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Iron complexation by phenolic ligands in seawater

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Abstract

Iron is an essential micronutrient for phytoplankton and can limit primary production in the ocean. Fe chemistry is highly controlled by its interaction with organic complexes (> 99%). It is still unknown which organic compounds produced by cells have the ability to bind Fe. Within the pool of organic ligands, polyphenols are known to be exuded by marine diatoms and, in this study, the role of three polyphenols ((±) – catechin, sinapic acid and gallic acid) was studied in terms of dissolved Fe complexation via kinetic and titration approaches, and also their role as a source of Fe(II) in seawater. The results demonstrated that these three polyphenols are weak L₂-type Fe-binding ligands according to the conditional stability constant, computed by using the kinetic approach ($\log K'_{\text{Fe'L}} = 8.86 - 9.2$), where the formation rate constant (k_f) was $3.1 \cdot 10^5 - 4.2 \cdot 10^5 \text{ M}^{-1} \text{ s}^{-1}$ and the dissociation rate constant (k_d) was $2.43 \cdot 10^{-4} - 4.4 \cdot 10^{-4} \text{ s}^{-1}$. The conditional stability was also computed from the titration approach with $\log K'_{\text{Fe'L}}$ from 8.6 to 9.5. These studied ligands also regenerated Fe(II) in seawater from 0.05% to 11.92%. The results obtained in this study suggest that polyphenols increase the persistence of dissolved Fe and should be considered as an important Fe-binding ligands in seawater to better understand the global biogeochemical cycles.

Keywords: Fe-binding ligands, complexation, polyphenols, Fe(III), Fe(II)

INTRODUCTION

Iron is involved in a variety of metabolic processes and is thus an essential micronutrient for marine microorganisms (Morel and Price, 2003). The low solubility of Fe in seawater (Liu and Millero, 2002) decreases the dissolved concentration of this metal, limiting the growth of phytoplankton in some areas of the ocean, in particular in high nutrient low chlorophyll oceanic regions (Martin et al., 1991). In seawater, the speciation of Fe is highly dominated by the complexation with organic complexes (> 99%), affecting its reactivity (Gledhill and van den Berg, 1994; Rue and Bruland, 1995) and increasing its solubility (Liu and Millero, 2002; Rue and Bruland, 1995), but most of the ligands are unknown. In general, the ligands are studied as natural ligands, a pool of organic ligands able to complex Fe in natural waters, participating in the Fe-uptake system of microorganisms (Shaked and Liss, 2012) by different processes. Adsorption, reduction of Fe(III) to Fe(II) in solution or in the cell wall and complexation of Fe in solution (Morel et al., 2008), and the production of organic ligands by microorganisms can make dissolved Fe (dFe) more bioavailable (Granger and Price, 1999; Hutchins et al., 1999; Maldonado and Price, 1999), although these mechanisms depends on the microorganisms and their environments (Shaked and Liss, 2012).

Fe-binding ligands are produced by cells; rupture of cells after grazing (Chase and Price, 1997; Hutchins et al., 1995; Hutchins and Bruland, 1994; Sato et al., 2007), viral lysis (Glober et al., 1997; Poorvin et al., 2011), transformation of organic materials (Boyd and Ellwood, 2010; Gerringa et al., 2006; Gledhill and Buck, 2012) and organic exudates from phytoplankton (González-Dávila et al., 1995; Rico et al., 2013; Santana-Casiano et al., 2014; Vasconcelos et al., 2002). In the ocean, the bulk of Fe-binding ligands can be ranked, according to the conditional stability constant (K'_{FeL}), as strong ligands ($L_1 - \log K'_{FeL} > 11$) and weak ligands ($L_2 < 11$) (Gledhill and Buck, 2012), where the ligand

concentration and the conditional stability constant of the formed complex is measured by competitive ligand exchange adsorptive cathodic stripping voltammetry (CLE-ACSV). However, this technique does not give any information about the molecular structure of organic ligands (Luther et al., 2001). In this sense, the identification of organic functional groups, which form part of the ligands in seawater or cultures, will be relevant in terms of trace metal chemistry.

There is a necessity of increase our knowledge about organic functional groups and identify them within the bulk of organic ligands in natural waters. In this sense, polyphenols have been measured in the exudates of two marine microalgae, the marine diatom *Phaeodactylum tricornutum* and the green algae *Dunaliella tertiolecta* (López et al., 2015; Rico et al., 2013; Santana-Casiano et al., 2014), as well as the effect of high metal exposition on the concentration of polyphenols excreted by the cells. The authors reported that the type of phenolic compounds and their concentrations are directly related to the metal concentrations in solution as a mechanism to keep Fe in solution or decrease the free copper concentration due to its toxicity. In addition, polyphenols such as catechol, (±) – catechin and sinapic acid can reduce Fe(III) to Fe(II) in seawater (Santana-Casiano et al., 2014).

Polyphenols, as other ligands produced by microorganisms (amino acids or polysaccharides), can complex Fe due to the hydroxyl and carboxyl groups on their molecular structures (Andjelković et al., 2006; Brown et al., 1998; Lodovici et al., 2001; Mira et al., 2002; Re et al., 1999; Sroka and Cisowski, 2003), but these Fe-binding complexes have never been studied in seawater in terms of Fe complexation. However, polyphenols have been described as strong dFe-binding ligands in acidic solutions (pH < 3; Hynes and O’Coinceanainn (2001, 2004)).

The goal of the current investigation is to measure the formation (k_f) and dissociation (k_d) rate constants and the conditional stability constants ($K'_{Fe'L}$ and $K'_{Fe^{3+}L}$) for the Fe-complexes with ($\pm$) – catechin (5,7,3',4'-tetrahydroxyflavan-3-ol), sinapic acid (3,5-dimethoxy-4-hydroxycinnamic acid) and gallic acid (3,4,5-trihydroxybenzoic acid) in seawater. These three compounds have been selected among a variety of polyphenols due to their presence in phytoplankton exudates (López et al., 2015; Rico et al., 2013; Santana-Casiano et al., 2014). These authors reported how these three organic compounds showed a higher concentration in seawater enriched with phytoplankton exudates than in natural seawater. These three compounds allow us to understand the role of three different molecular structures on the chemistry of Fe. In addition, Fe redox experiments were performed in seawater in order to determine the capability of these three model organic ligands to reduce Fe(III) to Fe(II) in seawater. These results will increase our knowledge about Fe biogeochemical cycle and the role of ligands in the chemistry of Fe in the ocean.

MATERIAL AND METHODS

Chemicals

Fe was determined by differential pulse cathodic stripping voltammetry (DP-CSV; Croot and Johansson (2000)). A 0.01 M solution of TAC (2-2(2-thiazolylazo)-*p*-cresol) (Sigma-Aldrich) was prepared in HPLC grade methanol (Sigma-Aldrich) once every two weeks and kept in the fridge when it was not in use (darkness and 4°C). An EPPS buffer solution (N-(2-hydroxyethyl)piperazine-N';2-propanesulfonic acid; SigmaUltra) was prepared at 1.0 M in 1.0 M NH₄OH (ultrapure, VWR) at pH 8.05. The buffer solution was cleaned three times through an 8HQ (8-Hydroxyquinoline) resin column. Fe(III) stock solutions (1.0 µM and 100 nM) were prepared weekly from a Fe standard solution for atomic

absorption spectroscopy (VWR) and acidified at pH 2 with HCl (ultrapure, VWR). The stock solution of Fe(II) ($4 \cdot 10^{-4}$ M) was daily prepared using ammonium Fe(II) sulfate hexahydrate (Sigma), acidified at a pH of 2 with Suprapur HCl (Sigma).

The selected organic ligands, ($\pm$) – catechin, sinapic acid and gallic acid (Sigma-Aldrich) were prepared weekly in HPLC grade methanol at 10^{-3} M and the second stock solutions were prepared, for each experiment, in MQ water (18 M Ω , Millipore) at 10^{-6} M. The molecules of these three selected organic ligands are presented in Figure 1.

The reagents were always prepared in 20 mL Teflon (Savilex) vials. These bottles were washed 5 times with MQ water and 2% HCl (suprapure, VWR). The Teflon vials were rinsed 5 times with MQ water prior to use.

The seawater used in the current experiments was collected during the oceanographic cruise “GEOVIDE” (station 77, 53.0 °N, -51.1 °E, 40 m) in the North Atlantic Ocean and was filtered on board through 0.2 μ m-pore size cartridge filters. The GEOVIDE seawater (GSW) was kept in the clean laboratory (Class 100) facilities (LEMAR-IUEM) with acid-clean carboy (25L) in the dark, at room temperature, until it was used.

Measurements of labile and dissolved Fe

Dissolved Fe (dFe), labile Fe (Fe’; all the inorganic species predominantly in the form Fe(OH)₃), and dFe-binding ligands (L_{Fe}) concentrations were determined by DP-CSV (Croot and Johansson, 2000) using a μ Autolab voltameter (Metrohm), with a static mercury drop electrode (Metrohm Model VA663), a double-junction Ag/saturated AgCl reference electrode with a salt bridge filled with 3 M KCl, and a glassy carbon rod as a counter electrode. The samples were always processed in a Class 100 clean laboratory at room temperature.

Labile Fe was measured in 10 mL seawater samples by adding 100 μ L of EPPS (final concentration 10 mM EPPS buffered to pH 8.05) and 10 μ L of 0.01 M TAC (final concentration 10 μ M). Samples were purged for 180 sec with dry nitrogen gas. A new Hg drop was formed at the end of the purging time. The Teflon vials were conditioned 5 times with GSW. The labile Fe concentrations were determined using the method of standard additions. TAC was added at the beginning of the purging time, then the contact time was 180 sec.

Dissolved Fe concentrations were measured following the same method, but the samples were previously UV-irradiated during 4 hours in quartz tubes. These tubes were soaked for one day in 10% HCl (suprapure, VWR) and washed with MQ water 5 times prior to use. They were also rinsed one more time with GSW. The Teflon vials were conditioned 5 times with GSW. The dFe concentration was determined using the method of standard additions. TAC was also added at the beginning of the purging time, then the contact time was 180 sec.

The voltammetric analysis was always the same for all the analysis. The deposition potential of -0.40 V was applied for 180 sec. The sample was stirred during the deposition time. At the end of the deposition time, the scan as a DP-CSV was applied with a modulation time of 0.01 sec, interval time 0.1 sec, initial potential -0.4 V, final potential -0.9 V, step potential 2.55 mV and modulation amplitude 49.95 mV.

Dissolved Fe organic speciation measured using the kinetic method

The kinetic of formation was prepared with 10 mL of UV-irradiated GSW (4 hours), 100 μ L of EPPS (1 M) buffered to pH 8.05, 10 nM of dFe, and 5 nM of the studied ligands in at least 14 Teflon vials. These experiments were performed with an excess of Fe

compared to ligand in order to have enough dFe to saturate the binding sites in the polyphenols and to achieve the maximum complexing capacity. The initial time (t_0) corresponds to the addition of the studied ligand. The addition of TAC (10 μ L of 0.01 M) was performed during the purge, 20 sec before starting the deposition time. This time was used to purge the sample after the addition of TAC. The Fe measured in the kinetic of formation experiments has to be considered as labile Fe (Gerringa et al., 2007), because there is no equilibration time between TAC and the studied ligands due to the short contact time between FeL and TAC. In irradiated seawater samples, this time was found to be enough to measure the labile Fe in solution.

The kinetic of dissociation of dFe-ligand complexes was prepared in at least 14 Teflon vials with 10 mL of UV-irradiated GSW (4 hours), 100 μ L of EPPS (1 M) buffered to pH 8.05, 10 nM of Fe and 5 nM of the studied ligand. The times of equilibration for each ligand were defined, according to the formation results, when the plateau was reached (4 hrs for ($\pm$) – catechin, 2 hrs for sinapic acid, and 15 hrs for gallic acid). Then 10 μ L of TAC (0.01 M) was added into the samples, corresponding with the initial time of dissociation (t_0). In this case, as TAC and the studied ligand can compete during the dissociation, the measured dFe is here called ‘TAC labile Fe’ (Gerringa et al., 2007). The addition of TAC will form an electroactive complex which can be detected in the mercury drop.

All the experiments were carried out in triplicate and the Teflon vials were conditioned 5 times with GSW.

Dissolved Fe organic speciation measured by DP-CSV

In a series of 14 Teflon bottles an aliquot of 10mL of natural seawater, 100 μ L of EPPS (1 M) buffered to pH 8.05, and different concentrations of dFe (from 0 to 15 nM) were pipetted into the bottles. For GSW titrations, the solution was left to equilibrate for one hour. Then, 10 μ L of TAC (0.01 M) were added and left to equilibrate overnight (Croot and Johansson, 2000). The samples were measured in a Teflon cell. First, two GSW subsamples were systematically analysed according to the recommendation of the GEOTRACES program, where two +0-dFe additions in the titration with at least eight dFe additions, for a total of 10 or more titration points will provide a better data interpretation (Garnier et al., 2004; Gledhill and Buck, 2012; Sander et al., 2011).

The dFe-binding capacities of the studied organic ligands, ($\pm$) – catechin, sinapic acid and gallic acid, were measured following the same method as indicated in the titration approach. A solution containing 10 mL of UV-irradiated GSW (UV-treatment for 4 hours), 100 μ L of EPPS (1 M) buffered to pH 8.05, 0 to 15 nM of Fe(III) and 2 – 11.8 nM of the studied ligand was prepared. These solutions were equilibrated for the same time as above and, 20 sec before the deposition time, 10 μ L of TAC (0.01 M) were pipetted into the Teflon cell for measurement. This short time was selected in order to avoid equilibrium between TAC and FeL, and because the interest of this work was to measure the equilibrium between Fe and polyphenols.

The titration data were processed using the ProMCC program (Omanović et al., 2014) in order to compute the concentrations of ligands and the conditional stability constants.

Fe(III) reduction experiments

The Fe(III) reduction experiments were carried out according to Santana-Casiano et al. (2010, 2014). Briefly, a 100 nM concentration of Fe(II) was used in 100 ml of seawater

in a 200 ml thermostatted vessel controlled to 25 ± 0.02 °C and pH 8.0 ± 0.01 . The pH of the solution was determined on the free scale, pH_F (Millero, 1986). The pH was adjusted to the desired value with additions of small amounts of 1 M HCl, automatically controlled by a 719 titrinoTM (Methrom) to keep it constant. This Fe concentration was used to reproduce the same experiments as carried out by Santana-Casiano et al. (2010, 2014) and to ensure that the preparation of the polyphenols stock solutions in methanol and MQ did not have any effect on the Fe(II) reduction process and be able to compare the current study with the literature. The solution was initially aerated with air for two hours under oxygen saturation conditions in order to fully oxidize the added Fe(II) solution. After that time, 10^{-4} M ferrozine (FZ) was added. Fe(II) was not detected in the absence of the phenolic compounds. The addition of 100 nM of the polyphenol corresponds to zero time of reaction. The Fe(II)-FZ complex was recorded over time at 562 nm in a 5-m-long waveguide capillary flow cell (LWCFC) from World Precision Instruments connected to the UV detector S4000 (Ocean OpticsTM). This technique allows to measure both Fe(II) and the organic compounds. Every acquisition time, the absorbance spectrum was analysed to correct the baseline (Santana-Casiano et al., 2010). These experiments will enable us to detect if Fe(II) is formed during the equilibration time between dFe and the studied polyphenols.

RESULTS

Dissolved Fe concentration in the seawater

The dFe in the natural seawater used in the current investigation was 0.36 ± 0.06 nM (n = 8) and the Fe' was 0.020 ± 0.004 nM (n = 8). Then, the FeL in the natural seawater sample was ~95%. Figure 2 shows one of the titrations carried out by additions of Fe (0 – 15 nM) to the GSW and the UV-irradiated GSW. These results show that the natural

ligand concentration calculated using the ProMCC code was 3.1 ± 0.7 nM ($n = 10$). This seawater contained 8 times more ligands than dFe. The conditional stability constant was $\log K'_{\text{Fe}^{\text{II}}\text{L}} = 11.5 \pm 0.4$ in respect to Fe^{II} and $\log K'_{\text{Fe}^{\text{III}}\text{L}} = 21.5$. Therefore, the ligands in GSW are ranked as L_2 -type ligands (Gledhill and Buck, 2012). No significant differences were observed between linear (van den Berg and Kramer, 1979) and non-linear treatment (Gerringa et al., 2014; Pižeta et al., 2015). The standard error from the ProMCC for each analysis in terms of $K'_{\text{Fe}^{\text{II}}\text{L}}$ and ligand concentration was always at least one order of magnitude lower than the same parameter.

Dissolved Fe speciation from the kinetic approach

In order to study the Fe-binding capacity of polyphenols in terms of kinetic experiments, the GSW was UV-irradiated during 4 hours. This was long enough to decompose all the organic ligands, as demonstrated by the linear dependence of the peak high (nA) and the Fe concentrations (Figure 2). The concentration of dFe did not exceed the solubility of Fe in our seawater samples because the standard additions were linear in the studied range (15 nM of dFe). However, the linear standard additions can also occur when formation kinetics are quick and TAC dissolves amorphous Fe oxides rapidly. The height peak (dFe concentration) after 10 nM of dFe addition was followed in UV-irradiated GSW by more than 24 h and it was stable. These experiments were also done by triplicate in at least 14 Telfon vials.

The kinetic approach was performed according with Witter and Luther (1998) and Wu and Luther (1995). The $K'_{\text{Fe}^{\text{II}}\text{L}}$ can be estimated from k_f and k_d by following Equation 1 where k_f and k_d were computed from the experimental results showed in Figure 3.

$$K'_{Fe'L} = k_f/k_d \quad (1)$$

In addition, the conditional stability constant with respect to Fe^{3+} ($K'_{Fe^{3+}L}$) can be calculated by assuming that the second-order rate constant is $k_{f,Fe^{3+}L} = 3.02 \cdot 10^{11} \text{ M}^{-1} \text{ s}^{-1}$, previously estimated from encounter theory (Luther and Wu, 1997).

$$K'_{Fe^{3+}L} = \alpha_{Fe'} K'_{Fe'L} \quad (2)$$

where $[Fe']$ is labile Fe as was described above and Fe^{3+} is the free inorganic Fe (Stockdale et al., 2016), $\alpha_{Fe'} = 10^{10}$ (Hudson et al., 1992; Sunda and Huntsman, 2003), is commonly used for pH 8 seawater. Then, the dissociation rate constant $k_{d, Fe^{3+}L}$ can be computed (Equation 3).

$$k_{d,Fe^{3+}L} = \frac{k_{f,Fe^{3+}L}}{K'_{Fe^{3+}L}} \quad (3)$$

The half-life time ($t_{1/2}$), for $Fe'L$ and $Fe^{3+}L$, has also been computed according to the pseudo-first kinetic equation, which can be expressed as: $t_{1/2} = \ln 2/k_d$.

In addition, the presence of organic ligands enhances Fe solubility by favoring the formation of small Fe soluble species (Kuma et al., 1996).

The results for the kinetic of formation and dissociation experiments are shown in Figure 3 and Table 1. They showed that the dFe – phenolic complexes are rapidly formed with $\log k_f$ between 5.50 – 5.62 (k_f in $\text{M}^{-1} \text{ s}^{-1}$, Table 1). On the other hand, the dissociation rate constants were also high; $\log k_d$ -3.36 – 3.61 (k_d in s^{-1} , Table 1) for these polyphenol

groups. Then, the $\log K'_{Fe'L}$ determined from the kinetic experiments were equal to 9.2 ± 0.1 ($(\pm)$ – catechin), 8.86 ± 0.04 (sinapic acid) and 9.01 ± 0.02 (gallic acid)), relative to the Fe' species. These Fe-organic complexes can be defined as weak ligands (L_2 -type ligands; Gledhill and Buck (2012)). According to the dissociation rate constants, the presence of polyphenols will keep dFe in solution from 11.3 min to 20.6 min, in respect to the complex of Fe' with sinapic acid or $(\pm)$ – catechin, respectively. This half-life time increased to the order of years (0.32 – 0.54) in terms of Fe^{3+} (Table 1). This difference is due to the calculation of half-life time for $Fe^{3+}L$ complexes assumes an upper diffusion control limit to formation of the complex rather than the activation control for $Fe'L$ (Witter et al., 2000).

Conditional stability constants from CLE-CSV

The total amounts of organic ligands in seawater can be computed using Equation (4).

$$[L_T] = [L'] + [FeL] \quad (4)$$

where $[L_T]$ is the total concentration of ligands, $[L']$ includes both free ligands and ligands complexed with other major cations in seawater (as Ca^{2+} , Mg^{2+} and H^+ ; Rue and Bruland (1995)), $[FeL]$ the concentration of Fe-binding ligands. In the same sense, the concentration of Fe in solutions can be considered as Equation 5.

$$[Fe]_T = [Fe'] + [FeL] \quad (5)$$

where $[Fe]_T$ represents the total dissolved Fe in seawater, $[Fe']$ represents the concentration of all the inorganic species (predominantly in the form $Fe(OH)_3$ (Stockdale et al., 2016). The equilibrium system between Fe and the organic ligand is:

where the conditional stability constant with respect to $[Fe']$ is expressed as Equation 7:

$$K'_{FeL} = \frac{[FeL]}{[Fe'][L']} \quad (7)$$

The relationship between Fe' and Fe^{3+} can be used as: $\alpha_{Fe'} = [Fe']/[Fe^{3+}]$, then $K'_{Fe'L} = \alpha_{Fe'} K'_{Fe^{3+}L}$, where $\alpha_{Fe'} = 10^{10}$ (Hudson et al., 1992; Sunda and Huntsman, 2003) is commonly used for pH 8 seawater and the $K'_{Fe'L}$ can then be calculated from the values of Fe' measured by CSV and the dFe and L concentrations using Equations 4, 5 and 7.

The complexation of dFe by the three different polyphenols, ($\pm$) – catechin, sinapic acid and gallic acid, was studied in UV-irradiated GSW (Table 2). Two different concentrations of the studied Fe-binding ligands were used in order to evaluate if the binding capacity was related to the concentration and to determine if the binding sites were saturated with those Fe levels in every sample. The ligand ($\pm$) – catechin was able to complex 1.6 ± 0.2 nM and 3.6 ± 0.5 nM of dFe when 5.7 and 11.8 nM of the studied ligand were present in solution, with a log $K'_{Fe'L}$ between 9.3 – 9.1, respectively. Sinapic acid complexed 3.6 ± 0.8 and 6.5 ± 0.7 nM of dFe when 5.0 and 9.9 nM of the ligand were added, with a log $K'_{Fe'L} = 8.6$. Finally, when 2.0 nM of gallic acid was added, 5.5 ± 0.3 nM of dFe was organically complexed and when 5 nM of gallic acid was added to the UV-seawater, 14 ± 2 nM of dFe were found complexed. The amount of Fe complexed or

removed by gallic acid was always over double the added ligand concentration. The log K'_{FeL} values were 8.8 and 9.5, respectively.

Taking into account the titration approach, the log K'_{FeL} was from 8.6 to 9.5 (Table 2). Accordingly, the studied ligands formed weak organic complexes with dFe in seawater, with a conditional stability constant ≥ 18 with respect to Fe^{3+} . Then, they can be classified as L_2 -type organic ligands (Gledhill and Buck, 2012).

Fe(III) reduction by polyphenols in seawater

Fe(III) is reduced by ($\pm$) – catechin, sinapic acid and gallic acid in UV-irradiated GSW at pH 8.0 and 25°C (Figure 4). The Fe(III) reduction rate constant (pseudo-first order rate constant) was $8.98 \pm 0.02 \cdot 10^{-7} \text{ sec}^{-1}$, $1.45 \pm 0.02 \cdot 10^{-7} \text{ sec}^{-1}$ and $3.90 \pm 0.04 \cdot 10^{-5} \text{ sec}^{-1}$ for ($\pm$) – catechin, sinapic acid and gallic acid, respectively. This means that 100 nM of polyphenol are able to reduce from 0.05 nM to 11.92 nM of Fe(III) in one hour of reaction in UV-irradiated GSW. Then, at equimolar concentration (100 nM of dFe and the studied organic ligand), the studied polyphenols were able to reduce Fe(III) to Fe(II) in seawater.

DISCUSSION

Polyphenols as dFe-complexing ligands in seawater

Recent investigations (López et al., 2015; Rico et al., 2013; Santana-Casiano et al., 2010, 2014) demonstrated that polyphenols have to be considered as important organic ligands within the Fe biogeochemical cycle due to their ability to reduce Fe(III) to Fe(II) in seawater as well as to be produced in the microalgae exudates as a response to the Fe exposition. According to the molecular structure (Figure 1), ($\pm$) – catechin is a flavonoid

type compound with a catechol moiety in the B-ring, a resorcinol group in the A-ring and a hydroxyl group at position 3 in the C-ring (Figure 1A). Sinapic acid is a phenylpropanoid compound, with 3,5-dimethoxyl and 4-hydroxyl groups substituting the phenyl group of the cinnamic acid (Figure 1B). Gallic acid is tri-hydroxybenzoic acid (gallyol moiety) (Figure 1C). Such acids are generally forming dimers such as ellagic acid. ($\pm$) – catechin, sinapic acid and gallic acid contain –OH groups in their molecules which at pH $\sim$ 8 (natural seawater pH) should be deprotonated forming anionic ligands capable of complexing metals, as the organic ligands at the cell surface of microorganisms (González et al., 2014). Accordingly, polyphenols at natural pH complex dFe in seawater, as it was previously reported at acidic pH and low ionic strength solutions (Andjelković et al., 2006; Brown et al., 1998; Hynes and O’Coinceanainn, 2004; Khokhar and Apenten, 2003; Mira et al., 2002).

In general, the dFe complexation by polyphenols is related to the presence of the *ortho*-di-hydroxy groups, mainly present in molecules bearing catechol or gallyol moieties (Khokhar and Apenten, 2003; Moran et al., 1997) and to the amount of –OH groups in each molecule. In fact, Andjelković et al. (2006) demonstrated that polyphenols bearing gallyol groups form stronger chelates than molecules with catechol moiety in aqueous solutions. The formation of complexes between dFe and the organic ligands proposed in this investigation showed that gallic acid is the most important phenolic compound in terms of dFe-binding capacity, where a stoichiometric ratio 1:1 can be assumed (Table 2). The differences in the complexing capacity could not only be understood as a role of the hydroxyl groups in the catechol or gallyol moieties in seawater. The presence of major ions such as Mg^{2+} and Ca^{2+} play a key role in the oxidation process of polyphenols, blocking the reaction to semiquinone and benzoquinone (Santana-Casiano et al., 2010, 2014). Then, some of the –OH groups are occupied by Mg^{2+} and Ca^{2+} , decreasing their

capacity to complex dFe (Santana-Casiano et al., 2010, 2014). In addition, the ability of gallic acid to form dimers in solution can be involved in the higher dFe-binding capacity compared to that for ($\pm$) – catechin and sinapic acid.

The kinetics of formation did not reveal a very strong decrease in the labile Fe in the first seconds (Figure 3), as it occurs for stronger ligands, L₁-type ligands, such as protoporphyrin IX (Witter et al., 2000), with a decrease of 2 nM of Fe within the first seconds of the reaction, supporting the experimental results for polyphenols that are ranked as weak ligands. The polyphenols studied in this manuscript progressively decreased the labile Fe over time (Figure 3). Other studied ligands have also been characterized in terms of formation rate constant in seawater (Witter et al., 2000) such as protoporphyrin IX, phaeophytin, apoferritin, phytic acid, alterobactin A and B, ferrichrome and desferrioxamine, with k_f from $0.93 \cdot 10^5$ to $19.6 \cdot 10^5 \text{ M}^{-1} \text{ s}^{-1}$. These authors (Witter et al., 2000), also reported the dissociation rate constant for the same organic binding ligands and ranked from $0.05 \cdot 10^{-6}$ to $15.8 \cdot 10^{-6} \text{ s}^{-1}$. The data collected from the literature for natural waters show a high variability (from $0.31 \cdot 10^{-6}$ to $39 \cdot 10^{-6}$; Luther et al. (1997) and Witter et al. (1998), which proves the huge diversity of ligands in the water.

The dissociation rate constant allows to compute the half-life time of Fe in solution, both as Fe' and Fe³⁺ (Table 1). In the presence of polyphenols, Fe' half-life time is 11 – 21 min, which increases to 0.3 – 0.5 years when Fe³⁺ species are considered. It means that Fe persists in solution longer periods when polyphenols are present. These $t_{1/2}$ are highly affected by the presence of organic ligands in the ocean and ranked from minutes to years (Witter et al., 2000).

The log $K'_{\text{Fe'L}}$ values estimated from the kinetic experiments for ($\pm$) – catechin, sinapic acid and gallic acid are weaker than other model ligands studied by Witter et al. (2000) which log $K'_{\text{Fe'L}}$ values were 10.8 – 13.0, and also with humic substances (log $K'_{\text{Fe'L}}$ =

11.1; Laglera et al. (2011)) and fulvic acids ($\log K'_{\text{FeL}} = 10.6-10.9$; Laglera et al. (2011; Laglera and van den Berg (2009), exudates from *Pseudonana antarctica* ($\log K'_{\text{FeL}} = 11.9$; Norman et al., 2015) and *Emiliania huxleyi* ($\log K'_{\text{FeL}} = 11.9$; Boye and van den Berg, 2000).

According to the recovery of ligands with respect to the initial addition, a tentative stoichiometry can be computed. The ($\pm$) – catechin and sinapic acid followed the same trend, the concentration of the recovered ligand was always lower than the added ligand, and the recovered ligand concentration in the titration approach (Table 2) is comparable with the FeL formation in the kinetic experiments (Figure 3). However, the addition of a certain concentration of gallic acid always resulted in a higher concentration of recovered ligand or FeL formation in both approaches (Table 2 and Figure 3). The addition of ($\pm$) – catechin was increased from 5.7 to 11.8 nM (2.07-fold) and the recovered ligands was 2.25-fold. Attending to sinapic acid, the added level increased by 2.0 (Table 2) and the recovered ligand increased by 1.8-fold. Then, a tentative stoichiometry of 1:1 ligand-to-metal complexes can be assumed. The gallic acid is the only case where the addition of a certain level resulted in a higher recovered concentration. Then, in this case, the relationship between FeL and added L was ~ 3 (Table 2). In the literature, there are conflicting results for Fe-gallic acid complexes where the stoichiometry can be from 1:1 to 1:3 metal-to-ligand complexes (Andjelković et al., 2006; Fazary et al., 2008; Powell and Taylor, 1982; Strlič et al., 2002) as well as 1:1 and 2:1 metal-to-ligand complexes (Hynes and O’Coinceanainn, 2001), demonstrating the complicated nature of these type of ligands in solution, where the binding capacity is function of the pH, the composition of the solution, the reactivity of the gallic acid to reduce Fe(III) to Fe(II) and the formation of other molecules such as quinone, semiquinone or tannic acids (Andjelković et al., 2006; Fazary et al., 2008; Powell and Taylor, 1982; Strlič et al., 2002) which could

complex Fe in a higher ratio. In addition, Strlič et al. (2002) reported the role of the Fe:gallic acid complexes in terms of the HO[•] production that also have an impact on Fe redox chemistry. Then, further experiments will be carried out in order to elucidate the Fe:gallic acid stoichiometry in seawater. For this purpose, an evaluation of binding capacity will be studied by using a combination of UV-Vis absorption spectroscopy (Andjelković et al., 2006) and voltammetric technique (this study) as a function of dFe and gallic acid concentration in seawater.

Polyphenols as a source of Fe(II) in seawater

The complexation of dFe with polyphenols can be considered as a first step in the process of internalization of Fe by phytoplankton, reducing Fe(III) to Fe(II) both in seawater (Santana-Casiano et al., 2010, 2014) and at cell surfaces (González et al., 2014). The same mechanism was also studied for catechol (Santana-Casiano et al., 2010), (±) – catechin and sinapic acid (Santana-Casiano et al., 2014) as a function of pH. Polyphenols form a complex with Fe(III) and reduce a fraction of Fe(III) to Fe(II), as it has been shown in the current manuscript. The reduction rate constants (in log k') for (±) – catechin and sinapic acid are comparable with those measured by Santana-Casiano et al. (2014), where k' was $7.02 \cdot 10^{-7} \text{ s}^{-1}$ and $2.67 \cdot 10^{-7} \text{ s}^{-1}$, respectively. Gallic acid showed a higher log k' that is in the same range as that for catechol (Santana-Casiano et al., 2010). In addition, the organic exudates excreted by *P. tricornutum* also retarded the Fe(II) oxidation rate constant in seawater (González et al., 2012; Santana-Casiano et al., 2014). From the current results, we can conclude that organic exudates such as polyphenols increase the persistence of Fe(II) in seawater, either by decreasing their oxidation rate constant to Fe(III) and/or by reducing Fe(III) to Fe(II).

439

440 **Possible discrepancies between kinetic and titration approaches**

441 The experimental methods used in the current manuscript will allow to determine the
442 conditional equilibrium constant for Fe-binding ligands as well as the concentration of
443 these ligands in solution. The calculation of these parameters is highly dependent on the
444 pH and major ions (Laglera et al., 2001).

445 In the current manuscript, the comparison between kinetic and titration approaches are in
446 a good agreement for ($\pm$) – catechin and sinapic acid, but there are significant differences
447 in the determination of FeL concentration when gallic acid was studied. These differences
448 can be understood as a consequence of the combination of a number of possible factors
449 acting on the experimental approach, such as Fe(II) production during the formation
450 reaction time, the oxidation of gallic acid to semiquinone or quinone groups and the
451 formation of other organic molecules from gallic acid. In the experimental approach, all
452 non-labile Fe is assumed to be complexed by the organic ligand, which can be the case
453 for catechin and sinapic acid, while for gallic acid, other processes could be acting.
454 Therefore, the constant provided in this study should be considered a global complexation
455 constant for any Fe present in the solution. There is an important discrepancy about the
456 Fe:gallic acid stoichiometry in the literature because its reaction mechanism dependent
457 on pH, ionic strength and concentrations of both chemical (Andjelković et al., 2006;
458 Fazary et al., 2008; Hynes and O’Coinceanainn, 2001; Powell and Taylor, 1982; Strlič et
459 al., 2002). Further experiments are necessary to properly define the Fe:gallic acid
460 stoichiometry in seawater by a combination of techniques and physico-chemical
461 conditions.

Accordingly, the Fe(II) regeneration during the formation reaction and during the equilibration between dFe and polyphenols can also invoke a slight discrepancies in terms of ligand concentration and conditional stability constant. During the equilibration time between Fe and the studied ligands (> 2 hours), a fraction of Fe(III) can be reduced to Fe(II) and when TAC is added to the titration vials, this fraction of Fe is not reacting with TAC as Fe(III). It was determined that during 1 h of equilibration between dFe and the studied polyphenols, from 0.05% to 11.92% of Fe(II) is formed. Then, the calculation of the conditional stability constant and ligand concentration from the titration can be overestimated.

In the case of polyphenols, the interaction with Mg^{2+} and Ca^{2+} affects the oxidation of the semiquinone radical to quinone and it may affect the Fe chemistry, both in terms of complexation and in terms of redox reactions, and it has been demonstrated for catechol, ($\pm$) – catechin and sinapic acid (Santana-Casiano et al., 2010, 2014). Moreover, during the equilibration time between Fe and the studied ligands, the competition with the MgL and CaL could affect the dFe-ligand complexation (Hering and Morel, 1988; Raspor et al., 1980; Wu and Luther, 1995), which becomes more relevant when weak ligands are in solution. This competition between Fe with Ca and Mg for the binding sites can introduce an interference for the calculation of the ligand concentration as well as of the conditional stability constant.

These artefacts are more relevant for weak ligands than for strong ligands. Therefore, the kinetic approach seems more consistent for use in future experiments defining the complexation of dFe and studied ligands in seawater.

CONCLUSIONS

Polyphenols as model dFe-binding ligands are excreted by phytoplankton and can play two different roles in terms of the Fe biogeochemical cycle. On one hand, the polyphenols can complex dFe in seawater, and on the other hand, these polyphenols can reduce Fe(III) to Fe(II). Among the pool of binding ligands, ($\pm$) – catechin, sinapic acid and gallic acid have been thermodynamically characterized. The formation rate constant (k_f) measured for the complex between dFe and ($\pm$) – catechin, sinapic acid and gallic acid, varied from $3.14 \cdot 10^5$ to $4.17 \cdot 10^5$ ($\text{M}^{-1}\text{s}^{-1}$). The dissociation rate constant (k_d) varied from $2.43 \cdot 10^{-4}$ to $4.4 \cdot 10^{-4}$ (s^{-1}). The conditional stability constants using the kinetic approach were also calculated ($\log K'_{\text{FeL}} = 8.86 - 9.2$). These conditional stability constants were compared with the titration approach, where the $\log K'_{\text{FeL}}$ values were from 8.6 to 9.5 for the three studied Fe-binding ligands. These polyphenols are within the L_2 -type ligands. Moreover, Fe(III) is reduced by ($\pm$) – catechin, sinapic acid and gallic acid in seawater, promoting the formation of Fe(II) from 0.05% to 11.92%. Taking these results into consideration, polyphenols should be considered as an important dFe-binding ligands in seawater in order to properly understand the biogeochemical cycle of Fe and organic ligands.

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Table 1. Formation and dissociation constants, conditional stability constant and half-life time determined using the kinetic method. The initial concentration of dFe = 10.36 nM and the initial concentration of studied ligand was 5 nM.

Studied ligand	$k_f \times 10^5$ (M⁻¹s⁻¹)	$k_d \times 10^{-4}$ (s⁻¹)	$\log K'_{Fe'L}$	$\log K_{Fe^{3+}L}$	Fe' t_{1/2} (min)	Fe³⁺ t_{1/2} (years)
(±)-Catechin	4.2±1.8	2.43±0.03	9.2±0.1	19.20	20.6	0.54
Sinapic acid	3.2±0.7	4.4±0.3	8.86±0.04	18.86	11.3	0.23
Gallic acid	3.1±0.4	3.2±0.1	9.01±0.02	19.01	15.9	0.32

Table 2. Concentration of studied ligand added and recovered after a titration, conditional stability constant determined using DP-CSV and calculated applying the ProMCC code. Errors represent the standard deviation from three replicates.

Studied ligand	[L]_{added} (nM)	[L]_{recovered} (nM)	$K'_{FeL} \times 10^9$	$\log K'_{FeL}$	$\text{Log} K'_{Fe^{3+}L}$
(±)-Catechin	5.7	1.6±0.2	2.0	9.3±0.1	19.3
(±)-Catechin	11.8	3.6±0.5	1.3	9.1±0.2	19.1
Sinapic acid	5.0	3.6±0.8	0.4	8.6±0.2	18.6
Sinapic acid	9.9	6.5±0.7	0.4	8.6±0.1	18.6
Gallic acid	2.0	5.5±0.3	0.6	8.8±0.2	18.8
Gallic acid	5.0	14±2	3.2	9.5±0.1	19.5

LIST OF FIGURES

Figure 1. Chemical structure of ($\pm$)-catechin (A), sinapic acid (B) and gallic acid (C)

Figure 2. Example of titration of natural seawater (dots) and 4 hours UV-irradiated seawater (squares).

Figure 3. Kinetic of formation of Fe complexes with ($\pm$) - catechin (A), sinapic acid (B) and gallic acid (C), where the concentration of dFe is expressed as labile Fe because of $\text{Fe}(\text{TAC})_2$ complex was measured after 20 sec. Kinetic of dissociation of ($\pm$) - catechin (D), sinapic acid (E) and gallic acid (F), where the concentration of dFe is expressed as TAC labile Fe due to the interaction between TAC and the studied ligands. Dashed lines correspond to the fitting of the results. All the experiments were carried out in triplicate in UV-irradiated seawater. The initial concentration of dFe was 10.36 nM. X-axis are in different scale for each polyphenol due to their reaction time.

Figure 4. Percentage of Fe(II) reduced from Fe(III) in UV-seawater at pH 8.0 and $T=25^\circ\text{C}$ in the presence of ($\pm$) – catechin, sinapic acid and gallic acid.

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