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The organic ties of iron

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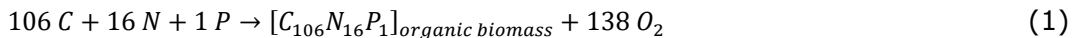
Chapter 1

Introduction

1.1. Iron and organic matter in seawater

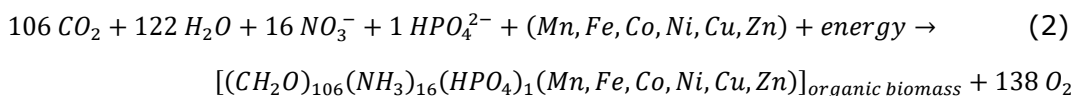
The basic building blocks of living matter are put together by photoautotrophs. These use the energy from light to carry out various cellular metabolic processes harvesting energy from sunlight to fix inorganic carbon (C) into organic matter. This photosynthesis process brings oxygen into the atmosphere. The photoautotrophic phytoplankton (unicellular algae; including photosynthetic bacteria) form the base of food webs in the Earth's oceans. Covering 70% of the globe's surface, marine primary production is responsible for about half the oxygen generated by photosynthesis on a planetary scale (Field et al., 1998).

The process of photosynthesis may be summarized in the following equation, reflecting the relative quantities of cellular carbon (C) in relation to the major nutrients nitrogen (N) and phosphorus (P), required for organic biomass (Redfield, 1958; Redfield et al., 1963):



Phytoplankton growth is when the availability of essential growth controlling variables is limited. An example of this so-called bottom-up control of primary production is the availability of inorganic nutrients. Depletion and limitation of major nutrients like nitrogen (e.g. nitrate) and phosphate are known to regulate seasonal phytoplankton production in coastal regions and open ocean, respectively (Timmermans et al., 2005; Veldhuis et al., 2005). However in about 40% of the oceans, the major nutrients nitrogen (as NO_3^-) and phosphate (as PO_4^{2-}) are abundantly available but phytoplankton growth is halted or forestalled for other reasons, these are the High Nutrient Low Chlorophyll (HNLC) regions (de Baar et al., 1995; Timmermans et al., 1998).

In order to better describe (and be more inclusive) phytoplankton stoichiometry, we must expand the above equation to include a more detailed representation of the elements involved in primary production, as here reproduced from de Baar et al. (2017):



Although the trace metals manganese (Mn), iron (Fe), cobalt (Co), nickel (Ni), copper (Cu) and zinc (Zn) in the equation are present in very low concentrations in the euphotic zone (the surface ocean where there is still light to allow phytoplankton primary production), these play a nontrivial role for enzymatic activity. Primary production is dependent specifically on the trace metal Fe for many cellular processes, e.g. it is an essential component for photosynthetic pigments, DNA replication enzymes and the reduction of reactive oxygen species

(Geider and La Roche, 1994; Netz et al., 2012; Twining and Baines, 2013; Zhang, 2014; de Baar et al., 2017). The next-limiting nutrient after major nutrients N and P is Fe (de Baar et al., 1990; Martin et al., 1990; Rijkenberg et al., 2018a), often depending on seasonality (Birchill et al., 2017). Co-limitation of Fe has been found to occur as well, with light (van Leeuwe and Stefels, 1998; Timmermans et al., 2001a) or co-limitation with a major nutrient or another trace nutrient (Browning et al., 2017). This ultimately results in HNLC regions where major nutrients are abundant but a lack of Fe limits primary production.

The solubility of Fe in seawater is low, resulting in low concentrations in natural seawater, and as such it is no surprise that Fe can be the limiting factor for primary production (de Baar et al., 1990, 2017; Martin et al., 1990; Tagliabue et al., 2017). The biochemical pathways that underlie the many Fe-requiring processes have been established millions of years ago, in a primordial ocean in which conditions were very different. Given a lack of oxygen then, metals that are now trace compounds were readily available in soluble forms (de Baar and La Roche, 2003). Only when oxygen was introduced into the system on a large scale through photosynthesis did these metals begin to be oxidised into particulates that subsequently sank out of the marine system. Therefore, the current situation has developed after biological processes became entirely dependent on these, now trace-, metals.

Fe can exist in two oxidation states, Fe^{2+} and Fe^{3+} , often also referred to as Fe(II) and Fe(III). In an oxygenated modern ocean, Fe^{2+} rapidly oxidizes to Fe^{3+} , to the point where the Fe^{2+} state is negligible. The ability of Fe^{3+} to dissolve in seawater (directly or bound by inorganic oxyhydrates) is as low as 0.1 nanomoles ($\text{nM} = 10^{-9} \text{ M}$; Liu and Millero, 2002), which essentially puts this in picomolar ($\text{pM} = 10^{-12} \text{ M}$) ranges. As a means to reflect these low concentrations, if one were to dissolve a paperclip in 15 Olympic size swimming pools the resulting Fe concentration would be 0.213 nM, or 213 pM (de Baar, *pers. comm.*). For phytoplankton growth to occur, Fe concentrations in the nM range are required (Larry E Brand, 1991; Sunda and Huntsman, 1997; Timmermans et al., 2001a, 2001b; Maldonado et al., 2005). Sources of Fe are diverse, including terrestrial (e.g. Klunder et al., 2012a; Rijkenberg et al., 2014), hydrothermal (Tagliabue et al., 2010; Klunder et al., 2012b) and aeolian sources either through deposition of volcanic ash or dust (Korte et al., 2017; Achterberg et al., 2018; Menzel Barraqueta et al., 2018).

To retain Fe concentrations in natural seawater beyond the inorganic solubility Fe needs to be bound to a soluble substance, the so called Fe-binding organic ligands. These are part of the dissolved organic matter (DOM) pool but are

largely still uncharacterized (Gledhill and van den Berg, 1994; Rue and Bruland, 1995; Gledhill and Buck, 2012; Hassler et al., 2017).

Microorganisms, heterotrophic bacteria as well as photoautotrophic phytoplankton, exchange DOM locally through uptake and excretion processes and cell death (Figure 1, ①). Fe-binding organic ligands are argued to be formed as a result and as such are part of this diverse pool of organic substances (Figure 1, "ligands"). DOM can be directly excreted by phytoplankton, e.g. under conditions of excess light and nutrient limitation. In these cases photosynthesis continues unabated but growth is not possible, and excess organic carbon may be excreted as extracellular polymeric substances (EPS), long-chain sugar like carbohydrates produced by microorganisms for protective and/or structural functions (Passow, 2002). Specifically, capsular material from bacterial cells has been found to contribute to the DOM pool (Stoderegger and Herndl, 1998). Bacteria may also produce DOM constituents (Reinthal et al., 2008; Jiao et al., 2010). Alternatively, dead cells may lead to the formation of DOM, transparent exopolymeric particles (TEP; Mari et al., 2005) and particulate organic debris (Figure 1, ② and ⑤). It has been speculated that marine viruses may have a fundamental role in the release of Fe-binding organic ligands. Infection of microorganisms by a lytic virus causes ultimately the infected host cell to lyse upon releasing newly produced progeny viruses. The remains of the host's cellular content, the cytosol, is released into the surrounding water (Figure 1, ⑤; Wilhelm and Suttle, 1999; Brussaard et al., 2008; Lønborg et al., 2013) and shown to increase the release of dissolved Fe (Gobler et al., 1997). Furthermore, Poorvin et al. (2011) showed that viral lysis of marine bacterium contributed to the ligand pool, and the Fe in these viral lysates seemed well bioavailable (Mioni et al., 2005; Poorvin et al., 2011). What is nevertheless still unknown is to what extent phytoplankton lysis specifically contributes to the ligand pool. Similarly unknown is the fate of Fe-binding organic ligands.

Heterotrophic bacteria subsequently modify the organic fraction through remineralisation of DOM and organic particulates to inorganic nutrients that may then be used again by phytoplankton (③ and ④). Heterotrophic bacteria are also known to excrete substances with the express purpose to bind Fe, the siderophores, discussed in more detail in the next section.

Furthermore, particulate organic matter (POM) is transferred to next trophic levels through feeding (⑥). Sloppy feeding by predators (Sarthou et al., 2008), production of fecal pellets (Laglera et al., 2017) and death tie higher trophic levels back to the particulate pool (⑦). Aggregation of particles exports POM down to the deeper seafloor (⑧). Alternatively, scavenging is a possible loss

factor for DOM, and by extension Fe-binding organic ligands, since dissolved substances are found to associate with aggregates and sinking particles.

The DOM in the oceans is formed largely in the biologically active upper ocean, the euphotic zone. DOM may also originate from terrestrial sources, entering the ocean via run-off and riverine input (Coble, 2007), although the majority of DOM flocculates and sinks out before reaching the open oceans (Sholkovitz, 1976). A group of Fe-binding organic ligands with a terrestrial origin that can contribute to the ligand pool the open ocean are the humic substances (HS). While local DOM production is greatest in areas where phytoplankton accumulates exponentially (Carlson, 2002), i.e. phytoplankton blooms, top-down growth control by grazing, viral lysis or sedimentation of algal cells may preclude bloom formation. However, in those cases DOM formation through excretion, fecal pellet production and cell death will still occur. Specific growth-limiting conditions such as trace-metal or light limitation are expected to affect ligand production and composition. For instance, siderophores have long been found to be excreted in higher concentrations in response to Fe-limitation (Wilhelm et al., 1998).

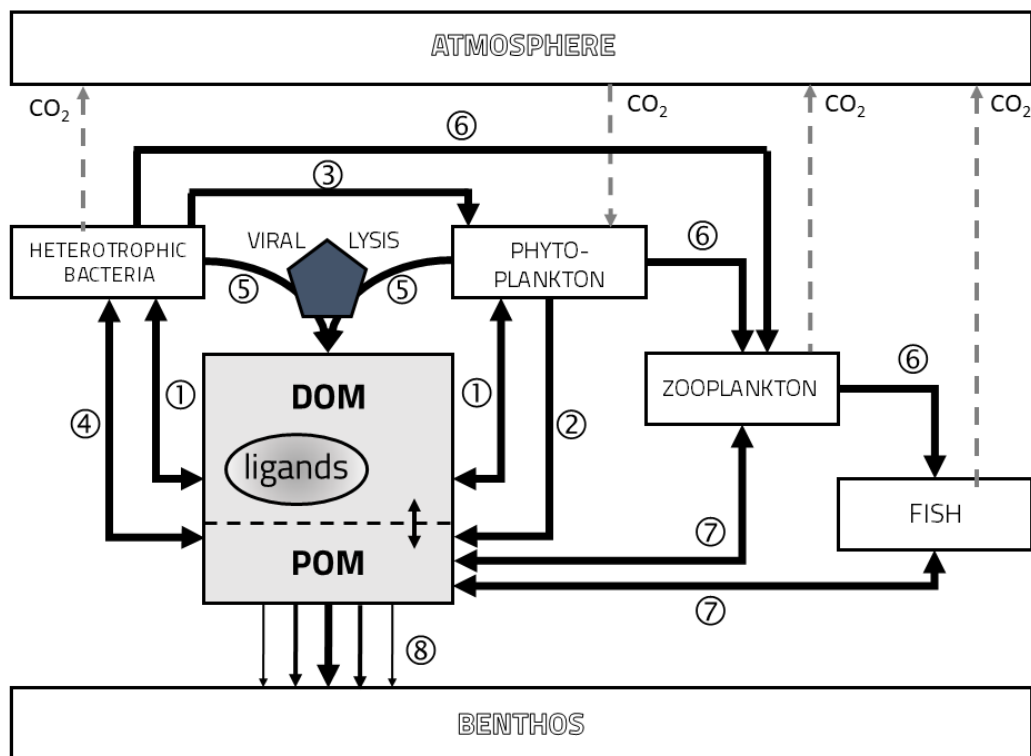


Figure 1 Roles of different trophic levels, dissolved and particle pools and interactions in the open ocean, relevant to the diverse origins and possible loss factors for Fe-binding organic ligands ("ligands" in the figure). At the centre of the diagram the pools of Dissolved Organic Matter (DOM), of which Fe-binding organic ligands are part, and Particulate Organic Matter (POM). Arrows indicate processes and their direction of influence discussed in this section (① to ⑧).

1.2. Fe-binding organic ligands

Organic ligands, i.e. the substances capable of binding the poorly soluble Fe, form a dissolved complex. The binding of Fe by ligands into an Fe-ligand complex is assumed reversible (equation 3). This by extension means that the different types of ligands form a somewhat undefined pool of binding sites that compete for Fe.



$$K' = \frac{[FeL]}{[Fe']+[L']} \quad (4)$$

$$[L_t] = [FeL] + [L'] \quad (5)$$

From the above equations, it becomes clear that the Fe-ligand complex (FeL) is made up from free Fe (i.e. the sum of Fe and inorganically bound Fe, denoted Fe') and free organic ligands (L'). To emphasise the additional capacity to keep Fe in solution L' is henceforth described as excess organic ligands.

The conditional stability constant K' is inferred from the mass balance in equation 3 as shown in equation 4. The stability constant indicates the strength of the Fe-ligand association. It is called conditional because it is given strictly for seawater conditions, here denoted with the apostrophe. The K' value is henceforth represented by its base-10 logarithm, for the Fe-ligand complex (FeL) and with respect to Fe' (with the exception of chapter 3, where before newer insights K' was still reported with respect to Fe^{3+}), denoted $\log K'_{Fe'L}$. This value in essence represents the average binding strength of a suite of organic Fe-ligand complexes, and typical ranges may be attributed to different ligand groups or types as discussed in the next paragraph. The total ligand concentration, denoted $[L_t]$, is the sum of Fe-ligand complexes and the excess organic ligand concentrations as equation 5 states (Gledhill and van den Berg, 1994; Gerringa et al., 2007, 2014).

Fe-binding organic ligands differ in origin, occurrence and binding strength (Gledhill and Buck, 2012; Hassler et al., 2017), but their relative contribution to the ligand pool as a whole is poorly understood. The earliest characterized examples are the siderophores, first isolated in soils by Francis et al. (1949).

These are molecules purpose-produced by bacteria (Figure 2, ①) to keep Fe in the local environment in a dissolved state. Siderophores were subsequently found to play a role as an Fe carrier in marine bacteria as well (Wilhelm et al., 1998; Butler, 2005; Hopkinson et al., 2009).

Microbial processes are another important influence on the ligand pool. As described in the prior section for DOM in general, infection of phytoplankton and bacteria by lytic viruses ultimately releases the cells' cytosol into the surrounding seawater (Figure 2, ②). Additionally, heterotrophic bacteria take up and metabolize DOM and POM (Figure 1) and therefore will influence the make-up of the ligand pool through local breakdown processes, potentially removing ligands through remineralisation. EPS contribute to the ligand pool although it is still unknown to what extent (Hassler et al., 2011a, 2011b; Figure 2, ③). Humic substances (HS) are hydrophobic breakdown products with a terrestrial origin. HS are also known to contribute to the ligand pool, possibly to a large extent (Laglera et al., 2011; Figure 2, ④). HS are operationally defined by how they bind to hydrophobic resins (Buffle, 1990; Bronk, 2002). Substances structurally similar to HS but produced locally are often called humic-like substances. The local production in the water column of humic-like substances by phytoplankton and bacteria also add to the ligand pool (Norman et al., 2015; Hassler et al., 2017).

What is nonetheless unidentified about the types of Fe-binding organic ligands here discussed is their relative contribution to the ligand pool. Typical measurements of Fe-binding organic ligands are a 'black box' approach, meaning the different types of Fe-binding organic ligands in a natural sample can only be described as a combined group. This inherent non-specificity of measurements makes it very difficult to explain the role played by different substances and processes. The only means currently available to separate substances and processes is by 1) analysis via multiple methods or varying implementations which reflect separate substances differently, and 2) experimentally separating different contributing processes. My thesis embraced both approaches, while focusing on the different sources of organic ligands and their ecological impact.

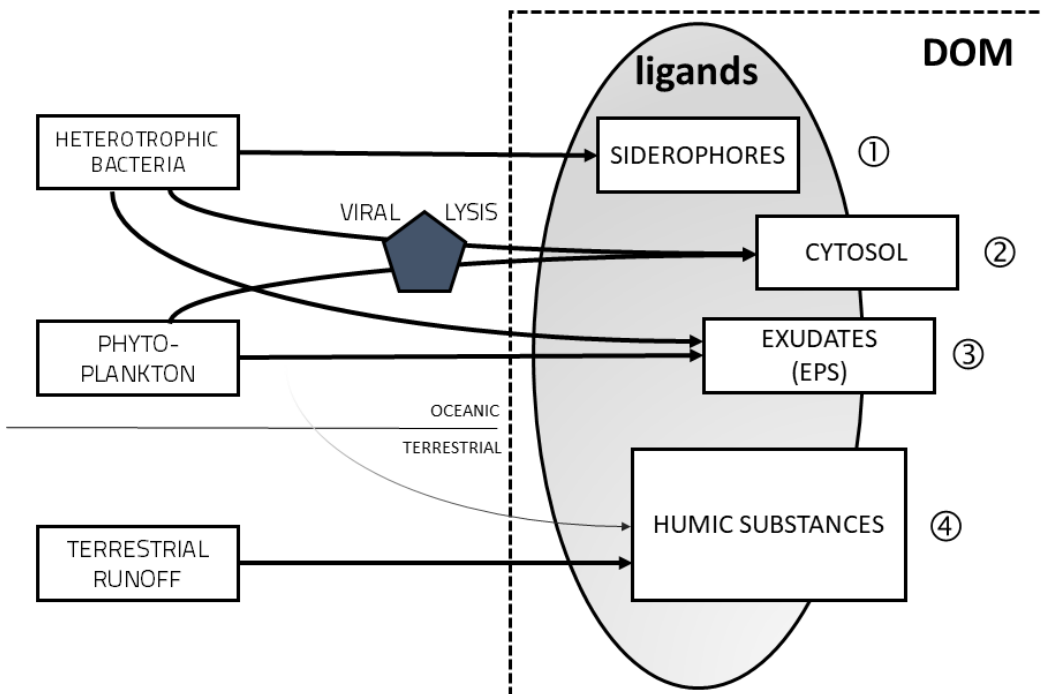


Figure 2 Different contributors to the Fe-binding organic ligand pool (referred to as ① to ④ in text) and their origins. Note the “DOM” and “ligands” sections which are also represented in Figure 1.

1.3. Thesis contents

With Fe being an essential trace nutrient for the basis of marine food webs, and its retention in seawater wholly dependent on Fe-binding organic ligands, the composition and cycling of the latter are essential to our understanding of ocean biogeochemistry. Thus far, most studies of Fe-binding organic ligands have been a ‘black box’ approach, with descriptors such as $[L_t]$ and $\log K'_{Fe'L}$ describing the ligand pool in its entirety, or in groups with similar binding strength. While Fe-binding organic ligands have been a long-studied subject, with total ligand concentrations and binding strengths reported for many marine regions and ecosystems, very little is known about relative contributors to the Fe-binding organic ligand pool (Gledhill and Buck, 2012; Hassler et al., 2017). The expectation is that different processes have a relative contribution to Fe speciation in the open ocean, mirroring the diversity in origins of DOM. However, what that relative contribution is, is currently unknown. Furthermore, modification of the Fe-binding organic ligand pool, e.g. loss factors, will also be an important factor in its composition. Again, these are part processes that remain veiled by study of the natural Fe-binding organic ligand pool as a whole.

This dissertation aims to generate more insight in the sources and sinks of Fe-binding organic ligands, and to that end has two objectives.

- 1 Identify different sources of Fe-binding organic ligands by *in-situ* study through correlations with descriptors of possible contributors and their relative importance to the Fe-binding organic ligand pool.
- 2 Narrow down the effect of specific microbial processes via experiments in culture in order to characterize their effect on the Fe-binding organic ligand pool, and to identify possible loss factors.

The methods most commonly used in this study are detailed in Chapter 2, explaining sampling and materials handling in trace metal clean conditions, culturing details and an overview of the electrochemical determination of Fe-binding organic ligands.

The numerous sources of Fe in the Mediterranean Sea (Gerringa et al., 2017) serve as an example for the diversity of Fe sources and complexation by ligands in Chapter 3. Here we describe how Fe-binding organic ligands support high dissolved Fe (DFe) concentrations in the surface from dust input, as well as deep patches of high DFe correlating with the occurrence of mud-volcanoes.

The relation between DFe and major nutrients under natural conditions in the Arctic Ocean is presented in Chapter 4. Under strong pressure of climate change, the Arctic Ocean is subject to rapid changes. Receding sea ice extent leads to increased light exposure and potentially increased net primary productivity in the surface water (Arrigo et al., 2008; Bhatt et al., 2014). High surface DFe (Klunder et al., 2012a; Slagter et al., 2017) is brought into the Arctic Ocean along the transpolar drift (TPD), a surface stream of sea ice and terrestrially influenced water from the Siberian shelf seas out to the north Atlantic Ocean (Gregor et al., 1998; Rudels, 2008). Even though DFe is assumed present in sufficient supply, we here show that Fe will be the next limiting factor in large parts of the Arctic Ocean when light stress is alleviated by sea ice melt.

The relatively high surface DFe indicated in Chapter 4 must be bound to Fe-binding organic ligands. The distribution of natural Fe-binding organic ligands in the Arctic Ocean is studied in detail in Chapter 5 (Slagter et al., 2017). Here the specific DOM signatures related to Fe-binding organic ligands found inside and outside of the TPD are studied. With the TPD carrying DOM with a terrestrial origin, we specifically explore the role of humic substances in the TPD's Fe-binding organic ligand pool. We illustrate the borders of the TPD flow path using multiple properties of DOM along two transects crossing the TPD.

Following this, Chapter 6 discusses specific ligands and their fate (Slagter et al., *in review*). A selection of the Arctic Ocean data from Chapter 5 was analysed

specifically for the role of humic substances, using a different application of our method of ligand analysis in comparison to the prior analyses. This approach reflects the natural Fe-binding ligands pool differently, with a stronger reflection of HS. We hope to clarify characterisation of Fe-binding organic ligands through the variations here observed in ligand properties such as L_t and $\log K'_{FeL}$.

Chapters 7 and 8 relate to the role of viral lysis in the formation and composition of ligands. Chapter 7 first establishes under controlled laboratory settings how Fe-limitation affects the interactions of virus and algal host (i.e., *Phaeocystis globosa* and *Micromonas pusilla*; Slagter et al., 2016). The time until the first progeny viruses are released from the algal host (latent period), the number of newly produced viruses per lysed host cell (virus burst size) and the degree of infectivity of these newly produced viruses are determined and compared to those under Fe-replete conditions. Since viral lysis may be a potential source of ligands and thus release Fe-stress, also the effect of partial alleviation of the Fe-stress on virus production was studied.

Chapter 8 gives an overview of ligand properties found in virally infected phytoplankton cultures as compared to non-infected controls (Slagter et al., *in prep.*). Special attention here is given to specific siderophores and hitherto uncharacterised siderophore-like substances, particularly upon aging of the non-infected and virally lysed cultures (to allow breakdown processes to run their course).

Finally, the thesis results and their scientific impact are summarized and discussed in Chapter 9, including a forward looking synthesis.