

Non-destructive on-line and long-term monitoring of *in situ* nitrate and nitrite reactivity in a clay environment at increasing turbidity



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ABSTRACT

Reliable, on-line and high frequency data are essential for increasing our knowledge regarding nitrate (and nitrite) reactivity in a clay formation surrounding a repository for nitrate-containing nuclear waste. In this paper, the performance and applicability of an on-line UV spectrophotometer and pH and redox probes for real-time monitoring of *in situ* nitrate and nitrite reactivity in Opalinus Clay water has been evaluated, as part of the Bitumen-Nitrate-clay interaction (or BN) experiment at the Mont Terri Rock Laboratory (Switzerland). Standardly, the spectrophotometer uses a multiwavelength-based algorithm combined with Partial-Least-Square regression analysis. This technique provides highly reliable measurements of the nitrate and nitrite concentrations (relative standard deviation of 0.3–1.7% for NO_3^- and 0.1–3% for NO_2^-) in artificially prepared Opalinus Clay water, even in the presence of high amounts of other UV absorbing species (e.g. chloride). Although a severe sensitivity of the nitrite measurements to turbidity was shown using the multiwavelength-based algorithm, accurate nitrite measurements based on UV absorbance spectra are still possible, even in turbid conditions, when applying a novel single wavelength-based algorithm including normalisation of the UV spectra for turbidity. A similar new algorithm was developed for accurate measurements of nitrate concentrations, higher than the limit of quantification using the multiwavelength-based algorithm and even in turbid conditions. Furthermore, as no organics or significant amounts of chloride are leaching from the pH and redox electrodes during long periods, its polymeric interface is proven very stable, demonstrating its long-term *in situ* applicability. Based on the results of an *in situ* test in the BN borehole in the Opalinus Clay, the combined on-line equipment provides detailed and accurate monitoring data of the *in situ* nitrate and nitrite reactivity. The probes immediately react upon changes in the nitrate and nitrite concentrations, enabling the detection and identification of intermediate and/or fast processes, which are frequently missed when only performing off-line analyses on sampled solutions. This is especially valuable for *in situ* experiments such as the BN experiment where the sampling frequency is necessarily limited and microbial processes can occur at a high rate. This on-line equipment is thus an efficient tool to assess the reactivity of nitrate and nitrite under *in situ* conditions.

1. Introduction

Nitrate and nitrite are important chemically and biologically reactive species to be monitored in the environment due to their direct toxicity and/or their role in the production of carcinogenic nitrosamines in e.g. food, but also as a source for denitrification for many wastewater treatment plants (e.g. WHO, 2016; Paredes et al., 2007). In addition, nitrate and nitrite are also key contaminants to be taken into account when considering disposal of nitrate-containing waste, either because they could enter and perturb the biosphere (especially in case

of near-surface disposal), or because they might affect the properties of the host rock of a deep geological disposal facility (in case of intermediate-level radioactive waste). For nitrate-containing bituminised intermediate-level long-lived waste, several countries like Belgium and France are investigating the possibility of a deep geological disposal inside a clay formation (Andra, 2005; ONDRAF/NIRAS, 2009).

To study the fate of nitrate and nitrite in the near field of such a waste repository, an *in situ* experiment named Bitumen-Nitrate-clay interaction (BN) experiment is currently being performed. In this experiment, (bio)chemical nitrate and nitrite reactivity and transport are

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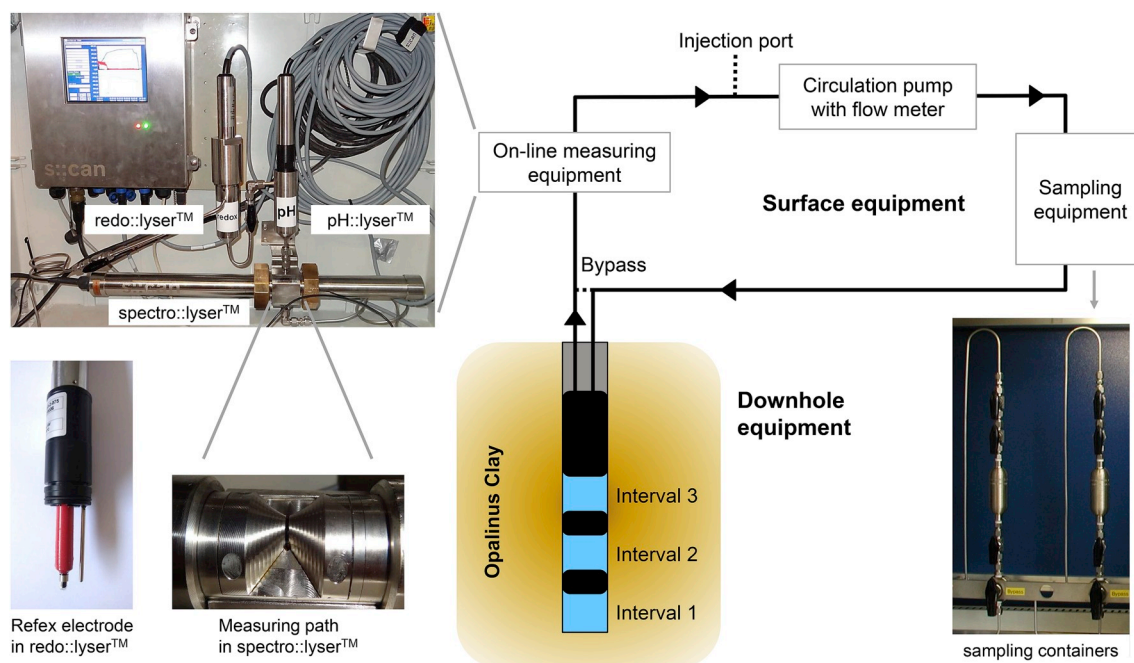


Fig. 1. Schematic overview of the set-up of the BN experiment. For more details the reader is referred to Bleyen et al. (2017).

studied inside closed-off anoxic intervals (or water chambers) in a borehole in the Opalinus Clay at the Mont Terri Rock Laboratory (Switzerland) (Bleyen et al., 2017). The solution is circulated continuously from the borehole to the surface equipment in a closed circuit, allowing both on-line monitoring of several important parameters as well as limited intermediate solution sampling and off-site analysis.

As microbial reactions can occur very fast, accurate and well-timed assessment of the nitrate (and nitrite) concentration is crucial. Numerous techniques are currently available for off-line measurement of nitrate and/or nitrite. Most are optimised for natural waters, biological fluids or water from waste water treatment plants: e.g. colorimetrically after reduction of nitrate to nitrite and diazotising the produced nitrite into an azo dye (Burakham et al., 2004; Colman, 2010), by chromatography (Helmke and Duncan, 2007; Jobgen et al., 2007; Schwarz et al., 2011; Tsikas et al., 2010), by chemiluminescence after reduction of nitrate to nitrite (Yaqoob et al., 2012), by capillary electrophoresis (Miyado et al., 2004) or by infrared spectroscopy (Choe et al., 2010; Jahn et al., 2006). However, these methods are only suitable for off-line application, because they require sample pre-treatment. In many (*in situ*) experiments, such as the BN experiment, sampling of the borehole solution is however limited to the removal of a fixed number of sampling containers from the circulation. Since reactivity of and in the clay surrounding the borehole is of interest, the number of sampling containers is limited to avoid decreasing the solid/liquid ratio. Direct sampling by opening the circuit could perturb the *in situ* system (*i.e.* causing volume, water pressure and/or redox condition changes) and is therefore not allowed. To be able to monitor the *in situ* nitrate and nitrite evolution without disturbing the system by sampling, high-frequency non-destructive and simultaneous analyses of nitrate and nitrite are essential. Because nitrate and nitrite have distinctive spectrophotometric properties and absorb light in the UV range [at 205.6 and 301.6 nm for nitrate and at 212.8 and 353.9 nm for nitrite (Sung, 2011; Thomas and Burgess, 2007)], a UV spectroscopy-based analysis system was chosen for the BN experiment.

(Bio)chemical reduction of nitrate leads to the generation of either nitrite, nitrogenous gases (denitrification) or ammonium (Madigan et al., 2000). Nitrite can thus be produced as intermediate product, but can itself also be reduced either microbially or purely chemically [*e.g.* abiotic vs biotic nitrite reactivity in clay water assessed by Bleyen et al.

(2016)]. Each of these processes can also perturb the pH and the redox potential of the system (Bailey and Beauchamp, 1971). Real-time follow-up of the pH and redox potential would therefore provide additional information when characterising nitrate and nitrite reactivity. However, certain electrodes contain organic polymers in the reference compartment, which release significant amounts of organic carbon (glycerol) when applied for long-term measurements (De Cannière et al., 2011). As this release of organics can stimulate certain microbial reactions, causing (bio)geochemical perturbations, any leaching of organics must be avoided.

In this paper, the performance of a real-time high-frequency UV spectroscopic method (using the spectro:lyser™; S:can Messtechnik GmbH, Austria) for long-term determination of nitrate and nitrite concentrations during *in situ* microbial nitrate reduction is evaluated and compared to traditional sampling and analysis by ion chromatography. Furthermore, the long-term applicability of on-line pH and redox electrodes (pH:lyser™ and redo:lyser™; S:can Messtechnik GmbH, Austria) is assessed. Although often used for effluent, surface and well water-quality monitoring (Grayson and Holden, 2012; Jeong et al., 2012; Langergraber et al., 2004; Rieger et al., 2004, 2006; Waterloo et al., 2006), *i.e.* dissolved organic carbon (DOC) measurements and follow-up of aerobic (de)nitrification processes, the selected UV spectrophotometer has never been used to follow up anaerobic nitrate reduction processes during long-term *in situ* experiments (without possibility for intermediate cleaning). The sensitivity of the nitrate and nitrite measurements to other important UV absorbing compounds in Opalinus Clay water (*e.g.* Cl^- , SO_4^{2-} , HCO_3^- , dissolved organic carbon and suspended solids) is investigated, as well as the cross-sensitivity. Furthermore, a new method for calculating the nitrite concentration based on normalised UV spectra was developed, to eliminate any interference of measurements due to turbidity. A similar procedure was established for the measurements of high nitrate concentrations (beyond the limit of quantification when using the default algorithm) in non-turbid and highly turbid solutions. Both methods are described and evaluated here. Finally, the application of this equipment and analyses methods in the BN experiment is discussed, showing its suitability to assess the fate of nitrate and nitrite in a clay environment.

Table 1

Chemical composition of the artificial pore water (APW) used to saturate the intervals and to calibrate the on-line UV spectrophotometer. The pH of this solution was 7.8.

Component	Concentration (mM)
Na ⁺	162
K ⁺	1.05
Ca ²⁺	12.6
Mg ²⁺	8.6
Sr ²⁺	0.4
Cl ⁻	181
SO ₄ ²⁻	12.28
TIC	2.8

2. Materials and methods

2.1. Design of the BN experiment

The BN *in situ* experiment is performed in a borehole drilled in the Opalinus Clay at the Mont Terri Rock Laboratory (Switzerland). The experimental set-up and its installation have been described in detail by Bleyen et al. (2017). Fig. 1 shows a schematic overview of the BN experimental set-up. Briefly, the downhole equipment consists of three anoxic test chambers (or intervals) of ~90 cm long, isolated from each other and from the gallery surface by inflatable packers to avoid both cross-contamination and oxygen ingress from the gallery atmosphere. The intervals are in contact with the surrounding clay through a cylindrical sintered stainless steel filter screen surrounding the central stainless steel tube. Stainless steel water lines connect each of the downhole intervals to a water sampling unit (with sampling containers and a sampling port) and an on-line chemical monitoring unit inside the gallery of the Rock Laboratory (see “surface equipment” in Fig. 1). To limit both desaturation and oxidation of the clay surrounding the BN borehole, the three intervals were filled anoxically with deoxygenated Artificial Opalinus Clay Pore Water (APW; Table 1), immediately after installation of the downhole equipment. The chemical composition of the APW was derived based on the relationship between sulphate, cations and the chloride content (Pearson et al., 2003) and the relationship between the chloride concentration and the location of the BN experiment within the Opalinus Clay at Mont Terri, as discussed in detail by Bleyen et al. (2017). This calculated chemical composition was validated by the composition of the interval solution after ~8 months of equilibration with the surrounding clay. No significant differences were found between the composition of the injected APW (and thus the composition of the pore water expected at the location of the BN experiment) and of the interval solutions, except for a higher inorganic carbon content in Interval 1 (5.2 mM inorganic carbon) and the presence of dissolved organic matter (DOM; ~0.8 mM organic carbon) in all intervals (Bleyen et al., 2017). This dissolved organic matter had probably diffused into the intervals during the equilibration period. Its concentration is in agreement with those found in other Opalinus Clay boreholes (Courdouan et al., 2007). Furthermore, the interval solutions are characterised by a near-neutral pH (pH between 7 and 8) (Bleyen et al., 2017).

The water in each of the intervals is continuously circulating from the downhole equipment to the surface equipment and back using a magnetic drive gear pump combined with a flow meter. This circulation results in a homogenous solution throughout the circuit of each interval and allows monitoring of the chemical composition of the interval solutions. Each circuit is equipped with five sampling containers (40 or 150 mL; Fig. 1), which can be safely disconnected when additional chemical and/or microbial analyses are required, without causing a perturbation to the geochemistry of the interval solution. Additional sampling is also possible by collecting the interval solution into an argon-flushed sterile septum bottle via a needle valve (“sampling port”

in Fig. 1). However, as sampling via the needle valves perturbs the water pressure and the total solution volume in the interval, this sampling method is only performed at the start and at the end of the injection tests. To monitor the real-time nitrate and nitrite concentrations and the pH and redox potentials in the intervals, a UV spectrophotometer and pH and redox probes (spectro::lyser™, pH::lyser™ and redo::lyser™ from S::can Messtechnik GmbH, Austria) are installed in the circuit (Fig. 1) inside gas-tight stainless steel flow-through cells (Section 2.3.1-2) at the time of the injection tests with nitrate (Section 2.2). The circulating solution flows continuously through the slit (*i.e.* measuring path) of the spectrophotometer and in the compartments of the electrodes, enabling real-time monitoring of the chemical composition.

This on-line monitoring equipment can be recalibrated and connected to another interval at the start of each injection test. At that time, the housing and the slit of the UV spectrophotometer and the electrodes are cleaned by rinsing with ethanol and later thoroughly with deionised water. However, during a single test, cleaning, and disconnecting of the equipment is not allowed, since this would cause perturbations of the system (*i.e.* water pressure changes, microbial and/or chemical contaminations, O₂ ingress etc.).

2.2. Injection of the borehole interval with nitrate

To assess the microbial nitrate reduction processes in Opalinus Clay water, several *in situ* tests were performed in the BN borehole. During these tests, the BN intervals were injected separately with APW solution containing nitrate, nitrite and/or acetate. One of these injection tests is discussed here, to illustrate the efficiency of the on-line monitoring equipment installed in the BN experiment. The injected APW solution was prepared anaerobically (Ar atmosphere with [O₂] < 5 × 10⁻⁴ vol %). Care was taken to prevent O₂ ingress and microbial contamination during injection.

The injection test discussed here was performed in Interval 2 (test code INT2_2013). For this test, the interval was injected with nitrate by replacing only the solution in the surface circulation loop with a new APW solution containing NaNO₃ while bypassing the downhole equipment (Fig. 1). More details on the procedure are described by Bleyen et al. (2017). The solution for injection of Interval 2 contained 59 mM NaNO₃. After replacement of the solution in the surface equipment, the bypass was opened and the solution was circulated at 40 mL min⁻¹ through the entire circuit to mix with the remaining solutions in the downhole equipment. After this overnight homogenisation period, the flow rate was decreased to 10 mL min⁻¹ for the remainder of the tests. The nitrate concentrations after overnight homogenisation are considered the starting concentrations of each test, *i.e.* about 27 mM NO₃⁻.

After 70 days of circulation, a pulse injection with acetate was performed to study the effect of acetate on the nitrate reduction processes. The pulse injection was performed by reconnecting one of the sampling containers (40 mL) filled with freshly prepared APW containing nitrate at the concentration present in the interval at the time of reconnection (*i.e.* ~16 mM NO₃⁻), and ~310 mM sodium acetate. After dilution of the injected acetate in the remaining interval solution by circulating the solution for 4 h at 40 mL min⁻¹, the concentration was ~80 times diluted to reach a final concentration of ~4 mM acetate. Afterwards, the flow rate was decreased again to 10 mL min⁻¹.

Throughout the test, the evolution of the nitrate and nitrite concentrations in the interval solution was monitored continuously using an on-line UV spectrophotometer with a 5 mm path length (Section 2.3.1). To evaluate the *in situ* robustness of these on-line measured nitrate and nitrite concentrations, the on-line results were compared with those obtained by ion chromatography (IC) performed on samples collected regularly (Section 2.3.3). The evolution of the pH and redox potential in the interval solutions was recorded with on-line pH and redox probes (Section 2.3.2).

2.3. Monitoring of the *in situ* chemical composition

2.3.1. On-line monitoring of nitrate and nitrite concentrations

A submersible multi-parameter UV spectrophotometer (spectro::lyser™) connected to the S::can computer system with data logger (Fig. 1) was used for the high-frequency on-line measurements of nitrate and nitrite. The spectro::lyser™ records absorbance spectra in the UV wavelength range between 200 and 400 nm, displays the results in real-time and saves them in an internal data logger connected to an S::can computer system (con::stat or con::cube; S::can Messtechnik GmbH, Austria). The probe is a dual beam 256-pixel UV spectrophotometer, with a Xenon lamp as a light source (Langergraber et al., 2003). The measurement range of the probe depends on the optical path length, which can range from 2 to 100 mm. For the BN experiment, two probes with different path lengths (5 or 10 mm) are used, with upper measuring limits (as specified by the manufacturer) of 29 mM NO₃⁻ and 54 mM NO₂⁻ (for 5 mm path length), and 15 mM NO₃⁻ and 27 mM NO₂⁻ (for 10 mm path length).

To determine the concentrations of nitrate, nitrite and other parameters simultaneously, the spectro::lyser™ standardly applies a multi-wavelength-based algorithm (or MW algorithm), correlating the concentration of the required determinants to the absorbance at certain wavelengths and using a Partial-Least-Square (PLS) regression for each of the parameters of concern (Langergraber et al., 2003; Rieger et al., 2004). In this MW algorithm, the nitrate and nitrite concentrations are determined using 5 wavelengths (between 230 and 245 nm) for each species (van den Broeke et al., 2006). As turbidity in the solution causes light scattering and shading, it may influence the absorbance over the entire spectrum (depending on the origin and the level of turbidity). The nitrate and nitrite concentrations are therefore calculated based on turbidity-compensated absorbance spectra, included in the spectro::lyser™ MW algorithm. This turbidity compensation is based on a mathematical equation describing the relationship between light scattering intensity and the wavelength as a function of the particle diameter (Langergraber et al., 2003). The value for turbidity itself is determined based on the estimated concentration of suspended solids, which is measured at 7 wavelengths ranging from 230 to 330 nm (Rieger et al., 2004).

Though the spectro::lyser™ is delivered with a default calibration of the MW algorithm (provided by S::can; taking into account the matrix composition for the BN experiment), the manufacturer encourages the user to verify and adjust this calibration slightly for local matrix effects. This ‘local’ calibration of nitrate and nitrite measurements was therefore performed before each BN injection test.

In some tests of the BN experiment, nitrate is injected at a concentration higher than the upper limit of quantification of the spectro::lyser™. To enable monitoring of the nitrate evolution in such cases, the nitrate concentrations could be determined based on the raw (non-compensated) UV spectra obtained with the spectro::lyser™. However, during long-term monitoring in the BN tests, microbial growth and the contact between the interval solution and the surrounding clay lead to a suspension of organic and clay particulates in the solution and an accumulation of these compounds in the slit of the spectro::lyser™. This accumulation perturbs the absorbance spectra by light scattering and results in an apparently high turbidity level. This perturbation is more intense in the UV than in the visible region (see Mendeley Dataset available in Bleyen et al., 2018), which is related to the organic nature of the particulates/colloids and their physical properties (Thomas and Burgess, 2007), but also causes an apparent increase in the absorbance peaks of nitrate. To avoid this, a new method to quantify ‘high’ nitrate concentrations in turbid solutions was developed, including a non-standard correction of the spectra for turbidity. For this, all acquired absorption spectra $[A(\lambda)]$ are first normalised by subtracting the baseline between 260 and 330 nm (derived by linear regression of absorbance A versus wavelength λ) from the measured UV spectrum:

$$A_{norm}(\lambda) = A(\lambda) - (b + m \lambda)$$

where b is the offset and m the slope of the baseline between 260 and 330 nm (see Mendeley Dataset available in Bleyen et al., 2018). This baseline was chosen based on the shape of the UV spectra for APW with different nitrate and nitrite concentrations and with/without turbidity. These two wavelengths correspond to absorption minima before and after the absorbance peak of nitrate at ~301 nm. The data provided in the Mendeley Dataset (Bleyen et al., 2018) demonstrate that the described normalisation of the UV absorbance spectra indeed provides turbidity-compensated spectra, which are suitable for calibration using the absorbances at certain wavelengths. Either the normalised absorbance at 230 nm $[A_{norm}(230)]$ or the maximum of the normalised absorbance peak at ~301 nm $[A_{norm}(301)]$ can be used in a univariate Least-Squares regression to calculate the nitrate concentration. Absorbances at wavelengths shorter than 230 nm (e.g. nitrate peak at ~206 nm) are not usable due to an oversaturation of the detector, especially in highly turbid solutions.

As a strong bias was observed for nitrite in turbid conditions when the MW algorithm was applied, a similar procedure was developed to calculate the nitrite concentration: normalisation of the UV spectra by subtracting the baseline between 300 and 390 nm, followed by calculation of the nitrite concentration based on the normalised absorbance at 245 nm $[A_{norm}(245)]$ (see Mendeley Dataset available in Bleyen et al., 2018).

In the remainder of this paper, we refer to the locally calibrated multiwavelength-based algorithm provided by S::can Messtechnik GmbH as the ‘MW algorithm’. The single-wavelength-based algorithm or ‘SW algorithm’ refers to the newly developed approach using normalised UV spectra as described above.

2.3.2. On-line monitoring of pH and redox potential

To follow-up changes in pH and redox potential due to nitrate reactivity in the interval solution, an on-line pH (pH::lyser™) and redox probe (redo::lyser™) were used, connected to the S::can computer system with a data logger (Fig. 1).

Both probes contain a Reflex reference system (Reflex Sensors Ltd, Ireland), which allows the separation of the interval solution from the Ag/AgCl reference electrode compartment by means of ceramic frit junctions covered with a Reflex polymeric interface (consisting of a polyvinyl acetate resin and doped with KCl). This double junction design eliminates the need for a standard porous liquid junction, thereby reducing the leaching of KCl from the electrode. Furthermore, due to the presence of KCl in the Reflex polymeric interface, the entire outside surface of the electrode is electrochemically active, which should provide a junction potential that is quick to stabilise and relatively constant with time, even in low ionic strength media (Diamond et al., 1994). To further stabilise the measurements, both probes are equipped with a temperature sensor and the pH and Eh measurement is corrected for the temperature.

2.3.3. Chemical analyses of sampled solution

After sampling of the interval solution, the samples were stored anaerobically ($[O_2] < 5 \times 10^{-4}$ vol%) at 4 °C until analysis, to slow down any reaction as much as possible until analysis. Subsamples of the solution were taken anaerobically and subsequently analysed at SCK-CEN by IC (Dionex™ DX600 with IonPac™ AS4A-SC column, Thermo Fisher Scientific, Waltham, USA) equipped with suppressed conductivity detection for NO₃⁻, NO₂⁻ and CH₃COO⁻ concentrations. The uncertainties on these concentrations are 5.5, 6.5 and 10% (for a 95% confidence interval), respectively.

2.4. Performance of the on-line equipment in APW

2.4.1. UV spectrophotometer

Local calibration of the spectro::lyser™ using the MW algorithm for

nitrate and nitrite measurements was performed using APW solutions with concentrations up to 40 mM NaNO₃ or 44 mM NaNO₂. Afterwards, the sensitivity of this method to organic compounds, chloride content in APW and cross-sensitivity between nitrate and nitrite measurements were evaluated. For this, APW solutions with broad ranges of NaNO₃ (0–40 mM), NaNO₂ (0–44 mM) and CH₃COONa (0–8.5 mM) were prepared and measured with the spectro:lyser™. In addition, the impact of changing the composition of APW was tested by preparing a range of diluted APW solutions (25–50 vol%) containing NaNO₃ (0–16 mM) or NaNO₂ (0–4.4 mM). The measured nitrate and nitrite concentrations were compared to the nominal concentrations of the prepared solutions. These nominal concentrations were verified by IC (see Section 2.3.3 for specifications) performed on randomly chosen APW solutions with nitrate and/or nitrite and on the stock solutions used to prepare the dilutions.

The effect of an increase in turbidity on the nitrate and nitrite measurements was studied by evaluating nitrate- (0–7 mM NO₃⁻) or nitrite- (0–11 mM NO₂⁻) containing APW to which nitrate reducing bacteria (NRB) were added (7×10^6 to 2×10^8 cells mL⁻¹), resulting in a turbidity of up to 70 FNU. The turbidity was measured using the spectro:lyser™ applying the MW algorithm (as discussed in Section 2.3.1). Note that this turbidity value was not verified for accuracy before testing. This value should therefore be considered as an estimated value. The NRB population was grown anaerobically by inoculating Boom Clay piezometer water in NRB medium (Mariën et al., 2011) for 10 days at 30 °C. Afterwards, the culture was centrifuged for 20 min at 4180 g (Hermle Labortechnik Compact Centrifuge Z206A) and the bacterial pellet was washed with APW (repeated twice). The nitrate and nitrite concentrations in the turbid solutions were measured with the spectro:lyser™ (using the MW algorithm) immediately after preparation, to avoid microbial nitrate or nitrite reduction before UV absorption measurement.

Furthermore, the effect of higher turbidity levels on the *in situ* measurement of nitrate and nitrite was assessed using real interval solutions containing nitrate (0–27 mM NO₃⁻) or nitrite (0–4 mM NO₂⁻), during the BN injection tests [more details provided by Bleyen et al. (2017)]. In these long-term *in situ* tests, turbidity values increased continuously, starting from 20 to 3500 FNU. The turbidity level and the nitrate and nitrite concentrations were determined with the spectro:lyser™ applying the MW algorithm (for turbidity, nitrate and nitrite) and the SW algorithm (for nitrate and nitrite). For all sampled interval solutions, the nitrate and nitrite concentrations determined spectrophotometrically were compared to those obtained by IC (see Section 2.4.3).

The uncertainties on the measured nitrate and nitrite concentrations are calculated as combined uncertainties (for a 95% confidence interval). This combined uncertainty takes into account the uncertainty on the standard solutions for calibration (5.5% for NO₃⁻ and 6.5% for NO₂⁻), the repeatability (expressed as relative standard deviation or RSD; n = 3 to 6) and the uncertainty on the calibrated data based on the least-squares method (Skog et al., 1996). The detection limits for nitrate and nitrite measurements are calculated according to the recommendations of the International Union of Pure and Applied Chemistry (IUPAC, 2014):

$$x_L = \bar{x}_{blank} + k s_{blank}$$

where x_L is the detection limit, \bar{x}_{blank} the average value for the blank solutions, k the statistical coverage factor ($k = 2$ for a 95% confidence interval) and s_{blank} is the RSD for the blank solution.

Sensitivity of parameters to other compounds (e.g. cross sensitivity between nitrate and nitrite) is assessed by performing a multiple regression analysis on all parameters including interaction terms and using a *t*-test (with H₀: no sensitivity; $\alpha = 0.05$) and by taking into account the combined uncertainties on the measurement values as calculated above.

2.4.2. pH and redox electrodes

To assess a possible leaching of dissolved organic matter and chloride from the pH electrode, two tests were performed. For this, the pH electrode was immersed at room temperature for 2 to 3 months in 840 or 305 mL of a continuously stirred test solution (1 mM KCl or deionised water respectively) in a glass recipient. Since the redox electrode is constructed with the same materials as the pH electrode, additional tests with the redox electrode were not deemed necessary. As light could cause photodegradation of polymers, both leaching tests were performed in the dark. Subsamples were taken from the leachant solution regularly (up to 110 days) and the Cl⁻ and total organic carbon (TOC) concentrations were determined by respectively IC (see Section 2.3.3 for specifications) and a standard TC/TIC analyser with UV/persulfate digestion. Evaporation of the solution was limited as much as possible by keeping the solution covered and was further taken into account by determining the weight loss of the solution. The stability and possible drift of the pH electrode was investigated by immersing the electrode in APW for ~4 days while continuously recording the pH (5-min measurement interval). Furthermore, the performance of both electrodes was tested during 2.5 months in the circuit of Interval 2. Finally, the variability of the measured redox potential was tested by measuring the redox potential every minute for 45 min (after reaching a stable Eh value) in a saturated quinhydrone solution buffered at pH 7. A possible drift of the redox probe after long-term measurements in the BN experiment was assessed by determining the Eh of saturated quinhydrone solutions buffered at pH 4 and 10 before and after one of the long-term injection tests (~1 year interval between both measurements).

3. Results and discussion

3.1. Performance of the UV spectrophotometer for nitrate and nitrite measurements using the MW algorithm

3.1.1. Single-component APW solutions

After local calibration of the on-line spectrophotometers used in the BN experiment, the uncertainty on the nitrate and nitrite concentrations measured in non-turbid APW solutions (turbidity < 30 FNU), is respectively 6–8% and 7–15% for concentrations higher than 0.8 mM nitrate or 1.8 mM nitrite, with an RSD of respectively 0.3–1.7% and 0.1–3% (Figs. 2 and 4). Below these threshold values (0.8 mM NO₃⁻ or 1.8 mM NO₂⁻), the uncertainty on the measured concentrations is higher, due to interference of the background noise on the measurements. This repeatability is comparable to that of destructive techniques using a cadmium or enzymatically catalysed reduction of nitrate to nitrite followed by a colorimetric reaction and a spectrophotometric determination of nitrite, which ranges from 0.1 to 2% RSD (Ayala et al., 2012; Burakham et al., 2004; Chen et al., 2000; Pons et al., 2008). Furthermore, the repeatability of the spectro:lyser™ is similar to those observed using other UV spectrophotometers: e.g. the ProPS on-line (TriOS GmbH) measures nitrate and nitrite concentrations with an RSD of 0.9–1.7% for nitrate and 2% for nitrite in natural waters or reference aqueous dilutions (Sandford et al., 2007; Snazelle, 2015); the Nitratax plus sc (Hach Lange) has been shown to measure NO_x⁻ concentrations with an RSD of 0.4% in reference aqueous dilutions (Snazelle, 2015) and 3.4% in a waste water matrix (Drolic and Vrotvsek, 2010); the Salatlantic SUNA can measure nitrate concentrations with an RSD of 4.1% in reference aqueous dilutions (Snazelle, 2015). Additionally, the combined uncertainties of the spectro:lyser™ measurements are similar to those obtained by IC performed on nitrate- and nitrite-containing BN interval solutions (i.e. 5.5% for NO₃⁻ and 6.5% for NO₂⁻).

The UV spectrophotometers used in the BN experiment are able to measure nitrate in APW (with turbidity < 30 FNU) accurately in the following ranges: 0.08–32 mM NO₃⁻ and 0.006–16 mM NO₃⁻ for a spectro:lyser™ with a path length of 5 and 10 mm respectively. For nitrite, the measuring ranges are 0.4–29 mM and 0.04–13 mM NO₂⁻ for

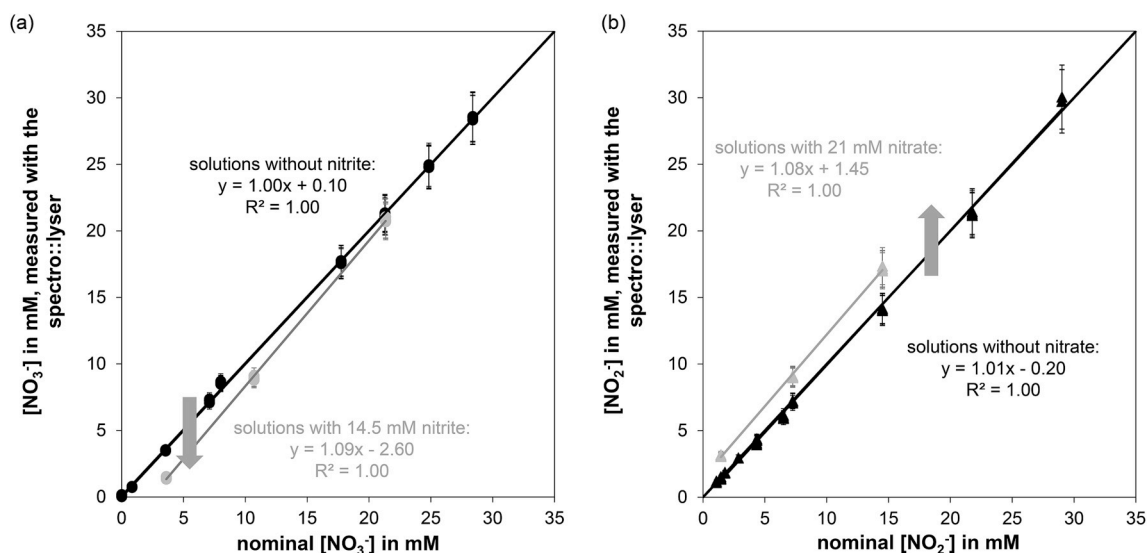


Fig. 2. Comparison of nominal nitrate (a) and nitrite (b) concentrations with those determined with the spectro:lyser™, using the MW algorithm. The nominal concentrations were verified by IC performed on the stock solutions of APW with nitrate and/or nitrite and randomly chosen dilutions. Black: measurements for solutions without nitrite (b) or nitrate (a). Grey: measurements for solutions with the highest concentration of nitrite (b) or nitrate (a) tested to investigate the cross-sensitivity of the spectrophotometrically determined nitrate and nitrite concentrations. The uncertainty bars indicate the combined uncertainties on the concentrations measured with the spectro:lyser™. The dotted line indicates $y = x$.

a spectro:lyser™ with a path length of 5 and 10 mm respectively. The detection limits are only slightly higher than the detection limit for nitrate and nitrite measurements in Opalinus Clay water obtained by IC, *i.e.* respectively 0.004 mM NO_3^- and 0.01 mM NO_2^- . A spectro:lyser™ with a longer path length will provide an even lower detection limit, as described in Beer's law. However, since the nitrate concentration can vary in the BN experiment from high (~50 mM) to zero, using a spectro:lyser™ with a longer path length would not be suitable for this experiment, since it would also lower the limit of quantification.

3.1.2. Cross-sensitivity between nitrate and nitrite

A possible cross-sensitivity between nitrate and nitrite measurements was investigated for APW solutions with varying concentrations of nitrate and/or nitrite. Such a cross-sensitivity could occur due to

significant overlapping of the absorbance spectra for nitrate and nitrite at high concentrations. Fig. 2a shows no effect of nitrite on the nitrate measurements at low nitrite concentrations (for $[\text{N-NO}_3^-]/[\text{N-NO}_2^-]$ ratio higher than 0.7), while higher concentrations of nitrite cause an increasing understatement of the lower nitrate concentrations, *e.g.* when 14.5 mM NO_2^- was added, NO_3^- was significantly understated (by ~2 mM NO_3^-) at concentrations below 11 mM NO_3^- . On the other hand, addition of nitrate to nitrite-containing APW causes an overstatement of the measured nitrite concentration compared to the nominal values. This overstatement is depending on the ratio of $[\text{N-NO}_2^-]/[\text{N-NO}_3^-]$ and is statistically significant (taking into account the combined uncertainty on the measured values) up to a ratio of 0.7. Above this ratio, the effect of the relatively low nitrate on the relatively high nitrite concentration is not statistically significant. Fig. 2b shows

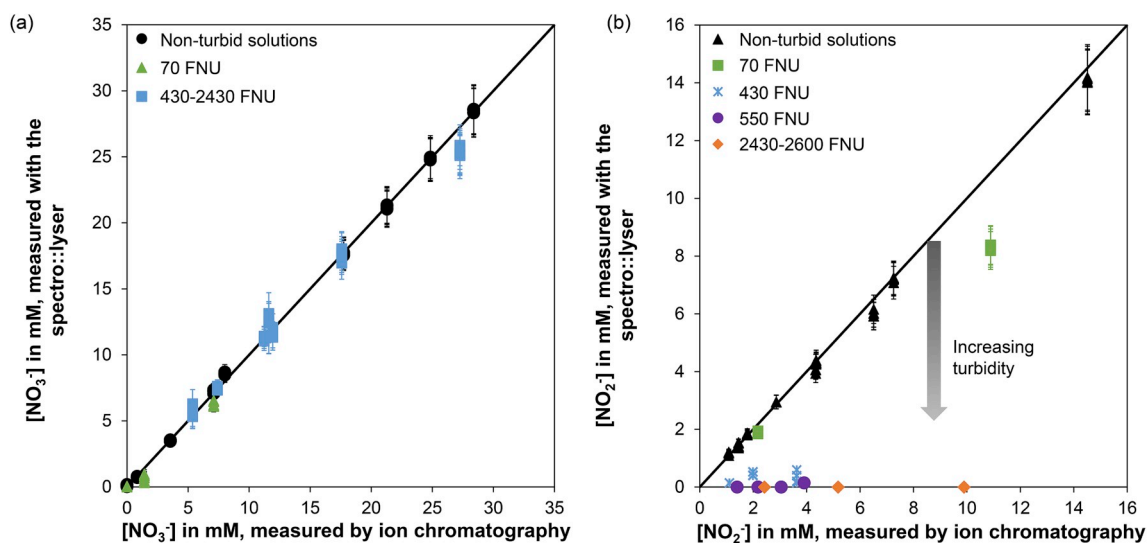


Fig. 3. Comparison of nitrate (a) or nitrite (b) concentrations measured by IC with those determined using the spectro:lyser™ and applying the MW algorithm. APW solutions with nitrate (0–28 mM NO_3^-) or nitrite (0–14.6 mM NO_2^-) and BN interval solutions with increasing turbidity (as indicated in the legend) were tested. For test solutions with nitrate (a), the $[\text{N-NO}_3^-]/[\text{N-NO}_2^-]$ ratio is higher than 0.7 and turbidity ranges up to 2430 FNU. In all test solutions with nitrite (b), the $[\text{N-NO}_2^-]/[\text{N-NO}_3^-]$ ratio is higher than 0.7 and turbidity ranges up to 2600 FNU. The uncertainty bars indicate the combined uncertainties on the concentrations measured with the spectro:lyser™. The dotted line indicates $y = x$.

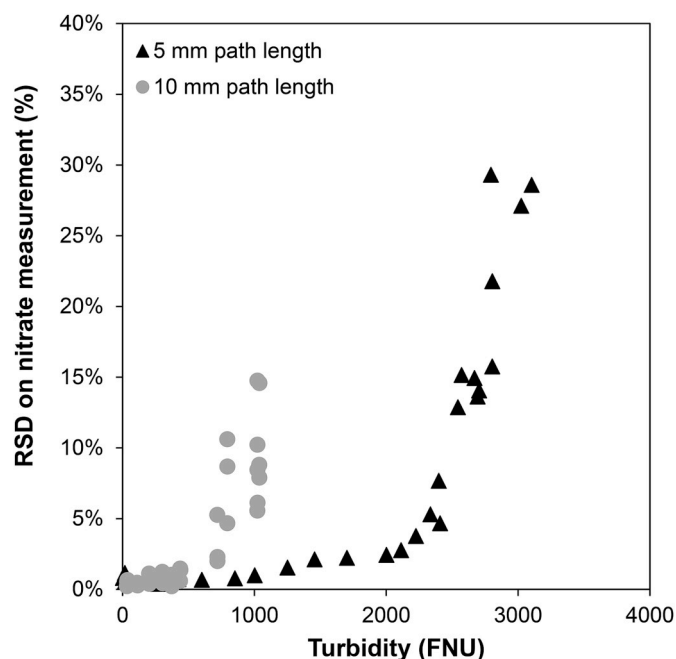


Fig. 4. Relationship between the RSD on the spectrophotometric nitrate measurements (applying the MW algorithm) and the measured turbidity of the solution for 2 spectro::lyser™ with a path length of 5 or 10 mm (as indicated in legend). The turbidity level was estimated spectrophotometrically based on the absorbance at 7 wavelengths ranging from 230 to 330 nm.

the overstatement of the spectroscopically measured nitrite concentrations due to the presence of 21 mM nitrate. This bias ranges from 2 to 4 mM NO_2^- for increasing nitrite concentrations.

3.1.3. Interferences with other components

As the Opalinus Clay pore water contains species that absorb UV light at similar wavelengths as nitrate and nitrite (e.g. Cl^- , SO_4^{2-} , HCO_3^- , dissolved organic carbon and suspended solids) (Thomas and Burgess, 2007), the BN multiwavelength-based algorithm was optimised specifically for APW, taking into account possible interferences of these species. To validate this, the sensitivity of the nitrate and nitrite measurements to these compounds was evaluated.

To investigate the interference of changes in the chemical composition of the background APW (with Cl^- and SO_4^{2-} as main contributors), nitrate and nitrite concentrations were measured in APW and 25 to 50 vol% dilutions of APW. Furthermore, the sensitivity of spectroscopic nitrate measurements to organic compounds was evaluated by measuring their concentration in APW solutions to which acetate was added (0–8.5 mM). Neither of these modifications to the chemical composition of the APW solution affects the measured nitrate and nitrite concentrations (data not shown).

Increasing the turbidity of the solutions by adding NRB (up to 2×10^8 cells mL^{-1} or 70 FNU) did not result in a significant bias of the measured nitrate concentration compared to the nominal concentration. During the injection tests of the BN experiment, the turbidity increased from ~20 FNU to ~3500 FNU, presumably due to continuous deposition of organic and/or clay particles in the measuring path of the spectro::lyser™. For such turbid solutions containing nitrate, the MW algorithm still deduces accurate values for the nitrate concentration (Fig. 3a). However, the precision of the measured nitrate concentrations decreases with increasing turbidity, e.g. the RSD increases from 0.5% for non-turbid solutions (turbidity < 30 FNU) to ~15% when the turbidity increases up to ~1000 FNU or ~2700 FNU for a spectro::lyser™ with a path length of 10 and 5 mm respectively, and even higher RSDs are observed at higher turbidity levels (Fig. 4). This also results in an progressively higher combined uncertainty on the measured nitrate

concentrations compared to those indicated in Section 3.1.1 (Fig. 3a) and a higher detection limit (e.g. detection limit is 4.1 mM in solutions with a turbidity of ~3400 FNU for a spectro::lyser™ with 5 mm path length). Additionally, some understatement (though not statistically significant) of nitrate concentrations close to the limit of quantification, e.g. an interval solution with 27.2 mM NO_3^- was measured with a bias of 7% using a spectro::lyser™ with a 5 mm path length; Fig. 3a).

Our results are comparable to those of Snazelle (2016), who observed little to no change in the measured nitrate concentration at increasing turbidity (below a turbidity of 1000 FNU) when using a spectro::lyser™ with path length of 5 mm. At 1000 FNU, the nitrate concentration was slightly understated by ~10%. For a spectro::lyser™ with a larger path length (35 mm), Huebsch et al. (2015) observed an increasing understatement of the measured nitrate concentration at increasing turbidity (due to accumulation of particles in the slit), starting from ~20 FNU. These literature data combined with our results thus indicate that even when a turbidity-compensating MW algorithm is applied, high turbidity values, especially due to fouling of the measuring path, can result in a bias and an increasing uncertainty on the measured concentration. The extent of this effect is positively related to the path length, as is the case for most interfering substances (Pellerin et al., 2013).

For nitrite, a significant understatement of the concentration can be observed once the turbidity exceeds 70 FNU (Fig. 3b). At rather low turbidity (~70 FNU), this effect is only observed for higher concentrations of nitrite (Fig. 3b). For solutions with higher turbidity levels this bias becomes increasingly severe and the nitrite concentration cannot be measured accurately using the MW algorithm. Indeed, when comparing the nitrite concentration measured either by IC in sampled solution or spectroscopically in one of the intervals of the BN experiment (with a turbidity of ~430 up to 530 FNU) during microbial nitrate to nitrite reduction, this severe understatement of the nitrite concentration was striking (Fig. 3b): a bias of 80% at 430 FNU to 100% at turbidity levels higher than 500 FNU was observed (with a spectro::lyser™ with a 10 mm path length). Also in test INT2_2013, where the turbidity reached up to 2600 FNU during microbial nitrate to nitrite reduction, nitrite concentrations up to 9.8 mM could not be detected anymore due to the high turbidity using a spectro::lyser™ with a 5 mm path length (Fig. 3b; data discussed further in Section 3.3). To be able to measure nitrite concentrations in highly turbid solutions more accurately, a new method based on normalised A(245) was developed (see Section 3.2.2).

3.2. Performance of the UV spectrophotometer for nitrate and nitrite measurements using the SW algorithm

3.2.1. Increasing the limit of quantification for nitrate at varying turbidities

In certain tests of the BN experiment, the BN intervals were injected with an initial nitrate concentration higher than the limit of quantification. Furthermore, turbidity was expected to be rather high due to microbial growth and clay particles potentially attaching to the slit of the spectro::lyser™ over the course of the long-term experiments. Therefore, a new procedure using normalised UV spectra (i.e. SW algorithm) was developed to measure high nitrate concentrations, even at high turbidity levels.

As shown in Fig. 5a, the SW algorithm using $A_{\text{norm}}(230)$ delivers accurate measurements of the nitrate concentration, even in highly turbid solutions (50 up to 3200 FNU). The combined uncertainty on the calculated value based on $A_{\text{norm}}(230)$ is 6–12%, though increasing for concentrations closer to the detection limit (i.e. 1.3 mM NO_3^-). The RSD of the measured nitrate concentration is 0.1–3%. This uncertainty and RSD are similar to the one obtained with the MW algorithm (see Section 3.1.1). The limit of quantification of this single-wavelength method is ~5 mM higher than for the MW algorithm.

When $A_{\text{norm}}(301)$ is used in the SW algorithm, the nitrate concentration can still be measured accurately, but with a combined

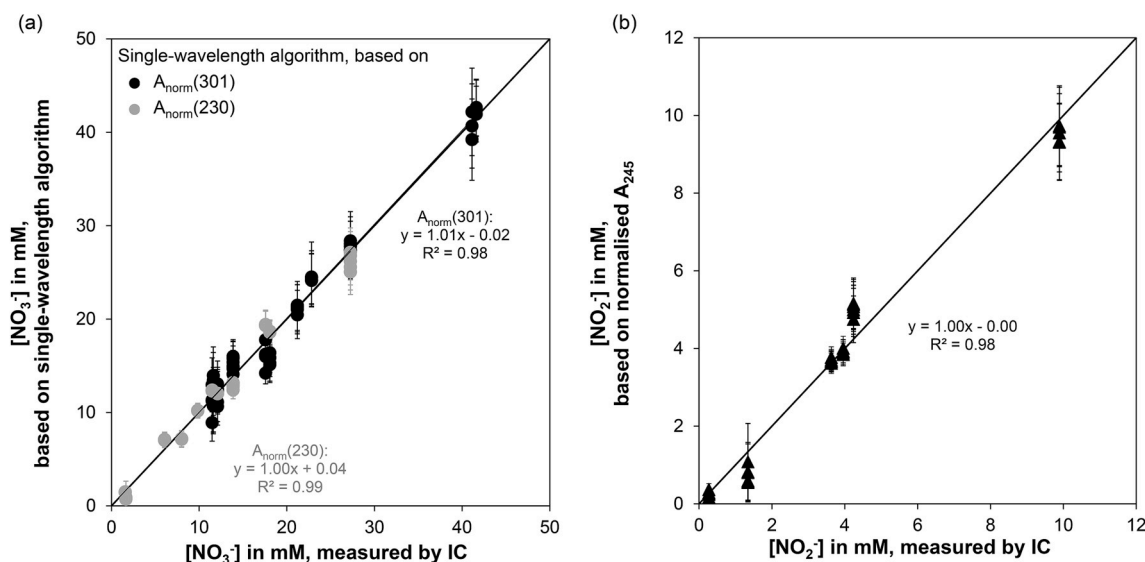


Fig. 5. Nitrate (a) or nitrite (b) concentrations in turbid interval solutions (for turbidity levels of 100 up to 3500 FNU), measured by IC compared to those determined using the spectro::lyser™ and applying the SW algorithm. The calculations are based on the normalised absorbance at 230 nm (●) or 301 nm (●) and 245 nm (▲) for measurements of nitrate and nitrite respectively. The uncertainty bars indicate the combined uncertainties on the concentrations measured with the spectro::lyser™. The dotted line indicates y equals x .

uncertainty and RSD slightly higher than when A_{norm}(230) is used, i.e. 7–19% combined uncertainty and 0.7–7% RSD. The detection limit for the SW algorithm using A_{norm}(301) is 12 mM NO₃⁻. The high detection limit for the method using A_{norm}(301) is due to the low intensity of the normalised absorption band at 301 nm. Nevertheless, the limit of quantification of this method is rather high: concentrations up to 45 mM NO₃⁻ could still be measured accurately (higher concentrations not tested).

No sensitivity to the presence of nitrite was observed when calculating nitrate concentrations using the A_{norm}(301) (data not shown). In contrast, nitrite causes an overstatement of the nitrate concentration in case the normalised absorbance at 230 nm is used (Fig. 6a), due to the presence of the nitrite absorbance peak at ~213 nm (Thomas and Burgess, 2007). This overstatement is positively related to the [N-NO₂⁻]/[N-NO₃⁻] ratio in the solution (Fig. 6a) and is statistically significant (taking into account the combined uncertainty on the measured values

or a bias higher than ~15%) as from a ratio of 0.14.

3.2.2. Nitrite in highly turbid solutions

For nitrite, the interference of a high turbidity on the nitrite measurements can be resolved by calculating the concentration from normalised A(245) values (Fig. 5b and Mendeley Dataset available in Bleyen et al., 2018). This method provides accurate nitrite concentrations with a combined uncertainty of 7–13% and a RSD of 1.5 and 3% for a spectro::lyser™ with a 10 or 5 mm path length respectively. For low concentrations of nitrite (close to detection limit), higher uncertainties are obtained (up to 90%) due to interference of the background noise on the measurement. The detection limit is depending on the path length of the UV spectrophotometer, ranging from 0.2 mM NO₂⁻ to 0.7 mM NO₂⁻ for a spectro::lyser™ with a path length of 10 and 5 mm respectively.

This SW algorithm has been shown to be insensitive to nitrate as

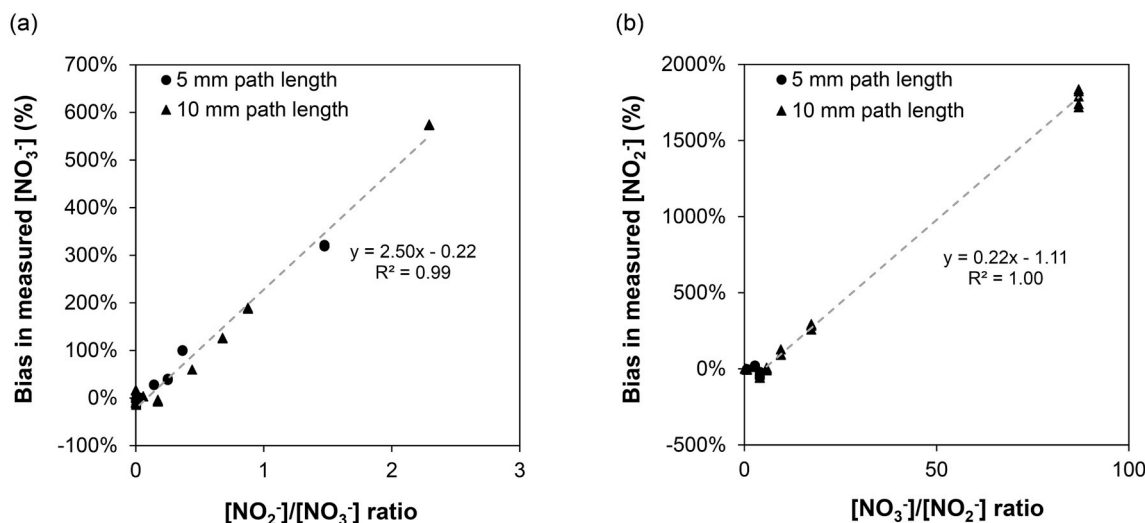


Fig. 6. (a) Relationship between the bias in the nitrate measurements (applying the SW algorithm and based on A_{norm}(230)) and the [N-NO₂⁻]/[N-NO₃⁻] ratio in the test solutions. (b) Relationship between the bias in the nitrite measurements (applying the SW algorithm and based on A_{norm}(245)) and the [N-NO₃⁻]/[N-NO₂⁻] ratio in the test solutions. Results for both spectro::lyser™ with a path length of 5 or 10 mm (as indicated in legend) are shown. The linear regression of these relationships is presented by the dashed line and takes into account all data points for which the bias is statistically significant (more details in Section 3.2).

long as the $[N-NO_3^-]/[N-NO_2^-]$ ratio does not increase above 6. Above this threshold of the $[N-NO_3^-]/[N-NO_2^-]$ ratio, the nitrite is significantly overstated (Fig. 6b). This overstatement is positively related to the $[N-NO_3^-]/[N-NO_2^-]$ ratio (Fig. 6b) and is due to the presence of the nitrate absorbance peak at ~ 206 nm (Thomas and Burgess, 2007).

3.3. Performance of the pH and redox electrode

3.3.1. Leaching behaviour

As the electrode in the pH::lyser™ and redo::lyser™ is lined with a Reflex polymeric interface (polyvinyl acetate) doped with KCl, degradation and leaching of organic molecules (e.g. acetate) and of KCl was considered possible, similar to the on-line pH electrodes used in other *in situ* experiments, e.g. the Pore water Chemistry experiment (PC) at the Mont Terri Rock Laboratory (Switzerland) (De Cannière et al., 2011) and the Coralus experiment at the HADES Underground Research Laboratory (Belgium) (Valcke et al., 2007). As leaching of chloride would ultimately result in instability of the electrodes and leaching of organics could artificially enhance the *in situ* nitrate and nitrite reactivity in the borehole, both leaching rates should be minimal, in order to consider the S::can probes as applicable for (bio)geochemical *in situ* tests such as the BN experiment. Therefore, two leaching tests were performed to determine the leaching rate of chloride and organic matter from two pH::lyser™ electrodes.

On average, the test electrodes leached chloride at a rate of $6.6 \mu\text{mol}$ per day or 0.5 mmol Cl^- in 80 days (Fig. 7). This rate is 23 times lower than the leaching rate of chloride from a glycerol-containing pH electrode (Xerolyt®, Mettler Toledo) discussed by De Cannière et al. (De Cannière et al., 2011), i.e. $\sim 11.5 \text{ mmol Cl}^-$ in 80 days. When only the initial leaching rate for the Xerolyt® electrode is taken into account, i.e. when the concentration gradient between the inner and the outer parts of the electrode is the highest, the Cl^- leaching rate of the electrode in the pH::lyser™ is ~ 50 times lower than that observed for the Xerolyt® electrode. As the Opalinus Clay water in the BN experiment contains high chloride concentrations ($\sim 200 \text{ mM}$; Table 1), the amount of chloride leaching from the pH::lyser™ and redo::lyser™ in the interval solutions is considered to be negligible, even for long-term experiments.

Furthermore, the TOC concentration in the leachant solution

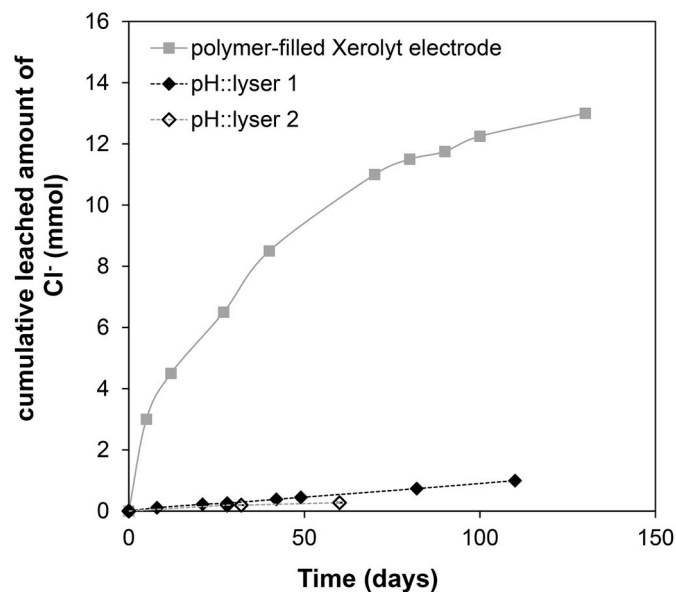


Fig. 7. Time evolution of the cumulative amount of chloride (determined by IC) leached from two pH::lyser™ probes (diamonds) and one polymer-filled Xerolyt® electrode (■) (data from De Cannière et al. (2011)) into the test solution (black closed markers: 1 mM KCl; open markers: deionised water). The uncertainty on the leached amount of chloride is 6%.

remained below the detection limit of 0.2 mM C , i.e. no leaching of organic compounds could be detected over the course of the leaching tests (data not shown). In contrast, considerable leaching of organic compounds (glycerol) from the Xerolyt® electrode used in the PC experiment was observed over time, i.e. an increase in TOC of $\sim 8 \text{ mmol C}$ after ~ 2 months (De Cannière et al., 2011). As leaching of biodegradable organic compounds stimulates microbial activity, the use of this kind of electrode should be avoided in long-term experiments. On the other hand, based on these results, the electrodes with a Reflex polymeric interface do not leach organics and are therefore considered highly useful for long-term *in situ* monitoring of biogeochemical processes.

3.3.2. Accuracy and stability of pH and redox measurements

The stability and possible drift of the pH measurements using the pH::lyser™ were investigated by immersing the electrode in APW (in equilibrium with air) for ~ 4 days while continuously recording the pH. Only a small uncertainty on the pH measurements was found, i.e. 0.8%. No drift of the pH electrode was observed after 4 days (Mendeley Dataset available in Bleyen et al., 2018). Furthermore, after immersion of the pH probe for 2.5 months in the circuit of Interval 2 in between 2 injection tests with nitrate (i.e. period without considerable microbial activity), no statistically significant drift of the electrode was observed (data not shown). For the redo::lyser, the short-term measurements indicate an uncertainty on the measured Eh of $\sim 3\%$. Verification of the redo::lyser after 1 year of immersion and measurement in one of the tests of the BN experiment showed only a small bias of the redox potential of $\sim 16 \text{ mV}$ at a pH between 4 and 10. This needs to be taken into account when interpreting the long-term evolution of the redox potential. The non-existing to small drift of long-term pH and Eh measurements is in line with the chemical stability of the polymeric interface, resulting in a very slow release of KCl from the reference compartment into the interval solution (see Section 3.2.1).

3.4. On-line nitrate, nitrite, pH and Eh measurements under *in situ* conditions

To validate the on-line monitoring of nitrate and nitrite concentrations and the pH and Eh under *in situ* conditions, the evolution of these parameters during one of the injection tests of the BN experiment will be discussed briefly. A detailed interpretation of the results regarding nitrate reactivity and the impact of the nature of the electron donor is given elsewhere (Bleyen et al., 2017).

In test INT2_2013, Interval 2 was injected with APW containing nitrate, and acetate was pulse injected ~ 70 days later (start concentration after dilution is 4 mM acetate). Fig. 8a shows the evolution of the nitrate concentration during the first 70 days after injection of nitrate, both measured with the BMW algorithm and deduced based on the normalised absorption at 301 nm (SW algorithm). At the start of this injection test (after overnight homogenisation of the injected solution), the nitrate concentration was 27 mM , which is rather at the high end of the measurable range, but which could still be measured accurately with a spectro::lyser™ with a 5 mm path length (Section 3.1.1). However, as the interval solution was not completely replaced by fresh APW solution at the start of the test, part of the clay and organic particles present in the borehole solution remained in suspension in the circulating water. This resulted in a rather high starting turbidity ($\sim 850 \text{ FNU}$) after overnight homogenisation of the interval solution, as estimated by the spectro::lyser™. Although this estimated turbidity appears to increase severely throughout the test (Fig. 8b), such high turbidity was not observed in the sampled solutions, demonstrating that continuous deposition of these particles in the slit of the spectro::lyser™ has resulted in the observed increase in measured turbidity levels. This fouling of the measuring path and thus apparently high turbidity caused a slight understatement of the highest nitrate concentration (for concentrations close to the limit of quantification) using the MW algorithm

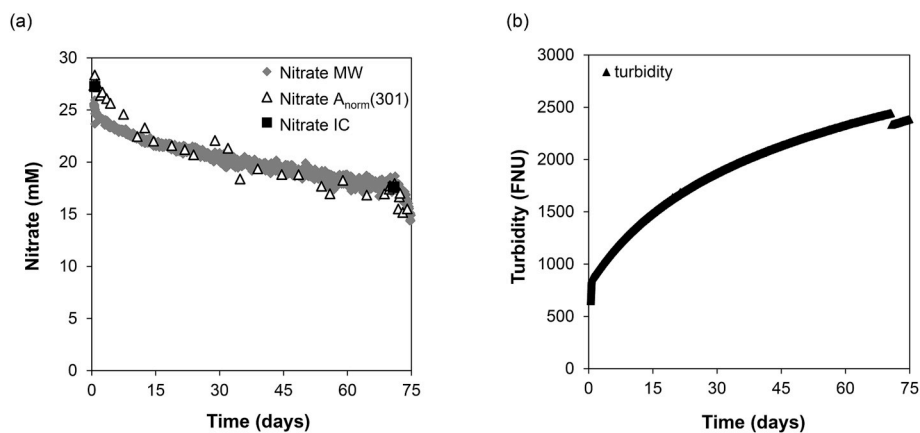


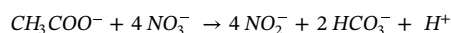
Fig. 8. Evolution of nitrate concentration and turbidity after injection of interval 2 with ~ 27 mM NO_3^- during the first 75 days. (a) The nitrate concentration in the interval solution was determined spectrophotometrically (using either the MW algorithm and averaged every 2 h (*i.e.* 6 measurements), or the SW algorithm based on $A_{\text{norm}}(301)$) or by IC, as indicated in the legend. (b) The turbidity level was estimated spectrophotometrically based on the absorbance at 7 wavelengths ranging from 230 to 330 nm.

(‘Nitrate MW’ in Fig. 8a) as already discussed in Section 3.1.3. This bias does not occur when using $A_{\text{norm}}(301)$ for deducing the nitrate concentration (‘Nitrate $A_{\text{norm}}(301)$ ’ in Fig. 8a), indicating that the SW algorithm is indeed suitable to determine relatively high nitrate concentrations in turbid solutions.

In Fig. 9 the evolution of the nitrate, nitrite and acetate concentrations and of the pH, Eh and turbidity level in the interval solution of test INT2_2013 during the acetate pulse is shown. During this pulse with acetate, the turbidity in the measuring path of the spectro::lyser™ decreased slightly as fresh and non-turbid APW with nitrate and acetate was injected (Fig. 9). Afterwards, the measured turbidity increased again to ~ 2500 FNU. Despite this high turbidity, nitrate was measured accurately using the MW algorithm. These high turbidity levels resulted in a significant underestimation of the nitrite concentration as stated in Section 3.1.3. Although nitrite concentrations up to ~ 10 mM were measured by IC (Fig. 9), nitrite was never detected in the interval solution spectrophotometrically using the MW algorithm. Nevertheless, the nitrite concentration can be determined accurately using the SW algorithm based on $A_{\text{norm}}(245)$. A comparison with the results obtained by IC clearly shows the high quality and accuracy of the nitrate and nitrite measurements using the UV spectrophotometer and applying the MW and SW algorithms respectively (Fig. 9). As the performance assessment of the spectro::lyser™ showed some sensitivity of low nitrate concentration to high concentrations of nitrite (Section 3.1.2), some understatement of the nitrate concentration at the time of maximal nitrite peak can be expected. However, due to the effect of the high turbidity on the precision of the measurement (*i.e.* higher RSD), the slight and temporary bias of the nitrate concentration due to the high

nitrite concentration seems to be masked by the higher combined uncertainty.

The results obtained by the on-line equipment clearly show a lag phase of about 2 days after pulse injection of acetate, during which the nitrate removal rate remained unchanged (Fig. 9). Afterwards, the added acetate caused a significant increase in the nitrate reduction rate and a rapid production of nitrite. On-line monitoring of the nitrate and nitrite concentrations allowed us to sample the interval solution on relevant time points (*e.g.* at the start, middle and end of the fast nitrate reduction phase), which provided additional information on other N species, electron donor and C source and on the overall chemical composition of the interval solution. During the acetate pulse, nitrate was reduced to mainly nitrite (90% of the decrease in the nitrate concentration), using acetate as the main electron donor (Fig. 9; more details in Bleyen et al., 2017). This reaction mechanism is further confirmed by the changes in pH accompanying the observed nitrate and nitrite evolution. Indeed, during the fast decrease in nitrate and increase in nitrite concentration, the pH of the interval solution decreased by 0.75 pH units, which is in agreement with the following reaction:



The observed changes in pH always occur at the same time as the metabolic changes, which confirms that the pH::lyser™ is able to provide accurate results under rapidly evolving pH conditions. These on-line pH measurements clearly support the data of the nitrate and nitrite evolution and therefore contribute to the overall understanding of the ongoing nitrate and nitrite reactivity.

The measured Eh of the solution remained more or less stable

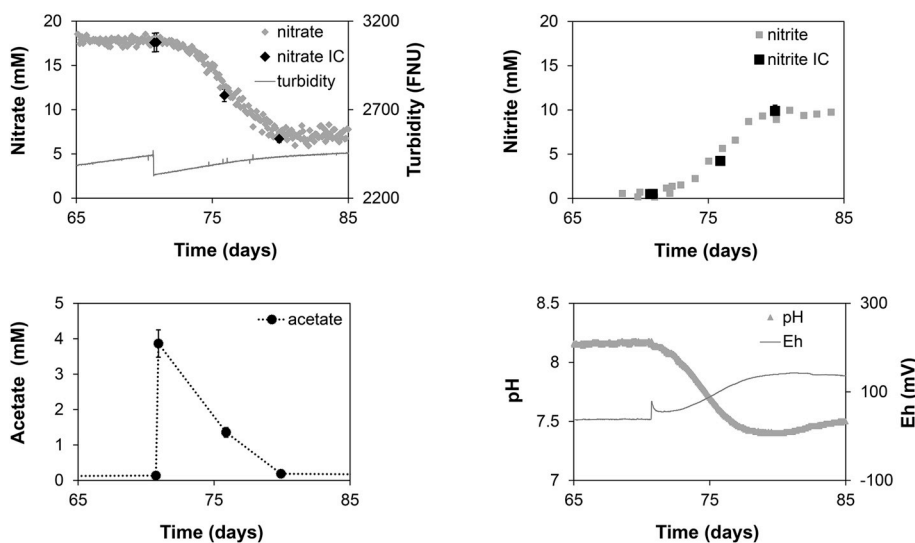


Fig. 9. Evolution of nitrate, nitrite and acetate concentrations, turbidity, pH and Eh after injection of interval 2 with ~ 27 mM NO_3^- only and pulse injection with acetate after 70 days. Only the evolution during the pulse of acetate (70 to ~ 80 days after injection of nitrate) is shown. Black: concentrations determined by IC; Grey: concentrations determined by spectro::lyser™ (nitrate, nitrite, turbidity), by pH::lyser™ (pH) or by redo::lyser™ (Eh). For the nitrate concentration measured spectrophotometrically, the MW algorithm was applied and averaged every 2 h (*i.e.* 6 measurements), while the nitrite concentration was calculated with the SW algorithm based on $A_{\text{norm}}(245)$.

during the lag phase, after a small re-increase due to injection with fresh APW with nitrate and acetate (Fig. 9). Afterwards, the fast nitrate reduction to mainly nitrite resulted in a fast increase in the redox potential of the interval solution, which is in agreement with previously reported lab experiments, where nitrate reduction to nitrite also resulted in an increasing redox potential (Percheron et al., 1998). Similar to the pH::lyser™, the redo::lyser™ is able to stabilise quickly under evolving redox conditions, thereby rapidly showing changes in redox potential of the solution according to the ongoing reactions.

After complete consumption of acetate (~80 days), the nitrate reduction rate decreased drastically and nitrite was no longer produced but removed from the interval solution. This significant change in nitrate reduction rate was accompanied by a drastic shift in the pH evolution, i.e. when nitrate was no longer rapidly reduced to nitrite, the pH no longer decreased but started to increase again. Also the redox potential of the interval solution stabilised when nitrite was no longer produced. The initial pH of the interval solution was slowly regained after several months (data not shown) due to the buffering capacity of the surrounding clay.

Based on these results, the on-line monitoring equipment and related calculation methods provide accurate results and an easy and non-destructive monitoring system for *in situ* nitrate reduction processes. As these reactions can occur very fast (depending on nutrient availability, electron donor and microbial population), blind sampling and chemical analysis of the solution would have missed certain processes (e.g. nitrite produced as intermediate reduced N species), which are detected by this on-line measuring equipment. This is especially true for *in situ* experiments such as the BN experiment where the liquid sampling frequency is necessarily limited. Furthermore, without on-line measurements, the length of the lag phase and the reaction rate would have been much more difficult to assess, as would be the case for mass balance calculations. Real-time monitoring of the nitrate and nitrite concentrations allows for a good timing of sampling, needed to evaluate the evolution of the entire chemical composition.

4. Conclusions

To investigate the fate of nitrate in a clay environment, an *in situ* experiment named BN experiment is currently being performed in the Opalinus Clay formation of the Mont Terri Rock Laboratory (Switzerland). This experiment is equipped with on-line monitoring tools for the real-time quantification of the nitrate and nitrite concentrations (spectro::lyser™) and of the pH (pH::lyser™) and Eh (redo::lyser™). Although promising based on their use for DOC and nitrate monitoring in effluents, surface and well waters, these devices have – to our knowledge – never been used for long-term *in situ* tests, monitoring anaerobic nitrate reduction. In this paper, the performance of this on-line monitoring equipment is shown to be highly satisfactory, especially in combination with new calculation methods to determine nitrate and nitrite concentrations.

Using its standard MW algorithm, the spectro::lyser™ provides an accurate measurement of the nitrate concentration in APW and *in situ* solutions (6–8% uncertainty; for solutions with turbidity below 30 FNU), even in the presence of UV absorbing compounds (sometimes present in the interval solution of the BN experiment (e.g. Cl⁻, SO₄²⁻, acetate). Nevertheless, some sensitivity of these measurements to nitrite (understatement of low nitrate concentrations) and turbidity (higher variability) has to be taken into account when interpreting the results of *in situ* tests. When higher nitrate concentrations need to be measured and/or in highly turbid conditions, the novel SW algorithm should be applied to determine the nitrate concentration accurately. This approach is based on either A_{norm}(230) (6–12% uncertainty) or – when nitrite is present in significant amounts ([N-NO₂]/[N-NO₃]⁻ ratio > 0.14) and for nitrate concentrations higher than 12 mM – on A_{norm}(301) (7–19% uncertainty).

The spectro::lyser™ also proved to be a suitable tool for monitoring

the real-time evolution of the nitrite concentration. Using the MW algorithm, nitrite can be measured accurately both in APW and under *in situ* solutions (7–15% uncertainty), but only when the ratio of [N-NO₂⁻]/[N-NO₃⁻] is higher than 0.7 and at low turbidity levels (turbidity < 30 FNU). In case of turbid solutions, the nitrite concentration should be determined with the SW algorithm based on A_{norm}(245) (uncertainty 7–13%). Additional turbidity compensation of the spectra using the SW algorithm is indeed critical to reducing the bias in the results, particularly during long-term monitoring periods, when particles are continuously deposited in the measuring path of the spectrophotometer. This newly developed calculation method has proven to be rather insensitive to the presence of nitrate, when nitrate remains below a certain threshold ([N-NO₃⁻]/[N-NO₂⁻] ratio < 6).

Overall, the spectro::lyser™ combined with the algorithms discussed here thus provided accurate measurements of both nitrate and nitrite concentrations, though which of these algorithms should be applied is depending on the conditions (e.g. low vs high turbidity or low vs high concentrations). Nitrate and nitrite measurements showed similar uncertainties compared to other on-line spectrophotometers and conventional methods, such as cadmium or enzymatically catalysed reduction of nitrate to nitrite followed by colorimetry or ion chromatography. This is especially surprising since BN solutions contain high Cl⁻ concentrations and turbidity levels. Both algorithms are believed to be applicable for other environmental water samples, although interferences with other site-specific compounds should be evaluated beforehand.

Furthermore, the applicability of the on-line pH and redox probes for long-term *in situ* tests in clay environments was assessed. These probes were found useful and stable for long periods of time under *in situ* conditions. In contrast to other electrodes used in previous *in situ* experiments, these electrodes do not leach organic compounds and show only a very low KCl leaching rate. Significant changes of the *in situ* pH and Eh were detected during the injection test, in agreement with the ongoing nitrate and nitrite reduction processes. These results thus confirm the benefit of these on-line pH and Eh probes during the investigation of biogeochemical processes. Their short response time to changes in the reactivity and thus pH and redox potential, demonstrates that the pH::lyser™ and redo::lyser™ are able to provide accurate results under evolving pH and redox conditions.

Based on this study, we can thus conclude that the combination of the on-line UV spectrophotometer and pH and redox probes used in the BN experiment provides a stable and accurate system, suitable for real-time monitoring of (rapid) *in situ* nitrate and nitrite reduction processes.

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References

- Andra, 2005. Dossier 2005 Argile – Référentiel du site de Meuse/Haute Marne. Andra, Paris, France C.R.P.ADS.04.0022.
- Ayala, A., Leal, L., Ferrer, L., Cerdà, V., 2012. Multiparametric automated system for sulfate, nitrite and nitrate monitoring in drinking water and wastewater based on sequential injection analysis. *Microchem. J.* 100, 55–60.
- Bailey, D., Beauchamp, E.G., 1971. Nitrate reduction and redox potentials measured with permanently and temporarily placed platinum electrodes in saturated soils. *Can. J. Soil Sci.* 51, 51–58.
- Bleyen, N., 2018. Data for paper: Non-destructive on-line and long-term monitoring of *in situ* nitrate and nitrite reactivity in a clay environment at increasing turbidity. Mendeley Data, v1. <http://dx.doi.org/10.17632/mn2pt5sz79.1>.
- Bleyen, N., Smets, S., Small, J., Moors, H., Leys, N., Albrecht, A., De Cannière, P., Schwyn, B., Wittebroodt, C., Valcke, E., 2017. Impact of the electron donor on *in situ* microbial nitrate reduction in Opalinus Clay: results from the Mont Terri rock laboratory (Switzerland). *Swiss J. Geosci.* 110, 355–374.

- Bleyen, N., Vasile, M., Marien, A., Bruggeman, C., Valcke, E., 2016. Assessing the oxidising effect of NaNO_3 and NaNO_2 from disposed EUROBITUM bituminised waste on the dissolved organic matter in boom clay. *Appl. Geochem.* 68, 29–38.
- Burakham, R., Oshima, M., Grudpan, K., Motomizu, S., 2004. Simple flow-injection system for the simultaneous determination of nitrite and nitrate in water samples. *Talanta* 64, 1259–1265.
- Chen, H., Fang, Y., An, T., Zhu, K., Lu, J., 2000. Simultaneous spectrophotometric determination of nitrite and nitrate in water samples by flow-injection analysis. *Int. J. Environ. Anal. Chem.* 76, 89–98.
- Choe, E., van der Meer, F., Rossiter, D., van der Salm, C., Kim, K.-W., 2010. An alternate method for fourier transform infrared (FTIR) spectroscopic determination of soil nitrate using derivative analysis and sample treatments. *Water Air Soil Pollut.* 206, 129–137.
- Colman, B.P., 2010. Understanding and eliminating iron interference in colorimetric nitrate and nitrite analysis. *Environ. Monit. Assess.* 165, 633–641.
- Courdouan, A., Christl, I., Meylan, S., Wersin, P., Kretzschmar, R., 2007. Characterization of dissolved organic matter in anoxic rock extracts and in situ pore water of the opalinus clay. *Appl. Geochem.* 22, 2926–2939.
- De Cannière, P., Schwarzbauer, J., Höhener, P., Lorenz, G., Salah, S., Leupin, O., Wersin, P., 2011. Biogeochemical processes in a clay formation in situ experiment: part C—organic contamination and leaching data. *Appl. Geochem.* 26, 967–979.
- Diamond, D., McEnroe, E., McCarrick, M., Lewenstam, A., 1994. Evaluation of a new solid-state reference electrode junction material for ion-selective electrodes. *Electroanalysis* 6, 962–971.
- Drolc, A., Vrtovsek, J., 2010. Nitrate and nitrite determination in waste water using on-line UV spectrometric method. *Bioresour. Technol.* 101, 4228–4233.
- Grayson, R., Holden, J., 2012. Continuous measurement of spectrophotometric absorbance in peatland streamwater in northern England: implications for understanding fluvial carbon fluxes. *Hydrol. Process.* 26, 27–39.
- Helmke, S.M., Duncan, M.W., 2007. Measurement of the NO metabolites, nitrite and nitrate, in human biological fluids by GC-MS. *J. Chromatogr. B Analyt. Technol. Biomed. Life. Sci.* 851, 83–92.
- IUPAC, 2014. *Compendium of Chemical Terminology - Gold Book*. pp. 1670. <https://goldbook.iupac.org>.
- Jahn, B., Linker, R., Upadhyaya, S., Shaviv, A., Slaughter, D., Shmulevich, I., 2006. Mid-infrared spectroscopic determination of soil nitrate content. *Biosyst. Eng.* 94, 505–515.
- Jeong, J.J., Bartsch, S., Fleckenstein, J.H., Matzner, E., Tenhunen, J.D., Lee, S.D., Park, S.K., Park, J.H., 2012. Differential storm responses of dissolved and particulate organic carbon in a mountainous headwater stream, investigated by high-frequency, in situ optical measurements. *J. Geophys. Res. Biogeosci.* 117, G03013.
- Jobgen, W.S., Jobgen, S.C., Li, H., Meininger, C.J., Wu, G., 2007. Analysis of nitrite and nitrate in biological samples using high-performance liquid chromatography. *J. Chromatogr. B Analyt. Technol. Biomed. Life. Sci.* 851, 71–82.
- Langergraber, G., Fleischmann, N., Hofstadter, F., 2003. A multivariate calibration procedure for UV/VIS spectrometric quantification of organic matter and nitrate in wastewater. *Water Sci. Technol.* 47, 63–71.
- Langergraber, G., Fleischmann, N., Hofstadter, F., Weingartner, A., 2004. Monitoring of a paper mill wastewater treatment plant using UV/VIS spectroscopy. *Water Sci. Technol.* 49, 9–14.
- Madigan, M.T., Martinko, J.M., Parker, J., 2000. *Brock Biology of Microorganisms*, ninth ed. Prentice-Hall, New Jersey, USA.
- Mariën, A., Bleyen, N., Aerts, S., Valcke, E., 2011. The study of abiotic reduction of nitrate and nitrite in boom clay. *Phys. Chem. Earth* 36, 1639–1647.
- Miyado, T., Tanaka, Y., Nagai, H., Takeda, S., Saito, K., Fukushi, K., Yoshida, Y., Wakida, S.-i., Niki, E., 2004. Simultaneous determination of nitrate and nitrite in biological fluids by capillary electrophoresis and preliminary study on their determination by microchip capillary electrophoresis. *J. Chromatogr. A* 1051, 185–191.
- ONDRAF/NIRAS, 2009. The Long-term Safety Assessment Methodology for the Geological Disposal of Radioactive Waste. ONDRAF/NIRAS, Brussels, Belgium ONDRAF/NIRAS Report NIRONDR TR- 2009-14 E.
- Paredes, D., Kuschik, P., Mbwette, T.S.A., Stange, F., Müller, R.A., Köser, H., 2007. New aspects of microbial nitrogen transformations in the context of wastewater treatment - a review. *Eng. Life Sci.* 7, 13–25.
- Pearson, F.J., Arcos, D., Bath, A., Boisson, J.Y., Fernández, A.M., Gabler, H.E., Gaucher, E., Gautschi, A., Griffault, L., Hernán, P., Waber, H.N., 2003. *Mont Terri Project - Geochemistry of Water in the Opalinus Clay Formation at the Mont Terri Rock Laboratory*. Geology Series. Federal Office for Water and Geology, Switzerland.
- Pellerin, B.A., Bergamaschi, B.A., Downing, B.D., Saraceno, J.F., Garrett, J.A., Olsen, L.D., 2013. *Optical Techniques for the Determination of Nitrate in Environmental Waters: Guidelines for Instrument Selection, Operation, Deployment, Maintenance, Quality Assurance, and Data Reporting: U.S. Geological Survey Techniques and Methods 1–D5*, pp. 37.
- Percheron, G., Michaud, S., Bernet, N., Moletta, R., 1998. Nitrate and nitrite reduction of a sulphide-rich environment. *J. Chem. Technol. Biotechnol.* 72, 213–220.
- Pons, C., Santos, J.L., Lima, J.L., Forteza, R., Cerdà, V., 2008. Multi-pumping flow system for the determination of nitrite and nitrate in water samples. *Microchim. Acta* 161, 73–79.
- Rieger, L., Langergraber, G., Siegrist, H., 2006. Uncertainties of spectral in situ measurements in wastewater using different calibration approaches. *Water Sci. Technol.* 53, 187–197.
- Rieger, L., Langergraber, G., Thomann, M., Fleischmann, N., Siegrist, H., 2004. Spectral in-situ analysis of NO₂, NO₃, COD, DOC and TSS in the effluent of a WWTP. *Water Sci. Technol.* 50, 143–152.
- Sandford, R.C., Exenberger, A., Worsfold, P.J., 2007. Nitrogen cycling in natural waters using in situ, reagentless UV spectrophotometry with simultaneous determination of nitrate and nitrite. *Environ. Sci. Technol.* 41, 8420–8425.
- Schwarz, A., Modun, D., Heusser, K., Tank, J., Gutzki, F.-M., Mitschke, A., Jordan, J., Tsikas, D., 2011. Stable-isotope dilution GC-MS approach for nitrite quantification in human whole blood, erythrocytes, and plasma using pentafluorobenzyl bromide derivatization: nitrite distribution in human blood. *J. Chromatogr. B* 879, 1485–1495.
- Skoog, D.A., West, D.M., Holler, F.J., 1996. *Application of Statistics to Data Treatment and Evaluation. Fundamentals of Analytical Chemistry*, seventh ed. Saunders College Publishing, USA.
- Snazelle, T.T., 2015. Results from Laboratory and Field Testing of Nitrate Measuring Spectrophotometers. 2015–1065. U.S. Geological Survey Open-File Report, pp. 39.
- Snazelle, T.T., 2016. The Effect of Suspended Sediment and Color on Ultraviolet Spectrophotometric Nitrate Sensors. 2016–1014. U.S. Geological Survey Open-File Report, pp. 10.
- Sung, W., 2011. Using UV-Vis spectrophotometry to estimate nitrite plus nitrate and monochloramine. *J. Am. Water Works Assoc.* 103, 97–103.
- Thomas, O., Burgess, C., 2007. *UV-visible Spectrophotometry of Water and Wastewater*. Elsevier.
- Tsikis, D., Mitschke, A., Gutzki, F.-M., Engeli, S., Jordan, J., 2010. Evidence by gas chromatography-mass spectrometry of ex vivo nitrite and nitrate formation from air nitrogen oxides in human plasma, serum, and urine samples. *Anal. Biochem.* 397, 126–128.
- Valcke, E., Smets, S., Labat, S., Lemmens, K., Van Iseghem, P., Gysemans, M., Thomas, P., Van Bree, P., Vos, B., Van den Berghe, S., 2007. CORALUS-II. An Integrated in Situ Corrosion Test on Alpha-active HLW Glass - Phase II. SCK-CEN, Mol, Belgium External Report SCK-CEN-ER-27.
- van den Broeke, J., Langergraber, G., Weingartner, A., 2006. On-line and in-situ UV/vis spectroscopy for multi-parameter measurements: a brief review. *Spectrosc. Eur.* 18, 15–18.
- Waterloo, M.J., Oliveira, S.M., Drucker, D.P., Nobre, A.D., Cuartas, L.A., Hodnett, M.G., Langedijk, I., Jans, W.W., Tomasella, J., De Araujo, A.C., 2006. Export of organic carbon in run-off from an Amazonian rainforest blackwater catchment. *Hydrol. Process.* 20, 2581–2597.
- WHO, 2016. *Nitrate and Nitrite in Drinking Water - Background Document for Development of WHO Guidelines for Drinking-water Quality*. WHO Document. World Health Organization, Geneva, Switzerland WHO/FWC/WSH/16.52. www.who.int.
- Yaqoob, M., Biot, B.F., Nabi, A., Worsfold, P.J., 2012. Determination of nitrate and nitrite in freshwaters using flow-injection with luminol chemiluminescence detection. *Luminescence* 27, 419–425.