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CENTRE D'ETUDE DE L'ENERGIE NUCLEAIRE

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SCK•CEN
Boeretang 200
BE-2400 Mol
Belgium

RDW

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SCK•CEN
Boeretang 200
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Belgium

Studiecentrum voor Kernenergie
Centre d'étude de l'énergie Nucléaire
Boeretang 200
BE-2400 Mol
Belgium

Phone +32 14 33 21 11
Fax +32 14 31 50 21

http://www.sckcen.be

Contact:
Knowledge Centre
library@sckcen.be

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1. Abstract

The dissolved organic matter (DOM) in Boom Clay pore water is one of the redox-active components causing the reducing environment in Boom Clay. As oxidised redox-sensitive radionuclides (*e.g.* actinides) possess a higher potential for migration, the reducing capacity of Boom Clay is an important chemical parameter for clays which are studied as potential host rocks for radioactive waste disposal. Certain perturbations caused by the presence of a geological disposal gallery for radioactive waste, such as reactions with $NaNO_3$ (and to a lesser extent $NaNO_2$) leached from Eurobitum (Belgian bituminised intermediate level radioactive waste) into the Boom Clay, could have an effect on the reducing capacity of DOM in Boom Clay. To study this, two methods for evaluating changes in the reducing capacity of DOM were optimised, discussed and compared in this report.

These two methods are based on the oxidation of DOM in real Boom Clay Water (RBCW) by an oxidant, *i.e.* either by ferricyanide or by ferric citrate. When ferricyanide is used as oxidant, the reducing capacity of oxidisable DOM in RBCW ranges from 3.4 to 6.1 meq/gC. For ferric citrate as oxidant, lower values of the reducing capacity of DOM in RBCW are obtained, ranging from 0.8 to 1.3 meq/gC. This lower value is attributed to the difference in standard redox potential and in the chemical structure between the oxidants, and to the effect of pH on the speciation of the Fe(III)-citrate complex. For both oxidants, the value of the reducing capacity can differ slightly depending on the batch of RBCW taken from the EG/BS piezometer, similar to the small differences in the concentration of total organic carbon (TOC) and functional groups that have been observed for different batches of RBCW.

As these methods would be applied to investigate whether a possible redox reaction between DOM and nitrate or nitrite would cause a significant change in reducing capacity of DOM in Boom Clay, the sensitivity of both oxidants to redox reactions with nitrate and/or nitrite was investigated. The results show that in case nitrite is present in the RBCW solutions, only ferricyanide can be used as oxidant to determine the reducing capacity of DOM in RBCW, as the observed redox reaction between nitrite and Fe(II) from ferrous citrate results in an underestimation of the reducing capacity. On the other hand, the presence of nitrate did not influence the value of the reducing capacity of DOM using either ferricyanide or ferric citrate.

Finally, for the abiotic test conditions considered in this study, the use of NaN_3 – added to inhibit microbial acitvity – resulted in a slight increase in reducing capacity of DOM determined with ferricyanide. Azide reduces to some extent the already oxidised functional groups in the DOM. These functional groups could then react (again) with ferricyanide, leading to an overestimation of the reducing capacity. On the other hand, when ferric citrate was used as oxidant, azide also lowered the concentration of the ferrozine-Fe(II) complex, which (partially) counteracts the overestimation caused by the reduction of DOM by azide.

2. Introduction

Boom Clay is considered as a potential host rock formation for the final disposal of EUROBITUM bituminised intermediate-level (IL) waste. This clay formation possesses favourable physico-chemical characteristics [*e.g.* reducing environment due to the presence of (dissolved) organic matter and minerals such as pyrite], which would limit and delay the migration of leached radionuclides to the biosphere over extended periods of time. Eurobitum consists of ~60 wt% hard bitumen (Mexphalt R85/40), in which the radioactive waste and high amounts of $NaNO_3$ (20 to 30 wt%) and other salts and (hydr)oxides (10 to 20 wt%) are homogeneously dispersed (Boulanger, 2011; Demonie, 1996). During and after saturation of the filled and closed disposal gallery, this bituminised waste will take up water, resulting in the dissolution of $NaNO_3$ crystals and the subsequent leaching of significant amounts of nitrate (Valcke *et al.*, 2009). A possible consequence of this release of $NaNO_3$ is the oxidation of the surrounding clay by nitrate and, possibly, nitrite, resulting in a lower reducing capacity of the clay towards redox-sensitive radionuclides, which in turn could have an impact on the migration of these radionuclides through the clay.

Although the Boom Clay formation contains relatively high levels of organic carbon, measurements on piezometer waters indicate that only a small fraction (0.01%) of the total organic carbon is present as mobile or dissolved organic matter (DOM) (Van Geet, 2004). The Boom Clay pore water sampled from the piezometers installed in the HADES Underground Research Laboratory (URL) at Mol contains 702 – 4425 mg/l HCO_3^-, a total inorganic carbon (TIC) concentration of 143 – 871 mg C/l, and has a dissolved organic carbon (DOC) concentration of 78 – 263 mg C/l (De Craen *et al.*, 2004). This dissolved organic fraction is rich in humic (HA) and fulvic acids (FA) with a wide molecular size spectrum. Humic acids are more highly aromatic, while fulvic acids are less aromatic and are richer in carboxylic acid, phenolic and ketonic groups (De Craen *et al.*, 2004; Kele *et al.*, 2006).

To evaluate the effect of nitrate and nitrite on the reducing properties of Boom Clay pore water, the evolution of the reducing capacity of dissolved organic matter (DOM), the main redox-active component of Boom Clay pore water, is determined during incubation of RBCW with either nitrate or nitrite. The reducing capacity of dissolved organic matter is defined as the amount of moles of electron charges per gram of carbon, which is transferred by DOM to an added oxidant [over a given (laboratory) timescale] (Peretyazhko and Sposito, 2006). The value of this reducing

capacity is controlled by the redox potential of the electron donor (DOM) and the electron acceptor used to measure it (Bauer and Kappler, 2009; Bauer *et al.*, 2007).

Investigating the reducing capacity of humic (HA) and fulvic (FA) acid fractions is commonly done by redox titrations with a suitable electron acceptor. Frequently, an Fe(III) compound is used: poorly crystalline Fe(III) hydroxides [$Fe(OH)_3$] or dissolved Fe(III) compounds such as $[Fe(CN)_6]^{3-}$, $FeCl_3$, or ferric citrate (Benz *et al.*, 1998; Pirlet, 2003; Matthiessen, 1995; Bauer and Kappler, 2009; Wolf *et al.*, 2009). The positive E_{SHE} values of dissolved Fe(III) compounds are substantially more favourable for reduction by HA and FA compared to Fe(III) minerals, whose E_{SHE} depends on their crystallinity (Bauer and Kappler, 2009). To detect possible decreases in the reducing capacity of DOM in Boom Clay pore water due to reaction with nitrate or nitrite, two oxidants were tested: ferricyanide and ferric citrate.

Ferricyanide, $[Fe(CN)_6]^{3-}$, is often used to determine the reducing capacity of dissolved organic matter owing to its favourable properties: (1) a constant redox potential (~430 mV) over a large pH range from 4 to 11, (2) a high complex stability ($K = 10^{42}$ M^{-5}) and (3) a one-electron reduction into $[Fe(CN)_6]^{4-}$, which can be followed by UV-VIS spectroscopy (absorption peak of $[Fe(CN)_6]^{3-}$ at 420 nm) (Pirlet, 2003; Matthiessen, 1995). Alternatively, ferric citrate can also be used to study the reducing capacity of DOM. The Fe(III)-citrate reduction (also a one electron reduction) can be monitored by complexation of the produced Fe(II) with ferrozine (Fe(II)-ferrozine$_3$ complex[1]; $K = 10^{15.53}$ M^{-3}). This complex can be detected by UV-VIS spectroscopy at 562 nm (Bauer and Kappler, 2009; Jiang and Kappler, 2008; Stookey, 1970). Note however that the redox potential of the Fe(III)/Fe(II) citrate couple depends on the speciation of the Fe(III) and Fe(II) citrate complexes. Several complexes (mono-, di- and polycitrate species) can be found in aqueous solutions, each with a different standard redox potential. Furthermore, the speciation of iron citrate is dependent on the pH of the solution. Therefore, the value of the redox potential (vs SHE) for Fe(III)/Fe(II)-citrate is dependent on the pH and on the ratio of Fe(III) to citrate and can range from ~0.3 to -0.2 V (Wang *et al.*, 2008; Vukosav *et al.*, 2012).

It should be noted that the use of ferricyanide leads to reported reducing capacity values of natural DOM that are about 2-10 times higher than those obtained with ferric citrate (Peretyazhko and Sposito, 2006; Bauer *et al.*, 2007). These differences are attributed to the difference in redox potential of both half reactions, in the chemical structure of the oxidants, and to the effect of pH on the speciation of the Fe(III)-citrate complex. Based on these differences,

[1] Ferrozine = 3-(2-pyridyl)-5, 6-bis (4-phensylsulfonic acid)-1, 2, 4-triazine monosodium salt

$K_3Fe(CN)_6$ is able to promote a broader range of oxidation reactions than ferric citrate, leading to a higher reducing capacity of DOM (Matthiessen, 1995; Bauer and Kappler, 2009; Peretyazhko and Sposito, 2006).

Finally, in order to evaluate the degree of oxidation of DOM exposed to $NaNO_3$ or $NaNO_2$, we need a procedure for determining the reducing capacity of DOM which:

(1) provides a reliable value for the reducing capacity of DOM in Boom Clay pore water (for a certain oxidant);

(2) is not influenced by azide (used as bacterial inhibitor), $NaNO_3$ and $NaNO_2$;

In this report, the optimisation and the final procedure for measuring the reducing capacity of DOM in RBCW is discussed. Furthermore, the sensitivity of this procedure to azide, nitrate and nitrite is assessed.

3. Experimental details

3.1. Types of dissolved organic matter

3.1.1. Hydroquinone as standard model for DOM

Among the functional groups of organic matter in clay water, quinones and hydroquinones are the most redox-active (Stevenson, 1982). The highly soluble monomers AHQDS/AQDS (anthrahydroquinone-2,6-disulfonate / anthraquinone-2,6-disulfonate) are also frequently used as a model compound for (hydro)quinone moieties in natural OM, *e.g.* to investigate chemical or microbial reduction of Fe(III) compounds by DOM (Kappler and Haderlein, 2003; Bauer and Kappler, 2009; Coates *et al.*, 2008). In our experiments, the hydroquinone/benzoquinone redox couple was used as a model for reduced/oxidised DOM to optimise the procedure for the determination of the reducing capacity of DOM in RBCW and to study the reduction of ferricyanide by DOM.

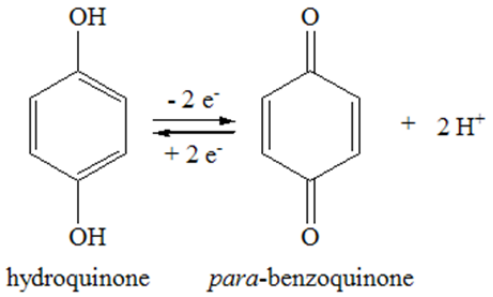

Figure 1: Half reaction of hydroquinone and p-benzoquinone (E^0 = 0.699 V)

In the reaction with ferricyanide, hydroquinone (H_2Q) is oxidised to (para-)benzoquinone (Q) (Figure 1). Note that the combination of hydroquinone and benzoquinone results in the formation of quinhydrone (Figure 2), a complex consisting of a reduced quinone (hydroquinone) interacting with an oxidised quinone (benzoquinone) through ring stacking (Regeimbal *et al.*, 2003). At alkaline pH and in the presence of light, the hydroquinone in quinhydrone is deprotonated and becomes a better charge-transfer donor, resulting in the characteristic red/purple quinhydrone complex (Kung and McBride, 1988; Regeimbal *et al.*, 2003). As the coloured quinhydrone complex hinders the UV-VIS measurements (Regeimbal *et al.*, 2003) necessary to determine the reducing capacity of hydroquinone, it is not opportune to perform the

tests at alkaline pH. On the other hand, acidification of a quinhydrone solution results in the formation of green/black precipitates (observed at pH ~4), which also interferes with the UV-VIS measurements. Furthermore, acidification leads to protonation of hydroquinone in the complex, which is a less efficient electron donor (Kung and McBride, 1988). Therefore, it was decided to buffer the hydroquinone solutions using a phosphate buffer at a circumneutral pH (0.05 M NaH_2PO_4 / Na_2HPO_4 buffer; pH ~7).

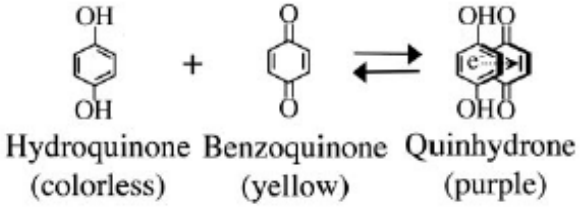

Figure 2: Complexation of hydroquinone and benzoquinone into quinhydrone. At alkaline pH and in the presence of light, the quinhydrone complex has a red/purple color (Regeimbal *et al.*, 2003).

3.1.2. Dissolved organic matter from Real Boom Clay Water (RBCW)

In the experiments discussed in this report, Boom Clay water from the EG/BS (Extension Gallery Bottom Shaft) piezometer (HADES underground research laboratory, Mol, Belgium) was used. This piezometer is vertically orientated located at the bottom of the First Shaft. It contains a stainless steel cylindrical filter screen placed in a coarse sand column (0.7-1.25 mm grain size) in the Boom Clay. The coarse sand (0.7-1.25 mm grain size) was used to enhance the water-draining capabilities of this piezometer and interconnects the pore water from the Boom Clay between septaria levels S40 an S60. This range encompasses also the silty "double band", which has a higher hydraulic conductivity than the rest of the Boom Clay and will therefore influence the chemical properties of the EG/BS pore water. Furthermore, influences of the coarse sand, the backfill material and the bentonite top-cover seal can be noticed in the geochemistry of the collected pore water (De Craen *et al.*, 2004). Nevertheless, pore water sampled from this piezometer (RBCW or Real Boom Clay Water) has been considered as a good representation of the aqueous phase in equilibrium with the Boom Clay solid phase.

The RBCW contains relatively high concentrations of dissolved organic matter (~130 mg C/l in average; De Craen *et al.*, 2004; Blanchart, 2011) and is rich in humic and fulvic acids (in approximately equal amounts) (Bruggeman and De Craen, 2012). Furthermore, the EG/BS pore

water contains many (unsaturated) fatty acids. The presence of such unsaturated fatty acids indicates an immature organic matter (Van Geet, 2004).

3.2. Prevention of microbial activity

When determining the reducing capacity of DOM in RBCW, care must be taken to avoid bacterial activity, which can perturb the results. Indeed, in preliminary tests (not discussed in this report), a significant increase in the reduction rate of ferricyanide was observed (incubation period > 50 hours), related to microbial reduction of ferricyanide by iron-reducing bacteria[2]. Electrons needed for this bioreduction are usually derived from the microbial oxidation of organic substrates (generic chemical formula CH_2O), which are passed down the electron transport chain (Morris *et al.*, 2005):

$$CH_2O + H_2O + 4\,[Fe(CN)_6]^{3-} \rightarrow CO_2 + 4\,H^+ + 4\,[Fe(CN)_6]^{4-}$$

To avoid such microbial activity in the clay water solutions containing ferricyanide, initially 0.1 to 1 wt% NaN_3 was added, since azide proved to be an appropriate inhibitor for obtaining abiotic conditions in clay water (Aerts, 2008; Mariën *et al.*, 2011). However, as later results showed the influence of NaN_3 on the reducing capacity value of DOM (see Section 4.1.5), it was decided to filter sterilise (filter with 0.2 µm pore size) the RBCW-ferricyanide solutions, instead of adding NaN_3.

As the reaction between ferric citrate and DOM reaches an equilibrium after 5 to 24 hours, a bacterial inhibitor (*e.g.* NaN_3) is not required. Indeed, microbial activity would occur only after a certain lag phase (Madigan *et al.*, 2000), which appears to take longer than the abiotic ferric citrate reduction by DOM in the RBCW solutions with ferric citrate. When using hydroquinone as a standard model for DOM, a bacterial inhibitor was not necessary either as insufficient nutrients were present for microbial growth.

For all types of solutions tested, the absence of bacteria was confirmed by Most Probable Number or MPN analyses (serial dilution series in specific bacterial growth medium) using NRP medium (Bleyen *et al.*, in preparation) or FC medium [adapted from Morris *et al.* (2005);

[2] The initial day(s) probably served as the lag time for the iron-reducing bacteria present in RBCW.

5 g/l yeast extract; 5 g/l NaCl and 10 g/l tryptone, 6.59 g/l $K_3Fe(CN)_6$, pH 7.8]. For each of these microbial analyses, 1 ml of slurry or 0.5 ml clay water was serially diluted (10×) in growth medium and incubated for 3 to 4 weeks at 30°C. The microbial concentration was then calculated based on the presence of gas and/or turbidity in the serial dilutions.

3.3. Determination of the reducing capacity of DOM using ferricyanide

For the determination of the reducing capacity of DOM using ferricyanide, the preliminary procedure from Pirlet (2003) was further optimised, keeping in mind its final use to evaluate possible changes in the reducing capacity of DOM exposed to nitrate and/or nitrite. For this, we followed the approach that is presented schematically in Table 1. All tests were performed under anaerobic conditions inside a glove box (N_2 atmosphere; [O_2] < 0.0004 vol%) to prevent air oxidation of DOM.

In general, the reducing capacity of DOM in RBCW was determined by monitoring the reduction of ferricyanide by DOM in time. For this, RBCW was diluted with synthetic clay water or SCW[3] and potassium ferricyanide [$K_3Fe(CN)_6$] and in some cases other additives ($NaNO_3$, $NaNO_2$ or NaN_3) were added (in case their impact was tested). Subsamples of these solutions were taken regularly and the concentration of ferricyanide is measured by UV-VIS (absorption peak at 420 nm). To minimise the delay between sampling and UV-VIS measurement and to prevent air oxidation, the UV-VIS measurements were performed inside a glove box under anaerobic atmosphere (N_2 atmosphere; [O_2] < 0.0004 vol%). When the reduction of ferricyanide by DOM was no longer significant (after 5 minutes for hydroquinone and ~50 hours for DOM in RBCW), the reducing capacity of DOM (in meq/gC) was calculated as the decrease in ferricyanide concentration divided by the concentration of DOM present in the solution.

[3] The composition of the used SCW is 0.044 g/l NaCl, 0.012 g/l $MgSO_4$, 0.0015 g/l Na_2SO_4, 0.020 g/l KCl, 0.008 g/l NaF and 1.25 g/l $NaHCO_3$.

Table 1: Approach that was followed to optimise the procedure for the determination of the reducing capacity of DOM for ferricyanide.

Question to be solved	Tests/approach to solve the question
Which reactions take place in a clay water – ferricyanide solution?	Tests with the hydroquinone/benzoquinone model as a standard for dissolved organic matter (DOM).
What is the kinetics of the ferricyanide reduction in clay water solutions?	Measurement at regular times of the ferricyanide concentration in clay water solutions.
What is the optimal ferricyanide/dissolved organic matter (DOM) ratio in clay water – ferricyanide solutions?	Measurement of the reducing capacity in solutions with different ferricyanide/DOM ratios. → The optimal ferricyanide/DOM ratio is established as the lowest ratio for which the maximal reducing capacity of DOM for ferricyanide can be obtained after a certain reaction period.
What is the effect of NaN_3 (bacterial inhibitor) on the ferricyanide reduction in clay water solutions?	Measurement of the reducing capacity in ferricyanide – hydroquinone (or clay water) solutions with and without NaN_3.
What is the effect of the presence of nitrate and nitrite on the ferricyanide reduction in clay water solutions?	Measurement of the reducing capacity in ferricyanide – hydroquinone (or clay water) solutions with and without nitrate or nitrite.
Is there an effect of ionic strength on the reduction of ferricyanide in clay water solutions?	Measurement of the reducing capacity of DOM in clay water with different ionic strength values using ferricyanide. The ionic strength is increased by addition of NaCl.

3.3.1. Direct reaction between NaN_3, $NaNO_3$ or $NaNO_2$ and ferricyanide / ferrocyanide

To study the possibility of a direct redox reaction between ferricyanide or ferrocyanide and azide, nitrate or nitrite, respectively NaN_3 (1 wt%), $NaNO_3$ (0.02 M) or $NaNO_2$ (0.02 – 0.1 M) was added to $K_4Fe(CN)_6$ or $K_3Fe(CN)_6$ solutions (1 mM final concentration; prepared in SCW). The evolution of the $[Fe(CN)_6]^{3-}$ or $[Fe(CN)_6]^{4-}$ concentrations were monitored in time by UV-VIS at respectively 420 nm (absorption peak of ferricyanide) and 323 nm (one of the absorption peaks of ferrocyanide).

3.3.2. Reduction of ferricyanide by hydroquinone (as standard model)

The reduction of ferricyanide by hydroquinone is represented by the following equation:

$$2\ [Fe(CN)_6]^{3-} + H_2Q \leftrightarrow 2\ [Fe(CN)_6]^{4-} + Q + 2\ H^+$$

Preliminary tests showed that its reaction rate is a function of temperature, solution pH and the ratio of ferricyanide / hydroquinone present in the system. As preliminary tests revealed that in unbuffered solutions green/black precipitates (quinhydrone complexes) are formed due to acidification of the solution by the redox reaction (see Section 3.1.1), a pH buffer (0.05M NaH_2PO_4/Na_2HPO_4 buffer at pH 7) was added to the solutions.

To determine the optimal ferricyanide / hydroquinone ratio, 1 mM $K_3Fe(CN)_6$ was added to hydroquinone with a concentration ranging from 13 to 56 mg C/l (Table 2). The reduction of ferricyanide by hydroquinone was monitored in time by UV-VIS (max ~300 hours).

Table 2: Set up of the optimisation test to define the optimal stoichiometric $[Fe(CN)_6]^{3-}/H_2Q$ ratio for the determination of the reducing capacity of hydroquinone for ferricyanide. The uncertainty on the H_2Q concentration (calculated based on TOC value) was ~10% (for a 95% confidence interval).

Solutions[a]	H_2Q (mg C/l)	$[Fe(CN)_6]^{3-}$ (mM)	stoichiometric $[Fe(CN)_6]^{3-}/H_2Q$ ratio
13 H_2Q_1mM FC	13	1	2.8
18 H_2Q_1mM FC	18	1	2.1
22 H_2Q_1mM FC	22	1	1.6
28 H_2Q_1mM FC	28	1	1.3
40 H_2Q_1mM FC	40	1	0.89
56 H_2Q_1mM FC	56	1	0.64

[a] All solutions were prepared in duplicate

To study the effect of azide, nitrate and nitrite on the value of the reducing capacity of hydroquinone, fresh $NaNO_3$ (0.1 M), $NaNO_2$ (0.1 M) or NaN_3 (0.1 or 1 wt%) was added to solutions with a stoichiometric ferricyanide / hydroquinone ratio of ~1.9 (1 mM $[Fe(CN)_6]^{3-}$ and 22 mg C/l hydroquinone). The ferricyanide concentration was monitored in time by UV-VIS spectroscopy (at 420 nm). The results were compared to the reducing capacity of hydroquinone without additives (using the same stoichiometric ferricyanide / hydroquinone ratio).

Furthermore, possible reactions between H_2Q and nitrate (0.1 M $NaNO_3$), nitrite (0.1 M $NaNO_2$) and azide (0.1 or 1 wt% NaN_3) were analysed by UV-VIS measurements and by chemical analysis (ion chromatography for measurement of nitrate, nitrite and azide).

3.3.3. Reduction of ferricyanide by DOM in RBCW

The reduction of ferricyanide by DOM in RBCW (*e.g.* humic acids or HA) is represented by

$$[Fe(CN)_6]^{3-} + \text{HA (reduced form)} \leftrightarrow [Fe(CN)_6]^{4-} + \text{HA (oxidised form)}$$

To evaluate the reduction of ferricyanide by DOM in time and to determine the optimal DOM / ferricyanide ratio and the optimal reaction time, solutions with different DOM concentrations (dilutions of RBCW in SCW) and different $K_3Fe(CN)_6$ concentrations were prepared as described in Table 3. To each of these solutions, 1 wt% NaN_3 was added to prevent microbial activity (see Section 3.1.2).

Table 3: Set up of the tests to optimise the procedure for the determination of the reducing capacity of DOM in RBCW for ferricyanide. In this test, different concentrations of $[Fe(CN)_6]^{3-}$ were reduced by different concentrations of DOM, to determine the optimal $[Fe(CN)_6]^{3-}$/DOM ratio. To each solution 1wt% NaN_3 was added.

Solutions[a]	$[Fe(CN)_6]^{3-}$ (mM)	$[Fe(CN)_6]^{3-}$/DOM ratio[b]
50 vol% RBCW dilution		
50% RBCW _0.25mM FC	0.25	0.9
50% RBCW _0.4mM FC	0.4	1.4
50% RBCW _0.8mM FC	0.8	3.2
50% RBCW _1.2mM FC	1.2	4.4
80 vol% RBCW dilution		
80% RBCW _0.4mM FC	0.4	0.9
80% RBCW _0.7mM FC	0.7	1.6
80% RBCW _1.2mM FC	1.2	2.8
27 vol% RBCW dilution		
27% RBCW _0.4mM FC	1	7.1

[a] All solutions were prepared in duplicate

[b] The $[Fe(CN)_6]^{3-}$/OM ratio is estimated by dividing the (theoretical) reducing capacity of DOM that would be measured in case that all added $[Fe(CN)_6]^{3-}$ would be reduced, by the experimentally determined maximal reducing capacity of DOM for $[Fe(CN)_6]^{3-}$ (6.1 meq/gC; determined in preliminary tests with RBCW).

3.3.3.1. Influence of azide, nitrate and nitrite on the reducing capacity of DOM measured using ferricyanide

The effect of azide, nitrate or nitrite on the reduction of ferricyanide by DOM was investigated by determining the reducing capacity of DOM in RBCW in the presence of different concentrations of NaN_3 (0 to 1 wt%), $NaNO_3$ (0.05 M) and/or $NaNO_2$ (0.005 to 0.025 M). The reducing capacity of DOM in these solutions was determined using the general procedure described in Section 3.3: 50 vol% dilutions of RBCW were prepared with SCW containing ferricyanide, $NaNO_3$, $NaNO_2$ and/or NaN_3, at final concentrations according to Table 4. The reduction of ferricyanide by DOM was monitored in time using UV-VIS measurements (max ~600 hours).

Table 4: Overview of the tests performed to study the effect of addition of azide, nitrate and/or nitrite on the reducing capacity of DOM in RBCW using ferricyanide.

Solutions[a]	DOM (mg C/l)	$[Fe(CN)_6]^{3-}$ (mM)	NaN_3 (wt%)	$[NO_3^-]$ (mM)	$[NO_2^-]$ (mM)
Without nitrate/nitrite					
50% RBCW_0% NaN_3	58	0.8	0	0	0
50% RBCW_0.1% NaN_3	58	0.8	0.1	0	0
With nitrate					
50% RBCW_$NaNO_3$_0% NaN_3	55	0.8	0	50	0
50% RBCW_$NaNO_3$_0.1% NaN_3	55	0.8	0.1	50	0
50% RBCW_$NaNO_3$_1% NaN_3	55	0.8	1	50	0
With nitrite					
50% RBCW_$NaNO_2$_0% NaN_3_a	52	0.8	0	0	5
50% RBCW_$NaNO_2$_0% NaN_3_b	52	0.8	0	0	25
50% RBCW_$NaNO_2$_0.1% NaN_3	52	0.8	0.1	0	25
50% RBCW_$NaNO_2$_1% NaN_3	52	0.8	1	0	25

[a] All solutions were prepared in duplicate

Moreover, to investigate the effect of NaN_3 further, the reducing capacity of DOM in RBCW solutions with and without 0.1 to 0.2 M $NaNO_3$ or 0.05 M $NaNO_2$ (freshly added or incubated up to 6 months) was determined with and without addition of NaN_3 (0.1 or 1 wt%) to the ferricyanide solutions at the time of measurement.

3.3.3.2. Effect of ionic strength on the reducing capacity of DOM measured using ferricyanide

The ability of organic matter to take part in redox reactions depends on its flocculation degree in the solution. Hence, differences in the ionic strength of the solution could affect the reducing capacity of the organic matter (Wittbrodt and Palmer, 1996). As the addition of nitrate, nitrite and/or azide (in different concentrations) to RBCW leads to differences in ionic strength, a test was performed to study the reducing capacity of DOM in RBCW at different ionic strengths.

To this end, the reduction of 0.8 mM $[Fe(CN)_6]^{3-}$ in 50 vol% diluted RBCW solutions was monitored in solutions with an ionic strength of 0.035 M to 0.20 M (adjusted with NaCl; Table

5). This range was chosen as it comprises all the ionic strengths of the solutions of the batch tests performed to study a possible oxidation of DOM by nitrate or nitrite (Bleyen *et al.*, in preparation). To prevent microbial activity, NaN_3 (0.1 wt%) was added to each solution.

Table 5: Set up of the test to study the influence of changes in the ionic strength on the reducing capacity of DOM in RBCW. Different concentrations of NaCl were added to 50 vol% RBCW solutions (OM concentration 58 mg C/l) with 0.8 mM $[Fe(CN)_6]^{3-}$ and 0.1 wt% NaN_3.

Solutions[a]	$[Fe(CN)_6]^{3-}$ (mM)	NaN_3 (wt%)	NaCl concentration (M)	Ionic strength (M)
50% RBCW	0.8	0.1	0	0.037
50% RBCW_0.01M NaCl	0.8	0.1	0.01	0.047
50% RBCW_0.02M NaCl	0.8	0.1	0.02	0.057
50% RBCW_0.04M NaCl	0.8	0.1	0.04	0.077
50% RBCW_0.06M NaCl	0.8	0.1	0.06	0.097
50% RBCW_0.16M NaCl	0.8	0.1	0.16	0.20

[a] All solutions were prepared in duplicate

3.4. Determination of the reducing capacity of DOM of RBCW using ferric citrate

Similar to the determination of the reducing capacity of DOM using ferricyanide, another existing procedure described by Bauer *et al.* (2007) using ferric citrate was further optimised. Again, the effect of nitrate, nitrite and azide on the measurement of the reducing capacity of DOM was investigated to assess whether this method could be applied to determine the reducing capacity of DOM, which has been incubated with these compounds. A similar approach as for ferricyanide (Table 1) was applied to optimise the procedure with ferric citrate (Table 6). Only RBCW was used here as the source of DOM during the optimisation, in contrast to the procedure with ferricyanide for which the hydroquinone / benzoquinone redox couple was used as a standard model for optimisation. All measurements were performed inside a glove box under an anaerobic atmosphere (N_2 atmosphere; $[O_2] < 0.0004$ vol%).

Table 6: Approach that was followed to optimise the procedure for the determination of the reducing capacity of DOM for ferric citrate.

Question to be solved	Tests/approach to solve the question
What is the optimal ferric citrate/DOM ratio?	Measurement of the reducing capacity in solutions with different ferric citrate/DOM ratios.
What is the kinetics of the ferric citrate reduction in clay water solutions?	Follow up of the reaction in detail during the first 72 hours after start.
What is the optimal pH environment for measuring the reducing capacity of DOM in RBCW?	Measurement of the reducing capacity in solutions with different initial pHs
What is the effect of NaN_3 (bacterial inhibitor) on the ferric citrate reduction (in clay water solutions)?	Measurement of the reducing capacity in • ferric citrate – RBCW solutions with and without NaN_3. • ferric citrate solutions with and without NaN_3 (effect on of ferric citrate on background) • RBCW solutions with and without NaN_3 (effect on background from RBCW)
What is the effect of the presence of nitrate and nitrite on the ferric citrate reduction (in clay water solutions)?	Measurement of the reducing capacity in • ferric citrate – RBCW solutions with and without nitrate or nitrite. • Ferric citrate solutions with and without nitrate or nitrite (effect on background)

In general, the reducing capacity of DOM was determined by monitoring the reduction of ferric citrate by DOM in time. For this, 50 vol% dilutions of RBCW were prepared with SCW to which ferric citrate and in some cases other additives ($NaNO_3$, $NaNO_2$ or NaN_3) were added (in case their impact was tested). This solution was acidified with 2 M HCl to reach the preferred pH. Immediately afterwards, ferrozine (freshly prepared in HEPES[4] buffer; final concentration 0.5 g/l) was added, which forms a complex with the produced Fe(II) [magenta coloured Fe(II)-$Ferrozine_3$ complex]:

$$\text{Fe(III) citrate} + \text{DOM (reduced form)} \leftrightarrow \text{Fe(II) citrate} + \text{DOM (oxidised form)}$$

$$\downarrow$$

$$\text{Fe(II)-Ferrozine}_3$$

[4] 6 wt% HEPES (or 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) in demineralised water.

Subsamples of these solutions were taken regularly and five-fold diluted to determine the concentration of the produced Fe(II)-Ferrozine$_3$ complex by UV-VIS spectroscopy (absorption peak at 562 nm).

When a significant reduction of ferric citrate no longer takes place, the reducing capacity of DOM is calculated by dividing the concentration of the produced ferrous iron by the concentration of DOM. To minimise the delay between sampling and UV-VIS measurement and to prevent air oxidation, the UV-VIS measurements were performed inside a glove box under anaerobic atmosphere (N_2 atmosphere; [O_2] < 0.0004 vol%).

3.4.1. Determination of optimal Fe(III) / DOM ratio and optimal incubation period

To study the reduction of ferric citrate by DOM and to determine the optimal DOM / Fe(III) ratio, 50 vol% RBCW dilutions were prepared to which ferric citrate was added at different concentrations (final concentration 1 or 2 mM). Each of these solutions was acidified to a pH 4.5 using 2 M HCl. After addition of ferrozine, the reduction of Fe(III) by DOM was monitored in time by measuring the produced Fe(II)-Ferrozine$_3$ complex using UV-VIS spectroscopy according to the general procedure described in Section 3.4.

3.4.2. Influence of the pH on the reduction of ferric citrate by DOM of RBCW

As the redox potential of the ferric citrate complex is depending on the pH of the solution due to pH-dependent shifts in its speciation (Wang *et al*, 2008; Vukosav *et al.*, 2012), differences in the ability of ferric citrate to be reduced by complex DOM are expected at different pHs. To study the effect of pH on the reduction of ferric citrate by DOM, some of the solutions containing ferric citrate (1 mM) and 50 vol% RBCW were acidified to pH 4.5. The initial pH of the remainder of solutions (not acidified) was ~7. After addition of the buffered ferrozine solution, the pH remained neutral during the reduction of ferric citrate. The production of Fe(II) was followed up in time by UV-VIS according to the general procedure described in Section 3.4.

3.4.3. Influence of azide, nitrate and nitrite on the reduction of ferric citrate by DOM in RBCW

A possible direct reaction between ferric or ferous citrate and either azide, nitrate or nitrite was investigating by adding respectively NaN_3 (0.1 wt%), $NaNO_3$ (0.1 M) and $NaNO_2$ (0.05 M) to 1 mM ferric citrate (with an initial background ferrous citrate = ~6% of total iron citrate content) or 6 µM $FeCl_2$, at pH 4.5 and/or pH 7. The evolution of the Fe(II) concentration was monitored by UV-VIS (at 562 nm) after addition of ferrozine (final concentration 0.5 g/l). Futhermore, the effect of azide, nitrate or nitrite on the reduction of ferric citrate by DOM was investigated by determining the reducing capacity of DOM in RBCW in the presence of different concentrations of freshly added NaN_3 (0 to 1 wt%), $NaNO_3$ (0.1 M) and/or $NaNO_2$ (0.0002 to 0.05 M) (Table 7), according to the general procedure described in Section 3.4.

Table 7: Overview of the tests performed to investigate the effect of addition of azide, nitrate and nitrite on the reducing capacity of DOM in RBCW using ferric citrate. For this, NaN_3, $NaNO_3$ and/or $NaNO_2$ was added to 50 vol% RBCW (55 mg TOC/l) and in the absence or in the presence of ferric citrate.

Solutions	Ferric citrate (mM)	NaN_3 (wt%)	$[NO_3^-]$ (mM)	$[NO_2^-]$ (mM)
Without nitrate/nitrite				
RBCW blanc	0	0	0	0
RBCW blanc_0.1% NaN_3	0	0.1	0	0
RBCW blanc_1% NaN_3	0	1	0	0
Ferric citrate blanc	1	0.	0	0
Ferric citrate blanc_0.1% NaN_3	1	0.1	0	0
Ferric citrate blanc_1% NaN_3	1	1	0	0
50% RBCW_0% NaN_3	1	0	0	0
50% RBCW_0.1% NaN_3	1	0.1	0	0
50% RBCW_1% NaN_3	1	1	0	0
With nitrate				
50% RBCW_0.1 M $NaNO_3$_0% NaN_3	1	0	0.1	0
50%RBCW_0.1 M $NaNO_3$_0.1% NaN_3	1	0.1	0.1	0
With nitrite				
50% RBCW_0.05 M $NaNO_2$_0% NaN_3	1	0	0	50
50%RBCW_0.05 M $NaNO_2$_0.1% NaN_3	1	0.1	0	50
50% RBCW_0.001 M $NaNO_2$_0% NaN_3	1	0	0	1
50% RBCW_0.0001 M $NaNO_2$_0% NaN_3	1	0	0	0.1
50% RBCW_0.0002 M $NaNO_2$_0% NaN_3	1	0	0	0.2

3.5. Proof of principle

To ensure that the two methods to determine the reducing capacity of DOM described in this report would allow us to determine decreases in this reducing capacity due to oxidation of DOM by nitrate or nitrite, an additional 'proof of principle' test was performed. For this, RBCW was stirred continuously in air, resulting in a fast oxidation of DOM by O_2. Subsamples were taken at different time intervals (up to 56 days) and brought into the glove box for the determination of the reducing capacity of the partially oxidised DOM using both ferricyanide and ferric citrate as oxidant (according to the final procedures in Annexes 1 and 2).

4. Results

4.1. Determination of the reducing capacity of DOM using ferricyanide

4.1.1. Kinetics of ferricyanide reduction by DOM

4.1.1.1. Hydroquinone as a model for DOM

In Figure 3, the reduction of ferricyanide (1 mM initial concentration) by hydroquinone (18 mg C/l) is shown. Similar results were obtained for solutions with other ferricyanide to hydroquinone ratios.

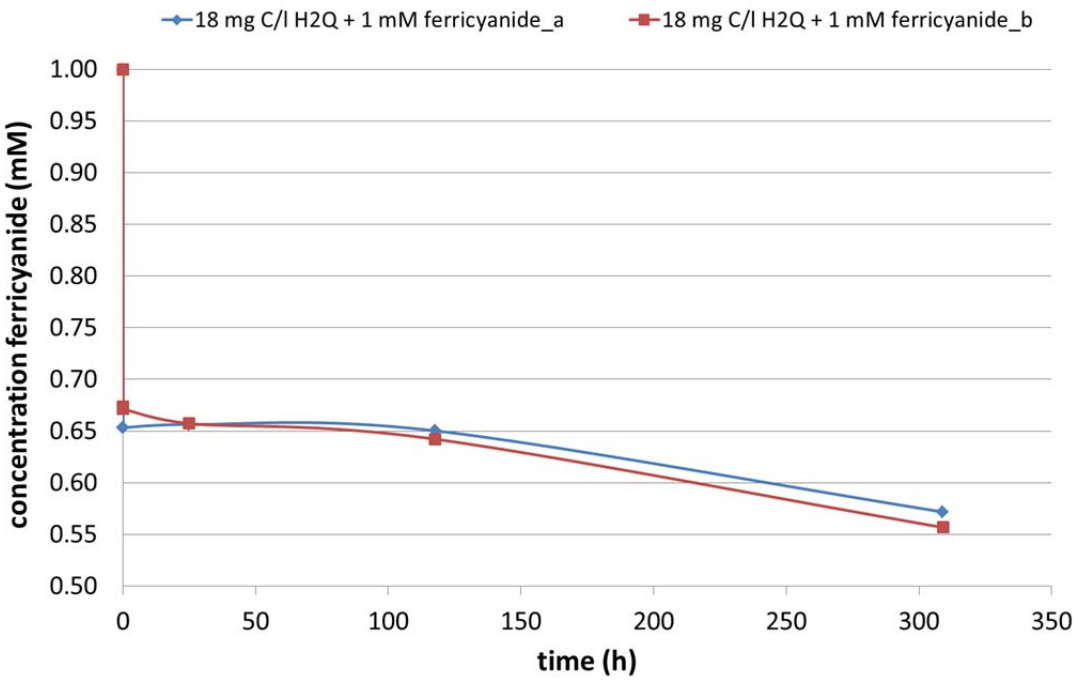

Figure 3: Time evolution of the $[Fe(CN)_6]^{3-}$ concentration (based on the absorbance at 420 nm) for a hydroquinone solution (18 mg C/l) with 1 mM $K_3Fe(CN)_6$ (results from duplicate solutions). The A_{420} at the start of the experiment could not be measured accurately due to a very rapid reduction of the ferricyanide, and was therefore considered to be the nominal A_{420} of a 1 mM ferricyanide solution in demineralised water. The error on the concentrations is 2% (for 95% confidence interval).

It is clear that the reaction is not characterised by a linear loss of $[Fe(CN)_6]^{3-}$ with time. The evolution of the $[Fe(CN)_6]^{3-}$ concentration suggests that the reduction is controlled by at least two different reduction mechanisms, which differ in their reaction kinetics. Initially, $[Fe(CN)_6]^{3-}$

is reduced very fast. Within a few minutes after addition of $[Fe(CN)_6]^{3-}$ to the hydroquinone solution a considerable amount (~35 mol%) of $[Fe(CN)_6]^{3-}$ is reduced. The reaction slows down significantly as more benzoquinone is formed, *i.e.* only ~10 mol% of $[Fe(CN)_6]^{3-}$ is reduced in the next 300 h.

The change in kinetics is probably due to the complexation of benzoquinone with (part of) the remaining hydroquinone (formation of a quinhydrone complex; Figure 2), which could hinder the reduction of $[Fe(CN)_6]^{3-}$ by hydroquinone (Kappelmeyer *et al.*, 2003). To investigate whether the formation of quinhydrone (resulting in a slightly reddish coloration of the solution) could also have influenced the absorbances at 420 nm (A_{420}), a deconvolution of the absorbance spectra (from 350 to 500 nm) was performed (using the interactive peak fitter 2.2 function in the Matlab software). The spectra from hydroquinone solutions (ferricyanide / H_2Q ratio ~2.0) at the moment of addition of ferricyanide and after different reaction times were analysed. This resulted in the detection of an absorption peak at 480 nm, which can be attributed to quinhydrone (absorption peak between 420 and 630 nm; Tossell, 2009). The value of A_{480} slowly increased over time, indicating oxidation of hydroquinone into benzoquinone and subsequent formation of quinhydrone (dimers). However, this peak did only interfere minimally with the peak at 420 nm from ferricyanide during the first ~800 hours after addition of ferricyanide to hydroquinone. On the other hand, long term measurements (over a few months) indicated that further oxidation of hydroquinone does result in a significant interference of the peak at 420 nm.
Nevertheless, as the oxidation of hydroquinone is hindered by the formation of quinhydrone, it is recommended to interpret the results only until ~100h after the start of the test, or prior to the first coloration of the solutions which indicates a significant formation of quinhydrone. As the main reduction of ferricyanide occurs during the initial (fast) reaction period, the reducing capacity of hydroquinone for ferricyanide is determined after ~5 minutes. In this case, the A_{420} values do not have to be adjusted for the formation of quinhydrone.

4.1.1.2. DOM in RBCW

Figure 4 shows the time evolution of the absorbance at 420 nm (proportional to the $[Fe(CN)_6]^{3-}$ concentration) in a 50 vol% RBCW solution with 0.8 mM $K_3Fe(CN)_6$. Similar kinetics have been observed for other ferricyanide / DOM ratios.
Pirlet (2003) and Matthiessen (1995) assessed the reduction of ferricyanide by DOM from RBCW (Pirlet, 2003) and natural organic matter (humic substances from podzol, sandy material and chernozem; Matthiessen, 1995). These researchers observed a significant reduction of

ferricyanide only during the first 24 hours of reaction. In contrast, the results of our tests suggest that the reduction of $[Fe(CN)_6]^{3-}$ by DOM in RBCW is characterised by two reaction mechanisms similar to what is observed for hydroquinone (Section 4.1.1.1): an initial fast reaction process that is attributed to the oxidation of phenolic functional groups of organic matter (*e.g.* hydroquinones) is followed by a second slow reaction process that involves less reactive functional groups. The latter functional groups are not yet identified, although some of these groups could be thiols and amines, as discussed by Stevenson (1982). On the other hand, the very slow reduction of ferricyanide, lasting for more than 500 h, might also be linked to the diffusion of electrons from complex DOM chains to $[Fe(CN)_6]^{3-}$. Plotting log[ferricyanide] versus time (data not shown), shows that both processes have a second order kinetics. Based on these results, the initial fast reaction process appears to dominate the system during the first 50 hours. Due to the (possible) formation of quinhydrone-like structures in the RBCW solutions (see Section 4.1.1.1), resulting in coloration of the solutions, it is recommended to interpret the results after the initial reaction period, *i.e.* 50 hours.

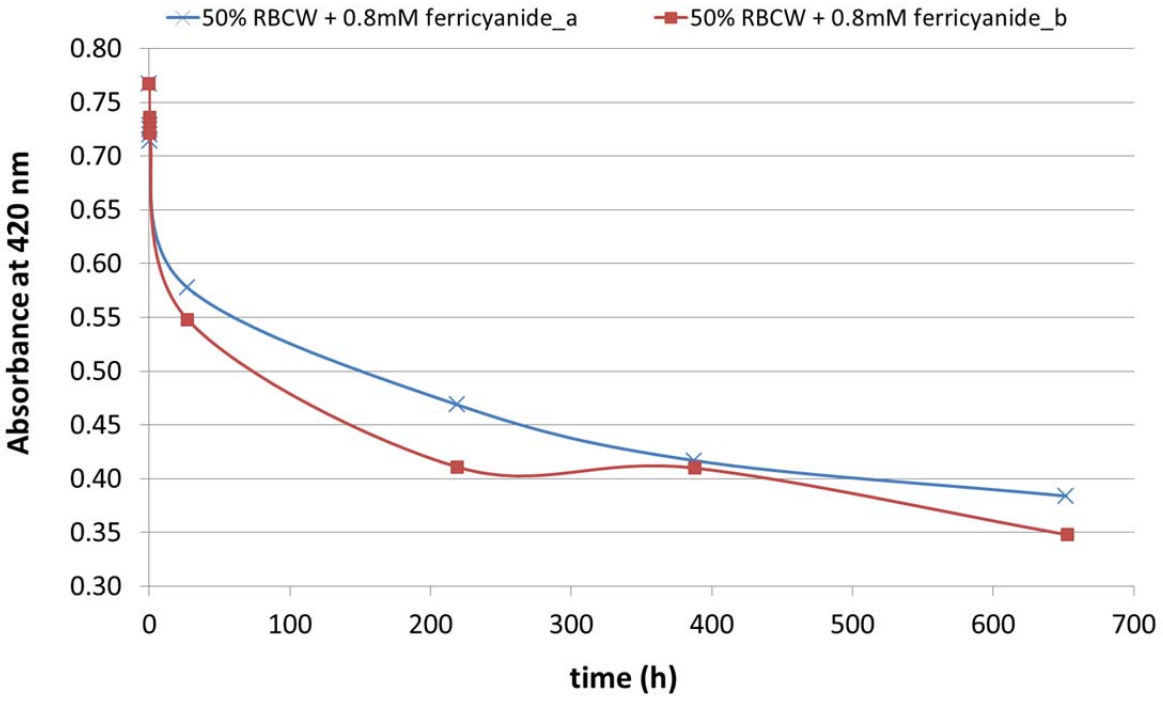

Figure 4: Time evolution of the absorbance at 420 nm (A_{420}), which is proportional to the $[Fe(CN)_6]^{3-}$ concentration, in two duplicate 50 vol% RBCW solutions with 0.8 mM $K_3Fe(CN)_6$. The A_{420} at the start of the experiment could not be measured accurately due to a very rapid reduction of the ferricyanide, and was therefore taken equal to the A_{420} of a 0.8 mM ferricyanide solution in demineralised water. The error on the absorbances is 1% (for 95% confidence interval).

4.1.2. Optimal ferricyanide to DOM ratio

To determine the optimal ferricyanide / DOM ratio, the reducing capacity of DOM was determined in solutions with different ferricyanide / DOM ratios, both for hydroquinone and for RBCW. The optimal ferricyanide / DOM ratio was established as the lowest ratio for which the value of the reducing capacity of DOM using ferricyanide is maximal. Based on this, a ferricyanide / H_2Q ratio of ~1 (*i.e.* 1 mM ferricyanide added to ~40 mg C/l hydroquinone) is considered as the optimal ratio to determine the reducing capacity of hydroquinone. For RBCW, a ferricyanide / DOM ratio of 3.2 (0.8 mM ferricyanide added to 50 vol% RBCW) is the optimal ratio for determining the reducing capacity of DOM in RBCW.

4.1.3. Reducing capacity of DOM in RBCW measured with ferricyanide

The reducing capacity of DOM in RBCW using ferricyanide ranges from 3.4 to 6.1 meq/gC. The value varies slightly depending on the batch of RBCW taken from the EG/BS piezometer, similar to the differences in TOC value and functional groups observed for different batches of RBCW (Van Geet, 2004; Van Geet *et al*., 2003). Also differences in pH of the RBCW can result in variations of the reducing capacity of DOM. Indeed, Matthiessen (1995) observed an increase in reducing capacity value of DOM with increasing pH, which is likely attributed to the dissociation of phenolic groups at increasing pH.

The expanded error on an individual reducing capacity value using the optimised procedure for ferricyanide (Annex 1) is 10-20% (for a 95% confidence interval), and is calculated for each value as the combined uncertainty, based on uncertainties on the TOC value and on the ferricyanide concentration.

4.1.4. Effect of nitrate and nitrite on the value of the reducing capacity of DOM

A possible influence of nitrate and/or nitrite on the (measured) $[Fe(CN)_6]^{3-}$ or $[Fe(CN)_6]^{4-}$ concentrations was investigated by monitoring both ferricyanide and ferrocyanide concentrations over time in the presence of $NaNO_3$ or $NaNO_2$. After 1 month of anaerobic incubation, no effect of either nitrate (0.02 M) or nitrite (0.02 M) could be observed on the ferrocyanide

concentration. Furthermore, no $[Fe(CN)_6]^{3-}$ reduction by 0.1 M $NaNO_2$ was measured after almost two weeks (300 hours) of storage in an anaerobic glove box.

4.1.4.1. Effect on reducing capacity of hydroquinone

Addition of 0.1 M $NaNO_3$ to hydroquinone does not have an effect on the reducing capacity value of H_2Q. In contrast, when nitrite is added to hydroquinone, the solution turns an orange colour, suggesting the formation of quinhydrone (-like compounds). Furthermore, a change in the absorbance spectrum of the solution was observed within the UV region (200-400 nm), especially around 280 nm, suggesting that some oxidation of hydroquinone occurs by nitrite. However, as the concentration of nitrite did not change significantly over ~7 months (determined by ion chromatography at SCK•CEN), the oxidation of hydroquinone by nitrite seems to be a very slow process. Such a reaction (at circumneutral pH) has been observed previously with phenolic compounds, resulting in the formation of organic nitrogen, nitroso- and nitro-compounds (Van Cleemput and Samater, 1996; Thorn and Mikita, 2000). At lower pH (< 5.5), this reaction appears to occur to a smaller extent, as chemodenitrification (abiotic production of gaseous N species) and decomposition of HNO_2 would prevail (Van Cleemput and Samater, 1996).

4.1.4.2. Effect on reducing capacity of DOM in RBCW

As a small effect of nitrite on the reducing capacity of hydroquinone was observed, a possible effect of nitrite on the reducing capacity of DOM in RBCW was investigated. For this, the UV spectra of the 50 vol% RBCW solutions containing 0.025 M $NaNO_2$ (incubated up to 2.5 years) were compared in time. In contrast to hydroquinone, these spectra do not indicate any significant changes of the A_{280}.

Furthermore, addition of either nitrate (0.05 M) or nitrite (0.005 M to 0.025 M) to RBCW did not result in significant differences in the measured reducing capacity of DOM of RBCW. The effect of nitrate and nitrite on the reducing capacity of DOM for ferricyanide is therefore considered to be negligible (within the time frame of the measurements).

4.1.5. Influence of the azide on the value of the reducing capacity of DOM

4.1.5.1. Effect on reducing capacity of hydroquinone

As shown in Figure 5, addition of NaN_3 to hydroquinone while measuring its reducing capacity with ferricyanide results in a faster reduction of ferricyanide. Nevertheless, no reaction between NaN_3 and ferricyanide was observed during more than 600 hours after addition of 1 wt% NaN_3 to 1 mM ferricyanide. These results suggest a reconversion of benzoquinone (Q) into hydroquinone (H_2Q) by NaN_3 (Couladouros *et al.*, 1997):

$$Q + 2\,N_3^- + 2\,H^+ \rightarrow H_2Q + 3\,N_2$$

The produced hydroquinone could then again reduce ferricyanide. These reactions ultimately result in the higher measured reducing capacity value of hydroquinone for ferricyanide compared to that of a hydroquinone solution without any NaN_3, *i.e.* a reducing capacity of H_2Q of 19 meq/gC, 46 meq/gC and 48 meq/gC was measured for respectively H_2Q without azide and with addition of 0.1 wt% and 1 wt% NaN_3.

In the hydroquinone solutions with the lowest NaN_3 concentration (0.1 wt% NaN_3), a similar equilibrium ferricyanide concentration to the one in the solutions with 1 wt% NaN_3 was reached, likely due to the excess of azide compared to DOM. On the other hand, the addition of 0.1 wt% NaN_3 led to a slightly slower initial reduction of ferricyanide (Figure 5), compared to when 1 wt% NaN_3 was added.

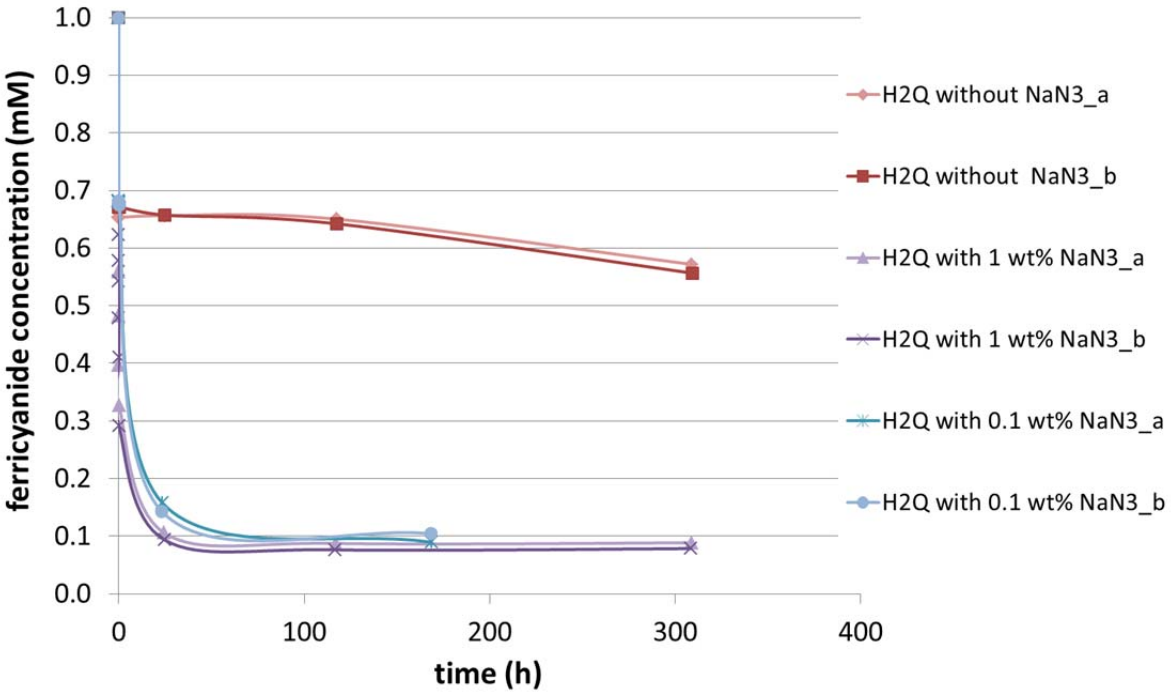

Figure 5: Time evolution of the ferricyanide reduction by hydroquinone (19 mgC/l), with or without addition of NaN_3 (0.1 or 1 wt%). The A_{420} at the start of the experiment could not be measured accurately due to the very rapid reduction of the ferricyanide. Therefore, the initial ferricyanide concentration was taken equal to 1 mM ferricyanide for all solutions. The error on the concentrations is 2% (for 95% confidence interval).

4.1.5.2. Effect on reducing capacity of DOM in RBCW

As hydroquinones and quinones are important redox-active compounds or functional groups in clay water solutions, the addition of azide while determining the reducing capacity using ferricyanide might also affect the measured value of the reducing capacity of DOM in RBCW. The possible impact of azide on the reducing capacity of DOM was therefore investigated further.

For this, the reducing capacity of DOM in RBCW solutions incubated with and without 0.1 M $NaNO_3$ or 0.05 M $NaNO_2$ was determined with and without addition of NaN_3 to the ferricyanide solutions at the time of measuring the reducing capacity. As can be observed in Figure 6, addition of NaN_3 appears to increase the reducing capacity slightly and this effect seems to be depending on the concentration of NaN_3. While addition of 0.1 wt% NaN_3 does not result in a statistically significant increase in the reducing capacity, addition of 1 wt% NaN_3 does (Figure 6). These results support the above mentioned hypothesis (see Section 4.1.3.1) explaining the effect of NaN_3 on certain phenolic (or hydroquinone-like) functional groups of RBCW.

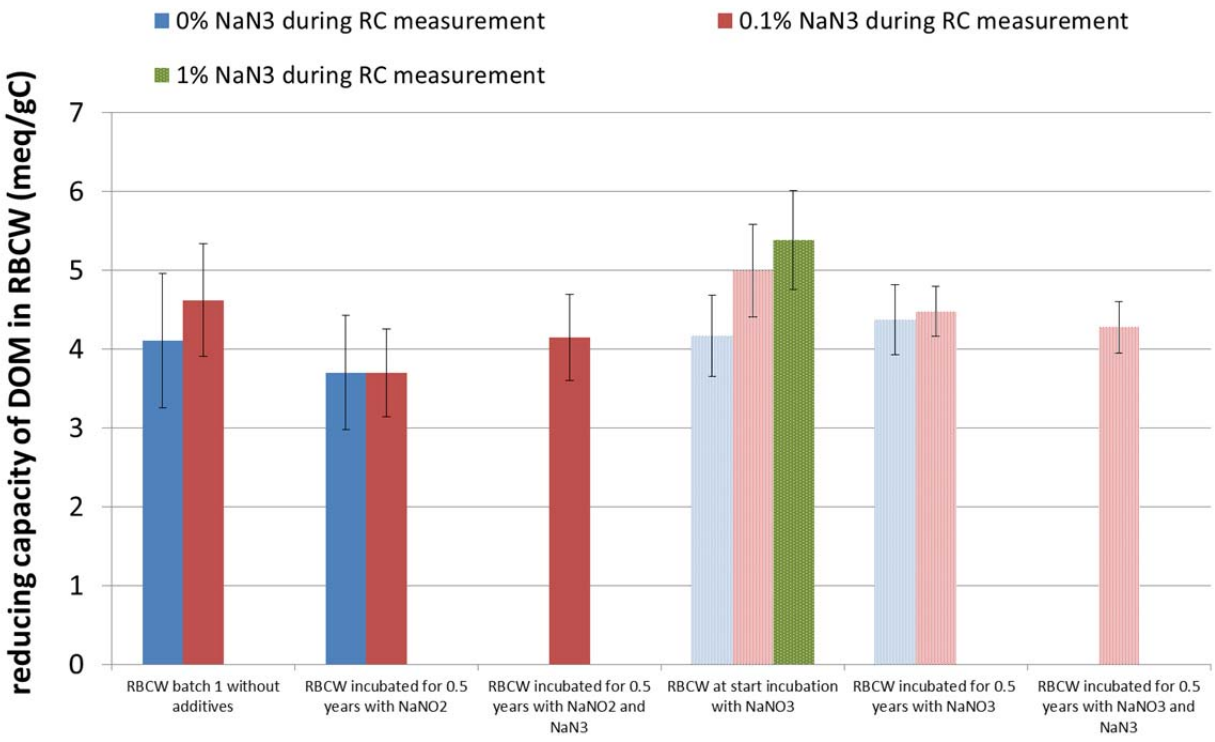

Figure 6: Reducing capacity values of DOM in RBCW incubated with 0.1 M $NaNO_3$ or 0.05 M $NaNO_2$, determined with $[Fe(CN)_6]^{3-}$ and with (0.1 or 1 wt%) or without addition of NaN_3 (concentrations indicated in the legend). Two batches of RBCW were used: batch 1 in full colours; batch 2 in dotted colours. All measurements were performed at least in duplicate. The average reducing capacity value for all replicates is shown. The error bars represent the 95% confidence interval.

The kinetics of the reduction of ferricyanide by DOM (Figure 7) suggest that the difference in reducing capacity of DOM is mainly caused by a higher degree in oxidation of the phenolic functional groups (hydroquinone-like groups) by ferricyanide (*i.e.* faster decrease in ferricyanide in the first 50 hours after addition). This supports the possible conversion of quinone-like groups into hydroquinone-like groups by azide (see Section 4.1.5.1), *i.e.* N_3^- could have reduced (some of) the functional groups of dissolved organic matter that were previously oxidised by ferricyanide. As the DOM in RBCW consists of different organic compounds, the effect of azide is less pronounced compared to its effect on pure hydroquinone and even not statistically significant for concentrations of NaN_3 below 1 wt%. However, a slight overestimation of the reducing capacity of DOM can occur when determining the reducing capacity in the presence of NaN_3. Therefore, it is recommended to avoid using NaN_3 as microbial inhibitor when determining the reducing capacity of DOM. As an alternative to NaN_3, filter sterilisation (0.22 µm filter) has proven to be efficient to prevent microbial activity. As this method does not perturb the measurement of the reducing capacity, it is the preferred sterilisation method.

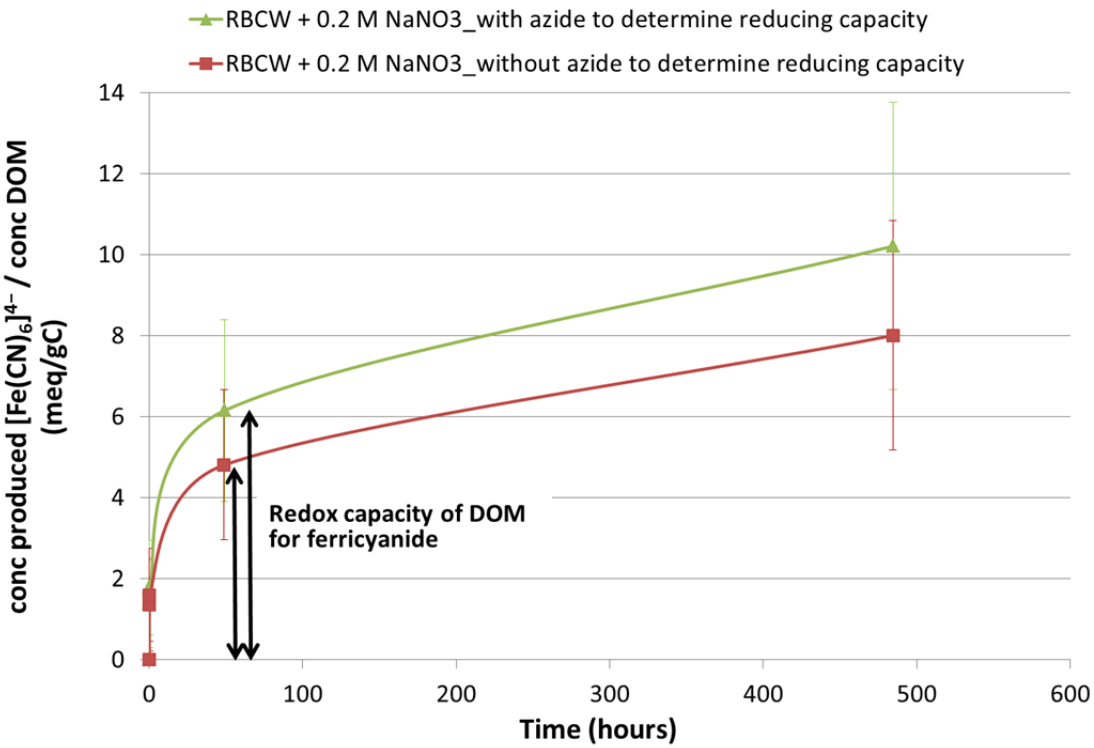

Figure 7: Evolution of the reduction of ferricyanide by DOM in RBCW (containing 0.2 M $NaNO_3$), normalised to the DOM concentration. The reduction process was monitored in both the presence (1 wt%) and in the absence of NaN_3 (as indicated in the legend). The error bars represent the 95% confidence interval.

4.1.6. Effect of ionic strength on the reducing capacity value of DOM of RBCW

Changes in ionic strength of the solution can cause conformational modifications in the DOM, due to changes in the thickness of the Humic Double Layer, and might cause flocculation of DOM. The extent of these changes depends on the valence of the counterions in the bulk solution (Jones and Bryan, 1998). These changes could therefore render the reactive functional groups of DOM either more or less accessible to $[Fe(CN)_6]^{3-}$, thereby possibly altering the rate of reduction. The effect of ionic strength on the reducing capacity of OM in the RBCW solutions was investigated by assessing the reduction of ferricyanide by OM in solutions with increasing ionic strength. A range of ionic strength values from 0.035 M to 0.20 M was tested (Figure 8), as this would be the range of ionic strengths in the experimental study in which the effect of nitrate and nitrite on the reducing properties of DOM in Boom Clay pore water was evaluated (Bleyen *et al.*, in preparation). The evolution of the ferricyanide reduction in time is similar for all solutions, so similar mechanisms are at work irrespective of the ionic strength value (ranging from 0.035 to 0.2 M). In general, a higher ionic strength leads to somewhat faster initial ferricyanide reduction kinetics and a higher resultant reducing capacity of OM.

Figure 8 shows the slight increase in the value of the reducing capacity of DOM in RBCW solutions with increasing ionic strengths (for ionic strengths of 0.097 M and 0.2 M), which suggests a (slightly) higher degree of available functional groups at higher ionic strengths. This observation is in contrast with the increasing degree in flocculation of DOM at higher ionic strength, as reported by Chang *et al.* (1993), Jones and Bryan (1998) and Sparks (2003), although it should be noted that at the low ionic strength values that were tested here, the effect of flocculation is expected to be limited. This is also confirmed by the TOC values of the ferricyanide solutions of this tests, which show no significant flocculation in the ferricyanide solutions with the highest ionic strength (0.2 M).

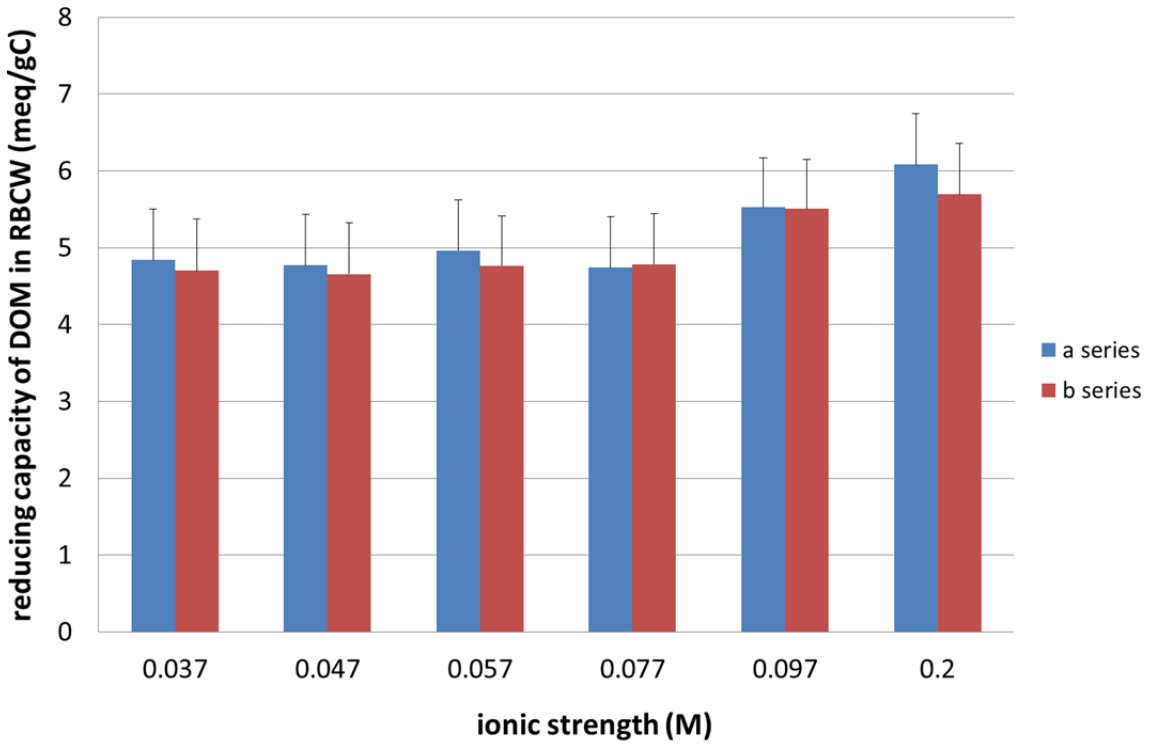

Figure 8: Comparison of reducing capacity values of DOM (in RBCW) for $[Fe(CN)_6]^{3-}$, measured in solutions with an increasing ionic strength (ranging from 0.035 to 0.20 M, by addition of NaCl). To determine the reducing capacity, 0.8 mM $K_3Fe(CN)_6$ was added to 50 vol% RBCW and 0.1 wt% NaN_3. The effect of the ionic strength was tested in duplicate. The error bars indicate the 95% confidence interval on the value.

It can be remarked that Tits (1990) measured an increase in ionisation of the functional groups of humic acids from a Podzol soil as the ionic strength increased from 0.01 to 1 M (at a given pH), due to the masking effect of the salt. Moreover, Matthiessen (1995) suggested that an increase in ionisation of the phenolic functional groups could explain the increase in reducing capacity of hydroquinone, which occurs with increasing pH. Hence, when the ionic strength increases (to values below 1 M), the higher number of ionised functional groups might result in conformational changes leading to a higher degree of available functional groups, explaining a higher reducing capacity. However, at this moment it is not yet clear whether the observations by

Tits (1990) and Matthiessen (1995) can be transferred to our tests to explain the increase in reducing capacity of DOM as the ionic strength increases from 0.04 to 0.2 M. No published results on the effect of ionic strength on the reducing capacity of DOM are known at the moment of writing this report.

To be able to compare the value of the reducing capacity of DOM in RBCW solutions containing additives (*e.g.* nitrate) with significant differences in ionic strengths, the ionic strength of the RBCW dilutions with ferricyanide should be adjusted to obtain equal ionic strength values in all solutions while determining the reducing capacity of DOM. However when the ionic strength of the solutions is below 0.1 M, adjustment of the ionic strength does not seem to be necessary.

4.2. Determination of the reducing capacity of DOM using ferric citrate

4.2.1. Kinetics of Fe(III) reduction by DOM of RBCW at different pHs

According to Bauer *et al.* (2007) and Chen *et al.* (2003), the rate and equilibrium of the redox reaction between ferric citrate and natural DOM is depending on the pH of the solution. This can be explained by the difference in the speciation of the Fe(III) and Fe(II) citrate complexes and in the standard redox potential of the corresponding reduction reaction. A lower pH of the solution seems to render a ferric citrate complex with a higher standard redox potential, and thus a higher oxidising capacity towards DOM (Wang *et al*, 2008; Vukosav *et al.*, 2012).
To investigate the pH effect further and to determine the optimal pH at which the reducing capacity of DOM in RBCW should be measured, the ferric citrate reduction by DOM was evaluated at different initial pHs, *i.e.* at pH 4.5 and pH 7. The production of Fe(II) was measured by UV-VIS after complexation with ferrozine. As the complexation of iron with ferrozine occurs in a HEPES buffer at pH 7 and the complex between Fe(II) and ferrozine is stable between pH 4 and 9 (Stookey, 1970), the tested initial pHs would only affect the speciation of the ferric citrate complex and not the stability of the Fe(II)-ferrozine complex and thus the measurement of the produced Fe(II).

As can be observed in Figure 9, ~50% less ferric citrate was reduced by DOM when the solution is initially acidified to pH 7 compared to pH 4.5, which is in agreement with the lower redox potential of the ferric citrate complex at a higher pH. As the concentration of Fe(II) measured by UV-VIS is higher at pH 4.5, the uncertainty on this concentration is lower (less influenced by background noise). Therefore, it is recommended to determine the reducing capacity of DOM for ferric citrate at an initial pH of 4.5. In the remainder of the tests described in this report, the initial pH was therefore always 4.5.

Similar to the reaction kinetics of the ferricyanide reduction by DOM (see Section 3.1.1), the reduction of ferric citrate by DOM in RBCW at pH 4.5 is also characterised by two processes: an initial fast reduction rate (increase in Fe(II) concentration) within the first hour is followed by a slower reduction process (Figure 9). After ~24 hours, no more statistically significant increase in Fe(II) concentration can be detected. Based on these results, it was decided to determine the value of the reducing capacity of DOM using ferric citrate after ~24 hours.

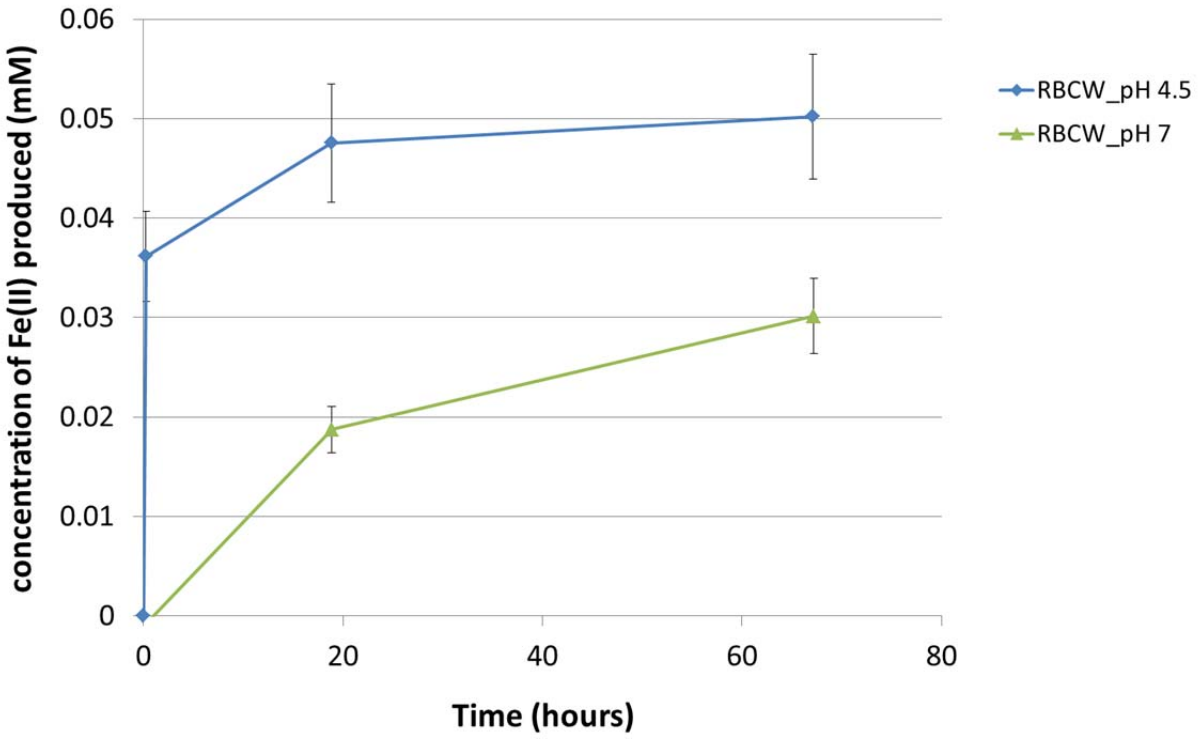

Figure 9: Time evolution of the Fe(II) concentration produced during the redox reaction between ferric citrate (starting concentration 1 mM) and DOM in RBCW (50 vol% diluted; 55 mg C/l). The effect of acidification (to pH 4.5 and 7) of the initial solutions with DOM and ferric citrate (before addition of ferrozine) is shown. The error bars represent the 95% confidence interval.

4.2.2. Determination of the optimal ferric citrate / DOM ratio

To determine the optimal ferric citrate to DOM ratio, two different concentrations of ferric citrate were added to 50 vol% RBCW: 1 and 2 mM. No significant differences in reducing capacity of DOM of RBCW were detected (Figure 10), indicating that addition of 1 mM ferric citrate suffices to achieve the maximal reducing capacity of DOM using ferric citrate.

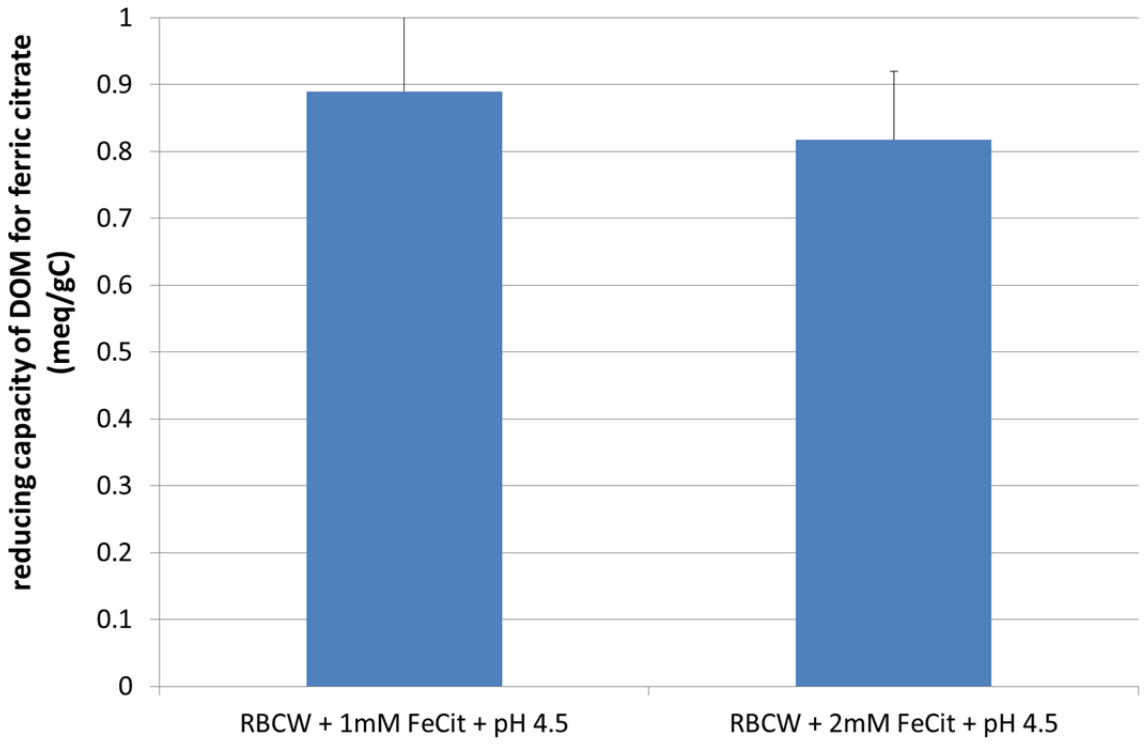

Figure 10: Comparison of the value of the reducing capacity of DOM in RBCW determined using 1 and 2 mM ferric citrate. The error bars represent the 95% confidence interval.

4.2.3. Reducing capacity of DOM in RBCW measured with ferric citrate

The reducing capacity of DOM in RBCW using ferric citrate ranges from 0.8 to 1.3 meq/gC. Similar to what has been observed for ferricyanide, the value of the reducing capacity of DOM in RBCW using ferric citrate varies slightly depending on the batch of RBCW. The expanded error on an individual reducing capacity value using the optimised procedure for ferric citrate is 12-15%, and is calculated for each value as the combined uncertainty (95% uncertainty), based on uncertainties on the TOC value and on the produced Fe(II) concentration.

Note that the value of the reducing capacity of DOM of RBCW for ferric citrate is ~5 times lower than the one obtained for ferricyanide. This is in agreement with previous observations for natural DOM (Peretyazhko and Sposito, 2006; Bauer *et al.*, 2007) and can be attributed to the difference in standard redox potential of both oxidants, in their chemical structure, and to the effect of pH on the speciation of Fe(III)-citrate (see Section 4.2.1).

4.2.4. Effect of nitrate and nitrite on the measurement of the reducing capacity using ferric citrate

To assess the effect of nitrate and nitrite on the measurement of the reducing capacity of DOM using ferric citrate, fresh $NaNO_3$ (final concentration 0.1 M) or $NaNO_2$ (concentration range 0.0002 to 0.05 M) was added to ferric citrate (1 mM) in the presence or in the absence of DOM (in 50 vol% RBCW). Based on the stability of ferric citrate and Fe(II) in the presence of $NaNO_3$ (0.1 M) and in the absence of a reductant (DOM), nitrate does not have a direct effect on Fe(III)-citrate or Fe(II). Furthermore, as the reduction of ferric citrate by DOM and the derived value of the reducing capacity of DOM in RBCW with 0.1 M $NaNO_3$ do not show statistically significant differences from those obtained in the absence of $NaNO_3$, addition of nitrate to RBCW does not seem to have a statistically significant effect on the value of the reducing capacity.

On the other hand, the presence of nitrite causes a significant and concentration-dependent decrease of the value of the reducing capacity of DOM in RBCW when using ferric citrate as oxidant (Figure 11). This decrease is statistically significant as from concentrations of nitrite higher or equal to 1 mM. This result can be attributed to the (re-)oxidation of Fe(II), produced during the reduction of Fe(III)-citrate by DOM, by nitrite (Percheron *et al.*, 1998 and Kampschreur *et al.*, 2011), which results in an underestimation of the amount of reduced ferric citrate during the measurement of the reducing capacity. This chemical reaction has been confirmed when comparing the UV-VIS spectra of $FeCl_2$ (6 µM) complexed with ferrozine with or without 0.05 M $NaNO_2$ at pH 4.5 and 7: a significant decrease in the A_{562} is observed in the presence of nitrite (Figure 12). This decrease is pH dependent: the oxidation of Fe(II) by nitrite is faster at low pH compared to neutral pH, most likely due to the formation of the more reactive HNO_2 at low pH.

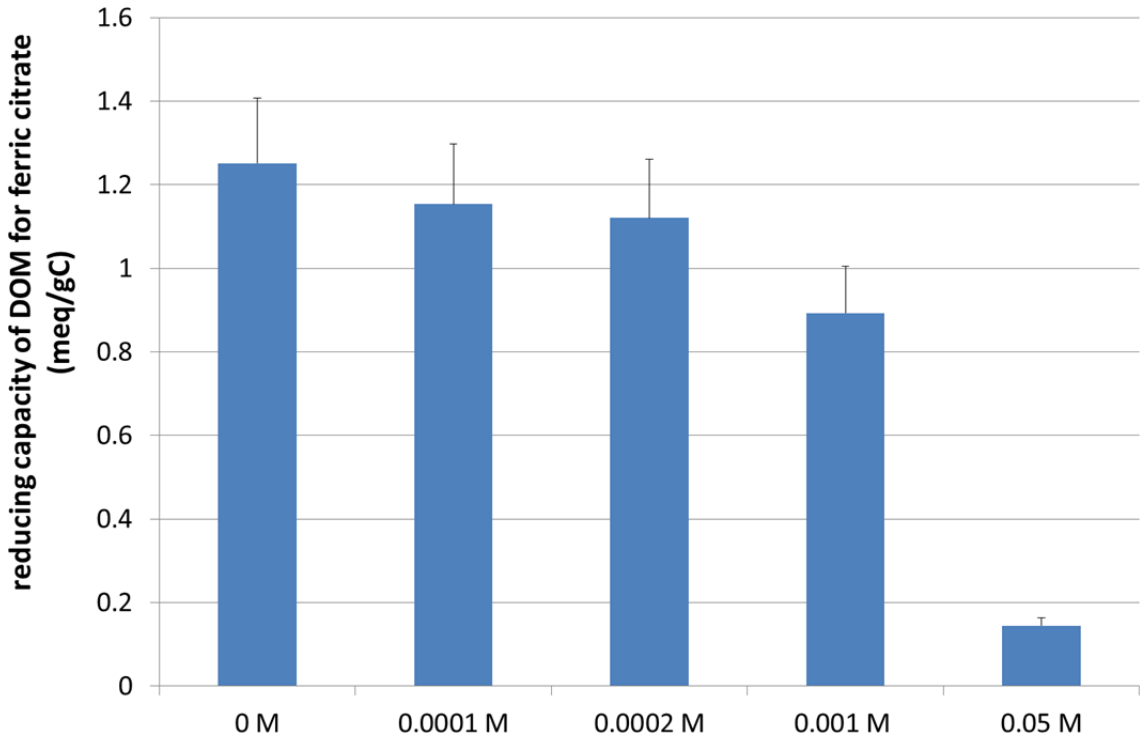

Figure 11: Influence of different concentrations of freshly added nitrite (concentrations indicated below the X axis) on the value of the reducing capacity of DOM measured with ferric citrate (using the procedure described in Annex 2). The error bars represent the 95% confidence interval.

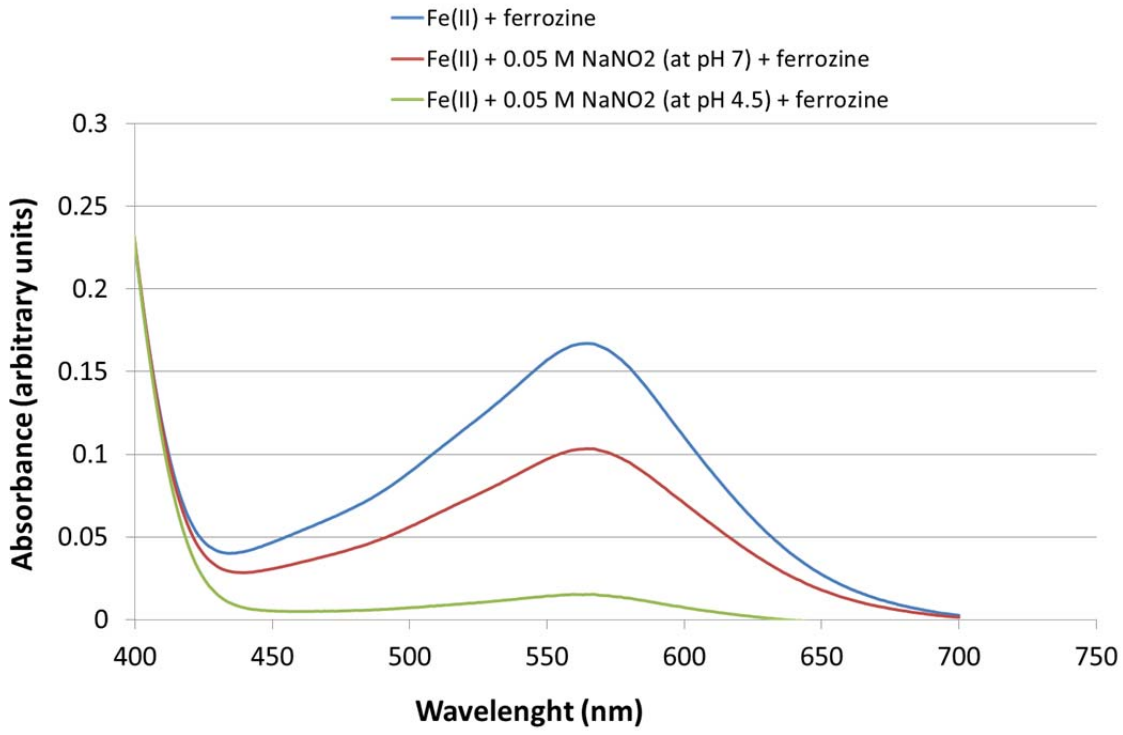

Figure 12: UV-VIS spectra showing the absorption peak of the Fe(II)-ferrozine complex in the absence and in the presence of nitrite (0.05 M). Nitrite was added to $FeCl_2$ at different pH (4.5 and 7) just before complexation of Fe(II) with ferrozine. The different conditions are indicated in the legend.

Due to the severe underestimation of the reducing capacity of DOM when using ferric citrate in the presence of nitrite (concentration ≥ 1 mM), it is advised not to use ferric citrate as oxidant to determine the reducing capacity of DOM in RBCW containing nitrite (for nitrite concentrations equal or higher than 1 mM).

4.2.5. Effect of NaN_3 on the measurement of the reducing capacity using ferric citrate

To study the effect of azide on the ferric (and the background ferrous) citrate itself, 1 mM ferric citrate solutions with and without 0.1% NaN_3 were prepared. The background concentration of Fe(II) was monitored in time by adding ferrozine and evaluating the UV-VIS spectra and the resultant Fe(II) concentration in the solutions. The results indicate a direct chemical reaction between Fe(II) and NaN_3, as shown by the decrease in the absorption peak at 562 nm in the presence of 0.1 wt% NaN_3 compared to in the absence of azide (Figure 13). For the studied conditions, this shift in absorption corresponds to a bias of 17% and a calculated concentration decrease of about 2.10^{-6} M of Fe(II). The reaction taking place is an oxidation of the background Fe(II) present in the ferric citrate solution by NaN_3, leading to a decrease in absorption since less Fe(II) is available to form the Fe(II)-ferrozine complex. When measuring the reducing capacity of an RBCW sample containing azide, this reaction might therefore lead to an underestimation which for our test conditions is in the order of 20%.

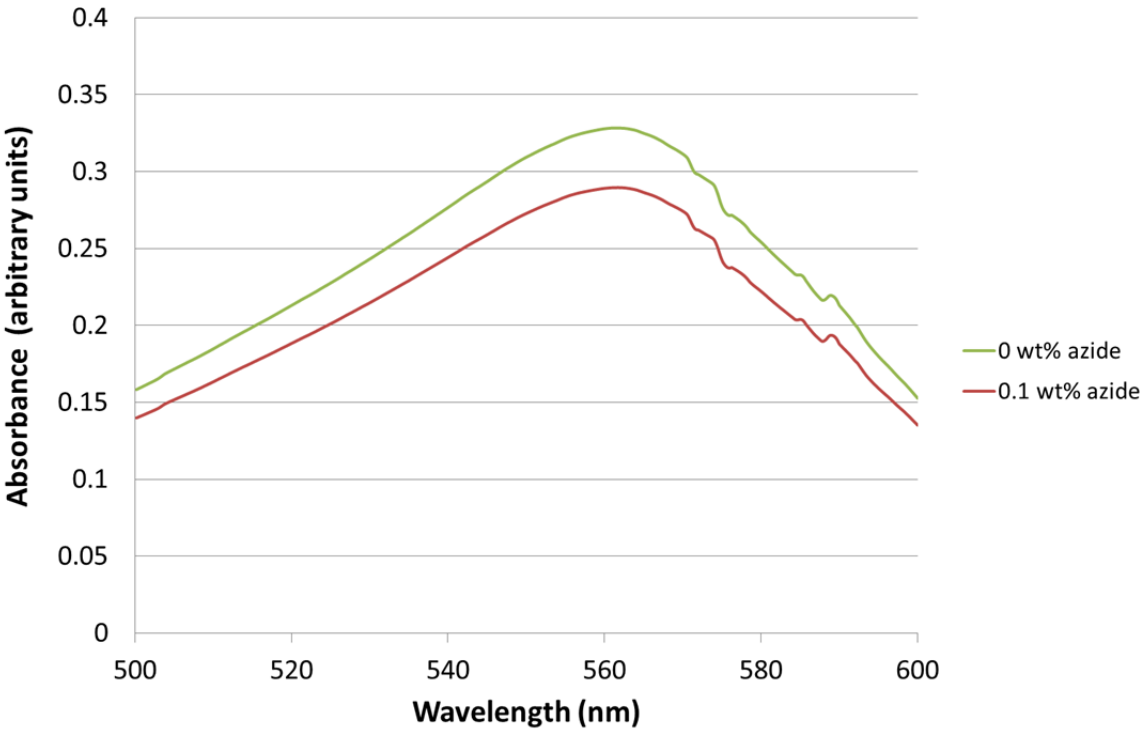

Figure 13: UV-vis absorption spectra showing the absorption peak of the Fe(II)-ferrozine complex to indicate the influence of the addition of NaN_3 on the absorption of a 1 mM ferric citrate solution.

However, as shown by the results with ferricyanide (Section 4.1.5), NaN_3 can also reduce the DOM to some extent, which would lead to an overestimation of the reducing capacity. To verify how much the addition of fresh NaN_3 during the measurement (as microbial inhibitor) affects the value of the reducing capacity of DOM, the reducing capacity of DOM in RBCW was determined in the presence and in the absence of 0.1 wt% NaN_3. The results indicate that freshly added NaN_3 (0.1 wt%) does not affect the value of the reducing capacity of DOM in RBCW significantly (Figure 14), as the two effects of freshly added NaN_3 seem to level out.

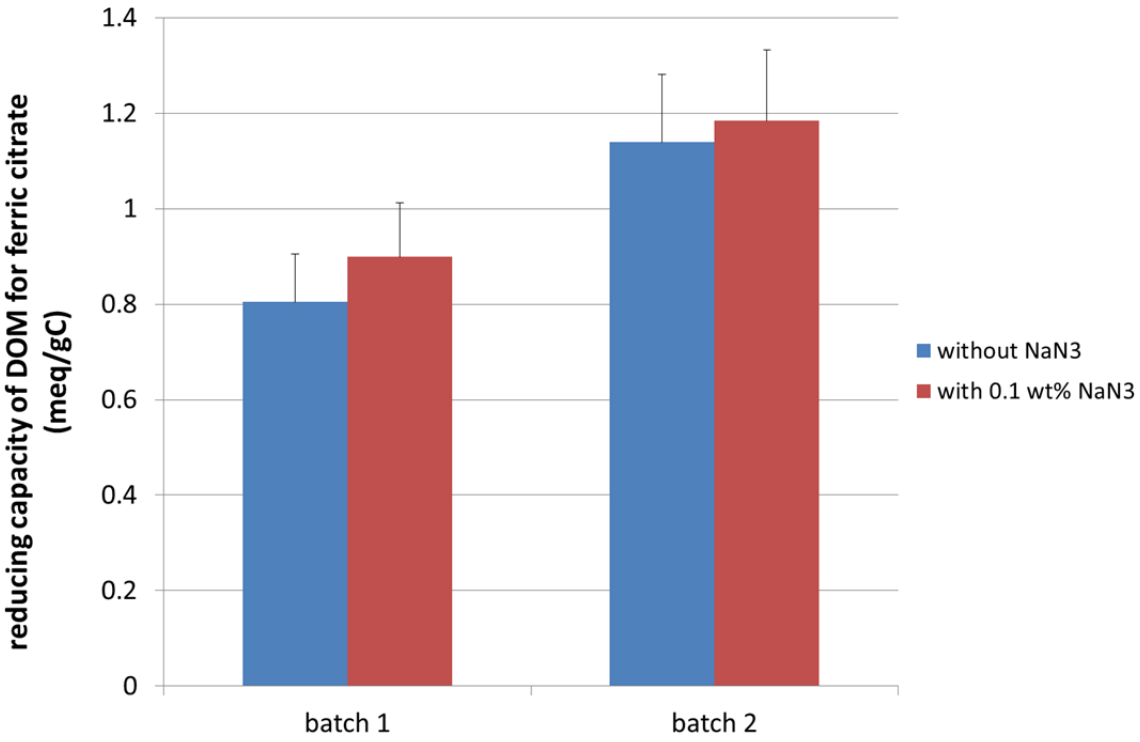

Figure 14: Influence of addition of fresh NaN_3 (0.1 wt%) during the measurement of the reducing capacity of DOM in two batches of RBCW using ferric citrate (using the procedure described in Annex 2). The error bars indicate the 95% confidence interval.

4.3. Proof of principle

In order to prove that the methods discussed in this report are reliable and useful to detect a decrease in reducing capacity of DOM due to oxidation, we performed an additional test where the DOM from RBCW was exposed to air for 56 days and the reducing capacity of the (increasingly oxidised) DOM was assessed in time, using both ferricyanide and ferric citrate. This additional test clearly demonstrates that oxidation of DOM can be detected as a decrease in reducing capacity of DOM using either ferric citrate or ferricyanide.

Different values for the reducing capacity were obtained when using either ferricyanide or ferric citrate as oxidant (Figures 15 and 16), similar to what was observed previously (Bauer et al., 2007; Peretyazhko and Sposito, 2006). Lower values were obtained when ferric citrate was used, which is in agreement with its lower redox potential compared to ferricyanide [0.43 V at pH 9 for ferricyanide/ferrocyanide; ~0.1 to -0.2 V for ferric citrate/ferrous citrate in solutions with pH ≥ 4.5 (Vukosav et al., 2012)], but can also be attributed to differences in the chemical structure of both oxidants, and to the effect of pH on the speciation of the Fe3+(citrate) complex (Bauer et al., 2007; Peretyazhko and Sposito, 2006; Vukosav et al., 2012; Wang et al., 2008).

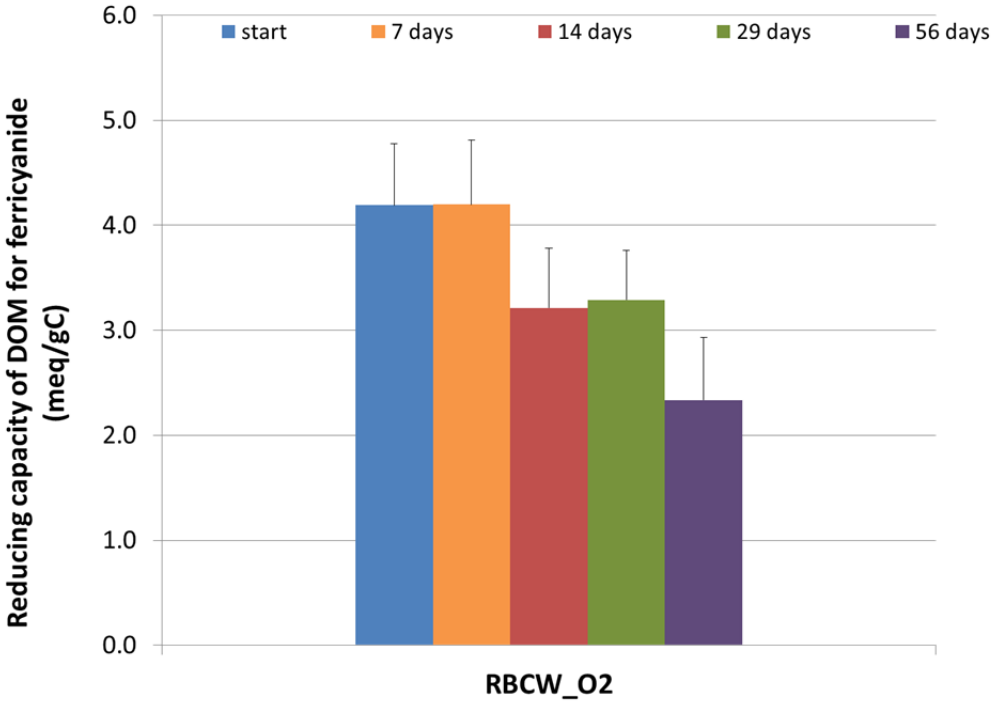

Figure 15: Decrease of reducing capacity of DOM in RBCW for ferricyanide in time due to oxidation of DOM by oxygen. The error bars represent the 95% confidence interval.

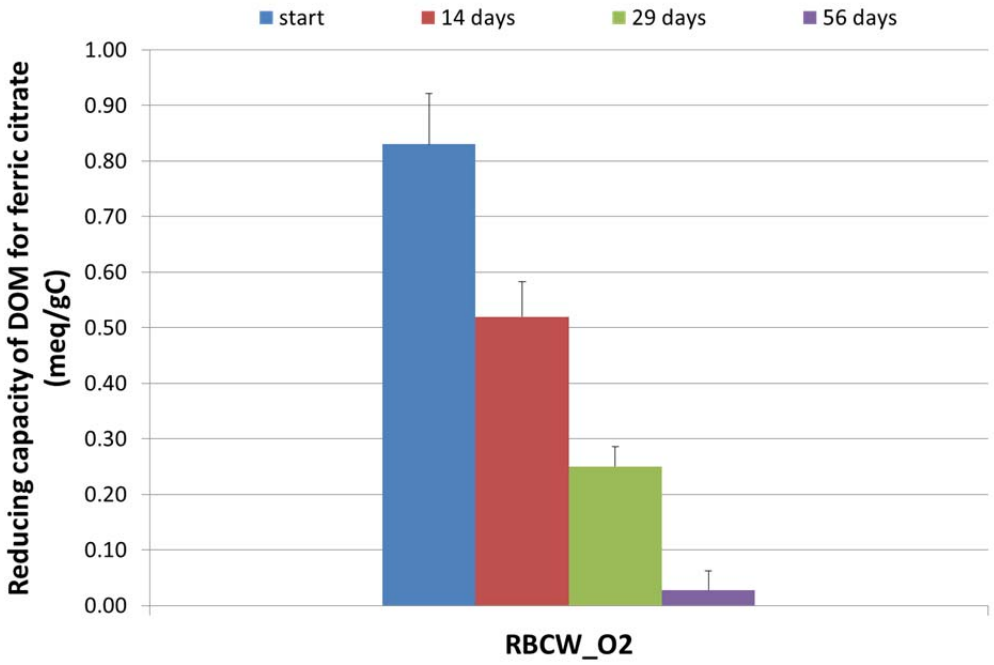

Figure 16: Decrease of reducing capacity of DOM in RBCW for ferric citrate in time due to oxidation of DOM by oxygen. The error bars represent the 95% confidence interval.

Using ferricyanide as oxidant, a decrease of 1.9 ± 0.8 meq/gC was observed after 56 days of air oxidation (Figure 15). A lower decrease (0.80 ± 0.05 meq/gC) in reducing capacity of DOM exposed to air for 56 days was observed when using ferric citrate (Figure 16). However, as the reducing capacity of DOM using ferric citrate had decreased to ~0 meq/gC after 56 days, the method had reached its limit of detection.

5. Conclusions

Using the above described optimisation and sensitivity tests, the procedure to determine the reducing capacity of DOM for both ferricyanide and ferric citrate was established and the limitations of both methods were determined. The final procedures are shown in Annexes 1 and 2. Tables 8 and 9 show the conclusions that can be drawn based on the results of the sensitivity tests when ferricyanide and ferric citrate are respectively used as oxidant to determine the reducing capacity of DOM in RBCW incubated with nitrate, nitrite and/or azide during tests to assess a possible oxidation of DOM by nitrate or nitrite.

Table 8: Overview of the conclusions drawn from the supporting tests to investigate the effect of several components on the determination of the reducing capacity of DOM (hydroquinone or DOM in RBCW) using ferricyanide.

Investigated component	Influence on ferricyanide reduction by DOM	
	Effect on ferricyanide / ferrocyanide	Effect on DOM during reducing capacity measurments
Nitrate	No	No
Nitrite	No	Yes (only significant after ~100 hours[a])
Microorganisms	Yes	Yes
NaN_3	No	Yes (+ possible reaction between azide and nitrite)
Differences in ionic strength	No	Yes: (slight) overestimation of the reducing capacity value of DOM (but only at ionic strength > 0.097 M)

[a] For hydroquinone solutions

Table 9: Overview of the conclusions drawn from the supporting tests to investigate the effect of several components on the determination of the reducing capacity of DOM in RBCW using ferric citrate.

Investigated component	Influence on Fe(III) reduction by DOM	
	Effect on Fe(III) or Fe(II)	Effect on DOM during reducing capacity measurments
Nitrate	No	No
Nitrite	Yes	Yes: underestimation of the reducing capacity value of DOM (only significant for nitrite concentrations ≥ 1 mM)
Microorganisms		No significant effect (due to lag phase)
NaN_3	Yes	No statistical significant effect within time frame of measurement but a trend towards overestimation of the reducing capacity value (but reaction between azide and nitrite)

The results show that in case nitrite is present in the RBCW solutions, only ferricyanide can be used as oxidant to determine the reducing capacity of DOM in RBCW. On the other hand, in the presence of nitrate, both oxidants can be used since the presence of nitrate would not influence the value of the reducing capacity of DOM using either ferricyanide and ferric citrate. Furthermore, NaN_3 should not be used as bacterial inhibitor while determining the reducing capacity of DOM as it could perturb the measurements due to a direct reaction of azide with Fe(II) and/or to some extent also with oxidised organic functional groups (*e.g.* hydroquinone).

It has been shown that the use of ferricyanide leads to reducing capacity values of natural DOM that are about 5 times higher than those obtained with ferric citrate, which is in agreement with previous studies (Peretyazhko and Sposito, 2006; Bauer *et al.*, 2007). These differences are attributed to the difference in the chemical structure of the oxidants and in redox potential of both half reactions, *i.e* $E_{ferricyanide/ferrocyanide}$ = 0.43 V and $E_{ferric\ citrate\ /\ ferrocitrate}$ = ~0.1 to -0.2 V for solutions with pH ≥ 4.5. Note that the redox potential of the Fe(III)/Fe(II) citrate couple depends on the speciation of the Fe(III) and Fe(II) citrate complexes, which is highly depending on the pH (*i.e.* the higher the pH, the lower the redox potential) and the Fe(III) to citrate ratio in the solution (Wang *et al.*, 2008; Vukosav *et al.*, 2012).

Based on the tests with air oxidised DOM, both methods can be applied to detect decreases in reducing capacity of DOM in RBCW due to oxidation. However, when investigating changes in the reducing capacity of DOM caused by incubation of the RBCW with nitrate, nitrite and azide,

the effect of azide and nitrite on the measurement of the reducing capacity of DOM should be taken into account when deciding which method to use. Furthermore, in case only small degrees in oxidation of DOM are expected, the method using ferric citrate is preferred as smaller differences in reducing capacity are likely masked by the uncertainty on the higher value of the reducing capacity of DOM when using ferricyanide.

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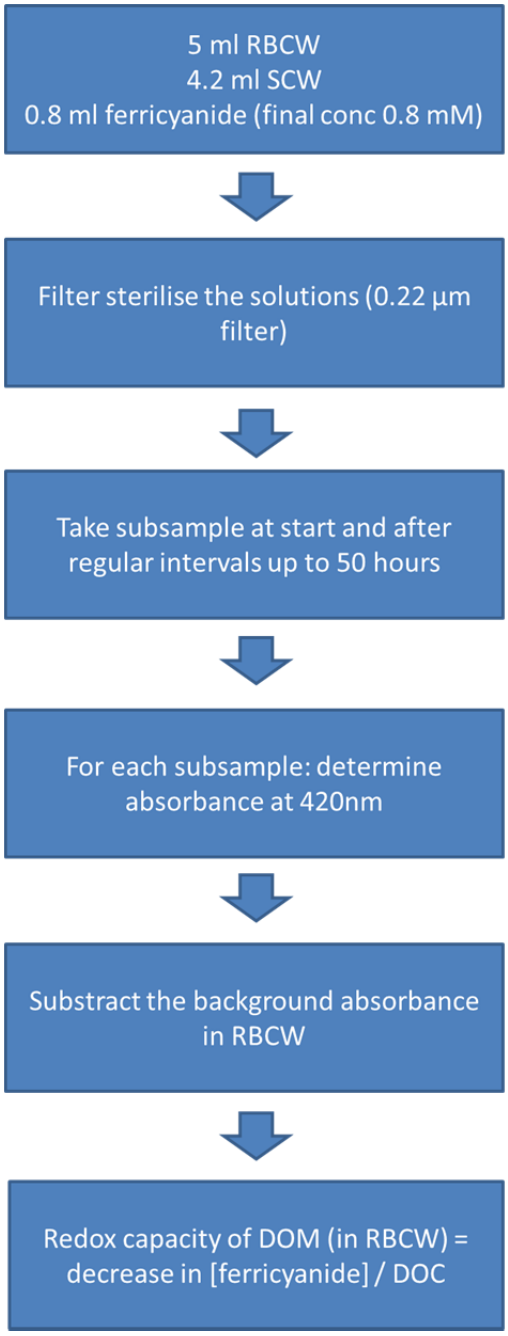
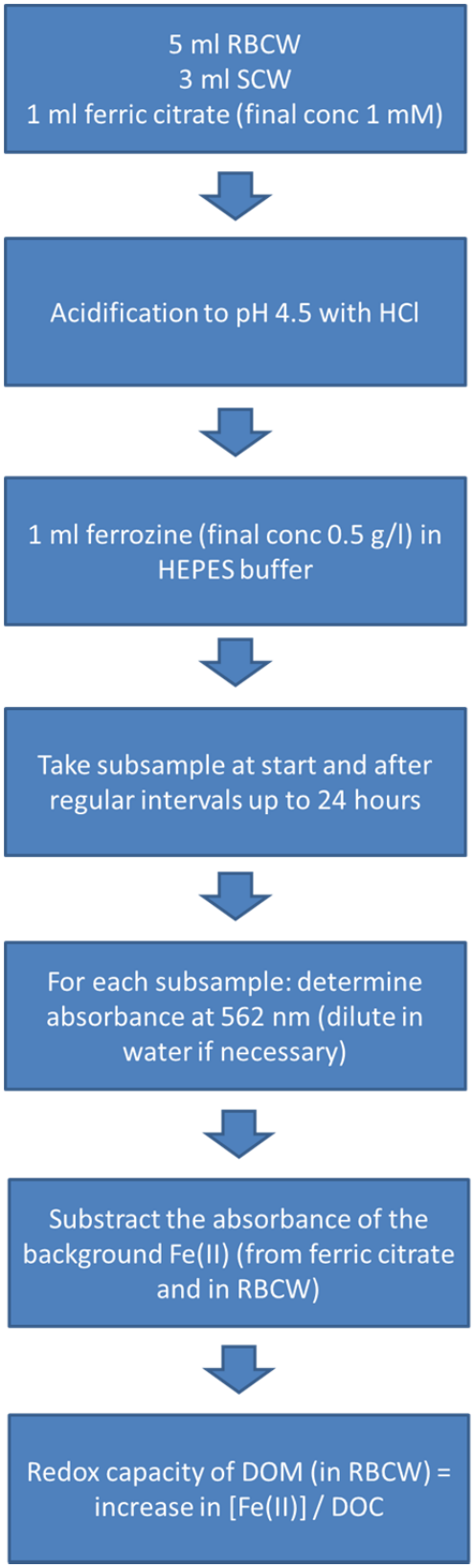
7. Annexes

Annex 1: Optimised procedure for determination of the reducing capacity of DOM in RBCW using ferricyanide as oxidant. A blanc (50 vol% RBCW without ferricyanide) is included to determine the background A_{420} in RBCW.



Annex 2: Optimised procedure for determination of the reducing capacity of DOM in RBCW using ferric citrate as oxidant. Two blancs should be included to substract the absorbance of background Fe(II) from the spectra: (1) ferric citrate in demineralised water; (2) 50 vol% RBCW



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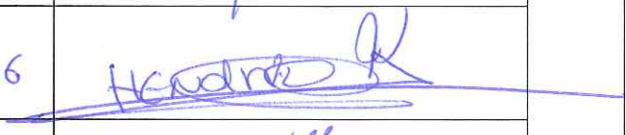
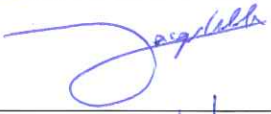
Verified by:	Katrien Hendrix	9/9/2016	
QA verification:	Elke Jacops	12/09/2016	
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