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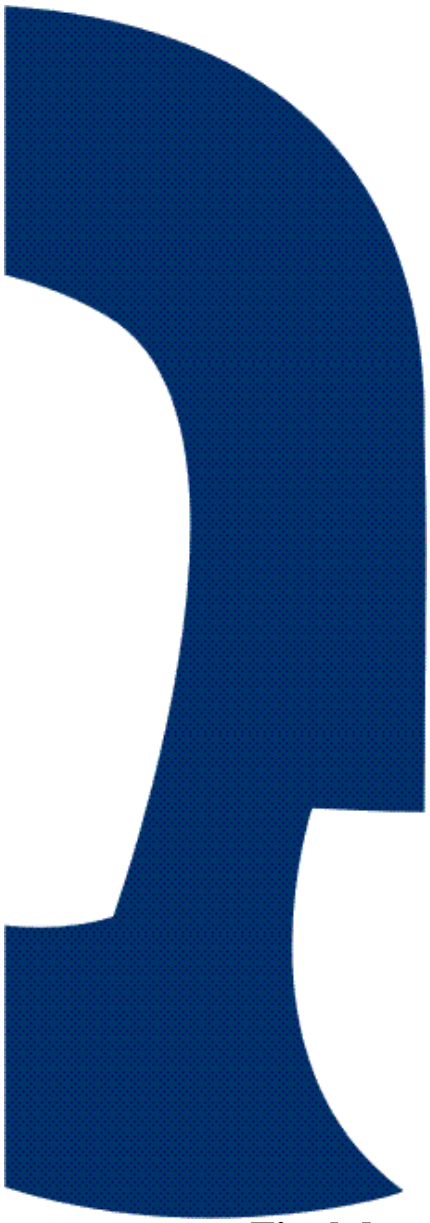

Determination of Conditional Stability Constants and Kinetic Constants for Phenolic Fe-Binding Ligands in Seawater

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ABSTRACT

Conditional stability constant and rates of formation and dissociation for the inorganic iron species (Fe′) and for Fe^{3+} complexation with three model polyphenols,(±) – catechin, sinapic acid and gallic acid were measured in UV-treated seawater by competitive ligand adsorptive stripping voltammetry (CLE-CSV) and kinetic approach using the iron binding ligand TAC (2-(2-thiazolylazo)-p-cresol). Conditional stability constants for the ligands ranged from log $K'_{Fe'L}$ 11.0 to 11.9. The stoichiometry of the complexes formed between dissolved Fe (dFe) and the polyphenols, in UV-seawater, was 3.5:1, 1.5:1 and 1:2.8 for (±) – catechin, sinapic acid and gallic acid, respectively. The formation rate constant varied from $3.82 \cdot 10^5$ to $4.16 \cdot 10^5$ $M^{-1}s^{-1}$ and dissociation rate constant $1.88 \cdot 10^{-4}$– $4.4 \cdot 10^{-4} s^{-1}$. The conditional stability constant (log $K'_{Fe'L}$) computed from the kinetic approach was from 8.96 to 9.4. The different dFe-binding capacity of polyphenols is highly affected by the interaction with Mg^{2+} and Ca^{2+} in seawater. Moreover, the dFe-binding ligands are able to keep Fe in solution for longer between 26 – 61 min for Fe′ species and 0.7 – 1.8 years for Fe^{3+}.

1. INTRODUCTION

Iron is an essential micronutrient for marine microorganisms because of it participates in a number of metabolic processes (Morel and Price, 2003), limiting the phytoplankton growth in high nutrient low chlorophyll oceanic regions (Martin et al., 1991). In the seawater, the speciation of Fe is highly dominated by the organic complexes (> 99%) affecting its reactivity (Gledhill and van den Berg, 1994; Rue and Bruland, 1995) and increasing its solubility in seawater (Liu and Millero, 2002; Rue and Bruland, 1995). Therefore, microorganisms have to use a variety of cellular tools in order to make the dissolved Fe (dFe) more bioavailable (Granger and Price, 1999; Hutchins et al., 1999; Maldonado and Price, 1999). In this sense, there are a high variety of possible dFe-binding ligands in natural waters, and the determination of the new ligands and the capacity to complex Fe is highly important to understand the biogeochemical cycle of Fe. In general, these organic ligands are produced by cells; rupture of cells after grazing (Chase and Price, 1997; Hutchins and Bruland, 1994; Hutchins et al., 1995; Sato et al., 2007), viral lysis (Gobler et al., 1997; Poorvin et al., 2011) and transformation of organic materials (Boyd and Ellwood, 2010; Gerringa et al., 2006; Gledhill and Buck, 2012). These organic ligands can be ranked according with the size of the molecule as highly specific low molecular weight to large molecules (Gledhill and Buck, 2012). The most accepted classification is according to the value of the conditional stability constant ($K'_{Fe'L}$) as strong ligands (L_1 - log $K'_{Fe'L} \geq 12$, L_2- log $K'_{Fe'L}11 - 12$) and weak ligands (L_3 - log $K'_{Fe'L}10 - 10.9$ and L_4 - log $K'_{Fe'L} < 10$) (Gledhill and Buck, 2012) where the dFe-binding ligands and the conditional stability constant can be measured by competitive ligand exchange adsorptive cathodic stripping voltammetry (CLE-ACSV).

International and global projects such as GEOTRACES made possible the measurements of Fe and ligands for most of the oceans around the word. The strong ligands are often measured in limiting Fe areas and surface waters (Buck et al., 2010; Maldonado et al., 2002; Mawji et al., 2011) with a biological origin (Gledhill and Buck, 2012; Hunter and Boyd, 2007), generally linked with the chlorophyll maximum (Boye et al., 2001; Croot et al., 2004; Ibisanmi et al., 2011; Wagener et al., 2008), but they can be in the whole water column as Gerringa et al. (2006) measured in the Atlantic Ocean. The weaker ligands should be composed by the photo-degradation of strong ligands (Barbeau, 2006), biological production (as exudates) (Boyd and Ellwood, 2010; Hassler

et al., 2011; Hutchins and Bruland, 1994) and humic-like substances (Laglera and van den Berg, 2009).

The structure of this organic compounds are still unknown (Luther et al., 2001), mostly the weak dFe-binding ligands, as saccharides, amino acids or phenolic compounds. The conditional stability constant has been determined for humic substances (log $K'_{Fe'L}$= 11.1; Laglera et al. (2011)), fulvic acids (log $K'_{Fe'L}$= 10.6-10.9; Laglera et al. (2011); Laglera and van den Berg (2009)) and specific model organic ligands (Witter et al., 2000) as protoporphyrin IX (log $K'_{Fe'L}$= 9.34), phaeophytin (log $K'_{Fe'L}$= 9.14), apoferritin (log $K'_{Fe'L}$= 8.67), phytic acid (log $K'_{Fe'L}$= 9.27), alterobactin A and B (log $K'_{Fe'L}$= 10.9 and > 11.0, respectively), ferrichrome (log $K'_{Fe'L}$= 8.51) and desferrioxamine (log $K'_{Fe'L}$= 8.56). Another groups as catecholate (log $K'_{Fe'L}$= 11.9) and hydroxamate (log $K'_{Fe'L}$= 11.5) have been determined (Macrellis et al., 2001). Moreover, the characterization of dFe-binding ligands have been also measured in natural incubations (log $K'_{Fe'L}$= 12.4; Buck et al. (2010)) and monocultures such as *Pseudomonas antarctica* (log $K'_{Fe'L}$ = 11.9; Norman et al. (2015)) or *Emiliana huxleyi* (log $K'_{Fe'L}$= 10.7 – 11.5; Boye and van den Berg (2000)).

Recent publications (López et al., 2015; Rico et al., 2013; Santana-Casiano et al., 2014) showed the phenolic profile produced by the marine diatom, *Phaeodactylum tricornutum*, and the green algae, *Dunaliella tertiolecta*. They reported that the type of phenolic compound and the concentration of them are directly related with the metal concentration in solution. In terms of complexation, phenolic compounds are formed by hydroxyl and carboxyl groups that can complex Fe in seawater affecting the solubility and the bioavailability for marine microorganisms (Onofrejová et al., 2010). In addition, some polyphenols have been described as strong dFe-binding ligands in acidic solutions (pH < 3; Hynes and O'Coinceanainn (2001); Hynes and O'Coinceanainn (2004)). These polyphenols are mainly involved in the scavenging of reactive oxygen species and metal complexation (Bentes et al., 2011; Neudörffer et al., 2006). Such as other organic ligands produced by microorganisms (amino acids or polysaccharides), polyphenols can form complexes with iron (Andjelković et al., 2006; Brown et al., 1998; Lodovici et al., 2001; Mira et al., 2002; Re et al., 1999; Sroka and Cisowski, 2003). Attending to the characterization of the exudates of culture of *P. tricronutum* in the presence of dFe in solution (Santana-Casiano et al., 2014) we have selected three phenolic compounds in order to study the dFe-binding capacity in seawater. These ligands are (±) – catechin

(5,7,3′,4′-tetrahydorxyflavan-3-ol), sinapic acid (3,5-dimethoxy-4-hydroxycinnamic acid) and gallic acid (3,4,5-trihydroxybenzoic acid).

The goal of the current investigation is to measure the conditional stability constants ($K'_{Fe'L}$ and K'_{Fe3+L}), and the formation (k_f) and dissociation (k_d) rate constants of (±) – catechin, sinapic acid and gallic acid iron complexes, as a model dFe-binding ligands that are present in the phytoplankton exudates. These measurements will contribute to increase the data base of possible dFe-binding ligands in natural waters and increasing our understanding of the Fe biogeochemical cycle.

2. MATERIAL AND METHODS

2.1. Chemicals

A 0.01 M solution of TAC (2-2(2-thiazolylazo)-*p*-cresol) (Sigma-Aldrich) was prepared in HPLC grade methanol (Sigma-Aldrich) every other week and kept in the fridge when it was not in use (darkness and 4˚C). A 1.0 M stock buffer of EPPS (N-(2-hydroxyethyl)piperazine-N';2-propanesulfonic acid; SigmaUltra) was prepared in 1 M NH_4OH (ultrapure, VWR) at pH 8.0. The buffer solution was cleaned three times in an 8HQ (8-Hydroxyquinoline) resin column. Iron stock solutions (100 nM and 1.0 μM) were prepared weekly from Fe standard solution for atomic absorption spectroscopy (VWR) and acidified at pH 2 with HCl (ultrapure, VWR). Model organic ligands, (±) – catechin, sinapic acid and gallic acid (Sigma-Aldrich) were weekly prepared in HPLC grade methanol at 10^{-3} M. Second stock solutions were daily prepared in MQ water at 10^{-6}M, following a modified protocol from Witter et al. (2000)**,** who used 1-nitroso-2-naphtol (1N2N) instead of TAC as artificial ligand to determine dFe. The dissolved iron was measured in the stock solutions and its concentration was negligible.

The seawater used in the current experiments was collected during the oceanographic cruise "GEOVIDE" (station 77, 40 m) in the North Atlantic Ocean and it was filtered by 0.2 μm with cartridge filters on board. The seawater was kept in the clean laboratory (Class 100) facilities (LEMAR-IUEM) with acid-clean carboy (25 L) under darkness conditions until it was used.

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). Before using, the Teflon vials were rinsed 5 times with MQ water.

2.2. Measurements of Labile and dissolved iron

Dissolved iron concentration and dFe-binding ligands were determined by differential pulse cathodic stripping voltammetry (DP-CSV; Croot and Johansson (2000)) by using a μAutolab voltameter (Metrhom), 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 counter electrode. The samples were always treated on a Class 100 clean laboratory at room temperature.

Labile Fe was measured in 10 mL seawater samples by adding 100 μL of EPPS (final concentration 10 mM EPPS buffered to pH 8.0) and 10 μL of 0.01 M TAC (final concentration 10 μM). Samples were purged for 180 sec with dry nitrogen gas. Thus, a new Hg drop was formed at the end of the purging time. 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 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. The dFe concentration was determined via standard additions method.

Dissolved Fe (dFe) concentration was measured following the same method but the seawater was previously irradiated with UV for 4 hours in quartz tubes. These quartz tubes were soaked for a day in 10% HCl (suprapure, VWR) and washed with MQ water 5 times before using. They were also rinsed one more time with the seawater used for the study.

2.3. Dissolved Fe organic speciation measured by DP-CSV

In a series of 11 Teflon bottles an aliquot of 10mL of natural seawater, 100 μL of EPPS (1 M) and different concentrations of Fe (from 0 to 15 nM) were pipetted into the bottles. Two cero values were always analysed according with the recommendation of GEOTRACES program (Gledhill and Buck, 2012). The solution was left to equilibrate for one hour and then 10 μL of TAC (0.01 M) was added and the resulting samples were equilibrated overnight. The samples were measured in a Teflon cell.

The dFe-binding capacities of the proposed model organic ligands: (±) – catechin, sinapic acid and gallic acid, were measured following the same method. In these cases, 10mL of UV-SW (UV treatment for 4 hours), 100 μL of EPPS (1 M), iron concentrations from 0 to 15 nM and 5-10 nM of the model ligand was added. These solutions were equilibrated and, 20 sec before the deposition time, 10 μL of TAC (0.01 M) were pipetted into the Teflon cell for the measurement. The equilibration time was always used according with the formation and dissociation experiments for each organic ligand.

The titration data obtained was analysed with the ProMCC program (Omanović et al., 2015) in order to compute the concentration of ligands and the conditional stability constants.

2.4. Dissolved Fe organic speciation measured using the kinetic method

The kinetic measurement for the dFe-ligand complex formation was prepared with 10 mL of UV-treated seawater (4 hours), 100 μL of EPPS (1 M) and 10 nM of dFe. The concentration of model ligand was always 5 nM. The initial time (t_0) corresponds with the addition of the model ligand. The addition of TAC (10 μL of 0.01 M) was 20 sec before starting the deposition time and during the purge. The concentration of dFe measured can be considered as labile Fe.

The kinetic of dissociation for dFe-ligand was prepared in Teflon bottles with 10 mL of UV-seawater (4 hours), 100 μL of EPPS (1 M), 10 nM of Fe and 5 nM of model ligand. They were equilibrated during a certain time, according to the formation results. The equilibration was 3 h for sinapic acid and overnight for (±) – catechin and gallic acid. Then 10 μL 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 model ligand can compete during the dissociation, the concentration of dFe measured is called TAC labile Fe.

All these experiments were always carried out by triplicate.

3. RESULTS

3.1. Dissolved Fe concentration in the seawater

The dFe in the natural seawater used in the current investigation was 0.36 ± 0.06 nM (n=4) and the labile dFe was 0.20 ±0.04 nM (n=5). These measurements were carried out via standard additions method (Fig. 1). According with these results, the seawater used in this study contained 0.16 nM of Fe organically complexed with natural ligands.

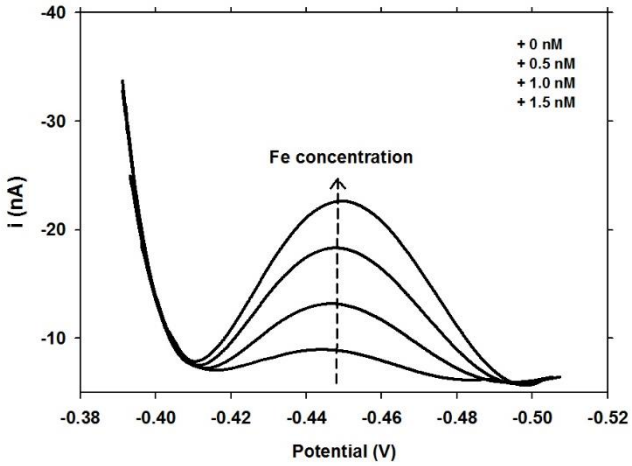

Fig. 1: Iron standard additions to a sample of 10 mL of natural seawater.

3.2. Conditional stability constants from CLE-CSV

The total amounts of organic ligands in seawater can be considered as the Equation (1) showing bellow.

$$[L_T] = [L^-] + [FeL] + [XL] \quad (1)$$

where $[L_T]$ is the total concentration of ligands, $[L^-]$ represents the free ligand, [FeL] the concentration of Fe-binding ligands and [XL] represents the complexes of the ligand with the major cations (as Ca^{2+}, Mg^{2+} and H^+; Rue and Bruland (1995)), commonly the last term is omitted in the mass balance. In the same sense, the concentration of Fe in solutions can be considered as Equation 2.

$$[Fe]_T = [Fe'] + [FeL] \quad (2)$$

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:

$$Fe' + L^{-} \leftrightarrow FeL \tag{3}$$

where the conditional stability constant with respect to [Fe′] is expressed as Equation 4:

$$K'_{FeL} = \frac{[FeL]}{[Fe'] + [L^{-}]} \tag{4}$$

The relationship between Fe′ and Fe^{3+} can be used as: $\alpha_{Fe'} = [Fe']/[Fe^{3+}]$, then $K'_{FeL} = \alpha_{Fe'} K'_{Fe^{3+}L}$, where $\alpha_{Fe'} = 10^{10}$ (Hudson et al., 1992; Sunda and Huntsman, 2003) is commonly used for pH 8 seawater.

The addition of the competing ligand TAC, is traduced in a new equilibrium between TAC and the organic ligands and Fe in solution (Croot and Johansson, 2000):

$$[Fe]_T = [Fe'] + [FeL] + [Fe(TAC)_2] \tag{5}$$

where the complexation of Fe′ by TAC and the side reaction coefficient, respect to Fe′, can be written as Equations 6 and 7, respectively:

$$\beta'_{Fe(TAC)_2} = \frac{[Fe(TAC)_2]}{[Fe'][TAC']^2} \tag{6}$$

$$\alpha_{Fe'(TAC)_2} = \frac{[Fe(TAC)_2]}{[Fe']} = \beta'_{Fe(TAC)_2}[TAC']^2 \tag{7}$$

As [TAC′] >>$[Fe]_T$, it is assumed that [TAC′] = $[TAC]_T$. The sensitivity (S) of the method can be determined as the relationship between the peak current (i_p) and the concentration of the complex Fe-TAC:

$$S = \frac{i_p}{[Fe(TAC)_2]} \tag{8}$$

Sensitivity can be computed for each sample by plotting the peak current vs the Fe concentration and measuring the slope in the region where the ligands are saturated (linear region at high Fe concentrations). According with this terminology, the relationship between the sensitivity and [Fe′] is:

$$[Fe'] = \frac{i_p}{S\alpha_{FeTAC_2}} \quad (9)$$

Then, we represent the data collected after linear transformation (Equation 10) in order to compare with the data previously published in the literature.

$$\frac{[Fe']}{[FeL]} = \frac{1}{K_{FeL}'[L_T]} + \frac{[Fe']}{[L_T]} \quad (10)$$

From the plot of [Fe′] / [L_T] vs [Fe′] is obtained a linear function provided that only one ligand complexed the Fe(III), where the slope represents de concentration of [L_T] and the conditional stability constant is obtained from the slope divided by the intercept (van den Berg and Kramer, 1979). In this study the program ProMCC is used for the calculation, where from the titration values the concentration of total binding ligand and conditional stability constant is calculated from the slope and the intercept (Omanović et al., 2015).

According with the theory of complexation, the complexing capacity of natural seawater was studied. Fig. 2 shows one of the titrations carried out by addition of Fe (0 – 15 nM) for the natural seawater and the same titration for the UV-treated seawater. However, 4 hours UV-irradiation was enough to discompose all these organic ligands and obtaining a linear response after Fe additions. In this case, a non-existence of organic ligands is demonstrated.

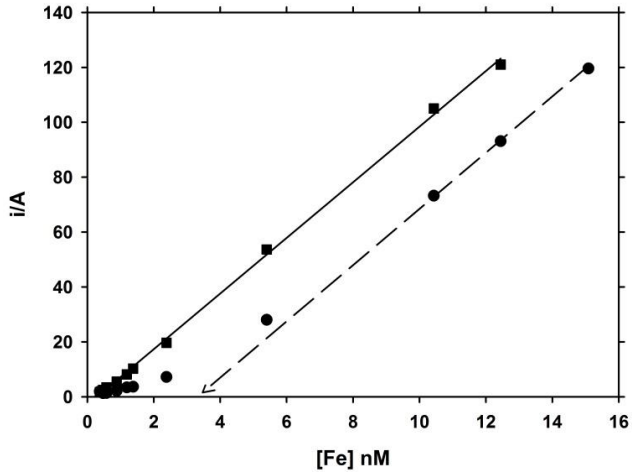

Fig. 2: Titration of natural seawater (dots) and UV-seawater (squares)

These results showed that the natural ligand concentration calculated using the van den Berg linearization was 3.1 ±0.7 nM (n=4). This seawater contained 8.6 times more ligands than dFe. The conditional stability constant was log $K'_{Fe'L}$=11.5±0.4, respect to Fe′ and log K'_{Fe3+L} = 21.5. Therefore, the ligands in the reference seawater are ranked as L_2-type ligands (Gledhill and Buck, 2012). In this case, no significant differences were observed between linear (van den Berg and Kramer, 1979) and non-linear treatment (Gerringa et al., 1995).

The complexation of Fe by the three different polyphenols (±) – catechin, sinapic acid and gallic acid (Fig. 3) was studied in UV-seawater. The results are presented in Table 1.

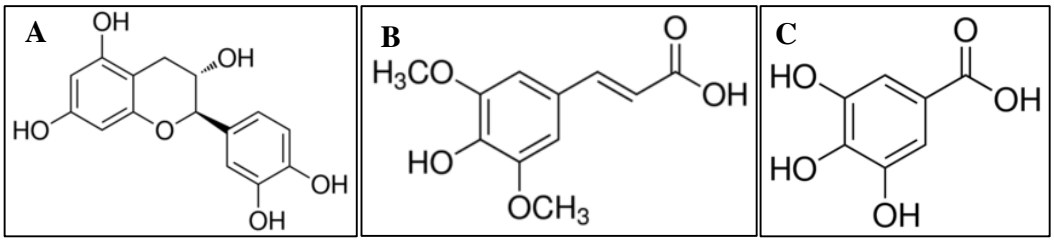

Fig. 3: Chemical structure of (±)-catechin (A), sinapic acid (B) and gallic acid (C)

These results demonstrated that dFe can be complexed by polyphenols in seawater. (±) – 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 model ligand was present in solution, with a log $K'_{Fe'L}$ between 11.7 – 11.5, respectively. In addition, sinapic acid complexed 3.6 ± 0.8 and 6.5 ± 0.7 nM of dFe when 4.95 and 9.91 nM of the model ligand were added to the UV-seawater. The complex dFe – sinapic acid was characterized by a log $K'_{Fe'L}$ = 11.0. The last model ligand, gallic acid, was the most relevant in terms of complexing capacity. In this sense, with only 1.99 nM of gallic acid, 5.5 ± 0.3 nM of dFe was organically complexed. When 5 nM of gallic acid was added to the UV-seawater, 14 ± 2 nM of dFe were also complexed. In this case, the log $K'_{Fe'L}$ was between 11.2 – 11.9 (Table 1). Accordingly, the model ligands studied in this current investigation formed weak complexes with dFe in seawater, with a conditional stability constant ≥ 21 respect to Fe^{3+}. Then, they can be considered as L_2-type organic ligands (Gledhill and Buck, 2012).

These results demonstrated a stoichiometry between the model ligands and dFe 3.5:1, 1.5:1 and 1:2.8, for (±) – catechin, sinapic acid and gallic acid in seawater, respectively.

Table 1. Concentration of model ligand added and recovered after a titration, conditional stability constant determined using DP-CSV and calculated applying the van den Berg- Ruzic linearization. Errors represent the standard deviation from three replicates.

Model ligand	$[L]_{added}$ (nM)	$[L]_{recovered}$ (nM)	K'_{FeL} x 10^{11}	$\log K'_{FeL}$	$\log K'_{Fe^{3+}L}$
(±)-Catechin	5.7	1.6±0.2	5.5	11.7±0.1	21.7
(±)-Catechin	11.8	3.6±0.5	3.2	11.5±0.2	21.5
Sinapic acid	4.95	3.6±0.8	1.0	11.0±0.2	21.0
Sinapic acid	9.91	6.5±0.7	1.0	11.0±0.1	21.0
Gallic acid	1.99	5.5±0.3	1.6	11.2±0.2	21.2
Gallic acid	5.00	14±2	8.7	11.9±0.1	21.9

3.3. Dissolved Fe speciation from kinetic approach

The conditional stability constant can be computed from the kinetic of formation and dissociation of the Fe′ and the model ligand, or natural ligands. In this study the conditional stability constants obtained from kinetic approach and from CLE-CSV have been compared. The kinetic approach has been performed according with Wu and Luther (1995), Witter and Luther (1998).

The kinetic approach provided two different constants, formation rate constant (k_f) and dissociation rate constant (k_d).

The formation rate constant can be experimentally determined from the initial rate of the complexation between Fe′ and the organic ligand studied, according with Equation 11.

$$Fe' + L \overset{k_f}{\rightarrow} FeL \quad (11)$$

Then, the kinetic equation can be expressed as (Equation 12):

$$\frac{\partial[FeL]}{\partial t} = k_f[Fe'][L] \quad (12)$$

where [Fe′] represent the initial dFe concentration used in this study ([Fe′] = 10 nM), and [L] is the concentration of model ligands able to complex a certain amount of dFe, calculated in the steady-state. The concentration of Fe was never at the saturation level, because of the standard additions were linear until 15 nM of dFe and the signal was stable for more than 24 h. The Fe measured in these experiments has to be considered as labile Fe, because there is not a competition between TAC and the model ligand.

The dissociation rate constant can be experimentally computed as the concentration of dFe recovered from the complex FeL after the addition of TAC. In this sense, the experiments were always performed for initial $[Fe]_T$ = 10 nM and 5 nM of the model ligand. This solution was pre-equilibrated according with the formation experiments. The addition of TAC will form an electroactive complex that can be detected in the mercury drop. Then, as there is a competition between TAC and the model ligand, the Fe measurable is TAC labile Fe. Then, the addition of TAC can be expressed as the new equilibrium (Equation 13).

$$FeL + TAC' \overset{k_{obs}}{\longrightarrow} Fe(TAC)_2 + L \quad (13)$$

where $Fe(TAC)_2$ is assumed as an stable complex that cannot release Fe′ to the solution. Then, the kinetic equation can be expressed as:

$$\frac{\partial[Fe(TAC)_2]}{dt} = -k_{obs}[FeL][TAC'] \quad (14)$$

$$\ln[FeL] = -k_{obs}[TAC']t \quad (15)$$

The overall reaction (Equation 13) can be written as two semi-reactions, the dissociation of the FeL complex and the formation of $Fe(TAC)_2$ complex.

$$FeL \overset{k_d}{\rightarrow} Fe' + L \quad (16)$$

$$Fe' + TAC' \overset{k_2}{\rightarrow} Fe(TAC)_2 \quad (17)$$

where the concentration of [TAC′] >> [L] and any Fe′ formed will rapidly react with TAC′ to form the irreversible complex $Fe(TAC)_2$, at least during the time scale and conditions used in this study. Accordingly, Equation 13 can be written as a pseudo-first order equation:

$$\frac{\partial[Fe']}{\partial t} = k_d[FeL] - k_f[Fe'][L] - k_2[Fe'][TAC'] \quad (18)$$

and

$$[Fe'] = \frac{k_d[FeL]}{\{k_f[L]+k_2[TAC']\}} \quad (19)$$

Therefore, the formation of $Fe(TAC)_2$ can be studied as:

$$\frac{-\partial[FeL]}{\partial t} = \frac{\partial[Fe(TAC)_2]}{\partial t} = k_2[Fe'][TAC'] \quad (20)$$

Operating with Equation 19 and 20:

$$\frac{-\partial[FeL]}{\partial t} = \frac{\partial[Fe(TAC)_2]}{\partial t} = \frac{k_2[TAC']k_d[FeL]}{\{k_f[L]+k_2[TAC']\}} \quad (21)$$

According to our experiments, $k_f[L] << k_2[TAC']$, because of the initial concentration of TAC used is 10^{-6} M and the concentration of L are at nM level. This assumption is also

in a good agreement with Wu and Luther (1995), and Witter and Luther (1998). Then, Equation 21 can be simplified to:

$$\frac{-\partial[FeL]}{\partial t} = \frac{\partial[TAC']}{dt} = k_d[FeL] \quad (22)$$

that allow us to integrate and obtain:

$$\ln[FeL] = k_d t \quad (23)$$

$$k_d = k_{obs}[TAC'] \quad (24)$$

Then, the dissociation rate constant can be calculated by plotting ln[FeL] vs time. The conditional stability constant via kinetic approach can be computed by:

$$K'_{Fe'L} = k_f/k_d \quad (25)$$

$$K'_{Fe^{3+}L} = \alpha_{Fe'} K'_{Fe'L} \quad (26)$$

where, as it was mentioned above, $\alpha_{Fe'} = 10^{10}$ (Hudson et al., 1992). In addition, we can also computed the conditional stability constant respect to Fe^{3+}, by assuming that the second-order rate constant is $k_{f,Fe3+L} = 3.02 \cdot 10^{11}$ $M^{-1}s^{-1}$, previously estimated from encounter theory (Luther and Wu, 1997). Then, the dissociation rate constant $k_{d,\ Fe3+L}$ can be computed. Accordingly:

$$k_{d,Fe^{3+}L} = \frac{k_{f,Fe^{3+}L}}{K'_{Fe^{3+}L}} \quad (27)$$

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

The results obtained for the kinetic of formation and dissociation experiments were showed in Fig. 4 and Table 2.

Table 2. Formation and dissociation constants, conditional stability constant and residence time determined using the kinetic method. The initial concentration of dFe = 10.36 nM and the initial concentration of model ligand was 5 nM.

Model ligand	k_f x 10^5 ($M^{-1}s^{-1}$)	k_dx10^{-4} (s^{-1})	log$K'_{Fe'L}$	log K_{Fe3+L}	Fe′ residence time (min)	Fe^{3+} residence time (years)
(±)-Catechin	4.16±1.8	1.88±0.03	9.4±0.1	19.40	61.4	0.66
Sinapic acid	4.0±0.7	4.4±0.3	8.96±0.04	18.96	26.3	1.83
Gallic acid	3.82±0.4	3.43±0.05	9.08±0.02	19.08	33.7	0.79

These results demonstrated that the formation of dFe – phenolic complexes are rapidly formed with log k_f between 5.58 – 5.62 (k_f in M^{-1} s^{-1}). On the other hand, the dissociation rate constant was also high; log k_d ~ 3.04 for these polyphenol groups. Then, the conditional stability constant determined from the kinetic experiments were 9.4 ± 0.1 ((±) – catechin), 8.96 ± 0.04 (sinapic acid) and 9.08 ± 0.02 (gallic acid)), in terms of Fe′ species. These complexes are also ranked as weak ligands (L_4-type ligands). According with the dissociation rate constant, the presence of polyphenols will keep dFe in solution for longer, from 26 min to 61 min, respect to the complex of Fe′ with sinapic acid or (±) – catechin, respectively. This half-life time increased to the order of years in terms of Fe^{3+}.

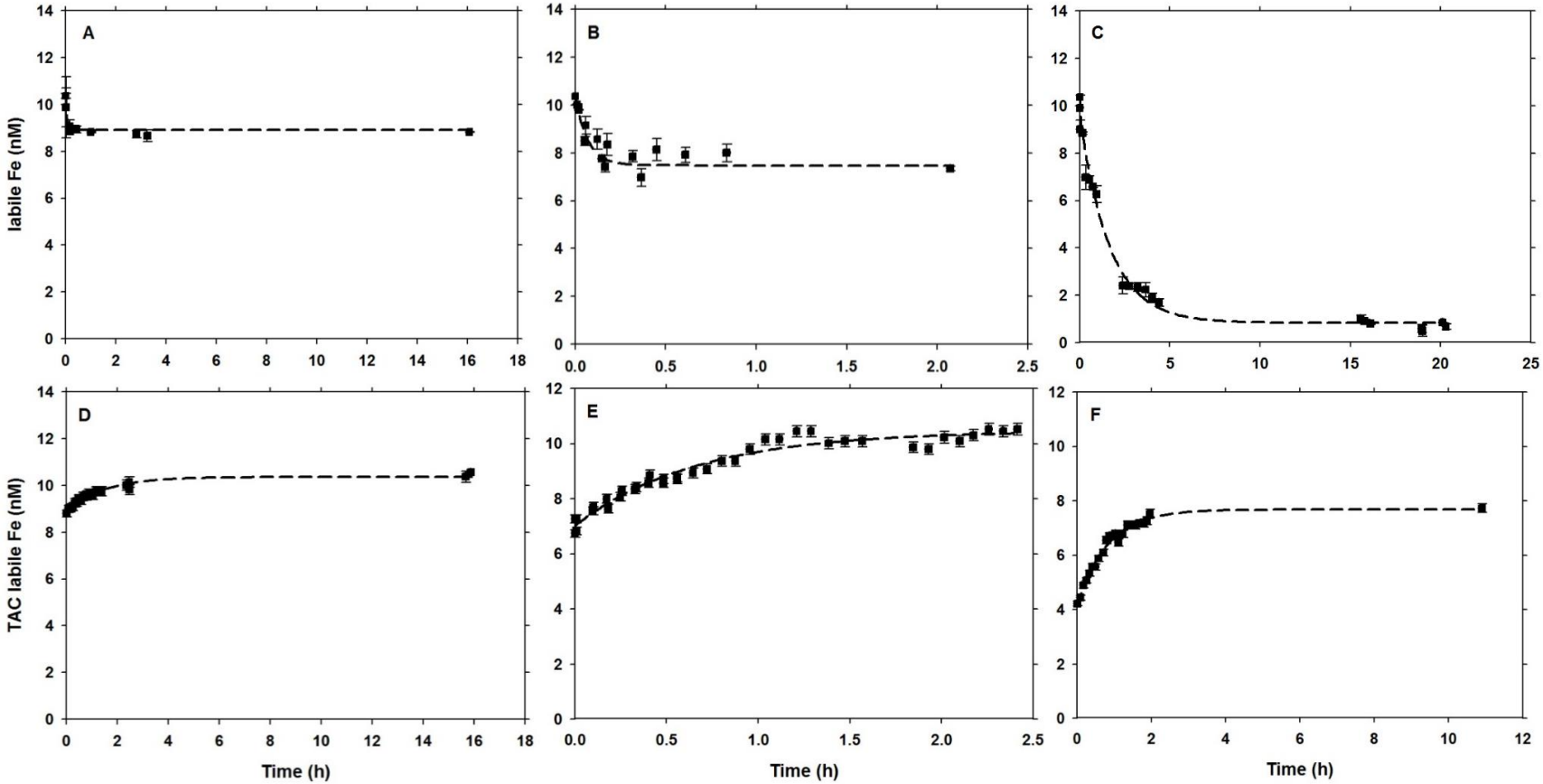

Fig 4: Kinetic of formation of (±) - catechin (A), sinapic acid (B) and gallic acid (C), where the concentration of dFe is expressed as labile Fe because of $Fe(TAC)_2$ complex was measured after 20 sec. Kinetic of dissociation of (±) - catechin (D), sinapic acid (E) and gallic acid (F), where the concentration of dFe is expressed as TAC labile Fe due to the interaction between TAC and the model ligands. Lines correspond with the fitting of the results. All the experiments were carried out by triplicate in UV-seawater. The initial concentration of dFe was 10.36 nM.

4. Discussion

4.1. Complexing capacity of polyphenols by CLE-CSV method

The concentration of dFe in surface waters is extremely low (< 1nM; de Baar and de Jong (2001)) and most of the dFe is organically complexed with organic ligands produced by microorganisms (Gledhill and van den Berg, 1994; Hunter and Boyd, 2007; Rue and Bruland, 1995). However, there are a variety of organic ligands that are forming the pool or dFe-binding ligands (Hunter and Boyd, 2007). Between these possible organic compounds, polyphenols have been identified as a source of ligands in the exudates of marine diatoms (Rico et al., 2013; Santana-Casiano et al., 2014) and green microalgae (López et al., 2015). In those studies, the authors demonstrated that the phenolic profiles, both type of polyphenols and concentration in solution, were directly related with high level of metal in solution. Then, polyphenols are playing a double role in solution, complexing metal to decrease the toxicity (in the case of copper) or to keep it in solution for longer increasing its bioavailability (in the case of Fe). In addition, the production of polyphenols is also to reduce the free radical reactions, which are produced in the redox reactions of trace metals in oxygenic seawater and known as Haber-Weiss mechanisms. These free radicals have negative impacts on the microorganisms (Lopes et al., 1999; Morel et al., 1994; Morel et al., 1993; Sugihara et al., 2001).

Moreover, (±) – catechin and sinapic acid have been studied in terms of Fe(II) regeneration in seawater (Santana-Casiano et al., 2014). These polyphenols are producing negligible Fe(II) levels (0.1 – 0.4%) in seawater at pH 8.0. The regeneration of Fe(II) was a pH-dependent mechanisms and at pH 6.0, the Fe(II) regeneration achieved 60% and 46% in the presence of (±) – catechin and sinapic acid, respectively. In that study, the authors demonstrated that (±) – catechin, sinapic acid and gallic acid increased their concentration in the exudates when high level of Fe was added to the cultures.

(±) – 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 the position 3 in the C-ring (Fig. 3A). Sinapic acid is a phenylpropanoid compound, with 3,5-dimethoxyl and 4-hydroxyl groups substituting the phenyl group of the cinnamic acid (Fig. 3B). Gallic

acid is tri-hydroxybenzoic acid (gallyol moiety) (Fig. 3C) that are generally forming dimers such as ellagic acid. (±) – catechin, sinapic acid and gallic acid contains –OH groups in their molecules that at pH 8 (natural seawater pH) should be deprotonated forming anionic ligands capable to complex metals, as the organic ligands in the cell walls of the microorganisms (González et al., 2014). Accordingly, one of the main role of polyphenols at natural pH is the formation of complexes with dFe. The complexation of dFe by polyphenols has been demonstrated by many authors (Andjelković et al., 2006; Brown et al., 1998; Hynes and O'Coinceanainn, 2004; Khokhar and Apenten, 2003; Mira et al., 2002) at acidic pH and low ionic strength solutions. There is a lack of information about the dFe-polyphenols complexation in natural seawater.

The complexation between polyphenols and dFe is related to the presence of the *ortho*-di-hidroxy groups, mainly present in molecules bearing catechol or gallyol moieties (Khokhar and Apenten, 2003; Moran et al., 1997) and with 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. 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 stoichiometry ratio was found as 1:2.8 (ligand:dFe), respect to that for (±) – catechin (3.5:1) and sinapic (1.5:1) (Table 1). The differences in the complexing capacity could not be only 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+} are playing a key role in the oxidation process of polyphenols, blocking the reaction to semiquinone and benzoquinone (Santana-Casiano et al., 2010; Santana-Casiano et al., 2014). Then, some of the –OH groups are occupied by Mg^{2+} and Ca^{2+}, decreasing their capacity to complex dFe. In addition, the ability of gallic to form dimmers in solution can be involved in the higher dFe-binding capacity respect to that for (±) – catechin and sinapic acid.

The conditional stability constant determined by CLE-CSV corresponds to the ligands L_2-type. The log $K'_{Fe'L}$ measured in the presence of (±) – catechin, sinapic acid and gallic acid are comparable with humic substances (Laglera et al., 2011), exudates from *P.antarctica* (Norman et al., 2015) and *E.huxlei* (Boye and van den Berg, 2000), alterobactin A and B (Witter et al., 2000), and catecholate and hydroxamate groups (Macrellis et al., 2001). Witter et al. (2000) studied the complexation of nine model

ligands in terms of CLE-CSV. They carried out the titration with 1N2N at pH 6.9. They reported the log $K'_{Fe'L}$ between 8.51 and 9.34 and log K'_{Fe3+L} from 21.6 to >24. In addition, these conditional stability constants computed here can be also found in most of the oceanic waters (Bundy et al., 2014; Gerringa et al., 2006; Gledhill and van den Berg, 1994; Rue and Bruland, 1995; Wu and Luther, 1995).

The results of this investigation, and the results reported above reported for other authors, confirm that CLE-CSV electrochemical measurements do not provide any information about the structure of dFe-binding ligands. It can be used as a method to determine the dFe-binding capacity of model ligands.

4.2. Complexing capacity of polyphenols determined by kinetic approach

The formation rate constant was higher in molecules with catechol moiety, like (±) – catechin than in gallic acid, with gallyol group, whiles the dissociation rate was higher in gallic acid than in (±) – catechin (Table 2).

The measurements did not revealed a very high decrease in the labile Fe in the first seconds (Fig. 4), as it occurs for stronger ligands such as protoporphyrin IX (Witter et al., 2000), where the concentration of labile Fe decreased 2 nM in the first seconds of the reaction, supporting the experimental results collected for polyphenols that are ranked as weak ligands. Witter and Luther (1998) described the complexation in natural seawaters in the water column, with $k_f = 4.78 \cdot 10^5 M^{-1}\ s^{-1}$ in the Northwestern Atlantic that are similar to our results. Witter et al. (2000) also reported that $k_f = 4.98$, 3.08 and $4.97 \cdot 10^5 M^{-1}\ s^{-1}$ for natural waters. Other model ligands have also been characterized in terms of formation rate constant in seawater (Witter et al., 2000) such as protoporphyrin IX ($k_f = 6.2 \cdot 10^5\ M^{-1}\ s^{-1}$) , alterobactin A ($k_f = 3.8 \cdot 10^5\ M^{-1}\ s^{-1}$) and ferrichrome ($k_f = 4.6 \cdot 10^5\ M^{-1}\ s^{-1}$).

The dissociation rate constant for (±) – catechin, sinapic acid and gallic acid was higher ($10^{-5}\ s^{-1}$) than the k_d reported for other model ligands as protoporphyrin IX ($0.7 \cdot 10^{-6}\ s^{-1}$), apoferritin ($0.08 \cdot 10^{-6}\ s^{-1}$), phytic acid ($0.51 \cdot 10^{-6}\ s^{-1}$), alterobactin A ($0.17 \cdot 10^{-6}\ s^{-1}$), alterobactin B ($0.25 \cdot 10^{-6}\ s^{-1}$), ferrichrome ($0.05 \cdot 10^{-6}\ s^{-1}$) and desferioxamine ($1.5 \cdot 10^{-6}\ s^{-1}$), but comparable with phaeophytin ($12.3 \cdot 10^{-6}\ s^{-1}$) and enterobactin ($15.8 \cdot 10^{-6}\ s^{-1}$) (Witter et al., 2000). 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 and Wu, 1997; Witter and Luther, 1998) that prove the huge diversity of ligands in the water.

The discrepancy between the conditional stability constant determined by CLE-CSV (Table 1) and the kinetic approach (Table 2) is interesting to the continue revision of the equilibration titration technique (Gerringa et al., 2007; Hunter, 2005; Town and van Leeuwen, 2005a; Town and van Leeuwen, 2005b; van den Berg, 2005; van Leeuwen, 2001). The titration method (CLE-CSV) assumes the 1:1 stoichiometry for the FeL, whereas other ratios can occur in natural waters. Gledhill (2001) observed in hydroxamate siderophores 1:2, 2:3 and 2:2 dFe-complexes. In this manuscript, stoichiometry of 3.5:1, 1.5:1 and 1:2.8 were measured for (±) – catechin, sinapic acid and gallic acid respect dFe, respectively. This assumption may affect the calculation of the conditional stability constant from the CLE-CSV analysis.

In fact, the chemistry of each ligand is different respect to others. In the case of polyphenols, the interaction with Mg^{2+} and Ca^{2+} affect the oxidation to quinone and it is affecting to the Fe chemistry, both in terms of complexation and in terms of redox reactions (Santana-Casiano et al., 2010; Santana-Casiano et al., 2014). Moreover, the formation and dissociation can be controlled by the competition with the MgL and CaL formed during the reaction (Hering and Morel, 1988; Raspor et al., 1980; Wu and Luther, 1995) that is becoming more relevant when weak ligands are in solution. These assumptions can have an important impact on k_f and k_d. Kinetic experiments were carried out with initial dFe concentration of 10.36 nM and model ligand concentration 5 nM. The stoichiometry between dFe and model ligand should be studied in order to know its effect on the binding process. On the other hand, the dissociation rate constant is computed as TAC (or other artificial ligand) labile Fe, because there is a competition between TAC and the model ligand to complex dFe. In the presence of weak model ligands this interaction is very relevant and the results can be also affected. In this sense, further investigation should be carried out with different stoichiometry ratios and different TAC concentrations (detection windows) during the kinetic of formation and dissociation reactions as well as the competition of Fe with Mg^{2+} and Ca^{2+} during the complexing reactions with polyphenols.

The dissociation rate constant allows to compute the half-life time of Fe in solution, both as Fe′ and Fe^{3+} (Table 2). In the presence of polyphenols, Fe′ residence time is 26 – 61 min that increased to 0.7 – 1.8 years when Fe^{3+} species are considered.

These residence times are highly affected by the presence of organic ligands in the ocean and ranked from minutes to years (Witter et al., 2000).

CONCLUSION

Polyphenols such as (±) – catechin, sinapic acid and gallic acid, are able to complex dFe in seawater. The conditional stability constant ($K'_{Fe'L}$) for the complexes Fe-model ligand was determined by CLE-CSV, where the values ranged from 11.0 to 11.9. Under the experimental conditions used in this investigation, the stoichiometry found between the model ligand and the dissolved iron, in seawater, was 3.5:1, 1.5:1 and 1:2.8, for (±) – catechin, sinapic acid and gallic acid, respectively. The different complexing capacity between polyphenols are also affected by the competitive role of Mg^{2+} and Ca^{2+} with dFe for the hydroxyl groups in the polyphenol molecular structures.

The formation rate constant (k_f) measured for the complex between dFe and (±) – catechin, sinapic acid and gallic acid, varied from $3.82 \cdot 10^5$ to $4.16 \cdot 10^5$ ($M^{-1}s^{-1}$). The dissociation rate constant (k_d) varied from $1.88 \cdot 10^{-4}$ to $4.4 \cdot 10^{-4}$ (s^{-1}). The conditional stability constant using the kinetic approach were also calculated (log $K'_{Fe'L}$ = 8.96 – 9.4). The discrepancy with the log $K'_{Fe'L}$ computed via CLE-CSV can be due to the titration method (CLE-CSV) assumes the 1:1 stoichiometry for the FeL, whereas other ratios can occur in this case. In addition, the chemistry of the model ligand in seawater that can be affected by the presence of Fe and other major ions in seawater as Mg^{2+} and Ca^{2+}, or the interaction between TAC and the model ligand during the dissociation reaction. Therefore, further investigations should be addressed in this direction.

The results of the present manuscript revealed that polyphenols which are produced by phytoplankton, are playing a key role in the biogeochemistry of Fe in natural waters and dFe can be complexed to remain in solution for longer.

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Appendix

Descripción detallada de las actividades desarrolladas durante la realización del TFT.

Como se trata de un trabajo experimental y bibliográfico, mientras realizaba la parte de experimentación aprendí a trabajar en un laboratorio limpio y en un entorno multidisciplinar, y como desarrollar el método científico en su totalidad, en el que pude diseñar experimentos en base a los resultados que se quiera obtener o los problemas que surgieron. Aprendí de un método analítico ampliamente utilizado química marina (Croot y Johansson, 2000) diseñado para medir la capacidad complejante de los ligandos orgánicos. La parte de búsqueda bibliográfica fue más extendido al principio ya que se trataba de un método que no conocía.

Después de la obtención de datos, mi tutor me ha enseñado como realizar el tratamiento de datos en el programa ProMCC que me ha ayudado a la evaluación de la calidad de los datos antes de su evaluación. Mientras se obtenían datos he ido escribiendo el TFT que a su vez era supervisado por el tutor.

La lectura de bibliografía que he realizado a lo largo de todo el trabajo ha sido fundamental para una mejor escritura del trabajo, además de haber aumentado mi nivel de conocimiento en el ciclo del hierro y la capacidad complejante estudiada por múltiples autores en diferentes condiciones, y como comparar estos datos con los que he obtenido. Dado que la parte experimental y bibliográfica la he efectuado en un centro extranjero he podido convivir en lengua extranjera y mejorar mis capacidades científicas.

Formación recibida.

- Funcionamiento y manejo de un laboratorio limpio, donde se me enseña las normas de seguridad, de contaminación y forma de trabajo.
- Aprendizaje y aplicación de un método analítico para medir la capacidad complejante de algunos ligandos orgánicos.
- Tratamiento de datos utilizando el software ProMCC.
- Como parte de mi formación para la escritura de artículos científicos se me facilita un libro del Profesor Joshua Schime de cómo escribir artículos científicos.
- Un curso que se impartió en inglés acerca de cómo escribir artículos científicos "Science writing boot camp 2016". Se realizó en la Universidad de Brest por el

Catedrático Linwood Pendleton y la profesora de Oceanografía Biológica de la Universidad Duke Cindy Van Dover, este curso tuvo una duración de 12 horas que se dividió en dos días.

Nivel de integración e implicación dentro del departamento y relaciones con el personal.

Durante el desarrollo del TFT me he integrado plenamente en el equipo de trabajo y en el laboratorio de acogida. Debido al carácter multidisciplinar del laboratorio hay un gran número de investigadores jóvenes de distintas nacionalidades que me ha facilitado la integración en el mismo, además me proporcionó la oportunidad de mejorar mi conocimiento en otras lenguas además de conocer distintas culturas.

Aspectos positivos y negativos más significativos relacionados con el desarrollo de las prácticas.

Obtener experiencia en trabajar en un laboratorio limpio es uno de los aspectos más positivos que he desarrollado a lo largo del trabajo experimental. Los reactivos y aparatos que fueron necesarios siempre se encontraron a mi disposición por lo que no he tenido ningún problema mientras realizaba la parte experimental del trabajo.

Valoración personal del aprendizaje conseguido a lo largo de la práctica.

A lo largo de todo el trabajo experimental y bibliográfico he tenido la oportunidad de aprender acerca del trabajo científico en grupo, de hacerme una idea más clara y más objetiva de cómo se desarrolla el mundo de la investigación ya que tengo particular interés en el mismo. Tener al tutor de empresa siempre disponible para resolver cualquier duda o inconveniente que se me presentase en el laboratorio (experimentación y teoría) ha sido fundamental a la hora del buen desarrollo de las dos partes del TFT. Integrarme en un ambiente donde la cultura, idioma y sistema de trabajo son diferentes ha sido enriquecedor a nivel personal y académicamente.