The organic ties of iron

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Chapter 2
Methods

This chapter details methods that are common to multiple chapters of this dissertation, and is modified from the peer-reviewed method sections of those chapters that have been published.

2.1. Material cleaning

Chemical preparation and bottle cleaning took place in an ISO class 7 ultra-clean (UC) laboratory environment (Interflow) with ISO class 5 workspaces. Culture handling and sampling were carried out in a 15°C climate chamber within the UC laboratory. When outside the UC environment, sample handling took place inside laminar flow hoods (ISO class 5, Interflow and AirClean systems). All material rinsing and chemical preparation was performed using ultrapure water (18.2 MΩcm, Milli-Q Element, Merck Millipore), further referred to as MQ. A Teflon® sub-boiling distillation apparatus (Savillex) was used to purify nitric acid (HNO_3) in triplicate, yielding Fe-free three-times distilled (3×D) HNO_3. A 0.3 M 3×D-HNO_3 solution was used to fill stored bottles for most purposes. A 2‰ v/v addition of ~10 M HCl (Suprapur, VWR) was used for FeL sample bottles. Labware was handled according to GEOTRACES protocols (Cuttler et al., 2010). Prior to use, general labware of high- and low-density polyethylene (HDPE and LDPE, respectively) and fluorinated ethylene propylene (FEP) was cleaned by pre-rinsing (5x) with MQ, followed by soaking in 6 M hydrochloric acid (HCl, Normapur, VWR) for a minimum of 24 h, and a final thorough (5x) MQ rinsing.Polycarbonate (PC) culture flasks (50-500 mL, VWR) and bottles (1-2 L, Nalgene) were acid cleaned with 1 M HCl (Normapur, VWR) for a minimum of 24 h, after which they were rinsed with MQ (5x). Finally, bottles were sterilized with a 10% volume of boiling MQ, i.e. the bottle with MQ was microwaved at 900 W to boiling point and left boiling for ~20 s. After vigorous shaking the hot MQ was poured out over the inverted lid. Culture vessels were then left to air-dry in a laminar flow bench for at least 2 h.

Nutrient and buffer stock solutions were cleaned by equilibration with a manganese dioxide (MnO_2) suspension and subsequent filtration after van den Berg and Kramer (1979). In short, the MnO_2 suspension was prepared by combining a 0.03 M $MnCl_2$ solution and a 0.02 M $KMnO_4$ solution, subsequent centrifugation in 50 mL tubes (VWR) at ~4000 rpm for 5 minutes using an Eppendorf 5810R centrifuge. The precipitate was resuspended in MQ and the centrifugation process was performed 3 times. Cleaning of solutions was performed twice, using a 100 µM final concentration of MnO_2 left to equilibrate in motion for ~8 hours to overnight and finally removed using 0.2 µm polycarbonate filters (Whatman, cleaned in 0.5 - 1 M HCl) in a polysulfone filter tower (Nalgene, also 1 M HCl cleaned; van den Berg, 2006).

2.2 Oceanic sampling

Samples were collected with a special ultra-clean sampling system (UCC) (Rijkenberg et al., 2015). Samples for the determination of dissolved Fe (DFe),

dissolved Fe-binding organic ligand characteristics [L_t] and log$K'_{Fe'L}$, and CDOM and HS measurements were collected through a 0.2 μm filter cartridge (Sartoban P, Sartorius) by nitrogen pressurisation (~2 bar) of the sampling bottles. All samples were taken in an ISO class 7 cleanroom environment which the UCC enters moments after arrival on deck (Rijkenberg et al., 2015).

2.3 Culturing

2.3.1. Strain information and conditions

Axenic phytoplankton cultures of the nano-eukaryotic Prymnesiophyte *Phaeocystis globosa* G(A) (culture collection of the University of Groningen, the Netherlands) and the pico-eukaryotic Prasinophyte *Micromonas pusilla* LAC38 (Marine Research Center culture collection, Göteborg University, Sweden) were maintained under trace metal clean conditions. Culture temperature was 15°C and irradiance was supplied at 90 μmol quanta m^{-2} s^{-1} under a 16:8 h light:dark cycle. Cultures were maintained semi-continuously to obtain and sustain constant and comparable physiology and growth, i.e. diluting the culture daily with new medium whereby the exact volume was determined by the maximum growth rate possible under Fe-limiting culture conditions without wash-out (Maat et al. 2014). The limiting Fe concentration determined the maximum cell abundance, which was measured before and after dilution using flow cytometry (Marie et al. 1999). Axenic viral lysate of the double-stranded DNA viruses PgV-07T (Baudoux and Brussaard 2005) infecting the host *P. globosa* G(A) and MpV-08T (Martínez Martínez et al., 2014) infecting the host *M. pusilla* LAC38 were used. Viral lysates were obtained by 10% v/v inoculation to exponentially growing phytoplankton host cultures and checked for full lysis by flow cytometry (FCM). Fe-limited viral lysates of both phytoplankton hosts were initiated by 1% v/v inoculations with Fe-replete lysates, after which a minimum of 5 subsequent 10% v/v inoculations followed before use for the experiment. This way the Fe concentration in the Fe-limited lysate was similar to the Fe concentration in the Fe-limited host culture.

2.3.2. Low-trace metal culture medium

A low Fe-containing medium (Low Trace: LT) based on natural seawater (DFe 0.2 nM, collected west of the Bay of Biscay in the Atlantic Ocean after Rijkenberg et al. 2012) was designed without metal chelators such as EDTA. Chelators as EDTA are used as metal buffers in culture media, and are added in μM to mM concentrations to ascertain that those transition metals added as micronutrients, e.g. Cu, Co, Fe, Mn, Mo, Ni, V, Zn, are kept in the dissolved phase through complexation by EDTA (Sunda and Huntsman 1995). However, these high concentrations of artificial ligands completely overrule the natural

chemistry of the metals, making the study of natural Fe binding organic ligands impossible (Gerringa et al., 2000). Therefore, an alternative medium needed to be designed. The medium was enriched with the macronutrients $NaNO_3$ (Sigma-Aldrich) and Na_2HPO_4 (Merck Millipore) to final concentrations of 128 and 8 µM, respectively. The following micronutrients were added: KBr, NaF, $CaCl_2 \cdot 2H_2O$, , $SrCl_2 \cdot 6H_2O$, $MgCl_2 \cdot 6H_2O$ and Na_2SeO_3; vitamins H, B1 and B12 and a combined Tris(hydroxymethyl)aminomethane and HCl buffer (Tris-HCl). Final concentrations are detailed in table 1. To maintain constant growth for multiple generations additional trace metals proved essential. These trace metal additions were kept to a minimum in order to avoid influencing Fe speciation, e.g. interactions between Cu and Fe (González et al. 2016). *P. globosa* received an additional trace solution containing final concentrations of 2.0 nM $ZnSO_4 \cdot 7H_2O$, 1.0 nM $CoCl_2 \cdot 6H_2O$, 4.6 nM $MnCl_2 \cdot 4H_2O$, and 0.6 nM $Na_2MoO_4 \cdot 2H_2O$. *M. pusilla* showed poor physiological condition and growth using this trace solution. Because it was impossible to maintain a steady state under these conditions, a slightly different trace solution was used, containing more comprehensive trace additions ($ZnSO_4 \cdot 7H_2O$, $CoCl_2 \cdot 6H_2O$, $CuSO_4 \cdot 5H_2O$, $NiSO_4 \cdot 6H_2O$, Na_3VO_4, K_2CrO_4, $MnCl_2 \cdot 4H_2O$, $Na_2MoO_4 \cdot 2H_2O$, and H_2SeO_3, concentrations detailed in Table 1). Fe was added from an acidified 3 µM FeCl stock solution made using a 1000 mg L^{-1} ICP stock (Fluka, Sigma-Aldrich). The Fe-limiting medium contained final concentrations of 1.0 and 3.0 nM Fe for *P. globosa* and *M. pusilla*, respectively (Chapter 7). The Fe-replete (control) medium (Chapter 7) contained 9.0 µM FeCl for both species. After the chapter 7 study, the LT medium was standardized after the modifications for *M. pusilla* (Table 1).

2.3.3. Enumeration of phytoplankton, bacteria and viruses

Phytoplankton cells in fresh samples were discriminated and counted based on Chlorophyll-a red autofluorescence using a FACSCanto flow cytometer (Becton Dickinson) equipped with a 17 mW 633 nm HeNe red laser. Viral abundances were also determined by flow cytometry using a FACSCalibur flow cytometer (Becton Dickinson) equipped with a 15 mW 488 nm argon-ion blue laser triggered on green fluorescence, following the protocol by Brussaard et al. (2010). In short, samples were diluted 200-1000-fold using a 2 M Tris-HCl buffer at pH 8 and viruses were stained using the nucleic acid-specific fluorescent dye SYBR Green I (Molecular Probes®, Life Technologies, Thermo Fisher). Raw data were analysed using Cytowin (Vaulot, 1989; Version 4.31 available at http://application.sb-roscoff.fr/Phyto/index.php), whereby PgV and MpV were easily discriminated by plotting green nucleic acid-specific fluorescence versus side scatter (A. C. Baudoux and Brussaard, 2005; Martínez Martínez et al., 2015).

Table 1 Low Trace (LT) medium constituents added to low-DFe natural seawater. See also MaCuMBA public deliverable D3.1 (www.macumbaproject.eu). All concentrations in M.

Major nutrients		**Trace nutrients**	
$NaNO_3$	1.28×10^{-4}	$FeCl_3 \cdot 6H_2O$	3.00×10^{-9}
$Na_2HPO_4 \cdot 12H_2O$	8.00×10^{-6}	H_2SeO_3	1.00×10^{-9}
H_3BO_3	3.75×10^{-6}	$CuSO_4 \cdot 5H_2O$	1.00×10^{-9}
Micronutrients		$ZnSO_4 \cdot 7H_2O$	4.00×10^{-9}
KBr	7.10×10^{-6}	$NiSO_4 \cdot 7H_2O$	1.00×10^{-9}
NaF	3.00×10^{-6}	Na_3VO_4	1.00×10^{-9}
$CaCl_2 \cdot 2H_2O$	1.79×10^{-6}	K_2CrO_4	1.00×10^{-9}
$SrCl_2 \cdot 6H_2O$	8.00×10^{-7}	$CoCl_2 \cdot 6H_2O$	5.00×10^{-9}
$MgCl_2 \cdot 6H_2O$	2.31×10^{-6}	$MnCl_2 \cdot 4H_2O$	9.00×10^{-9}
Na_2SeO_3	1.00×10^{-8}	$Na_2MoO_4 \cdot 2H_2O$	4.00×10^{-9}
Vitamins		**Buffer**	
Biotin (H)	2.03×10^{-9}	Tris-HCL	2.50×10^{-3}
Thiamin-HCL	2.96×10^{-7}		
Cyanobalamin (B12)	3.70×10^{-10}		

2.3.4. Photosynthetic capacity

Photosynthetic capacity (F_v/F_m) measurements were performed using a Chlorophyll Fluorometer with a red emitter-detector unit (Water-PAM, Waltz). Samples were kept in the dark for 30 minutes at culturing temperature, after which chlorophyll autofluorescence was measured in duplicate in the dark adapted state (F_0) and after a saturation pulse of 2.5 s (F_m). F_v is defined as the difference between F_m and F_0 (Genty et al. 1989).

2.4. Analyses related to Fe-binding organic ligands

2.4.1. Determination of DFe concentrations

Samples for DFe were collected from the Fe-binding organic ligand sample bottles at the time of voltammetric analysis and acidified with 2‰ v/v 12 M trace metal grade HCl (Seastar Chemicals). If immediate measurement of Fe-binding organic ligands was not possible the sample bottles were stored at -20

°C samples for DFe were taken at the moment of analysis after thawing. DFe concentrations (expressed in nM) were then measured using an automated Flow Injection Analysis system the same day (Klunder et al., 2011). The sample was transferred onto an iminodiacetic acid (IDA) column, binding only transition metals and serving to concentrate and desalt the retentate. The column was subsequently washed with MQ and eluted with HCl (0.4 M, Merck Suprapur). Luminol (0.6 mM, Aldrich), hydrogen peroxide (0.6 M, Merck Suprapur) and dilute ammonia (0.96 M, Merck Suprapur) are then mixed in. Fe catalyses the oxidation of luminol by hydrogen peroxide, producing blue light in correlation to the amount of catalyst present (Obata et al., 1993). The response of a photon counter is calibrated with a series of Fe standard additions (ICP standard, Fluka, Sigma-Aldrich). Samples are analysed in triplicate and reported with a standard deviation of the mean (SD). Quality control for the system was maintained by daily measurement of lab standards and regular measurement of certified reference material (Rijkenberg, Chapter 4).

2.4.2. Voltammetric determination of Fe-binding organic ligands

Total Fe-binding organic ligand concentration [L_t] and the conditional stability constant (K´) were measured with Competitive Ligand Exchange – Adsorptive Cathodic Stripping Voltammetry (CLE-AdCSV). Given that the diverse pool of organic Fe-binding organic ligands cannot be measured directly, a known ligand – the competitive ligand or added ligand (AL) – is added to the sample. As described in chapter 1, formation of Fe-ligand complexes is reversible. The diverse Fe-binding organic ligands making up the ligand pool in a sample are in competition with each other for Fe, resulting in an equilibrium over time. Therefore, the added ligand and the natural ligands in the sample need to be allowed to establish an equilibrium (Figure 1A). The sample is titrated with increasing additions of Fe. After a period of equilibration, an AdCSV scan is performed for each of these additions. During a voltammetric scan the surface active Fe-AL complex is initially deposited on a mercury (Hg) drop electrode at a (near) zero potential. This is followed by a quick potential sweep, during which the complex dissociates, which causes a current peak in nA which is representative of the amount of Fe-AL (Figure 1A, B). A titration curve resulting from these Fe additions typically changes in character after a number of additions. Initially the peak size in nA shows little to no increase with higher Fe additions. In this circumstance, the natural ligands in the sample and the AL compete for Fe. As the AL is added in excess, the titration curve changes direction once the natural ligands in the sample are saturated with Fe (Figure 1A, C).

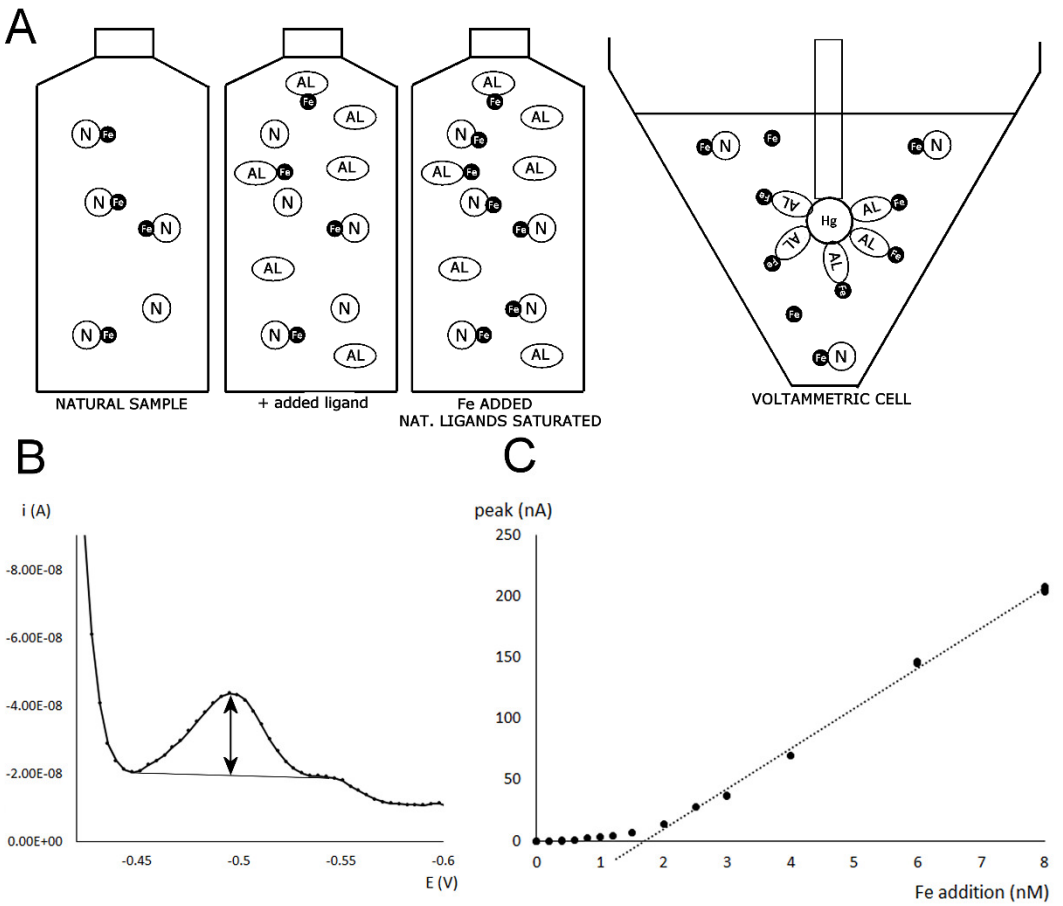

Figure 1 A) Cartoon representing Competitive Ligand Exchange Adsorptive Cathodic Stripping Voltammetry, showing 3 different stages in a titration with Fe and the principle of measurement (voltammetric cell); B) Voltammogram showing current I (A, vertical axis) vs. potential E (V, horizontal axis), in this case showing a current peak for $FeTAC_2$ at a higher Fe addition; and C) Titration plot showing current peaks of the FeAL complex, in this case for FeSA (nA, vertical axis) with increasing Fe concentration (nM, horizontal axis). The intercept of the linearization of the saturated phase with the horizontal axis gives a rough indication of natural ligand concentration.

In this dissertation two CLE-AdCSV methods are employed. One using 2-(2-Thiazolylazo)-p-cresol (TAC) after Croot and Johansson (2000), and the method using salicylaldoxime (SA; Rue and Bruland, 1995; Buck et al., 2015). For the TAC method a natural sample was left to equilibrate with the AL in the presence of a mixed boric acid - ammonia buffer (1 M, pH 8.05, Merck) and increasing standard additions of Fe(III). 30 mL PTFE cups (Savillex) were used to equilibrate 10 mL subsamples from a mix of natural sample, buffer (5 mM final concentration) and TAC (10 µM final concentration) with discrete Fe(III) additions of 0 (twice), 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.5, 2.0, 2.5, 3.0, 4.2, 6.0 and 8.1 (twice) nM. Equilibration lasted a minimum of 8 hours to overnight. The

resulting titration with Fe was analysed for $Fe(TAC)_2$ concentration using differential pulse voltammetry. TAC (Alfa Aesar) was dissolved in 3xD-methanol to a stock concentration of 0.02 M, Fe standards (1 and $3 \cdot 10^{-6}$ M) were prepared in MQ from a 1000 ppm ICP stock solution (Fluka) and acidified using 2xD-HNO_3. The voltammetric apparatus consisted of a 663 VA stand (Metrohm) equipped with a Hg drop multimode electrode with silanized capillary, double-junction Ag/AgCl reference electrode (KCl 3M) and glassy carbon auxiliary electrode in a polytetrafluoroethylene (PTFE) cell (all Metrohm), control hardware (μAutolab III, Metrohm Autolab B.V.) and a consumer laptop PC running Nova 1.9 (Metrohm Autolab B.V.). N_2 was used for purging and Hg drop formation. When measurements were performed at sea, interference from ship motion and vibration was minimized by suspending the VA stand in elastic bands. Any electrical interference was minimized using a consumer inline peak filter and an uninterruptible power supply with sinewave converter (Fortress 750, Best Power). Analysis was performed using a slightly altered version of the measurement procedure used by Croot and Johansson (2000): Purging for 180 seconds, no conditioning, deposition for 140 seconds at -0.4 V, a 5-second equilibration followed by a differential pulse scan from -0.4 to -0.9 V. The influence of high frequency vibrations from the ship's drivetrain was minimized by an increased scan rate of 39 mV s^{-1} (0.05 s interval and 0.004 s modulation time).

Our application of the CLE-AdCSV method using SA by Rue and Bruland (1995) and Buck et al. (2007) was modified from Abualhaija and van den Berg (2014), using the same equipment as the TAC method with the following modifications. SA was added from a stock prepared in 3xD-methanol in a final concentration of 25 μM (Abualhaija and van den Berg, 2014; Abualhaija et al., 2015). SA was added 15 minutes prior to a first analysis scan as done by Buck et al.(2007), after a minimum 1 h equilibration of the sample with buffer and discrete Fe additions in the same concentrations as above. The borate buffer for SA analyses was adjusted to a pH of 8.4 for better peak separation. The voltammetric apparatus was modified to purge with synthetic air as done by Abualhaija and van den Berg (2014) and Abualhaija et al. (2015), while still using nitrogen pressure for mercury drop formation. Analysis settings were deposition at 0 V for 240 s, followed by a differential pulse sweep from 0 to -0.7 V with a modulation time of 4 ms, an interval time of 100 ms, a step potential of 6 mV and 50 mV modulation amplitude, resulting in a 60 mV s^{-1} scan rate.

Titrations were analysed for natural ligand concentration and binding strength by a non-linear fit of the Langmuir model after Gerringa et al. (2014) using R (R Development Core Team, 2008). The conditional binding strength of the added ligand (AL) is given by the K'_{FeAL}, or $\beta'_{Fe(AL)2}$ in the case of bidentate

association. The detection window (D) is then given by the product of K'_{AL} and the free AL concentration, hence given as $D_{AL} = K'_{AL}[AL']$ in case of a single AL associating with Fe. When a the AL binds Fe in a pair D is given as $D_{AL} = \beta'_{AL}[AL']^2$. If both associations occur, $D_{AL} = (K'_{AL}[AL']) + (\beta'_{AL}[AL']^2)$. Given that AL is added in excess to the point where [AL'] does not significantly change due to formation of FeAL, [AL'] may be represented by the total [AL]. As mentioned with the definition of $\log K'_{Fe'L}$ in chapter 1, all conditional binding strengths are reported in relation to Fe'. Inorganic Fe [Fe´] is defined as the product of $[Fe^{3+}]$ and the inorganic side reaction coefficient (α_i), which depends on analysis pH as the inorganic side reactions are hydroxide complexations. For analyses at the seawater pH of 8.05, i.e. the TAC method, a value of 10^{10} was used after Liu and Millero (2002). However, as the pH for SA measurements was adjusted to 8.4, α_i needed to be recalculated. The inorganic side reaction coefficient assuming formation of FeOH and $Fe(OH)_2$ is given by equation 1:

$$\alpha_i = K_{FeOH} \times \frac{1}{[H^+]} + K_{Fe(OH)_2} \times \frac{1}{[H^+]^2} + 1 \quad (1)$$

Using $K_{FeOH} = 10^{-2.62}$ and $K_{Fe(OH)2} = 10^{-6}$ after Millero (1998), $\log\alpha_i$ for pH = 8.4 is 10.8. In chapter 3, and where the contrasting K'_{FeL} in relation for Fe^{+3} is relevant values are reported as $\log K'_{FeL,Fe3+}$ as opposed to $\log K'_{Fe'L}$. For the Langmuir fit of the TAC data a $\log\beta_{Fe'(TAC)2}$ of 12.4 was used after Croot and Johansson (2000). D is given by $D_{TAC} = \beta_{TAC}[TAC]^2 = 251.19$. For the Langmuir fit of the SA data a $\log\beta'_{Fe(SA)2}$ of 10.72 and $\log K'_{FeSA}$ of 6.52 were used, with the detection window given by $D_{SA} = (K'_{FeSA}[SA]) + (\beta'_{Fe(SA)2}[SA]^2) = 115.58$ (Abualhaija and van den Berg, 2014). As only FeSA is electroactive, this is the measured property. $FeSA_2$ is taken into account in the calculation of Fe' as $\beta'_{Fe'(SA)2}$ is part of the Langmuir fit from which $K'_{Fe'L}$ is determined. Where it was possible to resolve 2 ligand classes, the strongest class was L_1 and the weaker class designated as L_2 (Gledhill and Buck, 2012).

$[Fe^{3+}]$, [Fe'] and [L´] were determined from DFe, $[L_t]$ and K' by iterative calculations of the Fe speciation equilibrium with Newton's algorithm (Press et al., 2007), using an R implementation of the method described by Gerringa et al. (2014). In short, the following sequence is repeated 20 times:

$$x = x + \frac{1-f(x)}{f\prime(x)} \quad (2)$$

$$f(x) = \alpha_i \cdot x + \frac{K\prime\cdot[L_t]\cdot x}{1+x\cdot K'\cdot DFe} \quad (3)$$

$$f'(x) = \alpha_i + \frac{K\prime\cdot[L_t]}{1+x\cdot(K\prime\cdot DFe)} - \left(\frac{x\cdot(K\prime\cdot DFe)\cdot(K\prime\cdot[L_t])}{1+(K\prime\cdot DFe)\cdot x}\right)^2 \quad (4)$$

With initial condition $x = [Fe^{3+}] / DFe$, and the initial approximation of [Fe_{3+}] given by:

$$[Fe^{3+}] = \frac{DFe}{\alpha_i + K' \cdot [L_t]} \quad (5)$$

After the iterative process results are given as:

$$[Fe^{3+}] = x * DFe \quad (6)$$

$$[Fe'] = [Fe^{3+}] \cdot \alpha_i \quad (7)$$

$$[L'] = \frac{[L_t]}{K' \cdot [Fe^{3+}] + 1} \quad (8)$$

[L'] in equation 8 is the free ligand concentration. This represents ligands that do not bind Fe and are therefore typically referred to as excess ligands, indicating binding capacity beyond the DFe that is present. [L´] approaches 0 where [L_t] is insufficient to bind further Fe.

The ratio [L_t]/DFe reflects the surplus of Fe-binding organic ligands. Ratios >1 indicate that there is sufficient binding capacity to explain DFe, whereas a ratio between 0 and 1 would indicate a lack of binding capacity (Thuróczy et al., 2010). The reactivity of the ligands α_{FeL} is the product of K´ and [L´] (Ringbom and Still, 1972; Gledhill and Gerringa, 2017), expressed as the base-10 logarithm with respect to Fe´, further referred to as logα_{FeL}.

2.4.3. Voltammetric determination of humic substances

The concentration of humic substances (HS) was measured using cathodic stripping voltammetry (Laglera et al., 2007; Laglera and van den Berg, 2009). Samples were buffered with the same boric acid-ammonia solution (1 M, pH 8.05, Merck) as used for CLE-AdCSV using TAC, and saturated with Fe(III) by a 30 nM addition from the same $3 \cdot 10^{-6}$ M standard used for Fe additions in CLE-AdCSV. To increase the current signal of the dissociating Fe-humic complex at the electrode, $KBrO_2$ (13 µM final concentration) served as oxidizer. Standard additions of fulvic acid (Suwannee River Fulvic Acid Standard I, International Humic Substances Society (IHSS), St. Paul, USA, further referred to as SRFA) of 0.1 to 0.4 mg L^{-1} were used as a measure of equivalent quantification. Therefore [HS] is expressed in the equivalent mg L^{-1} of fulvic acid (Eq. mg/L FA) and is specific to the standard used (Sukekava et al., 2018). Analysis was performed with the same voltammetric equipment as above. The procedure employed a 180 s purge with N_2 followed by a 90 s deposition period at -0.1 V and a linear current sweep from -0.1 to -1.1 V at a scan rate of 100 mV s^{-1} (0.05 s interval).

2.5. Additional analyses

2.5.1. Spectrophotometric determination of Dissolved Organic Matter

The characterization of CDOM was performed after Stedmon et al. (2000). Absorbance spectra between 250 and 1000 nm, at 1 nm resolution, were recorded in a quartz cell with a 1 cm path length (SUPRASIL®, Hellma Analytics) using a Spectramax M2 multimode spectrophotometer (Molecular Devices). Daily measurements of MQ were used as blanks, the spectra of these were subtracted from the data as a baseline correction. A further baseline correction was performed by subtracting the mean absorbance between 450 and 500 nm in order to correct refractive index differences between seawater and the MQ blanks (H.E. Reader, pers. comm.). Extremely low values of absorbance at longer wavelengths, nearing the limit of detection, dictated the use of the 450 nm – 500nm window for that correction. The absorbance in RFU (A_λ) was converted into absorption coefficients (a_λ, m^{-1}) using path length (l, m) and ln(10) = 2.303, according to Stedmon et al. (2000):

$$a_\lambda = 2.303 \frac{A_\lambda}{l} \quad (9)$$

Spectral slopes for the intervals 275-295 nm (S_{275}) and 350-400 nm (S_{350}) were calculated by linear fitting to the ln(a) spectrum using R. Absorption values were recorded at wavelengths of 254 nm (a_{254}) and 300 nm (a_{300}) as indicators of complex organics (Helms et al., 2008).

The fluorescent fraction of DOM (FDOM) was analysed using UV fluorescence spectrophotometry (Mopper and Schultz, 1993). Emission spectra were recorded between 360 and 540 nm with an interval of 1 nm at an excitation wavelength of 250 nm using the same equipment as above. Emission at 450 nm using this excitation wavelength ($F_{250/450}$) was taken as a measure of humic-like FDOM (Coble et al., 1998; Coble, 2007). Daily measurements of a 1 mg L^{-1} quinine sulphate (QS) standard (Alpha Aesar, dissolved in MQ with 0.1 M H_2SO_4) served as an equivalent reference for expression of FDOM concentrations. FDOM measurements are thus expressed as equivalents of 1 ppb QS emission at 450 nm using 250 nm excitation, referred to as quinine sulphate units (QSU; Mopper and Schultz, 1993).

2.5.2. Nutrient analyses

Nutrient samples were analysed after Murphy and Riley (1962, phosphate), Strickland and Parsons (1972, silicate) and Grasshoff (1983, nitrate) using a TRAACS 800 auto-analyser (Technicon).