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# 1 Rapid and Simple Determination of Iron-porphyrin-like Complexes 2 (Fe-Py) in Estuarine and Marine Waters

3

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12 Abstract

13

14 This work presents a new method for determining iron-porphyrin-like complexes (Fe-Py) based on  
15 Continuous Flow Analysis (CFA) with chemiluminescence detection and its application to natural  
16 waters in an estuarine environment. The involved reaction is founded on luminol oxidation by  
17 hydrogen peroxide in the presence of Fe-Py complexes at pH 13. The detection limit is 7.2 pM hemin  
18 equivalent, the linear range extends to 150 nM and precision of the method is 6.4% at 0.25 nM (n=8).  
19 This new method's detection limit is 15 times lower than the previous analytical procedure of Vong  
20 et al. (2007), based on Flow Injection Analysis (FIA) and using different chemical conditions.  
21 Moreover, the presented method is fast (90s/analysis), involves a low consumption of reagents, a  
22 small sample volume, and simplified sample handling. The method was applied to natural samples  
23 collected along the temperate macrotidal Aulne estuary (Bay of Brest, France). Here, we report for  
24 the first time on the spatial distribution of the Fe-Py complex concentration (dissolved, reactive  
25 particulate) over the entire salinity gradient of a macrotidal temperate estuary. The Fe-Py  
26 concentrations in the riverine and marine end-members were  $0.873 \pm 0.007$  nM (S=0.92) and  $0.010 \pm$   
27  $0.004$  nM (S=34.86), respectively. Between these two salinities, non-conservative behaviour was  
28 observed, with an increase in Fe-Py concentrations to  $1.142 \pm 0.031$  nM at S=5.2 corresponding to  
29 the Maximum Turbidity Zone (MTZ), followed by a strong removal of Fe-Py in the salinity range 5-20.  
30 Then, the Fe-Py concentrations decreased linearly during mixing processes, reaching picomolar levels  
31 towards the coastal waters. The estimated entering flux from the river equaled  $240 \pm 2$  g.d<sup>-1</sup> whereas  
32 the net flux to coastal sea waters was  $95 \pm 10$  g.d<sup>-1</sup> leading to a loss of ~60 %. The estuarine system

33 globally acts as a sink for Fe-Py complexes, probably due to the aggregation of Fe-Py complexes on  
34 particles, to flocculation and/or sedimentation.

35

36 Keywords: Iron, heme, porphyrin, estuary, continuous flow analysis

37

## 38 1- Introduction

39

40 The importance of iron (Fe) in the oceanic carbon cycle has been clearly established through  
41 biogeochemical processes (Anderson, 2005). Iron is unquestionably one of the factors limiting  
42 primary production in more than 40% of the world ocean (de Baar et al., 2005; Geider and La Roche,  
43 1994; Moore et al., 2013). Despite advances in Fe biogeochemical involvement, there is an acute lack  
44 of data on its physico-chemical speciation in natural waters.

45 Its stabilisation in the water column, transport, transfer to the sedimentary compartment and  
46 capacity to be assimilated by marine microorganisms differ (Tagliabue et al., 2017) according to its  
47 form (free, inorganically or organically complexed). Iron is found in particulate, (PFe,  $>0.45 \mu\text{m}$ ),  
48 dissolved (dFe,  $< 0.45\mu\text{m}$ ), soluble (sFe,  $< 0.02\mu\text{m}$ ) and colloidal (cFe, between  $0.45$  and  $0.02\mu\text{m}$ )  
49 forms, operationally defined by the pore size of the filtration membrane (Worsfold et al., 2014).  
50 Dissolved iron is itself divided into two categories: free iron and complexed iron (Turner and Hunter,  
51 2001). The scientific community currently supports that dissolved iron is more than 99% complexed  
52 with organic ligands in oxygenated seawater (Gledhill and van den Berg, 1994; Witter et al., 2000)  
53 and estuarine waters (Batchelli et al., 2010; Muller, 2018; Rijkenberg et al., 2006). The organic pool is  
54 considered a key feature of iron biogeochemical processes (Völker and Tagliabue, 2015) as it  
55 increases the overall iron solubility (Johnson et al., 1997; Liu and Millero, 2002). However,  
56 characterisation of iron ligands, organic and inorganic in natural waters, is a major challenge. These  
57 ligands can be very different (Gledhill and Buck, 2012), with high concentrations in estuaries (e.g.  
58 humic substances, hundreds of  $\mu\text{g.l}^{-1}$  to  $\text{mg.l}^{-1}$ , Abualhaija et al., 2015; Laglera and Van Den Berg,  
59 2009; Riso et al., 2021) and extremely low concentrations in open ocean waters (e.g. siderophores,  
60 nM to pM, Boiteau et al., 2016; Bundy et al., 2014; Mawji et al., 2008). The ligands have varied  
61 sources such as terrestrial breakdown of plant materials: these can be produced *in situ* or degraded  
62 by different microorganisms. Natural waters can therefore contain a mixture of ligands such as  
63 siderophores (Bundy et al., 2018; Mawji et al., 2011; Vraspir and Butler, 2009), inorganic  
64 nanoparticles (Lough et al., 2019; Yücel et al., 2011), humic substances (Abualhaija et al., 2015;  
65 Dulaquais et al., 2018; Whitby et al., 2020) and/or porphyrins (Louropoulou et al., 2020, 2019; Vong

66 et al., 2007). According to their intrinsic nature, they can form strong and weak complexes with iron,  
67 such a qualification being operationally defined by competitive equilibrium voltammetric  
68 experiments (Gledhill and van den Berg, 1994; Witter et al., 2000). Witter et al., (2000) suggest that  
69 most unknown ligands in seawater could originate from porphyrin and siderophore-like compounds.  
70 Porphyrins, which include chlorophylls, chlorophyll breakdown products like phaeophytin, hemes,  
71 and vitamin B12, are produced by almost all living organisms (Gledhill and Buck, 2012). Hemes  
72 participate as enzymatic cofactors in processes like photosynthesis (cytochromes, globin), electron  
73 transfer (catalases, cytochromes P450, peroxidases), respiration, and nitrate assimilation (nitrate  
74 reductase, Hogle et al., 2014). Iron-porphyrin-like complexes such as heme b (Fe protoporphyrin IX)  
75 in seawater probably result from cell lysis, either through grazing or viral attack. (Witter et al., 2000)  
76 used tetrapyrrole molecules (phaeophytin, protoporphyrin IX and protoporphyrin IX dimethylester)  
77 to represent Fe-binding organic complexing ligands released into seawater from bacteria and  
78 phytoplankton degradation as part of their model. (Laglera et al., 2020) also suggest that the recycled  
79 iron ligands observed in their study were probably released during grazing and copepod fecal pellet  
80 cycling. They indicate that intracellular ligands such as hemes (pigments containing the iron binding  
81 moiety porphyrin) could be the cause of this class of ligands but without being able to characterise  
82 the true nature of these organic molecules. The different type of ligands could be released into the  
83 medium via cell lysis during sloppy grazing and recycling of fecal pellets (packed with phytoplanktonic  
84 cells). Despite their ubiquity in natural waters, Fe-Py compounds have been less studied than  
85 siderophores in the ocean. They are considered weaker binding ligands (Hassler et al., 2017;  
86 Schlosser and Croot, 2008; Völker and Tagliabue, 2015) with very low solubility in seawater at pH 8  
87 (Gledhill, 2007; Gledhill and Buck, 2012). However, several studies (Bellworthy et al., 2017; Isaji et al.,  
88 2020) have shown that heme and hemoproteins are relevant complexes in iron biogeochemical  
89 cycling as they persist in seawater and on marine particles. Recently, Hogle et al., (2014) and  
90 Louropoulou et al., (2020, 2019) highlighted the importance of such ligands in the iron  
91 biogeochemical cycle presenting an extensive dataset of particulate heme b abundance in the  
92 Atlantic Ocean. The authors suggest that heme b could account for between 0.17–9.1% of biogenic  
93 iron, a significant portion of the total biogenic iron pool in the Atlantic Ocean. The quantification of  
94 particulate heme b can be attained through High-Performance Liquid Chromatography (HPLC) –  
95 Diode Array Detection (DAD) or Electrospray Ionisation (ESI) - Mass spectrometry (MS) after solvent  
96 extraction (Gledhill, 2014, 2007; Gledhill et al., 2013; Louropoulou et al., 2020, 2019). These  
97 techniques present undeniable advantages in terms of sensitivity, reaching low detection limits  
98 ranging from 9 pM (Bellworthy et al., 2017) to 0.01-0.02 pM (Louropoulou et al., 2020) enabling the  
99 analysis of most seawater conditions. However, the overall heme b determination involves several  
100 steps of extraction and analysis. In 2007, Vong and co-authors published a method for determining

101 iron-porphyrin complexes based on flow injection analysis (FIA) and chemiluminescence detection  
102 using hemin (Chloroporphyrin IX iron (III)). These authors were able to detect heme-like  
103 compounds, called iron-porphyrin-like (Fe-Py) complexes in the Rhone river and the Berre pond.  
104 However, their detection limit (0.11 nM) did not allow the detection of Fe-Py complexes in the  
105 surface waters of the Southern Ocean.

106 In this study, we have optimised the method developed by Vong et al., (2007), moving from the  
107 flow injection technique to continuous flow analysis (CFA) with the addition of hydrogen peroxide as  
108 the reaction oxidant. Various interferences (organic and metallic compounds, pH, and salinity) were  
109 tested and figures of merits determined according to method modifications. This rapid method  
110 (90s/analysis) was applied to natural unfiltered samples collected along the land-sea continuum of a  
111 temperate macrotidal estuary (Aulne estuary, Bay of Brest, France), reporting for the first time Fe-Py  
112 (dissolved + reactive particulate) distribution throughout an entire estuarine system.

113

## 114 2- Experiment

115

116 The method used to analyse iron-porphyrin-like complexes (Fe-Py) is based on chemiluminescent  
117 detection and is derived from the work of Vong et al. (2007). The reaction relies on luminol oxidation  
118 in the presence of Fe-Py complexes at pH 13. The new reagent and apparatus conditions are  
119 described in the following paragraphs.

120 The concentrations of dissolved (DFe) and soluble iron (SFe) were obtained using a FIA technique  
121 based on colorimetric detection. This method is described in previous papers (Laës et al., 2005;  
122 Measures et al., 1995). Briefly, iron contained in an on-line buffer sample, is previously concentrated  
123 on a column, then eluted using hydrochloric acid (30% Sigma Aldrich suprapur<sup>®</sup>) diluted at a  
124 concentration of 0.11M in artificial seawater. The flow then passes through the manifold and the iron  
125 present in the carrier acts catalytically on the oxidation reaction of the amine (N,N-dimethyl-p-  
126 phenylenediamine (4-amino-N,N-dimethylaniline)dihydrochloride, Sigma Aldrich) at a specific pH of 9  
127 in presence of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>, 30% Suprapur, Merck) at a concentration of 1.6M. The  
128 detection limit for dissolved and soluble Fe detection by FIA was estimated to be 20.11 nM for direct  
129 sample determination and 0.13 nM when preconcentration step was used.

130 The Particulate Organic Carbon (POC) analysis was performed using a Thermo Flash 2000 elemental  
131 analyser (combustion of the sample packed in a tin combustion boat at 950°C, separation of the  
132 combustion gases via a gas chromatography column, then detection via a catharometer (Thermal  
133 Conductivity Detector, TCD)). The apparatus was calibrated according to several acetanilide

134 standards. For chlorophyll and pheophytin analysis, we followed the protocol developed by (Yentsch  
135 and Menzel, 1963). The sample filter was extracted in 90/10 acetone, and then stored in a cool and  
136 dark place for 12 hours. The fluorescence of the extract was than measured before and after  
137 acidification with HCl 0.1 M using a Turner Designs (10-AU) system with excitation and emission  
138 wavelengths of 450nm and 670nm, respectively.

139

## 140 2.1- Chemicals and reagents

141

### 142 2.1.1- Fe-Py determination

143 Aqueous solutions were prepared with ultrapure water (resistivity > 18.2 MΩ, Milli-Q 93 Element  
144 System, Millipore®, called UP-water hereafter). The method for determination of Fe -Py compounds  
145 is not as prone to contamination as iron analysis (Hogle et al., 2014). However, because metallic  
146 interference was studied and in pursuance of Fe-Py measurement quality, every plastic container was  
147 previously cleaned with hydrochloric acid 30% (Sigma Aldrich suprapur ®, pH 2), and rinsed three  
148 times using UP-water before being used for reagent preparation and sample collection. The same  
149 procedure was also applied to iron determination.

150 The alkaline luminol solution ( $1.5 \cdot 10^{-3}$  M) was prepared by dissolving 10 g of sodium hydroxide  
151 (Sigma-Aldrich®) in 300 ml of UP-water, then 88.6 mg of 5-amino-2,3-dihydro-1,4-phthalazinedione  
152 (Aldrich®) and 900 µL of linoleic acid (Fluka®) were added. The final solution was stirred until total  
153 dissolution before being completed to 500 ml. It was prepared the previous day and stored in the  
154 dark. The EDTA solution (0.25 M) was prepared in 1 l of UP-water to which were added 89.52 g of  
155 Ethylenediaminetetraacetic acid, trisodium salt hydrate (Across Organics®). The hydrogen peroxide  
156 solution (0.7 M) was obtained by diluting 35.75 ml of H<sub>2</sub>O<sub>2</sub> (for trace analysis Fluka®) in 500 ml of UP-  
157 water.

158 The Fe-Py standards were prepared by diluting a stock solution of  $1.5 \cdot 10^{-3}$  M hemin  
159 (Chloroporphyrin IX iron(III)). As described in Vong et al. (2007), the concentrations of Fe-Py  
160 complexes were determined by standard additions of hemin in artificial seawater. Consequently,  
161 concentrations of Fe-Py complexes are expressed in nanomolar of hemin equivalent. The stock  
162 solution was prepared by dissolving 0.0978 g of hemin (Sigma-Aldrich) in 100 ml of an alkaline  
163 solution of 0.1 M of sodium hydroxide. It was prepared daily, kept in the dark and at a low  
164 temperature. Tests were performed on the hemin response from a stock solution of hemin  
165 immediately, three hours and six hours after its preparation. No statistical differences were observed  
166 between the different tests. A daughter solution of hemin (2.4 µM) was prepared by diluting 80 µl of  
167 the stock solution in 0.1 M of sodium hydroxide. This solution was prepared daily and stored in the

168 dark. Artificial seawater (1l) was prepared from ten salts (NaCl © Suprapur: 23.9g, MgCl<sub>2</sub> 6H<sub>2</sub>O ©  
169 Normapur Analar: 10.8g, Na<sub>2</sub>SO<sub>4</sub> © Normapur Analar: 4g, CaCl<sub>2</sub> 2H<sub>2</sub>O © Emsure ACS : 3.08g, KCl ACS  
170 reagent: 0.7g, NaHCO<sub>3</sub> © Normapur Analar: 0.2g, SrCl<sub>2</sub> 6H<sub>2</sub>O © Normapur Analar: 0.025g, KBr ©  
171 Emsure ACS: 0.1g, H<sub>3</sub>BO<sub>3</sub> © Rectapur: 0.03g, NaF © Normapur Analar: 0.003g) dissolved in UP-water  
172 as indicated in Aminot and Kerouel (2007). Simplified artificial seawater (1 l) was composed of three  
173 salts (NaCl © Suprapur: 24.53 g, MgCl<sub>2</sub> 6 H<sub>2</sub>O © Normapur Analar: 11.1 g, Na<sub>2</sub>SO<sub>4</sub> © Normapur  
174 Analar: 9.4 g, CaCl<sub>2</sub> 2 H<sub>2</sub>O © Emsure ACS: 1.54 g) dissolved in UP-water. Before analysis, standards  
175 and samples were buffered using a mixture of NH<sub>3</sub>/NH<sub>4</sub> pH 9.5, prepared with 75 ml of suprapure®  
176 ammonia solution 25% (Merck) and 53 ml of suprapure® hydrochloric acid 30% (Merck).  
177 Nanomolar hemin standards were prepared by diluting the daughter solution in 10 ml of artificial  
178 seawater. A sample buffer of 750 µl was added to the standard solution before being pumped by the  
179 CFA apparatus. The same procedure was applied to the river, estuarine and seawater samples.

180 As the analytical method had been modified, the selectivity of this new procedure needed to be  
181 checked accordingly. A stock solution of bovine liver catalase (Sigma-Aldrich, 30 µM) was prepared in  
182 UP water and a stock solution of horseradish peroxidase (Apollo Scientific, 9 µM) was prepared in a  
183 Tris (hydroxymethyl) aminomethane buffer 0.25 M. As for hemin standards, nanomolar  
184 concentrations of the proteins were prepared by dilution in 10 ml of artificial water, following the  
185 procedure described above.

## 186 2.2 Interferences

187

188 Interfering metal solutions (100 mM, 25 ml of acidified UP-water pH 2) were prepared from 0.3407 g  
189 of zinc chloride (ZnCl<sub>2</sub>, Sigma-Aldrich), 0.9804 g of ammonium iron (II) sulfate hexahydrate  
190 (Fe(NH<sub>4</sub>)<sub>2</sub>(SO<sub>4</sub>)<sub>2</sub>, 6 H<sub>2</sub>O Sigma-Aldrich), 1.2055 g of ammonium iron (III) sulfate dodecahydrate  
191 (FeNH<sub>4</sub>(SO<sub>4</sub>)<sub>2</sub>, 12 H<sub>2</sub>O Sigma-Aldrich), 0.6242g of copper sulfate (CuSO<sub>4</sub>, 5H<sub>2</sub>O, Sigma-Aldrich) and  
192 0.4948 g of manganese chloride (MnCl<sub>2</sub>, 4H<sub>2</sub>O, Sigma-Aldrich). Intermediate solutions were prepared  
193 to complete the interference tests at different levels, i.e. 0, 350, 700 nM for Fe<sup>3+</sup>, Zn<sup>2+</sup>and Mn<sup>2+</sup> ; 0,  
194 50, 100 nM for Cu<sup>2+</sup>and 0, 25, 50 nM for Fe<sup>2+</sup>.

195

196 Two humic substances, three porphyrins (different from hemin), and three siderophores were tested  
197 as interfering organics. Humic substances were Elliot Soil Fulvic Acid (ESFA) and Suwannee River  
198 Fulvic Acid (SRFA, 4500 µgC/l, IHSS). Humic solutions were prepared by diluting a 256.12 mg.l<sup>-1</sup> stock  
199 solution (prepared by dissolving a dried mass of SRFA and ESFA in milliQ solution). Siderophore  
200 solutions were composed of ferrioxamine E (*Streptomyces antibioticus*, Sigma-Aldrich), deferoxamine  
201 for peak identifications CRS 1 (Sigma-Aldrich, metal uncomplexed), and a mixture of pyoverdine

202 (Sigma Aldrich P8124) mainly composed of succinic acid and succinimide. Siderophore solutions were  
203 prepared by dissolving respectively 1 mg of each compound in 100 ml of UP water. Daughter  
204 solutions of 6.67 nM were prepared to spike 10 ml of artificial seawater to reach 10 and 20 pM final  
205 concentrations for the interference tests. The three other porphyrin-like molecules tested were: (1) a  
206 magnesium complexed molecule: chlorophyll *a* (from spinach, Sigma Aldrich), (2) protoporphyrin IX  
207 (3,7,12,17-Tetramethyl-8,13-divinyl-2,18-porphinedipropionic acid, Sigma Aldrich), and (3) a  
208 manganese complexed porphyrin: 5,10,15,20-Tetraphenyl-21*H*,23*H*-porphine manganese (III)  
209 chloride (Sigma Aldrich). The Fe-Py solutions were prepared as for the stock solution of hemin using  
210 0.1 M sodium hydroxide. The masses were 0.0844 g for protoporphyrin IX and 0.1054 g for  
211 manganese complexed porphyrin. Finally, a chlorophyll-*a* solution (1 mg bulb Sigma Aldrich) was  
212 diluted in 100 mL 90% acetone. The final concentrations tested were supposed to be similar to those  
213 expected in an estuary media. Siderophores were tested at a final concentration of 0, 10 and 20 pM,  
214 porphyrins 0, 1 and 2 nM, Chlorophyll-*a* 0, 25 and 50  $\mu\text{g}\cdot\text{l}^{-1}$ , and humic substances 0, 2250, and 4500  
215  $\mu\text{g}\cdot\text{l}^{-1}$  for the Elliott Soil Fulvic Acid and 0, 1125 and 2250  $\mu\text{g}\cdot\text{l}^{-1}$  for the Suwannee River Fulvic Acid.

216 An interference test with Fe particles was performed with two solutions containing  $\text{Fe}_2\text{O}_3$  at  
217 concentrations of 3.25 and 6.5  $\mu\text{M}$  in artificial seawater, these solutions being prepared by diluting a  
218 stock solution made with 0.215g ferric oxide ( $\text{Fe}_2\text{O}_3$ , Sigma Aldrich powder < 5 $\mu\text{m}$  > 96%) in 100 ml UP  
219 water. The interference test with Fe colloids was performed with two solutions containing 0.35 and  
220 0.7  $\mu\text{M}$  of  $\text{FeCl}_3$  in artificial seawater; these solutions being prepared after two consecutive dilutions  
221 of a stock solution made with 0.219g of  $\text{FeCl}_3$  in 100ml of artificial seawater. The different stock  
222 solutions used here were maintained unstirred for 24 hours to enable the apparition of colloidal iron.  
223 The various interferences tests with Fe particles and Fe colloids were conducted at three different  
224 hemin concentrations: 0, 1 and 2 nM.

225 To complete the interference tests, a certified material for metal concentrations consisting of a  
226 powder of freeze-dried plankton from a freshwater pond (IRMM, BCR<sup>®</sup>-414) was tested. The tested  
227 concentrations of the BCR sample were 225 and 450  $\mu\text{g}\cdot\text{l}^{-1}$ . These levels were determined by (1)  
228 considering a chl-*a* concentration of 5  $\mu\text{g}/\text{l}$ , which is a typical high value for the Bay of Brest  
229 system (Le pape et al., 1996) and (2) that purely algal organic matter has a C/Chl-*a* ratio of 40 (Abril et  
230 al., 2000). Two types of phytoplankton cells belonging to the common microalgae population in the  
231 Bay of Brest were also tested for Fe-Py analysis. Dinoflagellate (*Scrippsiella*) and diatoms (*pseudo*  
232 *nitzschia*) were grown in the Ifremer laboratory. The average abundance for an usual winter period  
233 (October) was estimated between  $4 * 10^4$  cells. $\cdot\text{l}^{-1}$  (Queguiner and Tréguer, 1984) and  $2.4 * 10^5$  cells. $\cdot\text{l}$   
234  $^{-1}$  (Beucher et al., 2004). The tested concentrations were thus 1.24 and  $2.48 * 10^5$  cells.  $\cdot\text{l}^{-1}$  for the



235 dinoflagellates and  $0.7$  and  $1.4 \times 10^5$  cells.  $l^{-1}$  for the diatoms. Volumes of fresh cultures were added  
236 to the artificial seawater and then treated like other samples.

237

## 238 2.3- Study area and sample collection

239

### 240 2.3.1- Study area

241

242 The Bay of Brest and the Aulne river (Brittany, France, Fig.1) have been described in numerous  
243 studies (Marie et al., 2017; Raimonet et al., 2013; Riso et al., 2021, 2007; Waeles et al., 2013). Briefly,  
244 the Bay of Brest ( $48^{\circ}20'N$ ;  $04^{\circ}20'W$ , Brittany, France) is a 130-km-long semi-enclosed system  
245 connected to the coastal waters of the Iroise Sea by a narrow and deep strait. Its drainage basin  
246 covers  $1850 \text{ km}^2$  mainly influenced by agricultural activities. The Aulne estuary, the main river  
247 discharging into the Bay of Brest, is 35 km long. It is meandering and protected from the coastal  
248 influence by the Crozon peninsula. The water flux is primarily controlled by the river flow, 60% of the  
249 annual flow generally occurring in the December-March period. It is also dependent on the tidal  
250 range ( $1.2\text{-}7.3\text{m}$ ) of the Bay of Brest resulting in intense variations in water depth and sediment  
251 deposition and erosion in the Aulne estuary (Marie et al., 2017).

252

### 253 2.3.2- Sample collection

254

255 Sample collection was operated during the FeLINE cruise (Fer Ligands in AulNe Estuary) on 22  
256 October 2018 onboard the RV *Hésione* (INSU-CNRS-UBO). Twenty sub-surface (0.5 m depth) sampling  
257 points were dispatched along the Aulne river ( $S=0.92$ ) estuary, Bay of Brest, and Iroise Sea ( $S=34.86$ )  
258 covering the entire land-sea continuum (North Brittany, Atlantic Ocean, France, Fig. 1). The flow rate  
259 was minimal for this period with a value of  $4.85 \text{ m}^3 \cdot \text{s}^{-1}$ , compared to the average October flow rate of  
260  $9.71 \text{ m}^3 \cdot \text{s}^{-1}$  (Banque HYDRO, <http://www.hydro.eaufrance.fr>). The tidal coefficient was also low (39).

261 The sampling locations were selected with the aim of mapping the entire freshwater—seawater  
262 mixing zone. Twelve parameters were studied including Fe-Py complexes which will be discussed  
263 here. Conventional physicochemical parameters ( $T^{\circ}$ ,  $S$ ,  $\text{pH}$ ,  $\text{O}_2$ ) were measured in situ using an  
264 AQUAread AP-2000 probe calibrated on the day of sampling. The accuracy of the measurements is  $\pm$   
265  $0.1 \text{ g kg}^{-1}$  and  $\pm 0.05$  pH units for salinity and pH. A Maximum Turbidity Zone (MTZ) characterised by  
266 muddy waters was visible to the naked eye for salinities around five. The pH variation was confirmed.  
267 Sampling flasks for Fe-Py complexes (nalgene, HDPE, 30ml) were previously cleaned with  
268 hydrochloric acid (30%, suprapur®, Sigma Aldrich,  $\text{pH}=2$ ) and rinsed three times with UP water. They

269 were also rinsed three times with natural water before final collection. Fe-Py unfiltered samples were  
270 preserved by the addition of sodium azide (3µl NaN<sub>3</sub>, 0.01 M for 30 ml) and stored frozen before  
271 analysis. They were analysed in triplicates later in the laboratory using the method described  
272 hereafter. The fraction of Fe-Py complexes analysed in the paper will be considered as the dissolved  
273 plus the reactive particulate fraction and will be displayed in nM hemin equivalent.

274 Dissolved iron (DFe) samples were recovered after filtration with 0.45-µm-mixed-celullose ester  
275 membranes (HATF, Millipore). Soluble iron (SFe) samples were obtained after filtration of a dissolved  
276 aliquot through an Anotop syringe filter (0.02 µm). Both dissolved and soluble fractions were  
277 acidified with HCl (30%, suprapur®, Sigma Aldrich, pH=2) and stored in double plastic bags in the  
278 dark. The 0.45 µm HATF filters were processed to determine the fraction of particulate iron (PFe) as  
279 well as for chlorophyll and pheophytin determination. Dissolved and soluble iron were determined  
280 using the flow injection analysis method with colorimetric detection as described in (Measures et al.,  
281 1995). The lowest concentrations were detected using a preconcentration column composed of  
282 Toyopearl 650M.

283

#### 284 2.4- Apparatus

285

286 The apparatus used here is derived from the deep-sea chemical analyser CHEMINI (CHEMical  
287 MINIaturized analyser), previously described by Vuillemin et al., (2009). This laboratory version is  
288 based on Continuous Flow Analysis (CFA) with chemiluminescence detection.

289 Standards or samples, previously buffered at pH 9, were introduced to the manifold using a  
290 peristaltic pump (PA, pump A Fig. 2). They were then mixed with the EDTA (PB, pump B) in the first  
291 mixing coil (MC1). EDTA avoided precipitation of the major seawater ions as they met the reagents  
292 stream (H<sub>2</sub>O<sub>2</sub>, luminol, pH 12.6, RC, reaction coil, L=122 mm) pushed by PC (pump C). The mixture  
293 passed through the reaction coil before reaching the photomultiplier (PMT H5783-10, Hamamatsu  
294 Photonics). Finally, the light emitted from the oxidation of luminol in the presence of Fe-Py  
295 complexes was transformed into an electronic signal read on the data manager (Memograph M  
296 RSG45, Endress + Hauser). A standard analysis lasted 90 seconds and the voltage signal delivered by  
297 the datalogger was averaged over 50 values. The limit of detection was calculated as three times the  
298 standard deviation for the analysis of ten replicates of the blank (artificial seawater with buffer  
299 addition).

300

### 301 3- Results

302

#### 303 3.1- Impact of interferences

304

##### 305 3.1.1-Salinity and pH effect

306

307 The salinity effect was studied for two standards of hemin (1 and 5 nM) that were prepared in  
308 solutions with salinities varying from 2 to 35. The salinities were obtained by mixing artificial  
309 seawater with UP water. No statistical difference between the five salinities tested (S2, S5, S20, S25,  
310 S35, ANOVA one factor) was noticed, indicating that the analysis of the collected samples will not  
311 require any specific precaution regarding salinity.

312 pH variation along the Aulne transect was estimated between 8.2 and 7.3. The stability of the pH  
313 sample once buffered (pH 9.5 once added) was checked at the two pH values without any change in  
314 the signal response.

315

##### 316 3.1.2- Metallic interferences

317

318 The luminol oxidation reaction with hydrogen peroxide under acidic conditions is a well-known  
319 detection method for iron in seawater (Laës et al., 2007; Obata et al., 1993; Ussher et al., 2007). The  
320 method used here relies on the same process but uses a much higher pH of 13. The addition of EDTA  
321 inhibits the precipitation of the major ions of seawater thus permitting analysis of the Fe-Py  
322 complexes. It was however necessary to check if the initial metallic ion concentrations in the natural  
323 samples were not interfering with the analytical process. Therefore, a central composite design  
324 (CCD), using JMP software was conducted to rapidly test the influence of iron (Fe), manganese (Mn),  
325 zinc (Zn), and copper (Cu) at concentration levels commonly encountered in estuaries. For Fe<sup>3+</sup>,  
326 Zn<sup>2+</sup> and Mn<sup>2+</sup>, concentrations of 0, 350 and 700 nM were tested (Ouddane et al., 1999; Rijkenberg et  
327 al., 2006; van den Berg et al., 1987). Fe<sup>2+</sup> and Cu<sup>2+</sup> concentrations were respectively 0, 25 and 50 and  
328 0, 50 and 100 nM (Bordin, 1991; Rijkenberg et al., 2005). The maximum iron concentrations tested as  
329 a potential interferent have been determined according to the results obtained in this study. This test  
330 was conducted with three different hemin concentrations: 0, 1 and 2 nM. The final solutions were  
331 prepared in artificial seawater. Our results indicated that most of the metallic concentrations tested  
332 did not interfere with the analytical signal with and without the presence of hemin (p value < 0.001).  
333 Fe<sup>3+</sup> was the only exception, alone in the solution and coupled with hemin. Further extensive testing  
334 was performed using various Fe<sup>3+</sup> concentrations: 0, 87.5, 175, 350, and 700 nM in the absence or  
335 presence of hemin (2 nM). To ensure that the variation in the signal response was only due to Fe<sup>3+</sup> in

336 the solution, and not to a hysteresis nor a memory effect, a blank was analysed between each  
337 addition of iron. Moreover, the different iron concentrations tested were used randomly. The same  
338 procedure was achieved for the analysis of 2 nM + Fe<sup>3+</sup> solutions. In the presence of 2 nM hemin,  
339 none of the Fe<sup>3+</sup> concentrations impacted the hemin detection. The obtained signals were in  
340 accordance with the 2 nM signal detected before and after the analysis of iron (according to method  
341 uncertainties). However, in the absence of hemin, Fe<sup>3+</sup> gave a signal slightly above the blank value for  
342 a concentration of 700 nM. The difference in hemin concentration between the sample spiked with  
343 700nM Fe<sup>3+</sup> and the blank was estimated to be 5 pM hemin equivalent, which is lower than the  
344 detection limit. Therefore, it can be considered that Fe<sup>3+</sup> and the other ambient metallic ions should  
345 not impact Fe-Py determination in estuarine or marine waters. As already considered in Vong et al.  
346 (2007), the absence of interference by Fe<sup>2+</sup>, Mn<sup>2+</sup>, Cu<sup>2+</sup> and Zn<sup>2+</sup> on the Fe-Py signal likely results from  
347 non-optimum pH conditions.

348

### 349 3.1.3- Organic interference

350

351 There are hundreds and even thousands of discrete organic ligand types in seawater but we focused  
352 here on the main iron organic ligands already observed in estuarine and coastal waters. These ligands  
353 can potentially compete with Fe-Py compounds for the complexation of iron and can also directly  
354 affect the analytical response. Three different types of organic ligands were tested: humic  
355 substances, siderophores, and porphyrins. Muller, (2018) defines humic substances as complex,  
356 acidic organic molecules formed by biotic and abiotic processes following the decomposition of plant  
357 and microbial material. They form in soils and the water columns of lakes and oceans and have the  
358 potential to complex iron. In the estuarine and the marine environment, their composition and  
359 molecular structure can change in space and time making it complex to define a global intrinsic  
360 humic structure (Dulaquais et al., 2018; Riso et al., 2021). As they are considered an important  
361 component of the ligand pool in estuarine systems (Laglera and Van Den Berg, 2009), two models of  
362 humic substances were tested as potential interfering compounds: Elliott Soil fulvic acids (ESFA) and  
363 Suwannee River fulvic acids (SRFA). Gledhill & Buck, (2012) described two other biologically produced  
364 types of organics as major iron-binding ligands: siderophores and porphyrins. Siderophores are  
365 thought to be strong ligands produced by terrestrial and marine bacteria in order to sequester Fe  
366 from their environment (Wells and Trick, 2004) whereas porphyrins, produced by almost all living  
367 organisms, are mainly present as part of the cellular debris arising from mortality and heterotrophic  
368 activity (chlorophyll, phaeophytin, hemes and other porphyrins (Hutchins et al., 1999)). The potential  
369 competing/interfering behavior of siderophores also needed to be detected as they are considered

370 as strong iron-complexing ligands and may outcompete porphyrins. We also needed to evaluate if  
371 other porphyrin-like molecules, uncomplexed or complexed with other metals than iron, have the  
372 potential to interfere with the chemiluminescence signal.

373 Three central composite designs were conducted with hemin concentrations ranging from 0, 1 and 2  
374 nM. The final solutions were prepared in artificial seawater.

375 Our results indicated that none of the tested porphyrins nor the siderophores molecules interfered  
376 with the Fe-Py detection ( $p$  value  $< 0.001$ ). This is in accordance with the results obtained by Vong et  
377 al. (2007). However, Suwannee River fulvic acid (SRFA) showed slight interference on the Fe-Py  
378 signal. For the Elliott Soil Fulvic Acid (ESFA) no impact was observed. To estimate the impact of ESFA  
379 and SRFA, three calibration curves were established, the first with increasing concentrations of  
380 hemin alone (0, 0.5, 0.75, 1, 1.5 nM), the second with increasing concentrations of hemin in the  
381 presence of  $4500 \mu\text{g.l}^{-1}$  of ESFA and the last with increasing concentrations of hemin in the presence  
382 of  $4500 \mu\text{g.l}^{-1}$  of SRFA. The aim was to increase the tested hemin concentrations and to observe any  
383 variation in the chemiluminescence response with the highest humic concentrations encountered in  
384 the Aulne system ( $4500 \mu\text{g.l}^{-1}$ , (Riso et al., 2021)). The slopes and intercepts of the three calibrations  
385 were statistically identical ( $P > 95\%$ , ANOVA test and slope comparison) showing that none of the  
386 humic substances interfered with Fe-Py detection (Fig. 3). We are able to predict that none of the  
387 organic substances tested here and expected in the Aulne estuary will impact the detection of Fe-Py-  
388 like complexes.

389

#### 390 3.1.4- Colloidal Fe and particulate Fe interferences

391

392 To test the impact of colloidal Fe and particulate Fe interferences, a central composite design (CCD),  
393 using JMP software was conducted. Considering the maximum particulate iron concentration in the  
394 Aulne estuary ( $\text{PFe} = 4.3 \text{ mg.l}^{-1}$ ,  $S=5.2$ ) and that 24% of this PFe corresponds to iron oxides, we tested  
395 two different concentrations of  $\text{Fe}_2\text{O}_3$  solutions  $3.25$  and  $6.5 \mu\text{mol.l}^{-1}$ . The CCD was completed with  
396 the addition of a  $\text{FeCl}_3$  colloidal solution obtained 24 hours after dilution of  $\text{FeCl}_3$  in artificial  
397 seawater. The interfering concentrations were estimated at  $0.35$  and  $0.7 \mu\text{mol.l}^{-1}$ . This design was  
398 conducted with three different hemin concentrations: 0, 1, and 2 nM. The final solutions were all  
399 prepared in artificial seawater. Our results indicated that none of the particulate nor the colloidal  
400 solutions, tested at various concentrations, interfered with the analytical signal with and without the  
401 presence of hemin ( $p$ -value  $< 0.001$ ).

402

#### 403 3.1.5- Analytical response for powdered phytoplankton cells

404

405 The tested concentrations of the phytoplanktonic BCR sample were 225 and 450  $\mu\text{g.l}^{-1}$ . The CDD  
406 performed showed that none of these concentrations impacted the blank nor the hemin signal.  
407 Moreover, none of the fresh phytoplankton samples tested were considered as Fe-Py productive, the  
408 signal obtained in the presence and absence of hemin were statistically identical for the two  
409 phytoplankton concentrations tested. (Vong, 2008) highlighted an apparent and temporary  
410 production of Fe-Py complexes during a phytoplankton monitoring experiment (*Dunaliella salina*).  
411 This production was then rapidly followed by the complete disappearance of the complexes in the  
412 medium. Here, no production was detected during the active growth phase of phytoplankton cells.  
413 The fact that no Fe-Py signal was observed in our study could arise from the difference in tested  
414 algae species and probably also in the growth phases of phytoplankton development, our experiment  
415 being performed during the growth phase of *Scrippsiella* and *pseudo nitzschia* cultures. (Vong, 2008)  
416 also showed that grazing of phytoplankton by microzooplankton can be a significant source of Fe-Py  
417 and that the latter molecules do represent cellular degradation products. Complementary studies  
418 could be conducted to detect Fe-Py production by bacterial communities as well as by grazing  
419 activities, but this remains out of the scope of this study.

420

### 421 3.2- Selectivity of the method

422

423 The signal response to the addition of the macromolecular bovine liver catalase and horseradish  
424 peroxidase was estimated in the presence and in the absence of the hemin standard. As explained by  
425 (Khan et al., 2014), chemiluminescence emission of the luminol- $\text{H}_2\text{O}_2$  system for the determination of  
426 heme proteins is highly sensitive and should also be tested for heme other than hemin. The signal for  
427 a 2-nM concentration of a single protein gave  $0.53 \pm 0.02$  nM hemin equivalent for the catalase and  
428  $0.35 \pm 0.014$ nM hemin equivalent for the horseradish peroxidase, both in the absence and in the  
429 presence of hemin. This can be compared to the 1.54 nM hemin equivalent signal for the catalase  
430 and 3.35 nM hemin equivalent horseradish peroxidase obtained by (Vong et al., 2007). As exposed by  
431 previous authors there is an intra-variability of the Fe-Py signal within Fe-Py which is not related to  
432 the number of Fe-Py units contained in these molecules, or to the molecular weight of these  
433 proteins. It is worth noting that the signal ratio we observed between horseradish peroxidase and  
434 bovine liver catalase is 1.5 which is relatively close to the ratio of 2.2 found by (Vong et al., 2007).  
435 However, in our case, the proteins gave lower chemiluminescence signals which cannot be explained  
436 by the addition of hydrogen peroxide. Although catalase and peroxidase are known to catalyse the  
437 dismutation reaction of this strong oxidant (Vlasits et al., 2007), the tested concentrations of both  
438 enzymes are too low compared to the concentration of the  $\text{H}_2\text{O}_2$  stream (0.7 M). Another

439 explanation for such differences may arise from different chemiluminescence kinetics between Fe-Py  
440 molecules. Nevertheless, a mixture of different Fe-Py complexes present in a sample will provide  
441 different chemiluminescence responses. Unfortunately, we are not able, at this stage of  
442 development, to identify the nature of the porphyrin content. That is why it is necessary to express  
443 Fe-Py concentrations derived from natural samples as "nM hemin equivalent".

444

### 445 3.3- Figures of merits

446

447 The figures of merits of the Fe-Py method are summarised in Table 2. Figure 4 presents (a) the  
448 variation of the signal intensity obtained for a 0.14 nM hemin concentration and (b) the calibration  
449 curve obtained from different hemin concentrations (0, 0.07, 0.14, 0.25, 0.5, 1.25, 1.5 nM). It is  
450 worth noting that intensity signals were averaged over 50 points following the addition of the  
451 standard excluding the start and end artefacts.

452 For Fe-Py determination, the blank analysis was performed on buffered artificial seawater. To detect  
453 the potential contribution of the buffer to the blank value, increasing volumes of the ammonium  
454 buffer solution were added to a constant artificial seawater volume. The analytical response  
455 corresponding to the initial volume added to the standard and sample analysis (750  $\mu$ l) was  
456 estimated. Thanks to the method of standard addition, buffer contribution was estimated to be 5  
457 pM, which is below the detection limit (Table 2). The buffer contribution is therefore considered as  
458 negligible. Additional tests were conducted using different standard matrices: artificial seawater as  
459 described above, simplified artificial seawater as described in (Aminot and Kerouel, 2004) and UV-  
460 irradiated natural seawater used for trace metal analysis. Calibration slopes and intercepts obtained  
461 for the three media were statistically identical (p-value > 95%). The contribution of artificial seawater  
462 to the blank being negligible and comparable to UV-irradiated seawater, artificial seawater was used  
463 for the hemin standard preparation. The concentration of the Fe-Py complex in samples was  
464 obtained by subtracting the intercept of the calibration curve from the sample voltage signal and  
465 dividing the result by the calibration slope performed the same day of analysis using artificial  
466 seawater.

467 The limit of detection was calculated as three times the standard deviation for the analysis of ten  
468 replicates of the blank (artificial seawater with buffer addition). The detection limit was 7.2 pM, 15  
469 times lower than reported by Vong et al., (2007) (110 pM). This decrease in the detection limit results  
470 not only from the change in manifold architecture but also from the addition of hydrogen peroxide  
471 that increases the oxidation rate of luminol.

472 Several works (Table 3) have quantified particulate heme b using High Performance Liquid  
473 Chromatography (HPLC) with various detection methods: Diode Array Detection (DAD) or  
474 Electrospray Ionisation (ESI) coupled with Mass spectrometry (MS) after solvent extraction. Recently  
475 (Isaji et al., 2020) developed a new sample extraction (acetone and HCl) and analysis for dissolved  
476 heme b using LC-MS/MS. HPLC-DAD-MS and LC-MS/MS present undeniable advantages in terms of  
477 sensitivity, reaching picomolar detection limits (Bellworthy et al., 2017) or lower (Louropoulou et al.,  
478 2020) enabling the analysis of most types of seawater. Our method also demonstrates good  
479 sensitivity for detecting Fe-Py-like compounds and is applicable to estuarine and coastal  
480 environments (see section 3.3). Moreover, it shows additional strengths such as low reagent and  
481 sample consumption (for one analysis: EDTA: 1.2 ml, H<sub>2</sub>O<sub>2</sub> 0.6 ml, luminol 0.6 ml, sample 3 ml) with  
482 no preconcentration steps that might induce analytical bias, and simplified sample handling. Finally,  
483 as it requires no rigorous extraction nor time-consuming digestion techniques, which could degrade  
484 the hemin (Hogle et al., 2014), it provides direct and fast analysis (90 s) of Fe-Py complexes in  
485 riverine, estuarine and coastal waters. Certification of the analytical method was not possible since  
486 no certified reference material of Fe-Py-like molecules exists for particulate or dissolved matrices.  
487 Inter-comparison with existing methods based on HPLC -DAD or ESI-MS could have been an  
488 alternative, if the detected compounds were identical. Unfortunately, this study aims to detect the  
489 pool of Fe-Py-like complexes and is thereby not specific, whereas the above-cited references all  
490 specifically detect the heme b molecule (Table 3).

491

#### 492 3.4- Impact of NaN<sub>3</sub> addition

493

494 Instead of using HgCl<sub>2</sub> (Vong et al., 2007), the samples were poisoned with NaN<sub>3</sub>, also known as a  
495 preservative for metal binding ligands (Cotte et al., 2018). This was done directly after sampling, by  
496 adding 3 µl of NaN<sub>3</sub> 0.01 M to 30 ml of sample. The samples were then stored frozen at -20°C as the  
497 gap between sampling and analysis of hemin was relatively important (~6 months). To verify the  
498 impact of NaN<sub>3</sub> poisoning on hemin detection, the analytical response was studied on three hemin  
499 standards (1.66, 5 and 20 nM). The differences between no-addition and NaN<sub>3</sub> addition for the three  
500 hemin concentrations were found statistically negligible.

501

#### 502 3.5- Method application to the Aulne estuary – Bay of Brest system

503

504 Table 4 describes the Fe-Py, Chlorophyll a (µg.l<sup>-1</sup>), phaeophytin (µg.l<sup>-1</sup>) and POC concentrations (mg.l<sup>-1</sup>)  
505 as well as the C/N ratio (with associated pH and salinity values) obtained in the Aulne estuary- Bay



506 of Brest system. The unfiltered samples collected along the land-sea continuum were analysed in the  
507 laboratory after being defrosted at 4°C in dark conditions. We added 750 µl of buffer to 10 mL of  
508 sample and the solution was then analysed three times. The concentrations of Fe-Py compounds are  
509 expressed in heme equivalent (Fig. 5). SFe and DFe samples were analysed directly in the laboratory  
510 without any sample pre-treatment.

511

### 512 3.5.1 Comparison with other studies

513

514 As previously reported, the exact nature of the Fe-Py complexes in seawater is unknown and consists  
515 most probably of a mixture of various heme-like ligands, biosynthesised in organisms or present in  
516 the media via cell lysis. The Fe-Py concentrations obtained for the Aulne estuary-Bay of Brest system  
517 were compared here to the results obtained by other published methods. However, the comparison  
518 should be treated with caution because of the differences in the physical speciation (dissolved or  
519 particulate) and targeted analytes of each method. Here, we measured the dissolved fraction plus  
520 the particulate reactive fraction of Fe-Py-like complexes that could encompass heme b and other  
521 iron-type porphyrins soluble enough for detection. Vong et al., (2007) also focused on Fe-Py  
522 complexes but the study involved filtered samples. Conversely, Gledhill et al., (2013) and  
523 Louropoulou et al., (2020, 2019) analysed the particulate fraction of heme b, and (Isaji et al., 2020)  
524 reported heme b in both particulate and dissolved phases.

525 The Fe-Py concentrations varied from  $0.010 \pm 0.004$  nM at  $S=34.9$  to  $1.14 \pm 0.03$  nM at  $S = 5.2$ . They are  
526 lower than those reported by Vong et al., (2007) in the Berre pond (11.5 nM,  $S=22$ ) and in the Rhône  
527 river (9.2 nM,  $S=0$ ). However, they appear more consistent with the concentrations of Fe-Py  
528 observed in the Kourou river estuary (2.1 nM,  $S=30-34$ , Vong et al. 2007). At this stage, it is difficult to  
529 know whether the differences between studies are due more to analytical features or to the  
530 geographic/seasonal characteristics of the studied systems. Our values can also be compared with  
531 heme b concentrations reported for several oceanic systems. For the surface waters of the Atlantic  
532 Ocean, Louropoulou et al., (2020) reported particulate ( $>0.7$  µm) heme b concentrations varying  
533 from 0.10 to 33.7 pM (median = 1.47 pM,  $n = 974$ ). These concentrations, much lower than our  
534 values, correspond to typical open ocean waters of low turbidity and low productivity. The highest  
535 dissolved heme b concentrations reported in the literature were observed in the Gullmar Fjord ( $66 \pm$   
536 45 pM) and were associated with a Fe concentration of 4-40 nM (Louropoulou et al., 2019). In the  
537 Tokyo Bay, (Isaji et al., 2020) reported particulate heme b concentrations as high as 0.15 nM and the  
538 heme b concentration in the dissolved phase as low as 0.006 nM. It is worth noting that the  
539 concentrations we obtained in the Bay of Brest (i.e.  $0.010 \pm 0.004$  nM for  $S=34.9$  and  $0.013 \pm 0.002$  nM  
540 for  $S=34.0$ ) stand in the same order of magnitude as those of Isaji et al. (2020). Our values in the Bay

541 of Brest were associated with DFe concentrations of 6 nM whereas the highest Fe-Py concentrations  
542  $1.14 \pm 0.03$  nM (at S = 5.2) were associated with DFe concentrations of 730 nM. DFe and SFe ranges  
543 obtained here ([DFe]: 6-730 nM, [SFe] = 4-60 nM) are similar to those commonly observed in other  
544 estuaries of the area, *i.e.* [DFe]: 12-840 nM for Penzé estuary (Riso et al., 2006) and [FeT]: 10-140 nM  
545 for the Aulne estuary (Riso et al., 2007). Fe-Py concentrations appear much lower than those of  
546 dissolved iron ([Fe-Py] = 0.1 to 1.8% of [DFe]), in line with the results of Louropoulou et al. (2020)  
547 (heme b to biogenic iron ratio of 0.2–9.1%). (Isaji et al., 2020, 2019) also found similar results in the  
548 Tokyo Bay for which the heme b concentration range in the dissolved phase (47-71 pM)  
549 corresponded to only a few percent of typical dissolved iron concentration. At our furthest point of  
550 sampling, located in the Iroise Sea and a few kilometers from the Brittany coast, the concentration  
551 obtained was under the detection limit of the method. We infer that Fe-Py concentrations decrease  
552 to subpicomolar levels towards the open ocean. Further analytical improvement will be required for  
553 Fe-Py detection at nanomolar scale for a full application to coastal and open ocean waters. The  
554 addition of a pre-concentration step would probably help to widen such application.

555

### 556 3.5.2 Mixing behaviour of Fe-Py compounds

557

558 Figure 5 describes the variation of Fe-Py, Chlorophyll a, phaeophytin, Particulate Organic Carbon  
559 (POC) concentrations and the Carbon over Nitrogen ratio C/N along the Aulne estuarine gradient. To  
560 our knowledge, this is the first time such a Fe-Py distribution is presented. Our results indicate that  
561 Fe-Py compounds clearly exhibit non-conservative behaviour due to several processes, other than  
562 simple mixing of riverine and marine waters. A positive anomaly (of ~40%) was first observed in the  
563 upper estuary (salinity range 2-8) corresponding to the Maximum Turbidity Zone (MTZ) where low  
564 pH values were also found (pH<7.3). These maximum concentrations of Fe-Py corresponded to the  
565 inner part of the estuary where the highest values of Chlorophyll a ( $1.36 \mu\text{g.l}^{-1}$ ), phaeophytin ( $5.45$   
566  $\text{mg.l}^{-1}$ ) and POC ( $3.79 \text{mg.l}^{-1}$ ) were also found in accordance with previous studies (Chauvaud et al.,  
567 2000; Queguiner and Tréguer, 1984). An important negative anomaly was detected in the salinity  
568 range 5-20, highlighting significant elimination of Fe-Py compounds following this enrichment. At  
569 salinities above 20, the Fe-Py concentrations display a conservative behaviour ( $\text{Fe-Py} = -$   
570  $0.0097\text{S} + 0.348$ ,  $R^2 = 0.983$ ). A similar conservative behaviour was also displayed by DFe and SFe  
571 concentrations (not shown) indicating these Fe fractions and Fe-Py compounds are mainly diluted in  
572 the lower part of the estuary.

573 From salinities from 0 to 22.5, the POC/Chla ratio above 200 indicates the occurrence of a degraded  
574 material from resuspension or river input (Savoye et al., 2003). For the highest salinities the  
575 POC/Chla ratio was generally lower than  $200 \text{g.g}^{-1}$  displaying a high contribution of 'living' or non-

576 degraded phytoplankton (Table 4). The low Chl $a$ /Phaeo ratios (<1) in the inner estuary (salinity  
577 between 0 and 22.5) should be related to grazing activity which could also explain the highest Fe-Py  
578 concentrations. C/N values were generally in the range 6-10, indicating that phytoplankton is the  
579 major contributor of particulate organic matter in the estuary (Liénart et al., 2017). Stronger C/N  
580 values obtained at lower salinities could argue for a more terrestrial origin influenced by other  
581 factors such as microzooplankton, heterotrophic microbes and terrestrial organisms (Huang et al.,  
582 2017). The positive anomaly of Fe-Py compounds at low salinity is probably the result of two  
583 combined processes commonly found in tidal estuaries: (1) the accumulation of particles (including  
584 those containing particulate reactive Fe-Py compounds) and/or (2) the heterotrophic activity that can  
585 potentially produce Fe-Py compounds (Servais and Garnier, 2006). Indeed, Knicker, (2004) shows that  
586 such organic matter mineralisation can lead to the production of humic-like compounds as well as  
587 amino acids, pyrimidines, purines or porphyrin structures.

588 Conversely, the negative deviation of Fe-Py compounds at higher salinities (22.5) associated with the  
589 strong decrease in POC and phaeophytin concentrations could correspond (1) to sedimentation of  
590 the particulate fraction containing the Fe-Py compounds in a less turbulent area of the estuary  
591 and/or (2) to flocculation of dissolved Fe-Py compounds that strongly tend to aggregate (Gledhill,  
592 2007) as do other Fe colloids in estuaries as a result of neutralisation of their negatively charged  
593 surface by seawater cations (Sholkovitz et al., 1978).

594

### 595 3.5.3 Fluxes

596

597 Fluxes of Fe-Py compounds across the Aulne estuary were estimated for this specific time of the year.  
598 The linear curve drawn for the conservative part of the gradient ( $S > 20$ ) was used to estimate the  
599 theoretical Fe-Py concentration (Fe-Py<sub>th</sub>). This concentration, which results from a hypothetical  
600 conservative behaviour, corresponds to the intercept of the linear curve to fluvial salinity (Delmas  
601 and Tréguer, 1983). The entering flux equals the river flow rate the day of sampling (Banque HYDRO,  
602 <http://www.hydro.eaufrance.fr>) multiplied by the Fe-Py concentration at  $S = 0$  and by the molar mass  
603 of hemin considered here as the standard reference for Fe-Py complex estimation. Similarly, the net  
604 flux equals the flow rate of the river multiplied by Fe-Py<sub>th</sub> and by the molar mass of hemin. The loss  
605 of Fe-Py compounds within the estuary was estimated from the difference between the net flux and  
606 the entering flux. Figure 8 summarises the estimated values and the potential associated processes.  
607 The estimated entering flux through river runoff equals  $240\text{g} \pm 2 \text{ g}\cdot\text{d}^{-1}$ , whereas the net flux to the  
608 Iroise sea is  $95 \pm 10 \text{ g}\cdot\text{d}^{-1}$  leading to a global loss of  $60.2 \pm 0.0\%$ . This result indicates that the removal  
609 of Fe-Py compounds is of higher magnitude than the enrichment of Fe-Py compounds in the MTZ.  
610 The Aulne estuary globally acts as a sink for Fe-Py complexes.

611

612 4- Conclusion

613

614 Moving from a Flow Injection Analysis (FIA) technique of the initial method (Vong et al., 2007) to a  
615 Continuous Flow Analysis (CFA) with the addition of hydrogen peroxide as a reaction oxidant  
616 improved the sensitivity of the technique and expanded the linear range with rapid detection  
617 (90s/analysis). Fe-Py (dissolved + reactive particulate) distribution was reported for the first time  
618 along the land-sea continuum of the temperate macrotidal Aulne estuary (Bay of Brest, France). The  
619 Fe-Py distribution showed non-conservative behaviour due to multiple and variable modification  
620 processes in addition to the simple mixing of natural and marine waters. The related entering and  
621 removal fluxes were also estimated. The highest Fe-Py concentrations were observed in the  
622 Maximum Turbidity Zone, revealing enrichment in Fe-Py probably via resuspension of sediment.  
623 Losses of Fe-Py complexes (60%) for salinities higher than five were probably due to aggregation on  
624 the particles and to flocculation. Fe-Py concentrations decreased towards the ocean during mixing  
625 processes. Analysis of the other components of the iron cycle as part of the FeLINE project (in  
626 progress) will allow to closely link the behaviour of ligands to that of iron and to fix the balance of  
627 biogenic iron in the Aulne estuary.

628 The next challenge will be to study the physical speciation (particulate/colloidal/soluble fraction) of  
629 Fe-Py complexes in the Aulne estuary to better identify and distinguish the active processes between  
630 the dissolved, colloidal and particulate phases occurring in the land-sea continuum as described by  
631 (Isaji et al., 2020). Moreover, this sensitive chemiluminescence technique for the detection of Fe-Py  
632 complexes in seawater offers the potential for ship-board and coastal platform analysis as well as  
633 sensor development. Further analytical work will be required on detection limit improvement for Fe-  
634 Py detection at nanomolar scale and to widen its application to open ocean waters. In the near  
635 future, the use of this method to rapidly detect average concentrations of batch Fe-Py complexes  
636 could be a useful and complementary tool to combine with HPLC methods more focused on heme b  
637 detection in natural samples. A comparison of these two analytical methods would also be of major  
638 interest.

639

640 Author statement

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647

648 Declaration of competing interest

649 The authors declare that they have no known competing financial interests or personal relationships  
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652

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659 5- References

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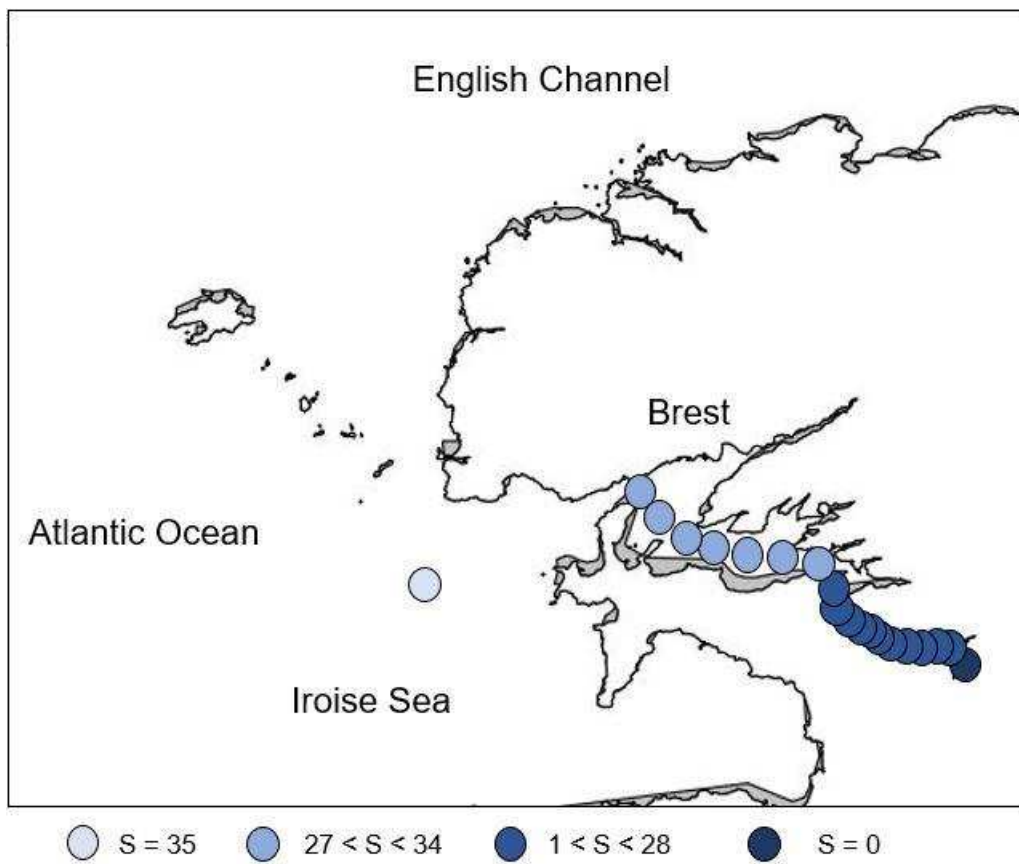
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930 Figures :

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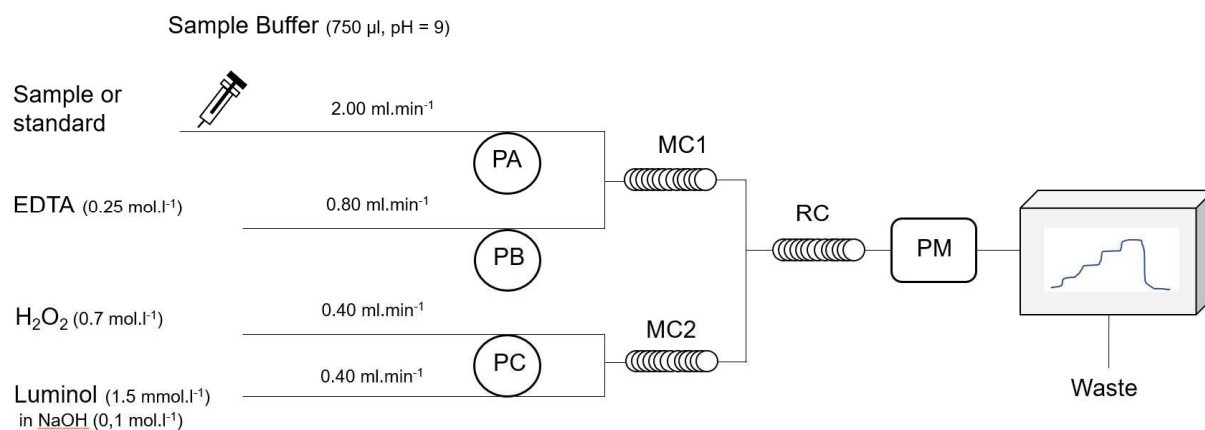
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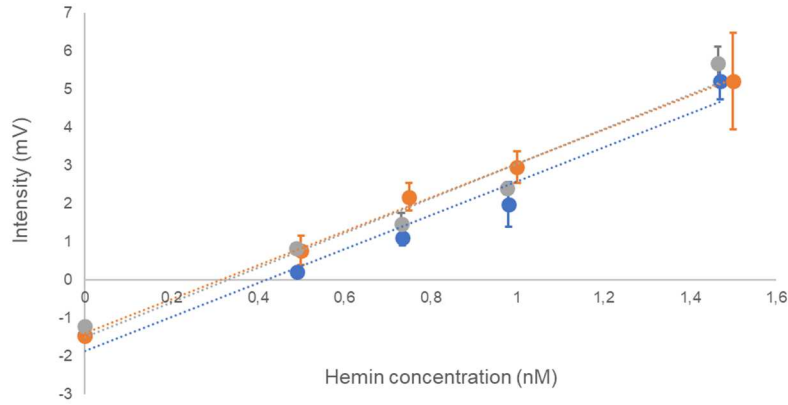
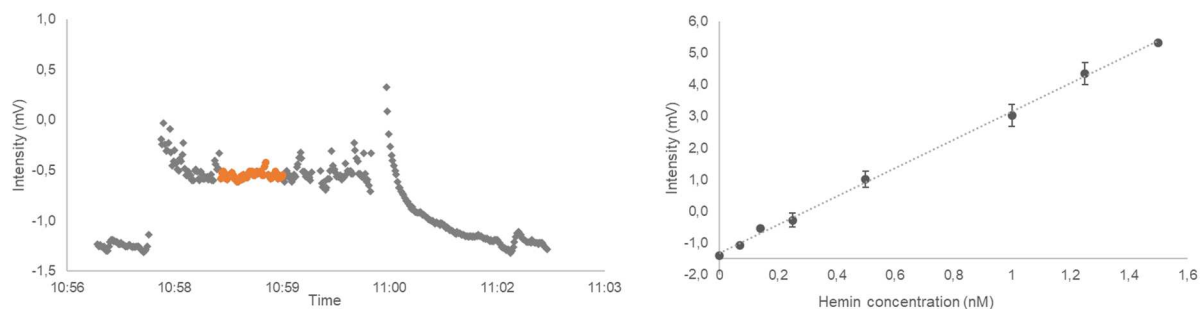


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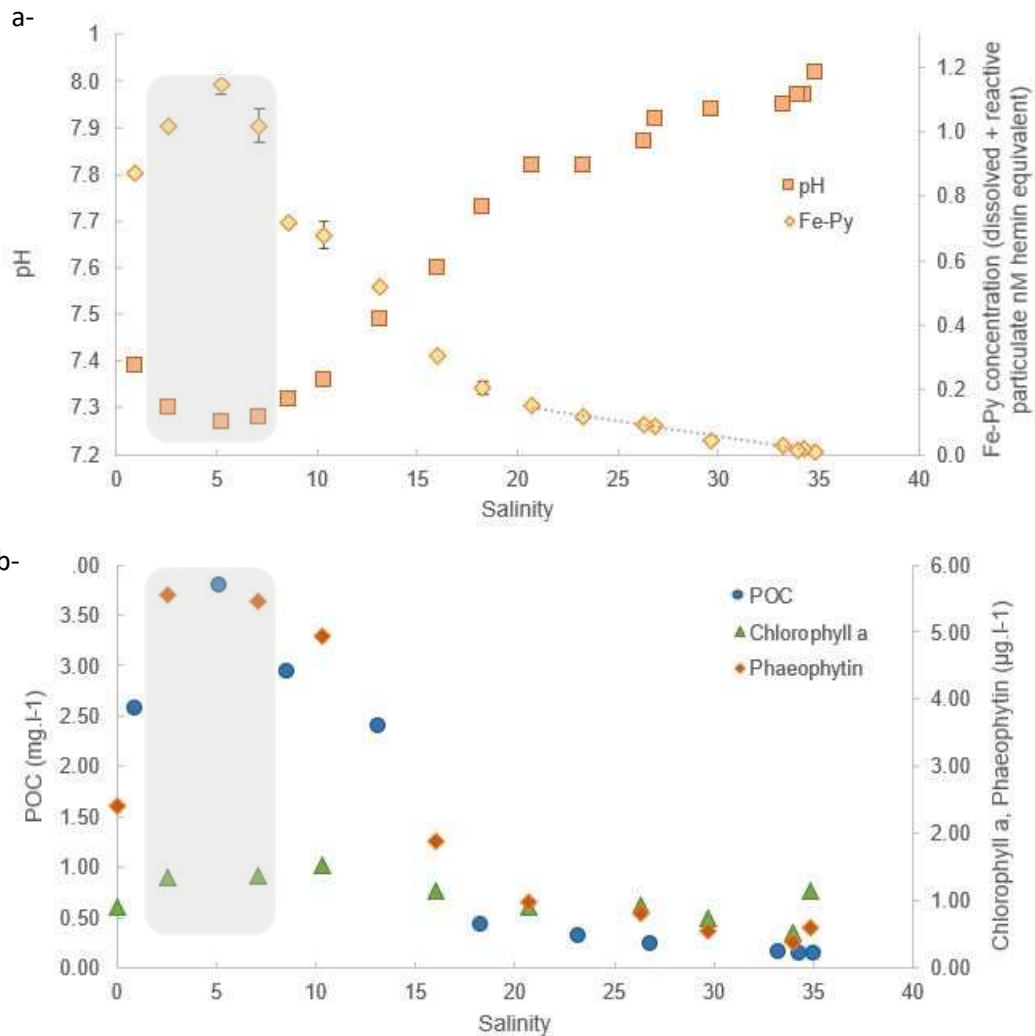
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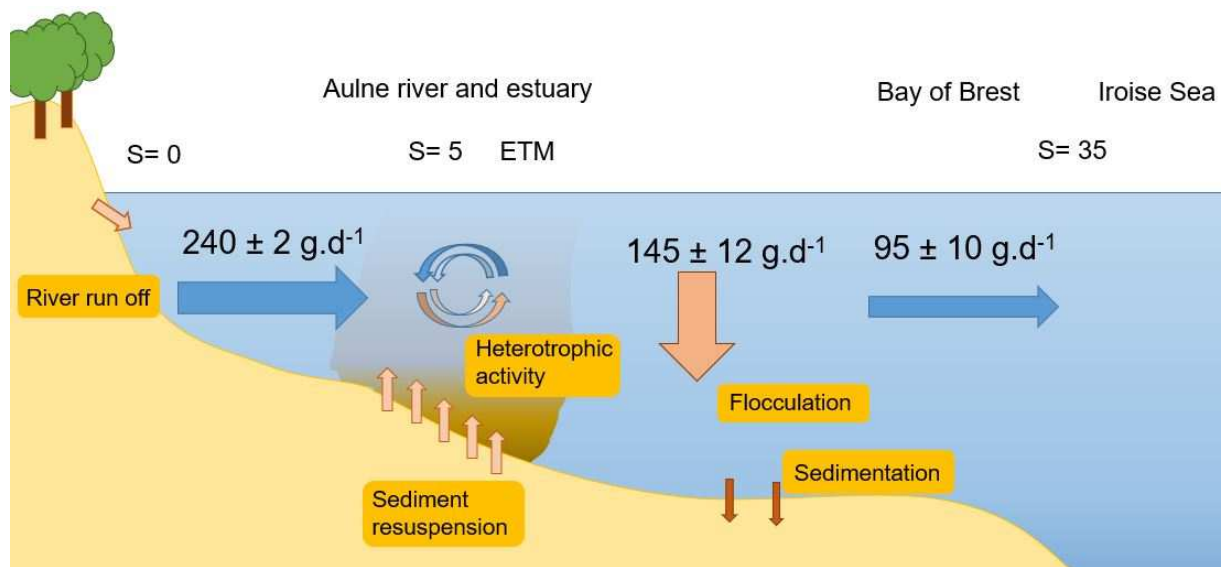


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Fig. 5: a- Evolution of the concentrations of Fe–Py complexes (yellow diamonds nM hemin equivalent,  $n=3$ ), pH (green squares), b- Evolution of the concentrations of POC ( $\text{mg}\cdot\text{l}^{-1}$ , blue circles) and Chlorophyll a ( $\mu\text{g}\cdot\text{l}^{-1}$ , green triangles) and phaeophytin ( $\mu\text{g}\cdot\text{l}^{-1}$ , red diamonds) for the samples collected along the salinity transect of the Aulne river, estuary and Bay of Brest (shaded rectangle: Maximum Turbidity Zone, MTZ), linear regression for the conservative part of the gradient a- Fe-Py = -  $0.010S+0.348$ ,  $R^2 = 0.98$ ,  $n= 9$ , Student test = 2.36



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*Fig. 6: Mass Balance of Fe-Py complexes and the potential associated biogeochemical processes in the Aulne Estuary-Bay of Brest system*

Tables

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1043 *Table 1: Various organic potential interferents, the tested concentrations in the experimental designs*  
 1044 *and the associated response*

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Nature of the potential interferent	Name of the compounds	Range of concentrations	Interference
<b>Metals</b>	Fe <sup>3+</sup>	0, 350, 700 nM	None
	Fe <sup>2+</sup>	0, 25, 50 nM	None
	Mn <sup>2+</sup>	0, 350, 700 nM	None
	Zn <sup>2+</sup>	0, 350, 700 nM	None
	Cu <sup>2+</sup>	0, 50, 100 nM	None
<b>Porphyrins</b>	Chlorophyll a (Mg)	0, 25, 50 µg.L <sup>-1</sup>	None
	Protoporphyrin IX (no metal)	0, 1, 2 nM	None
	Tetraphenylporphine Mn(III) chloride	0, 1, 2 nM	None
<b>Siderophores</b>	Ferrioxamine E (Fe)	0, 10, 20 pM	None
	Deferoxamine	0, 10, 20 pM	None
	Pyoverdines	0, 10, 20 pM	None
<b>Humic Substances</b>	Elliott Soil fulvic acid (ESFA)	0, 2250, 4500 µg.l <sup>-1</sup>	None
	Suwannee River fulvic acid (SRFA)	0, 1125, 2250 µg.l <sup>-1</sup>	None

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1048 *Table 2: Figures of merits of the Fe-Py method*

Analytical parameters	Fe-Py response
Calibration slope	4.467*10 <sup>+9</sup> ±0.069*10 <sup>+9</sup>
Calibration curve intercept	-1.321±0.055
Regression coefficient	0.9986
Linearity range	0.024 – 150 nM
Detection limit (3 x sd, blank n=10)	7.2 pM
Precision (% , 0.25 nM, n=8)	6.35 %
Time of analysis	90 s

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Table 3: Compilation of existing techniques for heme b or Fe-Py analysis in seawater

Reference	Analysis time	Method	Sample pre-treatment	Heme detection limits when available or detection levels	Volume sample	Nature of the sample
(Gledhill, 2007)	>1 hour	High Performance Liquid Chromatography Diode Array Spectrophotometry (HPLC-DAD)	Extraction from filtered (0.8-0.2 $\mu$ m) cells (2.5% octyl $\beta$ -glucopyranoside in 0.02 M ammonium hydroxide), ultrasonication and centrifugation, and filtration (0.25 $\mu$ m) before injection	0.08 pmol or 1.57 nM (50 $\mu$ L injection volume)	30-50 ml	Particulate material from phytoplankton culture
(Honey et al., 2013)	>1 hour	High Performance Liquid Chromatography Diode Array Spectrophotometry (HPLC-DAD)	For seawater samples: filtration (glass microfiber), extraction (ammoniacal EMPIGEN detergent solution), for phytoplankton cultures: extraction from filters (glass microfibers) using acid acetone (acetone:HCl) and extraction (ammoniacal detergent), for both ultrasonication, centrifugation and filtration (0.2 $\mu$ m) before injection	Not indicated	2-4 l	Particulate material from batch culture and Celtic Sea and subtropical north Atlantic samples
(Gledhill et al., 2013)	>1 hour	High Performance Liquid Chromatography Diode Array Spectrophotometry (HPLC-DAD)	Filtration (0.7 $\mu$ m glass fiber), storage (< 80°C), extraction (0.02M NH <sub>4</sub> OH and zwitter-ionic detergent EMPIGEN), centrifugation, and filtration (0.2 $\mu$ m) before analysis	1.6nM	1-4 l	Particulate material from Atlantic Ocean seawater (Iceland Basin, tropical northeast Atlantic, Scotia Sea)
(Gledhill, 2014)	>1 hour	High Performance Liquid Chromatography (HPLC) Electropray Ionisation Mass spectrometry (ESI-MS)	Filtration (0.7 $\mu$ m), storage at -80°C, extraction (ammoniacal OGP), ultrasonication on ice, centrifugation and filtration (0.2 $\mu$ m) before analysis	43.5 $\pm$ 12.5 fmol (DAD) 1.2 $\pm$ 0.5 fmol (ESI-MS)	2l	Particulate material from phytoplankton culture and seawater samples from North Atlantic
(Gledhill et al., 2015)	>1 hour	High Performance Liquid Chromatography Diode Array Spectrophotometry (HPLC-DAD), Electropray Ionisation Mass spectrometry (ESI-MS)	Filtration (0.7 $\mu$ m glass fiber), extraction into ammoniacal detergent (octyl $\beta$ -glucopyranoside), storage -80°C prior to analysis	Not indicated, lowest concentration detected on particulate material in culture (10 $\pm$ 3 pM)	Not indicated	Particulate material from phytoplankton culture with Southern Ocean seawater
(Bellworthy et al., 2017)	>1 hour	High Performance Liquid Chromatography Photo-Diode Array detector (HPLC-PDA), Electropray Ionisation Mass Spectrometer (ESI-MS)	Filtration (0.7 $\mu$ m glass microfiber), storage at -80°C, extraction into ammoniacal detergent (2.5% Octyl gluco pyranoside in 0.02 mol L <sup>-1</sup> NH <sub>4</sub> OH), centrifugation, filtration (0.2 $\mu$ m) before analysis	9 $\pm$ 8 pmol heme b L <sup>-1</sup> seawater	1 l	Particulate material from Gullmar Fjord off Sweden mesocosm seawater
(Louropoulou et al., 2019)	>1 hour	High Performance Liquid Chromatography (HPLC) Electropray Ionisation Mass spectrometry (ESI-MS)	Filtration of seawater on glass fiber filters (GF/F, pore size 0.7 $\mu$ m), storage at -80 °C, extraction (w-v Octyl $\beta$ -D-glucopyranoside-OGP, NH <sub>4</sub> OH), centrifugation ultrasonication on ice, and filtration (0.22 $\mu$ m) before analysis	0.01 to 0.02 pM for field samples	Not indicated	Particulate material from subpolar and subtropical North Atlantic Ocean seawater

(Isaji et al., 2020, 2019)	>1 hour	Ion-exchange open column chromatography, High Performance Liquid Chromatography Diode Array Spectrophotometry (HPLC-DAD) or liquid chromatography mass spectrometry (LC-MS/MS), nano-elementary analyser isotope ratio mass spectrometry (EA/IRMS)	Filtration of seawater on glass fiber filters (GF-75, pore size 0.3 $\mu\text{m}$ ), for biological samples and sediment frozen ( $-20^{\circ}\text{C}$ ) Multi-step extraction with acetone or acetone/0.5 M HCl (4:1), ultrasonication 5 min on ice and centrifugation, extraction in a 1:2 mixture of DCM and 0.1 M HCl. DCM fraction dried under Ar gas at $30^{\circ}\text{C}$ . For filtered seawater, 30 mL mixed with 1 mL of 12 M HCl and 6 mL of acetone, extracted three times with 6 mL of DCM. DCM fractions dried under Ar gas.	HPLC DAD: picomole level, MRM LC-MS/MS 1fM of heme injected	30 ml for the dissolved phase	Particulate and dissolved material from coastal seawater of Tokyo Bay
(Louropoulou et al., 2020)	>1 hour	High Performance Liquid Chromatography Diode Array Detection (HPLC-DAD), Electrospray Ionisation Mass Spectrometry (ESI-MS)	Filtration of seawater on glass fiber filters (GF/F, pore size 0.7 $\mu\text{m}$ ), storage at $-80^{\circ}\text{C}$ , extraction (w-v Octyl $\beta$ -D-glucopyranoside-OGP), centrifugation, and filtration (0.2 $\mu\text{m}$ ) before analysis	1.57 nM (HPLC DAD), 190 pmol heme b L <sup>-1</sup> (HPLC ESI) 32 pmol heme b L <sup>-1</sup> (MS)	Not indicated	Particulate material from subpolar North Atlantic to the subtropical South Atlantic seawater
(Vong et al., 2007)	3 minutes	Flow injection analysis (FIA) with chemiluminescence detection	Filtration (0.2 $\mu\text{m}$ ), buffer addition $\text{NH}_3/\text{NH}_4$ before direct injection or storage with $\text{HgCl}_2$ at $-20^{\circ}\text{C}$ , buffer addition $\text{NH}_3/\text{NH}_4$ before analysis	0.11 nM	3.5ml	Dissolved material (<0.2 $\mu\text{m}$ ) from Guiana, Rhone and Berre river samples and phytoplankton cultures
<i>This study</i>	4 minutes	Continuous flow analysis (CFA) with chemiluminescence detection	Storage with $\text{NaN}_2$ at $-20^{\circ}\text{C}$ , buffer addition $\text{NH}_3/\text{NH}_4$ before analysis	7.2 pM	10ml	Dissolved and particulate material from the Aulne estuary North Atlantic seawater

Table 4: Salinity, pH, Fe-Py (nM hemin equivalent), Chlorophyll a ( $\mu\text{g.l}^{-1}$ ), phaeophytin ( $\mu\text{g.l}^{-1}$ ), POC concentrations ( $\text{mg.l}^{-1}$ ) and C/N ratio observed along the Aulne estuary- Bay of Brest system

Sample	Salinity	pH	Fe-Py (nM hemin equivalent)	sd	Chlorophyll a ( $\mu\text{g.l}^{-1}$ )	Phaeophytin ( $\mu\text{g.l}^{-1}$ )	POC concentrations ( $\text{mg.l}^{-1}$ )	C/N ratio
19	35.05	8.09	< LD				0.03	5.63
18	34.86	8.02	0.010	0.004	1.16	0.61		
17	34.3	7.97	0.019	0.002			0.026	6.53
16	33.98	7.97	0.013	0.002	0.53	0.39		
15	33.27	7.95	0.029	0.003			0.029	6.33
14	29.68	7.94	0.045	0.002	0.75	0.54		
13	26.83	7.92	0.087	0.003			0.043	6.74
12	26.3	7.87	0.092	0.003	0.94	0.82		
11	23.22	7.82	0.117	0.007			0.048	7.98
10	20.71	7.82	0.154	0.010	0.91	0.98		
9	18.26	7.73	0.206	0.019			0.059	8.48
8	16	7.6	0.307	0.004	1.14	1.89		
7	13.12	7.49	0.516	0.003			0.255	11.00
6	10.34	7.36	0.678	0.043	1.52	4.95		
5	8.59	7.32	0.718	0.004			0.316	10.85
4	7.1	7.28	1.016	0.054	1.36	5.45		
3	5.2	7.27	1.142	0.031			0.414	10.67
2	2.6	7.3	1.012	0.003	1.33	5.53		
1	0.92	7.39	0.873	0.007			0.292	10.30

## Figure legends

Fig 1: Map of the study area (Western Brittany, France) with the 20 sampling points along the saline gradient from freshwater in the Aulne, towards the Bay of Brest and finally the Iroise sea.

Fig. 2: Schematic view of the manifold for the Fe-Py complex determination by Continuous Flow Analysis and chemiluminescence detection (PA, PB, PC: peristaltic pumps, MC1: mixing coil 1, L=4500 mm, ID = 0.8 mm, MC2: mixing coil 2, L= 500 mm, ID = 0.8mm, RC: reaction coil, 122mm, ID = 0.8mm; PM: Photo multiplier). All the tubings were made of tygon (VWR) except the spiral cell located in front of the photomultiplier which was made of Teflon (Upchurch ID 0.8 mm).

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### Highlights:

- Rapid and simple detection of Fe-Py complexes was developed for marine waters
- Fe-Py complexes distribution was reported for the first time in a salinity gradient
- Aulne estuary acts as a sink for Fe-Py, decreasing their input to the Atlantic Ocean