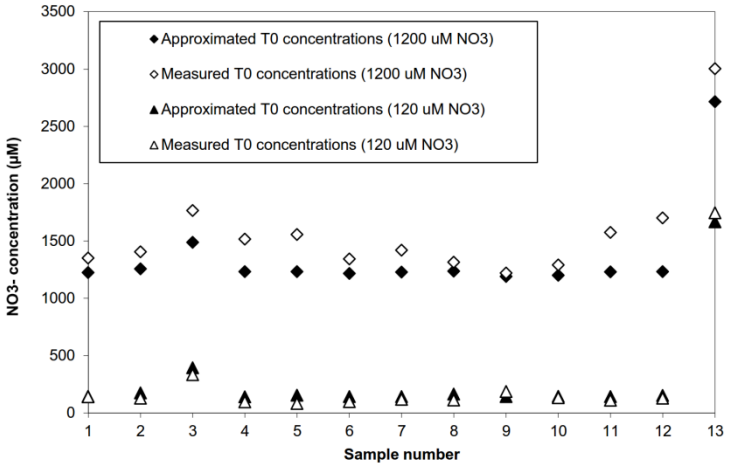


Fig C.1: Difference between measured and approximated (based on calculations) NO_3 concentrations

Table C.1: Difference between measured and approximated Cl concentrations

Sample	Prior to incubation	Post incubation	Expected based on addition of unfiltered groundwater
	Cl (µmol/L)	Cl (µmol/L)	Cl (µmol/L)
1	1896	3481	3241
2	471	1993	1966
3	87	1967	1681
4	891	2778	2468
5	2487	4114	3779
6	935	2746	2425
7	50077	47117	46013
8	484	2265	1990
9	258	2029	1733
10	17214	17199	16850
11	32424	31088	30226
12	95011	86769	85576
13	1054	2751	2501

Table C.2: Difference between measured and approximated F concentrations

Sample	Prior to incubation	Post incubation	Expected based on addition of unfiltered groundwater
	F (µmol/L)	F (µmol/L)	F (µmol/L)
1	0	51	1
2	21	2	20
3	2	45	3
4	0	0	1
5	27	19	25
6	0	49	1
7	0	0	1
8	0	42	1
9	0	40	1
10	35	71	32

11	0	13	1
12	23	50	21
13	21	2	20

Table C.3: Coefficients of the double linear model describing removal trends of NO_3

	NO_3 initial rate						NO_3 final rate					
Sample	A (slope)			B (intercept)			A (slope)			B (intercept)		
1	-22.4	±	29.6	1375.6	±	200.0	-13.2	±	1.7	1118.8	±	62.6
2	-32.4	±	40.9	1495.4	±	276.6	-10.0	±	3.8	1052.1	±	141.3
3	-54.3	±	10.4	1845.0	±	70.3	-9.0	±	0.7	1336.6	±	27.6
4	-42.4	±	7.9	1484.4	±	53.4	-10.4	±	1.4	1200.5	±	51.4
5	-44.7	±	8.7	1520.2	±	58.5	-8.1	±	0.8	1170.7	±	29.5
6	-36.2	±	10.7	1385.6	±	72.4	-11.1	±	1.7	1107.6	±	63.0
7	-74.0	±	9.9	1380.4	±	66.8	3.3	±	0.6	557.4	±	18.1
8	-24.6	±	15.7	1250.1	±	106.4	-7.9	±	0.8	1112.1	±	28.3
9	-43.8	±	14.1	1276.7	±	95.4	-10.1	±	2.3	930.3	±	86.3
10	-50.1	±	22.5	1200.1	±	152.1	-7.2	±	3.0	875.2	±	109.4
11	-78.1	±	5.3	1553.6	±	35.7	-9.8	±	2.6	908.0	±	94.8
12	-93.6	±	11.1	1655.9	±	75.3	-3.1	±	3.3	664.0	±	120.5
13	-130.5	±	124.3	3505.0	±	840.0	-4.0	±	4.9	1841.8	±	179.6

Table C.4: Coefficients of the double linear model describing production trends of NO_2

	NO_2 initial rate						NO_2 final rate					
Sample	A (slope)			B (intercept)			A (slope)			B (intercept)		
1	8.5	±	1.5	0	±	NA	7.8	±	1.4	79.4	±	50.5
2	6.3	±	1.0	0	±	NA	2.3	±	2.0	103.4	±	72.9
3	7.4	±	0.6	0	±	NA	1.1	±	0.1	71.2	±	3.0
4	7.2	±	1.4	0	±	NA	1.0	±	0.5	93.4	±	19.8
5	-0.1	±	0.6	5.9	±	4.0	5.8	±	1.3	-72.8	±	49.3
6	11.5	±	1.3	0	±	NA	-0.9	±	1.6	96.4	±	60.6
7	8.8	±	0.5	0	±	NA	3.5	±	1.2	115.7	±	42.5
8	2.5	±	0.5	0	±	NA	2.3	±	0.7	32.9	±	24.8
9	25.0	±	5.1	0	±	NA	7.9	±	0.9	212.5	±	34.3
10	14.2	±	0.6	0	±	NA	1.5	±	0.6	118.4	±	21.1
11	14.9	±	0.8	0	±	NA	-0.6	±	0.6	195.2	±	23.3
12	11.1	±	2.1	0	±	NA	7.2	±	1.7	57.6	±	60.9
13	3.3	±	0.5	0	±	NA	1.8	±	12.0	125.5	±	441.2

Table C.5: Coefficients of the linear regression for NH_4

	NH_4^+ linear regression	
Sample	A (slope)	B (intercept)
1	0.8 ±	63 ±
2	-0.6 ±	86 ±
3	-0.2 ±	40 ±
4	-0.3 ±	71 ±
5	0.0 ±	35 ±
6	0.0 ±	48 ±
7	-2.1 ±	1200 ±
8	0.3 ±	38 ±
9	-0.9 ±	198 ±
10	-8.3 ±	1431 ±
11	-2.1 ±	380 ±
12	-3.6 ±	1124 ±
13	0.0 ±	50 ±

Table C.6: Coefficients of the linear regression for DOC

	DOC linear regression					
Sample	A (slope)			B (intercept)		
1	-1.2	±	1.2	1254.1	±	37.9
2	0.6	±	6.7	5806.8	±	213.2
3	-2.5	±	1.4	1637.6	±	44.5
4	-3.4	±	1.9	974.1	±	62.3
5	-9.9	±	4.9	4757.9	±	158.2
6	-2.4	±	1.5	1991.1	±	49.1
7	-1.5	±	4.3	4301.8	±	137.6
8	-2.2	±	1.2	635.0	±	37.2
9	-3.0	±	2.3	1487.7	±	75.0
10	5.7	±	3.7	3374.2	±	117.0
11	-2.5	±	2.4	3016.8	±	75.6
12	-1.3	±	2.4	2026.8	±	78.0
13	-6.9	±	2.7	3356.2	±	85.2

Table C.7: Coefficients of the double exponential model describing trends of NO_3 and NO_2

Sample	**NO_3**			**NO_2**		
	k_1	**k_2**	**α**	**k_1**	**k_2**	**α**
1	0.019	0.019	0.099	0.00004	0.020	0.578
2	0.029	0.010	0.550	0.00004	0.050	0.177
3	0.117	0.008	0.724	0	0.179	0.045
4	0.383	0.012	0.827	0.00013	0.075	0.091
5	0.299	0.009	0.776	0.00268	0.003	0.949
6	0.020	0.020	0.091	0.00013	0.040	0.052
7	0.220	0	0.444	0	0.047	0.222
8	0.200	0.009	0.847	0.00023	0.034	0.121
9	0.119	0.019	0.869	0.00163	0.034	0.572
10	0.200	0.010	0.682	0.00139	0.240	0.088
11	0.242	0.022	0.652	0	0.148	0.115
12	0.175	0.002	0.339	0.00412	0.025	0.115
13	0.070	0.001	0.500	0	0.054	0.082

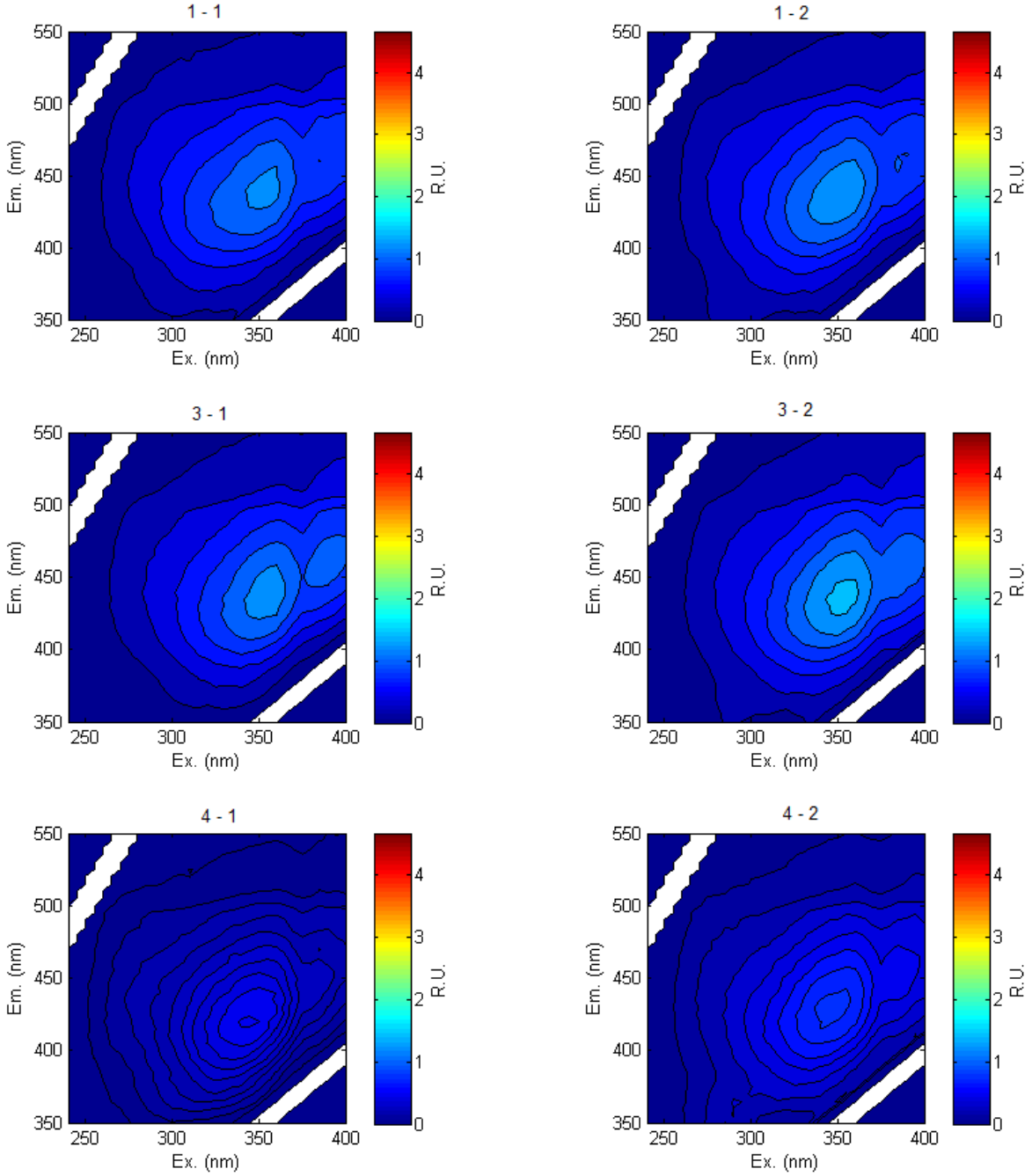
Appendix D: Supplementary results of DOM characterization

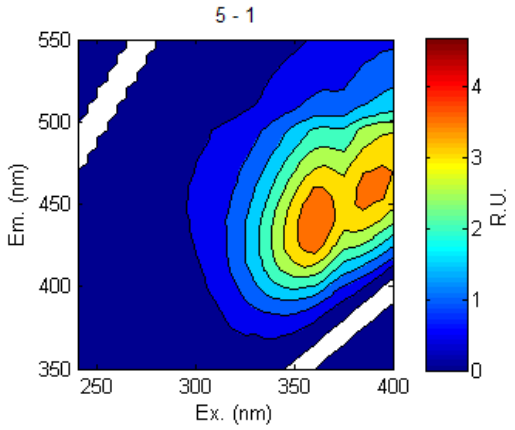
UV-Vis absorbance

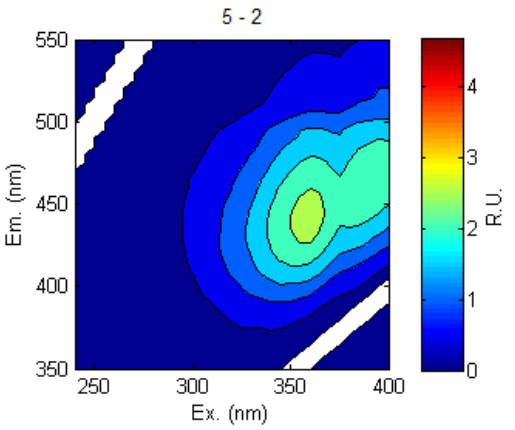
	Before denitrification experiment			After denitrification experiment		
Sample	a_{365} (cm^{-1})	a_{465} (cm^{-1})	a_{665} (cm^{-1})	a_{365} (cm^{-1})	a_{465} (cm^{-1})	a_{665} (cm^{-1})
1	0.088	0.022	0.009	0.101	0.02	0.004
2	0.767	0.138	0.016	0.644	0.142	0.031
3	0.139	0.019	0	0.149	0.03	0.003
4	0.081	0.019	0.011	0.055	0.015	0.008
5	0.378	0.038	0.001	0.326	0.055	0.015
6	0.539	0.179	0.057	0.127	0.015	0
7	0.22	0.033	0.01	0.215	0.023	0
8	0.019	0.013	0.013	0.025	0.002	0
9	0.201	0.043	0.006	0.202	0.051	0.017
10	0.497	0.147	0.036	0.438	0.115	0.02
11	0.178	0.021	0.001	0.188	0.021	0
12	0.076	0.007	0.001	0.119	0.014	0.001
13	0.246	0.028	0	0.235	0.032	0

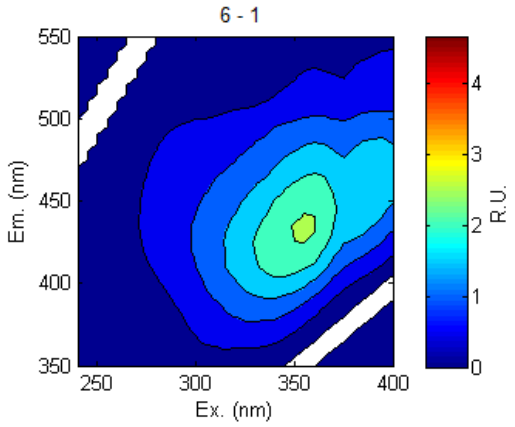
Fluorescence spectroscopy

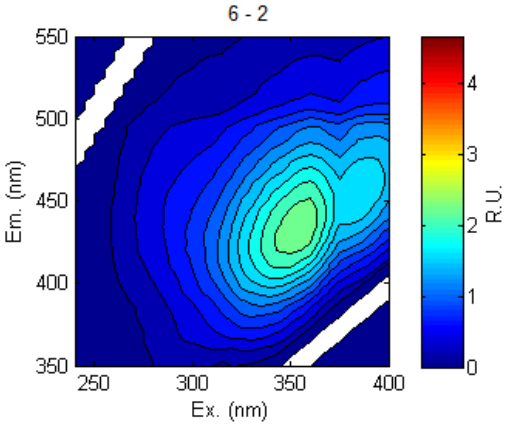
EEMs of all samples on a fixed scale

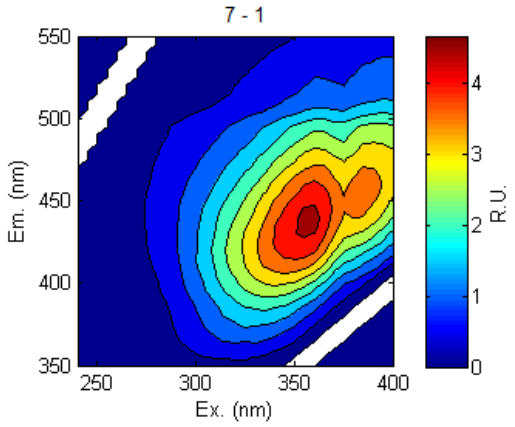


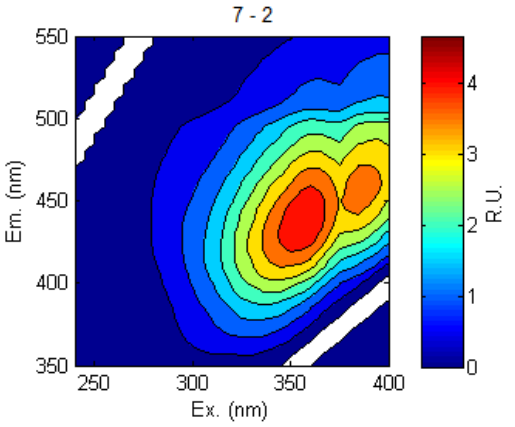

5 - 1
Em. (nm)
Ex. (nm)
R.U.

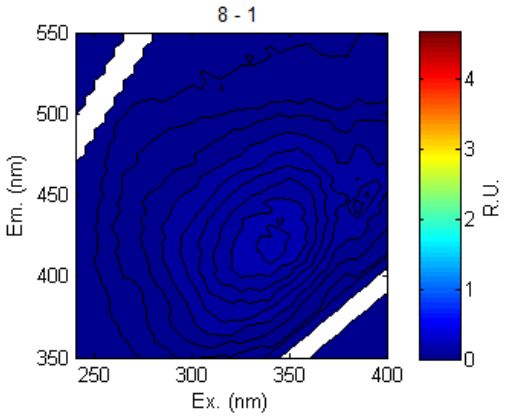

5 - 2
Em. (nm)
Ex. (nm)
R.U.

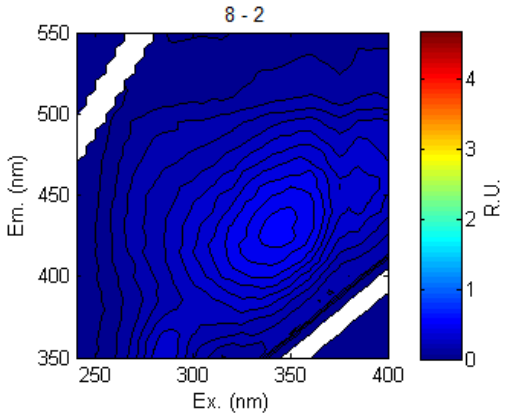

6 - 1
Em. (nm)
Ex. (nm)
R.U.

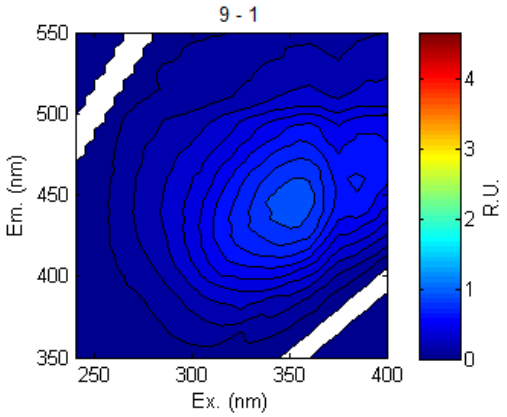

6 - 2
Em. (nm)
Ex. (nm)
R.U.

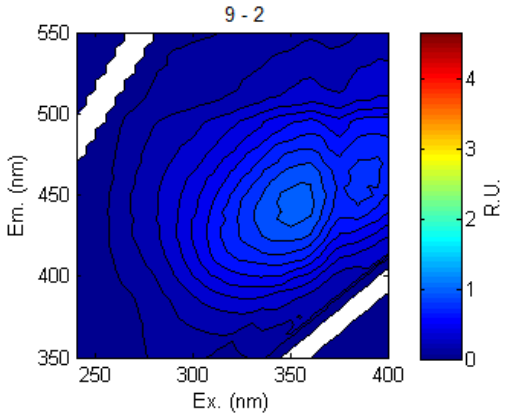

7 - 1
Em. (nm)
Ex. (nm)
R.U.

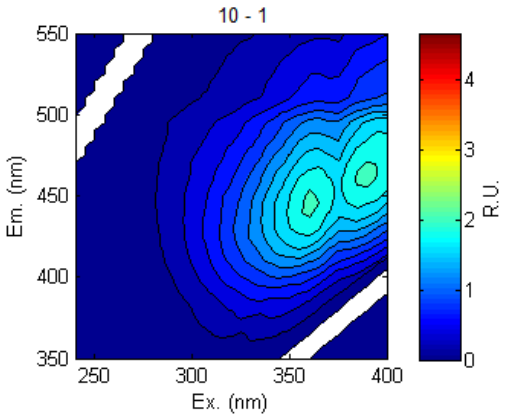

7 - 2
Em. (nm)
Ex. (nm)
R.U.

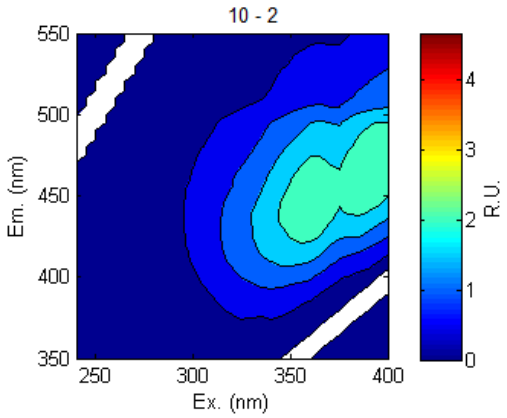

8 - 1
Em. (nm)
Ex. (nm)
R.U.

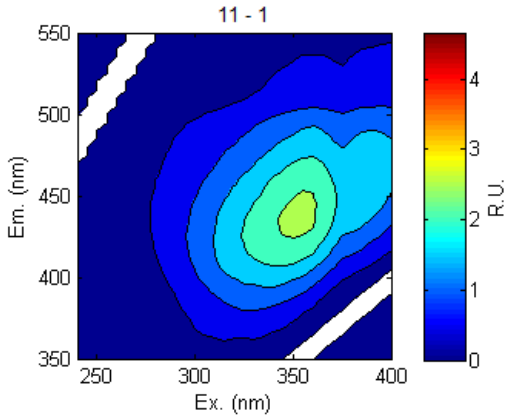

8 - 2
Em. (nm)
Ex. (nm)
R.U.

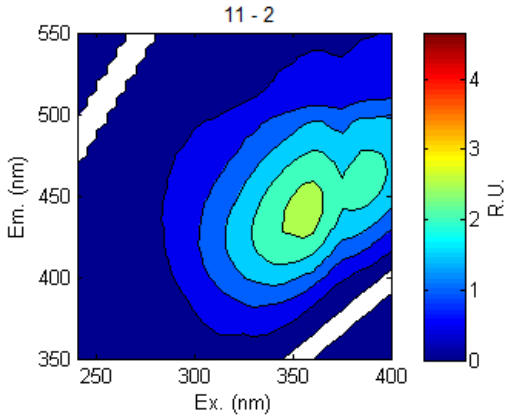

9 - 1
Em. (nm)
Ex. (nm)
R.U.

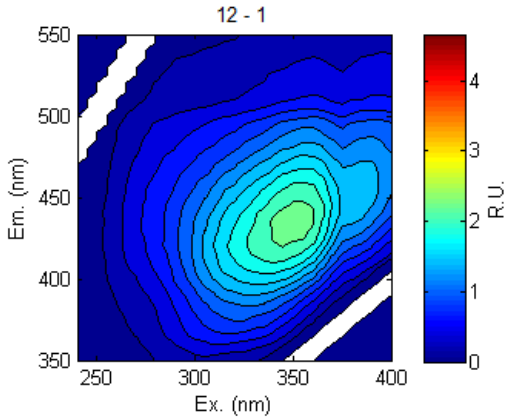

9 - 2
Em. (nm)
Ex. (nm)
R.U.

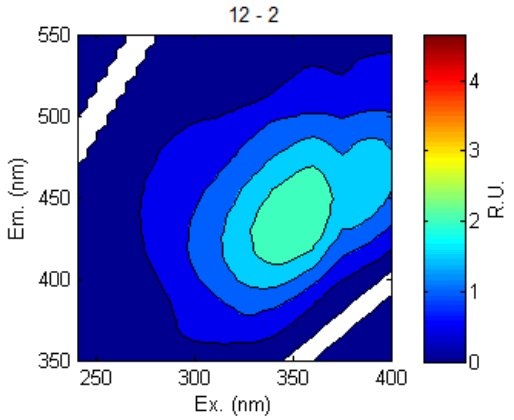

10 - 1
Em. (nm)
Ex. (nm)
R.U.

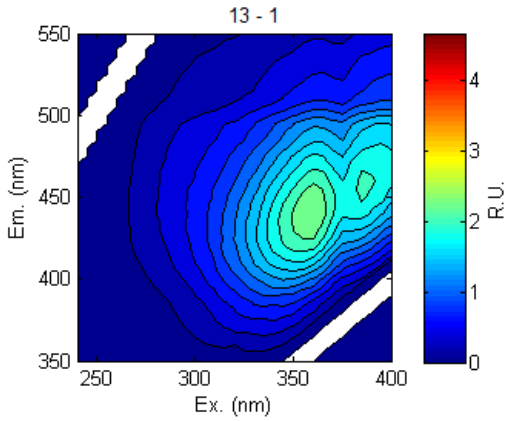

10 - 2
Em. (nm)
Ex. (nm)
R.U.

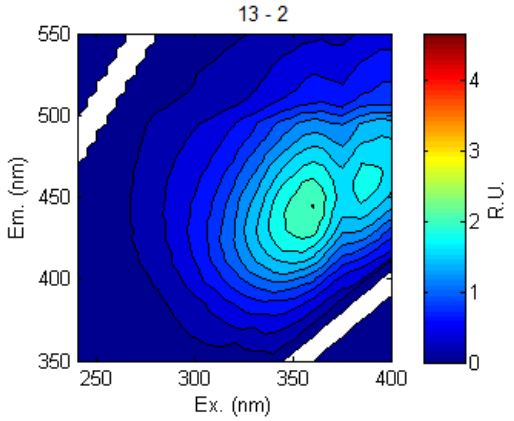

11 - 1
Em. (nm)
Ex. (nm)
R.U.


11 - 2
Em. (nm)
Ex. (nm)
R.U.

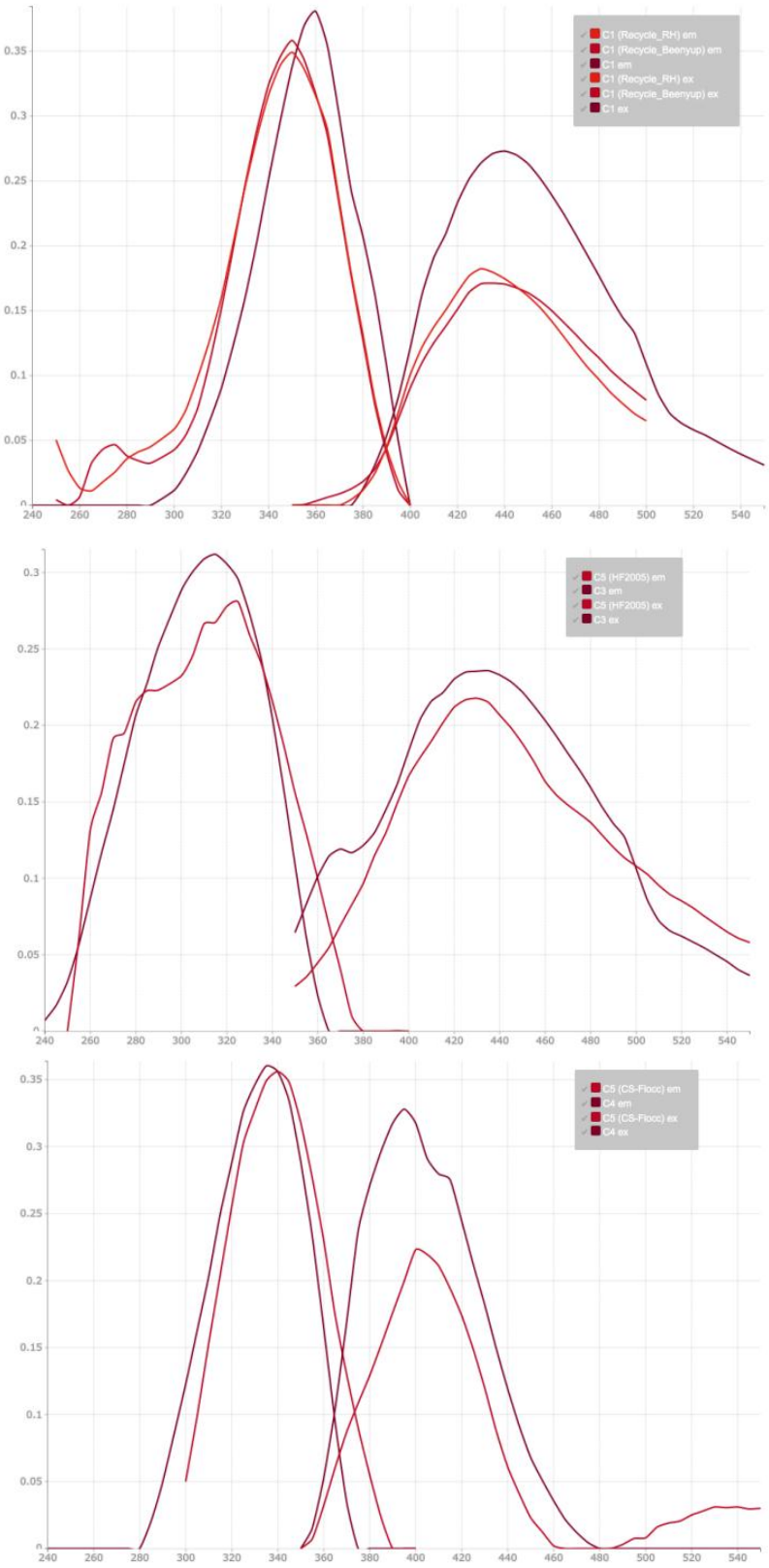

12 - 1
Em. (nm)
Ex. (nm)
R.U.


12 - 2
Em. (nm)
Ex. (nm)
R.U.


13 - 1
Em. (nm)
Ex. (nm)
R.U.


13 - 2
Em. (nm)
Ex. (nm)
R.U.

Correlations between excitation and emission fluorescence of PARAFAC components obtained in this study and components from previous studies accessible in Openfluor



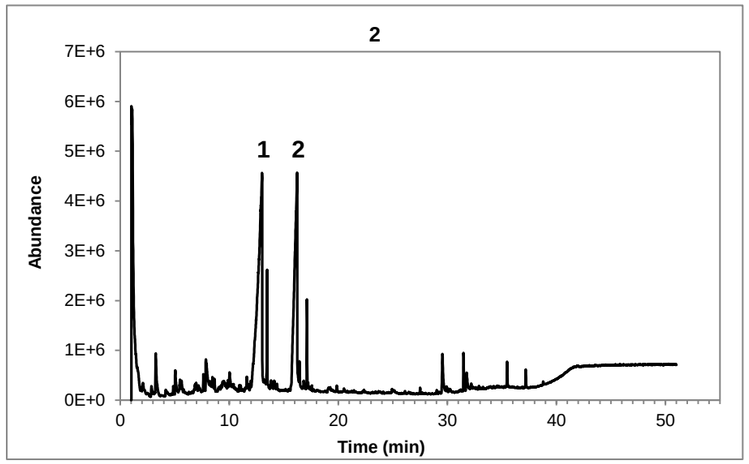
Appendix E: Pyrolysis Gas Chromatography Mass Spectrometry Results

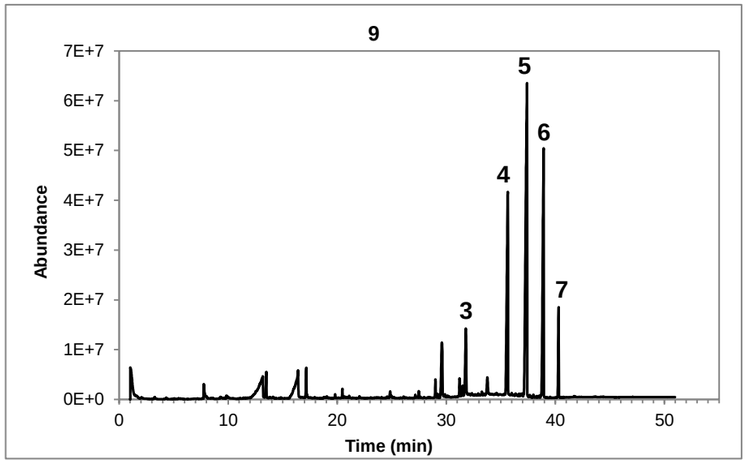
Groundwater samples 2, 3, 7, 9 and 13 were freeze dried and pyrolyzed using a Curie-Point pyrolyzer. Chromatograms for these samples can be found in fig E.1. Seven peaks were identified and the corresponding mass spectrum of each peak is shown in fig E.2. Compound analysis was performed in MassLab using a NIST library. Results are provided in table E.1

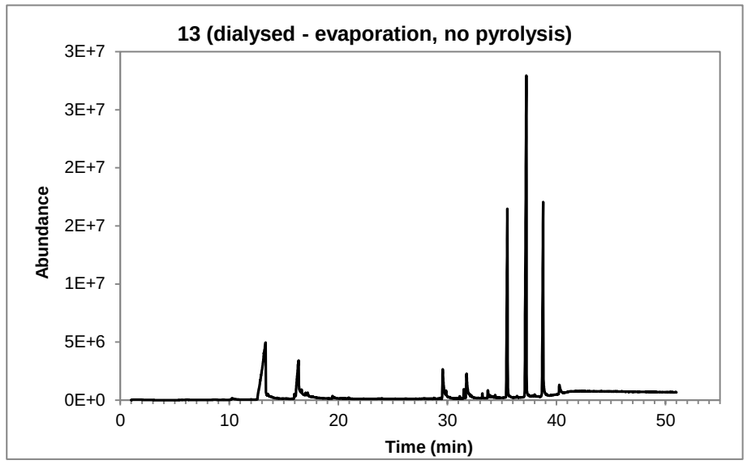
Table E.1: Identification of organic compounds

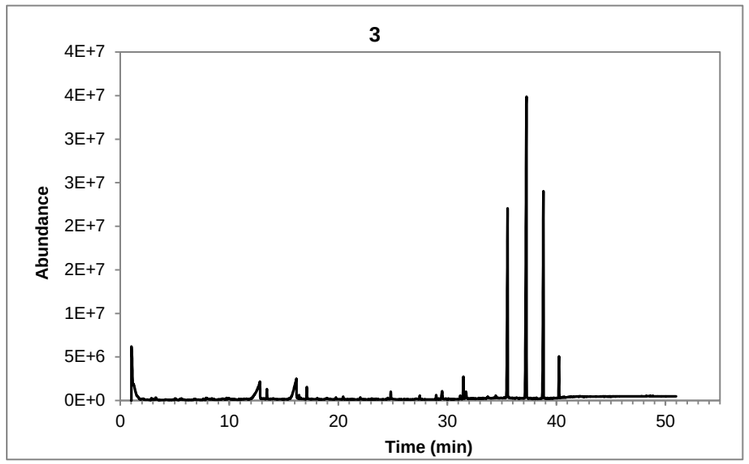
peak	Chromatogram reference time (min)	Organic compound (derived from NIST library)
1	13	Octanoic acid
2	16	Decanoic acid Glycine
3	31	Benzenedicarboxylic acid 3-nitro and other benzenecarboxylic acid groups Glycerol tricaprylate
4	36	Glycerol tricaprylate
5	37	Glycerol tricaprylate
6	39	Glycerol tricaprylate
7	40	Octanoic acid Decanoic acid, 1,2,3-propanetriyl ester Glycerol tricaprylate

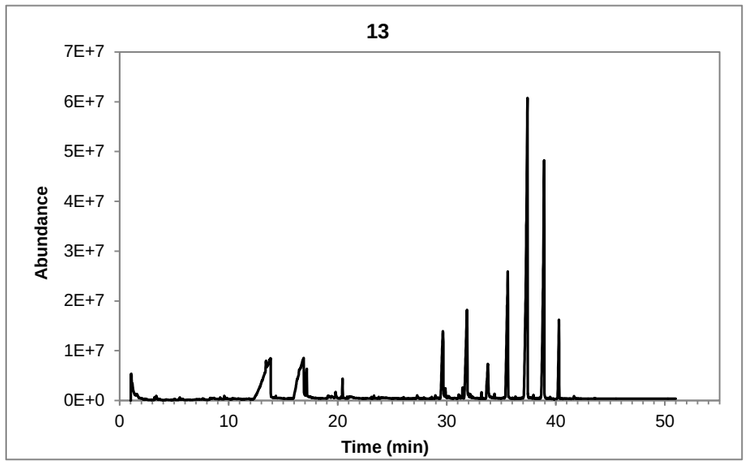
The pyrolysis signal is very uniform for all samples and is unlikely to be a natural source due to the distribution of sampling locations.
Dialysis tubing seemed to be a source for contamination (NPOC 25.88 mg/L when soaked in demi water for two days and water was analysed for TOC). Washing the tubing reduced the amount of TOC released from the tubing (again soaked in demi for two days, NPOC 0.9229 mg/L) but the pyrolysis signal on the chromatogram remained the same. Sample 13 was pyrolyzed without dialysis (fig) and the contamination remained. It was also attempted to evaporate the contamination by initially running the Py/GC/MS programme without pyrolyzing the sample, followed by the full pyrolysis procedure (fig C.1). Unfortunately removing the contamination by evaporation did not improve the chromatogram signal.
The contamination is characterized as octanoic or decanoic acids either free or bound to glycerol. It is currently unknown where the contamination in the pyrolysis signal is originating from. Possible sources include sampling equipment and freeze drying equipment. Natural sources for these substances are seed oils.

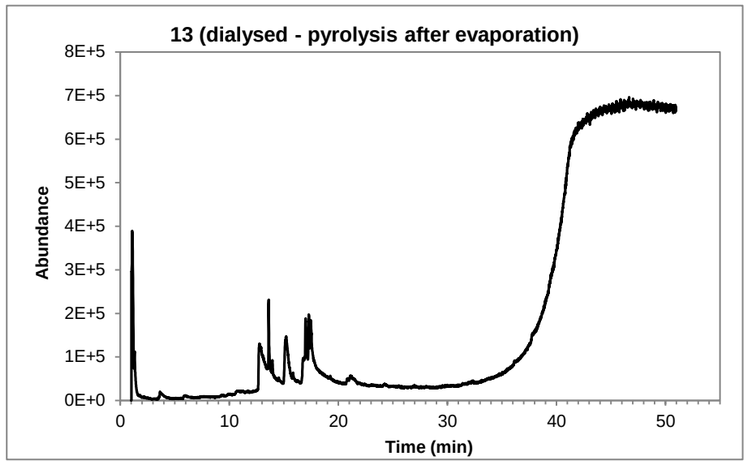


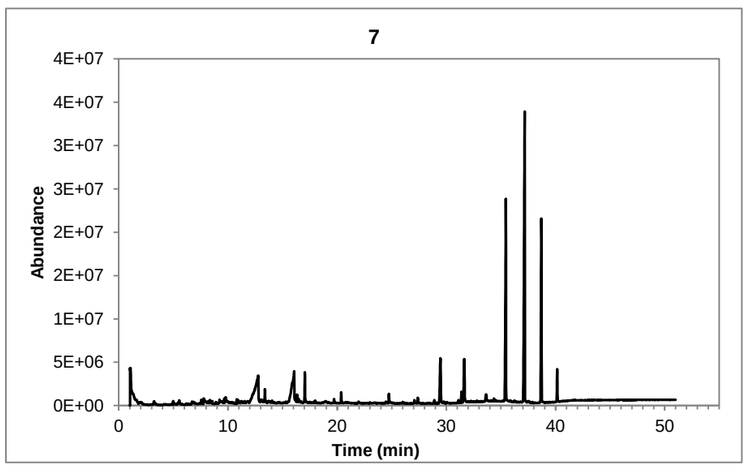












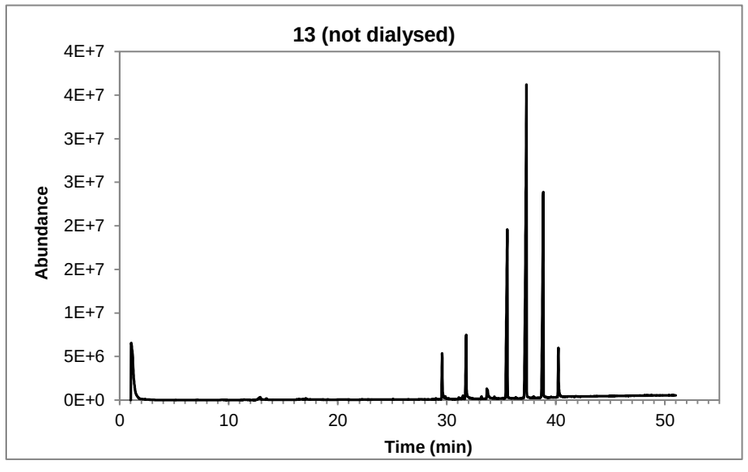
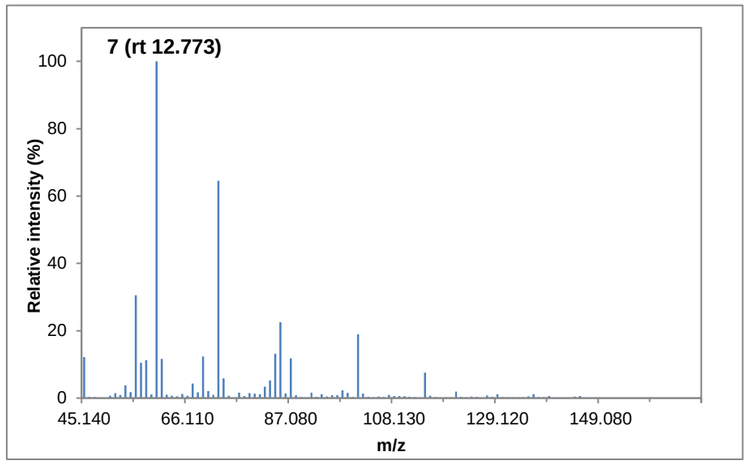
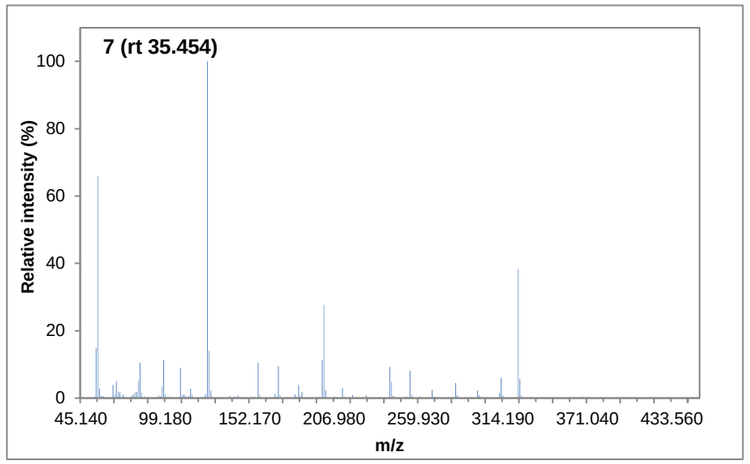
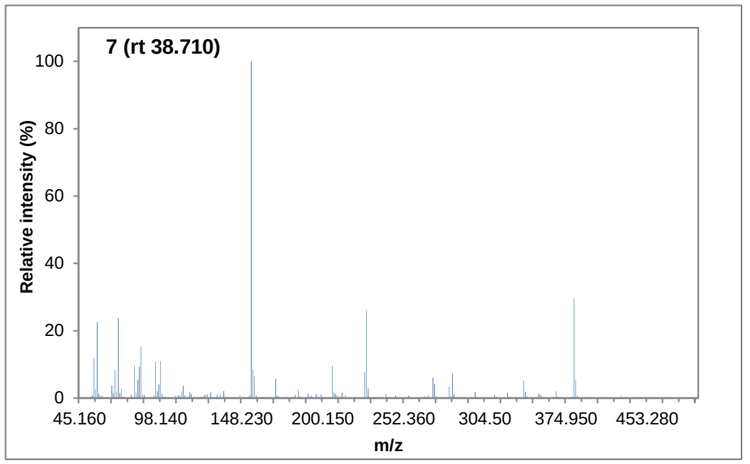
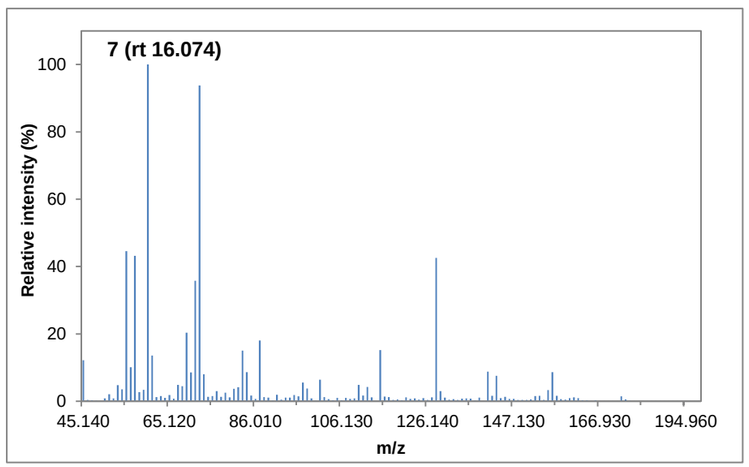


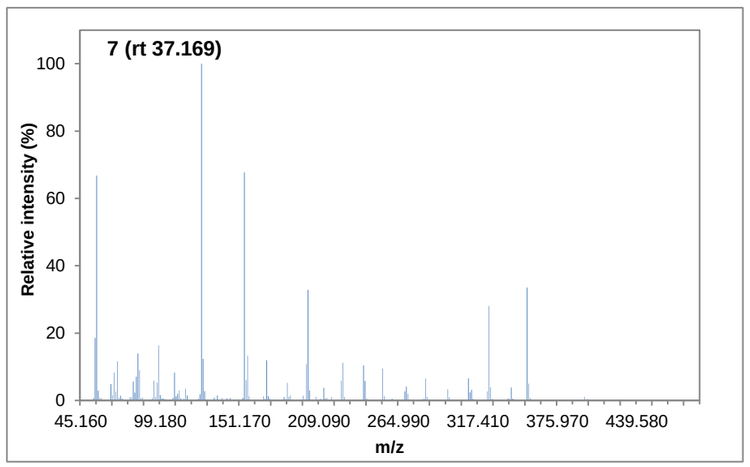
Fig E.1: Chromatograms for groundwater sample 2, 3, 7, 9 and 13.

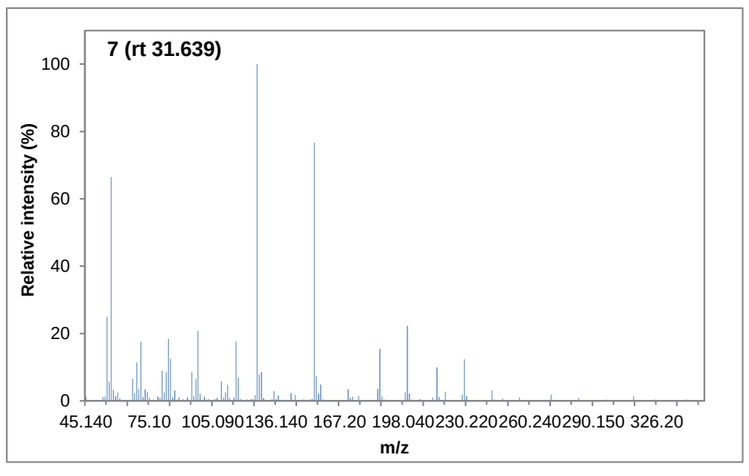












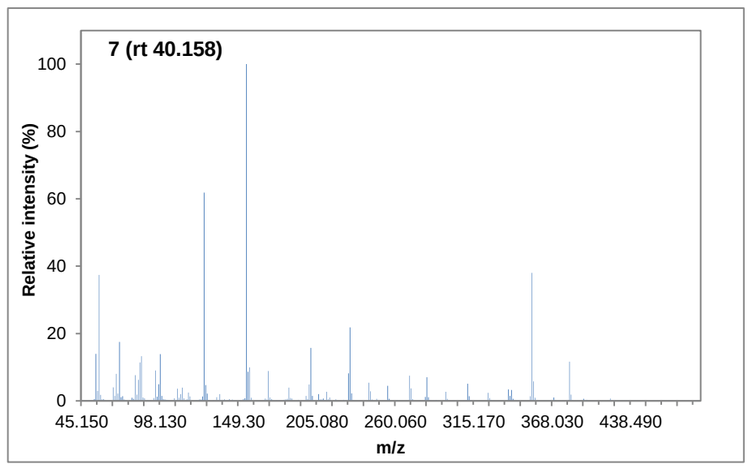


Fig E.2: Mass spectra for 7 peaks found in the chromatograms. In this case the mass spectra of sample 7 were used.

Appendix F: Electron balance

Electron consumption and production were calculated from concentration changes measured during the denitrification experiment.
Electron consumption was quantified by:

(1) The production of NO_2 by reduction of NO_3 to NO_2

$$NO_3^- + 2e^- \rightarrow NO_2^-$$

using the total amount of NO_2 produced (in µmol/L) at the end of the denitrification experiment and multiplying it by 2.

(2) Further reduction of the remaining NO_3 removed (remaining NO_3 = $TOT\Delta NO_3$ – $TOT\Delta NO_2$) to either N_2O or N_2,

$$2NO_3^- + 8e^- \rightarrow N_2O$$

$$2NO_3^- + 10e^- \rightarrow N_2$$

by substracting the amount of NO_2 produced from the amount of NO_3 removed and multiplying it by either 4 (for N_2O) or 5 (for N_2).

Electron consumption was then calculated by adding the amount of electrons from both pathways.

Electron production was quantified by:

(1) The loss of DOC due to mineralization

$$CH_2O \rightarrow CO_2 + 4e^-$$

using the amount of DOC decrease (in µmol/L) from the denitrification experiments and multiplying it by 4.

(2) Fe(II) oxidation

$$Fe^{2+} \rightarrow Fe^{3+} + e^-$$

using Fe(II) concentrations measured before the experiment and after the experiment.

(3) Anammox

$$2NH_4^+ \rightarrow N_2 + 6e^-$$

using the amount of NH_4 decrease (in µmol/L) from the denitrification experiment and multiplying it by 3. Note that anammox bacteria use NO_2 as electron acceptor ($NH_4^+ + NO_2^- \rightarrow N_2 + 2H_2O$) and no decrease in NO_2 is observed during the denitrification experiments. However as the observed NH_4 decrease is much smaller than NO_2 increase, it is assumed that NO_2 production from NO_3 reduction is much larger than NO_2 removal by anammox, and is not reflected in NO_2 measurements.

Electron production was then calculated by adding the amount of electrons from all three pathways.