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## The organic ties of iron

Slagter, Hans Arent

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# Chapter 9

## Synthesis

## 9.1. Summary of conclusions

### 9.1.1. Mediterranean Fe and Fe-speciation

Sources and sinks of Fe and Fe-binding organic ligand in the Mediterranean Sea are diverse and do not necessarily share their origin. DFe was high at the surface, explained by atmospheric dust input primarily from North Africa. As the dust alone does not sufficiently contribute to the Fe-binding organic ligand pool to support DFe concentrations, ligands must have other origins, such as local microbial production or fluvial sources. Fe-binding organic ligands were near saturation and elevated in concentration. As our understanding evolved as detailed in Chapters 5 and 6, we now know that part of the humic Fe-binding organic ligand pool is not reflected by our measurements. As primary production was limited by nitrogen rather than Fe as found by a parallel study (van de Poll et al., 2015), microbial sources of Fe-binding organic ligands were expected to be only processes not specifically triggered by Fe-limitation such as grazing and lysis. The input of dust is in turn found to cause low DFe deeper in the water column due to scavenging and ballasting, which both act as a potential sink for Fe-bound ligands as well as emptying binding sites due to the removal of and downward transport of Fe.

DFe in the deeper Mediterranean Sea was not uniformly low, localized patches of high DFe occurred. These were found to relate to known locations of seamounts and mud volcanoes. Fe-binding organic ligands were present uniformly at relatively constant concentrations at depths >100 m and were completely saturated at these deep high-DFe patches. Given that DFe beyond the solubility provided by Fe-binding organic ligands will precipitate,  $[L_t]$  limits deep DFe in these areas. A steady state between ligand-facilitated solubility and scavenging is expected for the deep Mediterranean water column, where outside of the high-DFe patches  $[L_t]/DFe$  ratios increase, making Fe-binding ligands an important determining factor for physical and chemical speciation of Fe throughout the Mediterranean Sea (Chapter 3).

### 9.1.2. The Arctic Ocean

The Arctic Ocean is another mediterranean system with a comparatively large shelf-to-basin ratio. This results in a strong terrestrial influence, especially at the surface Arctic Ocean by means of the Transpolar Drift. This surface current is known to carry terrestrial water originating from the large rivers surrounding the Arctic Ocean from the shelf seas across to the North Atlantic. These rivers have their catchments inside the Arctic Circle, where the negative feedback system of permafrost loss due to climate change is expected to rapidly increase riverine output of DOM to the shelf seas.

High DFe is carried by the TPD, causing a localized surface enrichment in the Arctic Ocean along the TPD flow path. Outside of the TPD, under-ice phytoplankton over the Nansen basin was Fe limited, and elsewhere in the Arctic Ocean nutrient ratios indicated that surface Fe is expected to be the next-limiting factor after light with the exception of the strongly enriched TPD. With the rapid changes in the Arctic Circle, alleviation of light limitation due to loss of sea ice cover may soon be reality (Chapter 4).

Given that DFe cannot be present in high concentrations without Fe-binding organic ligands, these must be present in sufficient concentrations to support the Fe enrichment found in the Arctic Ocean. Indeed, the surface enrichment of DFe in the TPD was supported by high ligand concentrations. The Fe-binding organic ligands in the Arctic Ocean, in contrast to the Mediterranean Sea, have their origin in common with DFe, as both are primarily introduced via the TPD. The TPD is known to carry 'Gelbstoffe' after the earliest mentions (e.g. Kalle, 1937, 1949), as HS are known for their effect on ocean colour. These may be detected as Chromophoric Dissolved Organic Matter (CDOM), which can be measured at specific wavelengths of absorbance or fluorescence spectra as well as direct voltammetric measurements of HS. Among the parameters thus describing the TPD flow path, a practical measure was chosen to constrain the border of the TPD in the form of a CDOM fluorescence sensor used to target the TPD for sampling. With the high data density this offered, a value of 0.5 a.u. was used to constrain the TPD in this work. Finally, while the Fe-binding organic ligand measurements correlated well with the CDOM and humic substance parameters, there were occurrences of ligand (over)saturation, which is thermodynamically improbable. Hence, our methodology employed on-board was missing part of the, very probably humic dominated, Fe-binding organic ligand pool (Chapter 5).

Expanding on the on-board measurements that were suspected not to reflect the Fe-binding organic ligand pool in the Arctic Ocean surface entirely, a subset of the data was reanalysed from stored samples using a variation of the CLE-AdCSV method with another competitive ligand. Hence, two established CLE-AdCSV methods are compared, as well as direct voltammetric measurements of humic substances using a fulvic acid (FA) as a standard. The latter data was augmented and confirmed with measurements in our colleagues' lab at the university of the Balearic Islands (UIB), where additional samples had arrived, using an evolution of the technique employed on board (Sukekava et al., 2018). Both datasets had excellent agreement and have been merged in Chapter 6. Ligand concentrations differ strongly between CLE-AdCSV methods but both show similar linear relations with HS though one reflects 60% of the other, detailed further in §9.2. With remarkable similarity in  $\log K'_{FeL}$  determined by

either CLE-AdCSV method despite a large diversity of sample origin (inside and outside of the TPD), characterization of the Fe-binding ligand pool beyond the humic fraction remains veiled by methodological non-specificity. Additionally, we must question if the specific FA standard commonly used for these analyses is sufficiently representative of the HS that are to be determined. However, valuable insight is gained in the Fe-binding organic ligand pool in the Arctic Ocean. We can conclude that in the Arctic Ocean surface, Fe is primarily kept in solution by humic substances. This was not limited to the TPD alone, in the surface outside the TPD boundary humic substances also play a major role. In a similar set of measurements in the Mediterranean Sea, Dulaquais et al. (2018) found that local production was the major source of humic substances. As we cannot distinguish terrestrial humic substances from marine humics, no conclusions about the exact sources of HS can be made based on direct voltammetric measurement of HS. Although the complete ice cover of our stations does not make it likely that a local biological source offers a dominant contribution to the Fe-binding organic ligand pool, it cannot be excluded. However, given that ligand classes derived from analysis using a 2-ligand Langmuir model partly differed between CLE-AdCSV methods, the Arctic Ocean ligand pool is not limited to either a single class of humic substances, or humics alone. Furthermore, it is possible that stronger ligands such as siderophores are present but saturated and veiled by high concentrations of humic substances dominating the Fe-binding organic ligand pool. Ultimately, neither voltammetric method is able to give a complete picture, as CLE-AdCSV using TAC underestimates the contribution of humic substances but CLE-AdCSV using SA may well overestimate  $[L_t]$  (Chapter 6).

### 9.1.3. Fe speciation in phytoplankton cultures

Microbiota have been shown to contribute to the Fe-binding organic ligand pool. Specifically, production of siderophores by heterotrophic bacteria is a long-recognized process and it has been shown that viral lysis of phytoplankton cells contributes to Fe bioavailability, and is presumed to release ligands. However, before we could investigate the possible contribution of viral lysis to the Fe-binding organic ligand pool, we had to investigate the effect Fe limitation has on viral lysis. While the effect of major nutrient limitation on viral lysis of a phytoplankton host has been studied in some detail, the effect of Fe-limitation in this regard was thus far unknown.

Monocultures of the cosmopolitan phytoplankton species *Micromonas pusilla* and *Phaeocystis globosa* were cultured in a specially developed low-trace-metal medium, as well as their specific viral lysates. Fe-limited and Fe-replete cultures were infected with their respective lysates to study differences in the infection

process. These were also cross-inoculated to ascertain the effect of Fe limitation on the quality of the virus progeny and the effect of Fe added to limitation-adapted hosts at the time of infection. It was found that burst sizes were strongly reduced under Fe stress, as well as infectivity of the *M. pusilla* virus progeny. Introduction of Fe during the lytic cycle only partly alleviated the lowered production of virus progeny, indicating that host condition plays an important role as well. Overall the effects of Fe limitation were very similar to major nutrient limitation, though without affecting the latent period of the lytic cycle. The lowered burst size and infectivity of the progeny viruses under Fe limitation diminish viral control of phytoplankton. The viral shunt mobilizing nutrients (Wilhelm and Suttle, 2000) and increasing bioavailable Fe (Poorvin et al., 2011) may therefore be less important in low-Fe regions (Chapter 7). This stresses the importance of the viral shunt for Fe speciation, as there is a negative feedback on Fe cycling and release of Fe-binding organic ligands if these are indeed part of phytoplankton lysates.

In order to elucidate the role of viral lysis on Fe speciation, Fe-binding organic ligands were studied in Fe-limited cultures of *P. globosa* and *M. pusilla* during viral infection. The cultures and lysates were then left for a 4 week senescence period to allow for possible modification by heterotrophic bacteria present in the cultures. Both at the end of the experiment and the senescence period, samples were collected for ESI-MS analysis of siderophores and siderophore-like substances. As of yet unknown siderophore-like substances were found both after growth, lysis and senescence, with a number of unique substances after senescence though in lower abundance suggesting a changing Fe-binding organic ligand pool with a loss factor in bacterial breakdown of ligands.

An increase in  $[L_t]$  was found upon lysis of *P. globosa*, indicating that viral lysis of phytoplankton releases Fe-binding organic ligands in the surrounding seawater. As no increase in  $[L_t]$  was found upon lysis of *M. pusilla*, ligand release may be highly dependent on phytoplankton host species. Conditional binding strength also differed between phytoplankton host species, with no significant change in binding strength in lysed cultures compared to controls. The  $\log a_{Fe'L}$  showed similar relations to  $\log K'_{Fe'L}$  between cultures and treatments. The  $\log a_{Fe'L}$  was considerably higher than oceanic samples (4.36, SD = 0.49, N = 14 for experimental samples; 2.85, SD = 0.33, N = 47 in Chapter 6 SA data). The  $a_{Fe'L}$  is dependent on both  $K'$  and  $L_t$ . The  $[L_t]$  observed in the cultures was higher than typical open ocean values by an order of magnitude, which results in high  $\log a_{Fe'L}$ . However, culture cell density will also be considerably higher than oceanic conditions.

## 9.2. Implications and recommendations

### 9.2.1. Surface waters and microbial activity

Both the Mediterranean Sea and the Arctic Ocean are examples of shelf-surrounded oceans with a strong terrestrial influence. In both, and in contrast to other world oceans, surface DFe is high and therefore the ligands to support it are present in relatively high concentrations. In the Arctic Ocean, humic substances are of particular importance for the Fe-binding organic ligand pool. We now also know from colleagues' efforts that this is also the case for the Mediterranean Sea (Dulaquais et al., 2018). They found, similarly to our first findings in the Arctic Ocean in Chapter 5, a clear relation between our  $[L_t]_{TAC}$  measurements (Chapter 3) and HS. Their HS measurements explained  $L_t$  in the surface of the Mediterranean Sea in part, though with lower HS concentrations (ranging roughly 10-80  $\mu\text{g SRFA L}^{-1}$ , compared to  $\sim 10\text{-}300 \mu\text{g SRFA}^{-1}$  in the surface Arctic Ocean from Sukekava et al., 2018). Therefore, a revisit of Fe-speciation in the entire Mediterranean Sea using methods more representative of humic substances is called for. In the Arctic Ocean, CDOM and FDOM have good agreement with  $L_t$  from CLE-AdCSV. An improved method for direct voltammetric determination of humic substances also correlates well with ligand and DOM measurements, as confirmed by Sukekava et al. (2018). Together these properties and methods should be used to investigate the influence of HS in the Mediterranean Sea in more detail, as well as in other regions.

To constrain the flow path of the TPD, the choice was made to use an uncalibrated CDOM fluorescence sensor for its practicality and high sample density, which worked very well. However, for purposes of intercomparison and reproducibility it is recommended to use a metric that is less arbitrary. A common calibration scheme or another widely published spectral metric of terrestrial substance is recommended. Possible means to refine spectral data is the use of a specific absorbance wavelength (e.g. Coble, 2007), or if more precise equipment is available the use of spectral slopes (Helms et al., 2008). However, the use of a fast-response sensor system will still be vital to target sampling on-board.

Future analyses should focus on further elucidating the role of humic substances both inside and outside the TPD, as well as beyond the Arctic Ocean, e.g. in the North Atlantic where the TPD ultimately leads. Now that we know the extent of the TPD's influence on Fe speciation by crossing it, changes in the Fe-binding ligand pool along the length of the TPD flow path are important to describe. Gerringa et al. (2015) found that  $[L_t]$  decreases along the flow of NADW which is directly influenced by Arctic outflow, illustrating the far reach of the TPD.

Climate change has a strong influence on the composition of DOM. Both conditions in the central Arctic Ocean (Bhatt et al., 2014), and the catchments of the source areas of the TPD (Peterson et al., 2002; Schuur et al., 2015) are already changing rapidly. As we found that Fe is potentially growth limiting once light limitation gives way in a future Arctic Ocean surface outside of the continuous supply of Fe inside the TPD, a time series of DFe, nutrients and Fe speciation measurements is warranted. It is imperative to not only elucidate these processes for predictive purposes, but also to track change as it happens.

Additionally, to pry apart the relative contribution of remotely produced terrestrial humics, their modification and local production of humic-like substances, these studies must be combined with detailed microbial surveys. For instance, while Gerringa et al. (2015) described the course of  $L_t$  in the west Atlantic, Achterberg et al. (2018) did not find an influx of DFe in Arctic water in the high latitude north Atlantic. This may very well be due to immediate utilization for primary production. The nutrient utilisation ratios used in Rijkenberg et al., 2018 (Chapter 4) to predict future Arctic Ocean supplies are highly dependent on the phytoplankton community. Thus, future studies must disparage chemical oceanography with microbiology in regard to Fe speciation.

In our efforts to identify microbiological interactions with the Fe-binding organic ligand pool, we found that viral lysis of phytoplankton and bacterial presence modify and contribute to the Fe-binding organic ligands pool. To this end we employed a novel culture medium without interfering chelates. This medium is ideally suited for future study of Fe-speciation in phytoplankton cultures. The contribution of siderophore-like Fe-binding organic ligands as well as the loss factor posed by heterotrophic bacteria may be strongly dependent on the bacterial community. In our study, we were limited to the natural bacterial community present in our non-axenic cultures. The composition of this bacterial community was unknown and future studies need to include genotyping of the bacterial phyla present. In order to gain a more conclusive insight in the effect of bacterial community composition it would be beneficial to control that community more precisely. Apart from study of bacterial monocultures (e.g. *Vibrio* sp., Gauglitz et al., 2012), it would be of great interest to further study natural bacterial communities and their interactions with phytoplankton (such as done by Sheik et al., 2014) in Fe-limited cultures. Such control of the bacterial communities would be a prime avenue of future study, as at present it is prohibitively difficult to maintain axenic cultures under a low trace metal regime for long enough. If this were attained, addition of specific bacterial phyla may elucidate the role of bacteria for the Fe-binding organic ligand pool further. Such studies should include the role of heterotrophic bacteria in breakdown as

a loss factor of Fe-binding organic ligands or conversion into substances of a possibly lower binding strength.

Furthermore, our results from culture experiments in Chapter 8 suggest a strong host species dependence of conditional binding strengths and of the occurrence of ligand increase in viral lysates. Studies with a more diverse set of phytoplankton species will help us understand this phytoplankton host species dependence. For one, cell size may be an important factor. Additionally, it will be of great interest to involve a diatom representative, given diatom importance in marine phytoplankton community succession as well as their divergent genetic background from secondary endosymbiosis. The latter may have led to siderophore-related pathways being present in diatoms.

### 9.2.2. *Deep waters*

Lateral transport is expected to play a major role in distributing DFe in the deep Mediterranean Sea, transporting Fe and possibly Fe-binding organic ligands from their sources. We suggest a link between mud volcanoes and Fe-speciation, as these were present in the area of the deep high-DFe patches found. Further study is required to confirm these possible sources. Similarly, hydrothermal sources are important point sources along the ridges separating the deep basins of the arctic. The importance of hydrothermal sources is well known for DFe (a.o. Tagliabue et al., 2010; Klunder et al., 2012). Fe-binding organic ligands near hydrothermal sources are typically at saturation (Bennett et al., 2008; Hawkes et al., 2013; Gerringa et al., 2015). Our measurements of Fe-binding organic ligands in Chapter 5 did not have the required resolution to reflect the hydrothermal DFe source described in Chapter 4. In order to better describe deep Fe-speciation, deep sampling resolution should be increased, for instance by pairing sampling density with source distance. The importance of scavenging for Fe-speciation was visible in our measurements in the Mediterranean Sea and deep Makarov Basin of the Arctic Ocean, reflected by an increased ratio  $[L_i]/DFe$ .

Furthermore, while we did not take HS samples beyond 200 m depth, HS profiles seemed to settle at values between 10-30  $\mu\text{g SRFA L}^{-1}$ . Similar values were reported in the deep Atlantic near the Strait of Gibraltar by Dulaquais et al. (2018), indicating that the recalcitrant HS play a role in deep Fe speciation. To what extent HS are contributing to the ligand pool at depth is a lingering question, in the Arctic Ocean as well as elsewhere. With more detailed studies of deep Fe-speciation in shelf-surrounded systems, both in resolution and diversity of measurements, we may start to unravel the interactions between point sources of DFe and possibly Fe-binding organic ligands, scavenging and ballasting, and the reach of humic substances at depth.

### 9.2.3. Methodological considerations

A number of voltammetric methods using different competitive ligands are employed in studies of Fe-binding organic ligands. Earlier intercomparisons show that open ocean studies have been found to give similar results within acceptable tolerances (Buck et al., 2012, 2016). However, in our study of Fe speciation in the Arctic Ocean where there is a strong influence of HS, a clear discrepancy in results between different CLE-AdCSV methods was found. The competitive ligand TAC has been previously reported to suffer from interference when measuring HS (Laglera et al., 2011), particularly the commonly used SRFA standard, as we have also concluded (Chapter 5). However, we find that measurements using TAC in fact do respond to changes in a HS-dominated system, and reflects terrestrial influences at least in part. Assuming measurements using SA measure the humic influence in entirety, measurements using TAC only reflect 61% (Chapter 6).

CLE-AdCSV using SA evidently reflects humic substances in a more complete manner. However, we found a large offset with an HS-derived measure of  $[L_t]$  in a HS-dominated system (0.88 nM). In the application of CLE-AdCSV using SA, only the FeSA complex is measured voltammetrically. The formation of the non-electroactive FeSA<sub>2</sub> (Abualhaija and van den Berg, 2014) is subtracted from the signal in the Langmuir model: the sum of both  $K'_{FeSA}$ , and  $\beta'_{Fe(SA)_2}$  are taken into account together with  $\alpha_i$  to calculate  $[Fe']$ . Our choice was to follow Buck and used an equilibration time of 15 minutes, assuming a plateau in FeSA<sub>2</sub> formation, to minimize the effect on sensitivity. Overnight equilibration would ensure the kinetics of SA to run their course as they do with the TAC method (Croot and Johansson, 2000; Gerringa et al., 2007). However, the existence of an equilibrium state is essential to use the Langmuir equation (Langmuir, 1916; Gerringa et al., 2014; Laglera and Filella, 2015). The assumption of a plateau in FeSA<sub>2</sub> formation may be matrix-dependent, and use of SA may lead to an overestimation of  $[L_t]$ . The intricacies of SA kinetics are subject to ongoing research and these questions need to be resolved in order to gain confidence in the method. In all, it may be required to rely on multiple added ligands in CLE-AdCSV to come to a complete picture of Fe speciation. To what extent different CLE-AdCSV methods represent the Fe-binding organic ligand pool in a dissimilar manner must also be extended beyond our comparisons of TAC and SA as added ligands. To this end a revisit of methods employing 2,3-dihydroxynaphthalene (DHN; van den Berg, 2006) or 1-nitroso-2-naphthol (NN; Gledhill and van den Berg, 1994) would increase our insight and allow for comparison against earlier studies.

In order to confidently compare methods, their implementation in different labs as well as identify matrix dependence, intercalibration standards such as those available for DFe (Johnson et al., 2007) are direly needed. Adoption of multiple methods in different labs will also assist in increasing confidence in their application. In order to readily adopt these methods, it is imperative to pay very close attention to detail in the descriptions of method application, with special attention to preparatory steps and material handling, which is often highly lab-dependent and in our experience has a profound influence on results.

In the studies here described, we found a remarkable lack of variation in  $\log K'_{\text{Fe}^{\text{L}}}$  both in *in-situ* samples and in cultures of diverse treatment. Attempts are being made to translate specific ranges of  $\log K'_{\text{Fe}^{\text{L}}}$  to relatively specific ligand classes and locales (e.g. Hassler et al., 2017). However, the extent to which these may be applied to *in-situ* sampling given the mixed character of Fe-binding organic ligands in the oceans is questionable. In the studies combined in this dissertation, we could not identify such specific locales based on  $\log K'_{\text{Fe}^{\text{L}}}$ . Characterisation of the ligand pool may depend more on the identification of specific substances through other means (E Mawji et al., 2008; Helms et al., 2008; Stedmon et al., 2011; Boiteau et al., 2013; Sukekava et al., 2018). When such analyses yield insight into the relative importance of those substances, CLE-AdCSV methods may in turn provide measures of binding capacity and strength of a ligand pool as a whole, or specific isolates where available.

### 9.3. Concluding remarks

This dissertation has set out to generate more insight in the sources and sinks of Fe-binding organic ligands with the twofold approach of *in-situ* study and experiments in culture. By correlating measurements of possible contributors with traditional measurements of Fe-binding organic ligands, we have found that humic substances make a particularly important contribution to the Fe-binding organic ligands pool in the surface waters of the Arctic Ocean, both inside and outside of the avenue of terrestrial substances formed by the TPD. In deeper waters no strong correlations with DOM could be identified, but a smaller concentration of recalcitrant humics may play a role here.

Concentrations of Fe-binding ligands increase upon viral lysis of phytoplankton, dependent on phytoplankton species. Additionally, senescence of cultures in the presence of heterotrophic bacteria indicated that these can be both contributors and a loss factor for specific substances. To what extent the Fe-binding ligand pool can be diminished requires further study. Identification of loss factors due to the activity of heterotrophic bacteria via ligand concentration and conditional binding strength in cultures is severely complicated by the lack of sensitivity in

electrochemical methods in the 'organic soup' resultant from these processes. More substance-specific methods such as LC-ESI-MS as used in Chapter 8 may assist in these efforts. However, more control over bacterial communities is required.

We have identified that methodologically, the study of Fe-binding organic ligands will strongly benefit from diversity in methods, as none are capable of elucidating the Fe-binding organic ligand pool in its entirety alone. Profiling of ligands into specific regimes based on electrochemical parameters is at odds with their dependence on method, application and inconsistent reflection of different contributors to the ligand pool.



