



Supplement of

Comparing CLE-AdCSV applications using SA and TAC to determine the Fe-binding characteristics of model ligands in seawater

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**Supplementary information
Tables**

Table S1: Equipment and chemicals. Instrument setting and chemical concentrations of the three applications, TAC, SA5 and SA25 on two voltammetric set-ups, Metrohm and BASi. The TAC application was used only with the Metrohm.

Titration with SA were done with the BASi, kinetics with both set-ups.

¹: see Table S3; ² the pH of the buffers was checked regularly by preparing a 10 ml sample (as for the titrations) and with the Fe addition that consisted of the addition of the largest volume of this acidified solution. ³: 0 to 1.2 nM with 0.2 nM intervals, ⁴: 0 to 3.0 nM with 0.5 nM intervals

	TAC Metrohm	SA Metrohm	SA BASi
electrode stand	VA663	VA663	controlled growth mercury electrode
voltammeter interface	µAutolab III, IME663	µAutolab III, IME663	Epsilon □2 (BASi)
reference electrode	Ag/AgCl with KCl	Ag/AgCl with KCl	RE-5B Ag/AgCl (3M KCl)
auxillary electrode	glassy carbon	glassy carbon	platinum, MW-1032
purge	nitrogen	air	non, open to the atmosphere
stirring	yes	yes	yes
Stirring	rod	rod	bean
dropsize	1 ¹	1 ¹	10 ¹
Software	Nova 1.9	Nova 1.9	ECDsoft
AL	(2-(2-thiazolylazo)-p-cresol (TAC)	Salicylaldoxime (SA)	
producer	Alfa Aesar	Acros Organics	
concentrations	10 µM	5 and 25 µM	
buffer	NH3/NH4OH borate buffer, pH=8.05 ²	NH3/NH4OH borate buffer, pH=8.2 ²	
producer	Merck, Merck supra pur NH4OH		
Fe standards	0-10.2 nM in 15 steps ³	0-10.2 in 12 steps ⁴	

Table S2 Electrochemical settings for the three setups

CSV parameters	Metrohm TAC	Metrohm SA	BASi SA
mode	differential pulse	differential pulse	differential pulse
purge time	120 s, 25s for duplicate	60 s, 25 s for duplicate	non
Deposition potential	-0.4V	0 V	0 V
deposition time	140 s	90 s	90 s
quiet time	5 s	5 s	10 s
Initial potential	-0.4V	0 V	-0.15 V
Final potential	-0.65 V	-0.7 V	-0.75 V
step potential	-0.004 V	-0.003 V	-0.006 V
Modulation amplitude	-0.02505 V	-0.05 V	-0.03 V
modulation time	0.004 s	0.004 s	
interval time	0.05 s	0.1 s	0.2 s
scan rate	40mV s ⁻¹	40mV s ⁻²	30 mVs ⁻¹

Table S3: Mercury drop volumes and surfaces calculated by weight for three electrodes and different mercury drop sizes. Size reflects the number indications on the electrode (1-14 for BASi, 1-3 for Metrohm). Size 10 was used at the BASi electrode for all other measurements, size 1 at the Metrohm electrode. N the number of drops collected. For the Metrohm this number can be ± 1 .

Metrohm electrode 2 was used for the SA, 4 for the TAC application.

Electrode	size	Total weight mg	N drops	mg per drop	volume mm ³	surface mm ²
BASi	14	442.4	75	5.90	0.422	2.72
BASi	14	347.2	60	5.79	0.414	2.69
BASi	10	243.0	60	4.05	0.290	2.12
BASi	10	245.9	60	4.10	0.293	2.14
BASi	5	68.1	63	1.08	0.077	0.88
BASi	5	60.2	59	1.02	0.073	0.84
Metrohm electrode 2	3	25.2	100	0.25	0.018	0.33
Metrohm electrode 2	3	24.5	100	0.25	0.018	0.33
Metrohm electrode 4	3	20.5	100	0.20	0.015	0.29
Metrohm electrode 2	1	11.2	99	0.11	0.008	0.19
Metrohm electrode 4	1	7.7	100	0.08	0.005	0.15

1. Tests

1.1 Test to observe the potential influence nitrogen with SA25 in a Metrohm stand

SA25 kinetics experiment in a Metrohm stand, with air purge and N for drop formation. Nitrogen did not leak into the headspace of the sample from the tube that pulsed the knocker. However, drop formation did emit pulses of nitrogen ending up in the headspace of the sample, purging with air before each measurement removed N.

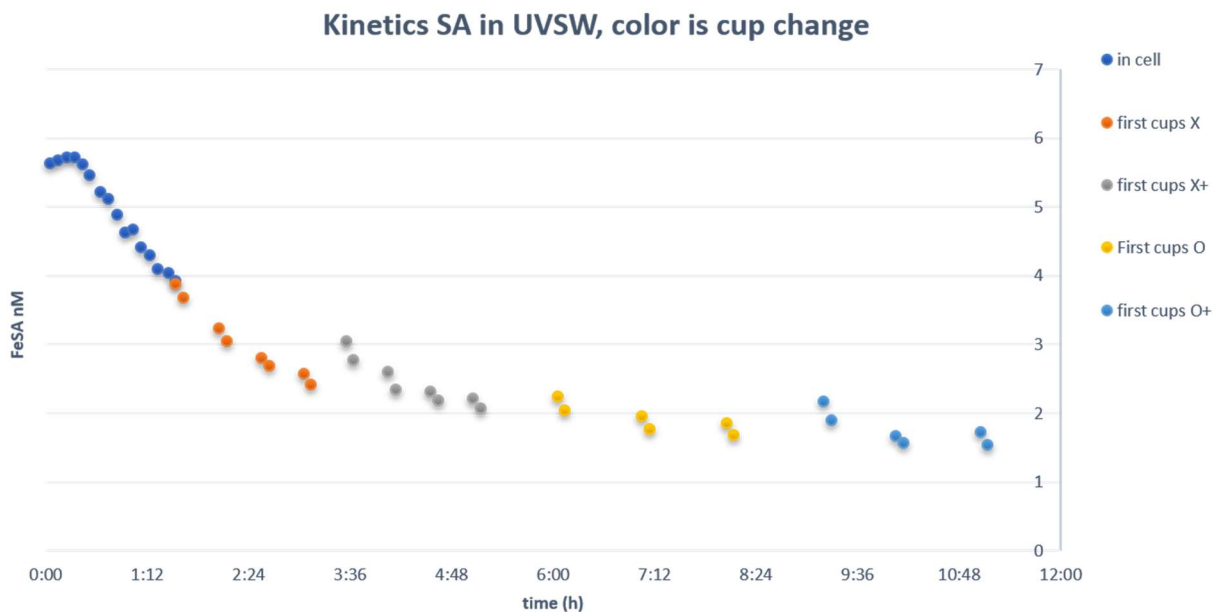


Figure S1: Kinetic experiment with SA25, using the Metrohm stand. Added DFe=7 nM. Every color show measurements of a specific sub-sample in a conditioned vial, all vials had the same t=0, when SA was added. The first measurements can be called in-cell. Upon changing sub-samples tiny shifts occurred, which might be due to changes in DFe and contamination during sub-sample change. Before every measurement the sample in the cell is purged with air, but pulses of nitrogen are introduced in the headspace of the sample when drops are formed. However, the decrease in signal with time is independent of whether the vials were positioned in the cell or not, which suggests that the reduction in FeSA was not related to any small ingresses of nitrogen that might result from the use of nitrogen during drop formation. Standard additions were done at the end of the experiment on kept samples and concentrations were calculated from peak heights.”

1.2 Purge step

For the effect of purging in the SA application an extra purge step with air during 60 s was introduced in the protocol for the BASi electrode, whereas for Metrohm the purge time was set to 0 instead of the normal 60 s purge time. Measurements were repeated continuously during 1 h in the same 10 ml volume (protocol 1 in section 2.4.1 of the main text and Tables S1 and S2). Results are shown in figure S1.

We hardly noticed an effect of purging in sensitivity with SA5. At the end of the experiments, peaks from experiments that were purged were 1.6 times higher than when not purged using the Metrohm and 4 times higher when using the BASi instrument (Figure S1). However, the decreases were very similar, with or without purge. It must be noted that we did not compare air versus nitrogen as was done by Abualhija and Van den Berg (2014), but an air purge versus no purge. Nitrogen did not leak into our cell and did not blanket the sample during the test.

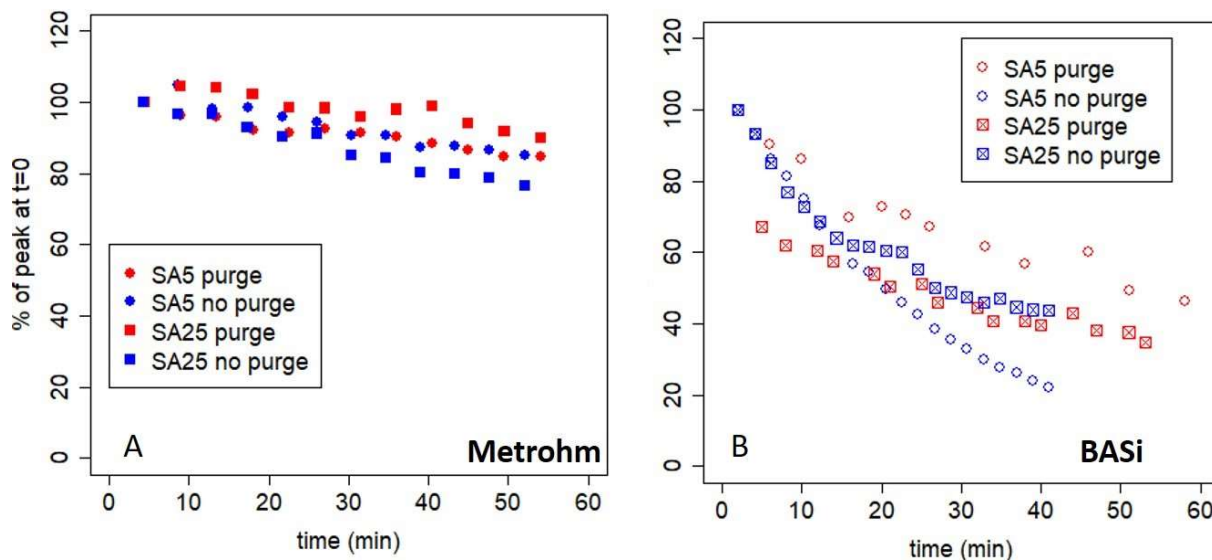


Figure S2: Measurements using SA5 and SA25 in UV irradiated seawater with 6 nM DFe of FeAL with time (s) with and without a purge step in both setups, A: Metrohm, B: BASi. The data is in % of the first recorded peak height. Drop size 1 for Metrohm, size 10 for BASi.

1.3 Mercury drop size and effect of mercury accumulation on cell bottom

We needed to know the drop size to connect the variability in sensitivity and study the impact of mercury on the bottom of the cell on the decrease of the peak height. We determined the mercury drop size by releasing and weighing drops with a micro balance ($SD = 3 \mu\text{g}$) of both electrodes at different sizes, assuming the formation of a perfect sphere (Table S3).

For testing the effect of mercury accumulation on the cell bottom the voltammetric procedure was repeated over a 40 to 50 min period in UV-irradiated water containing 6 nM extra added Fe. The number of repetitions depended on the duration of the measurements and was 9 times for the Metrohm setup and 20 times for the BASi setup. For BASi drop sizes 5, 10 and 14 (= maximum size) were applied, for Metrohm drop sizes 1 and 3 (= maximum size) (Figure S4).

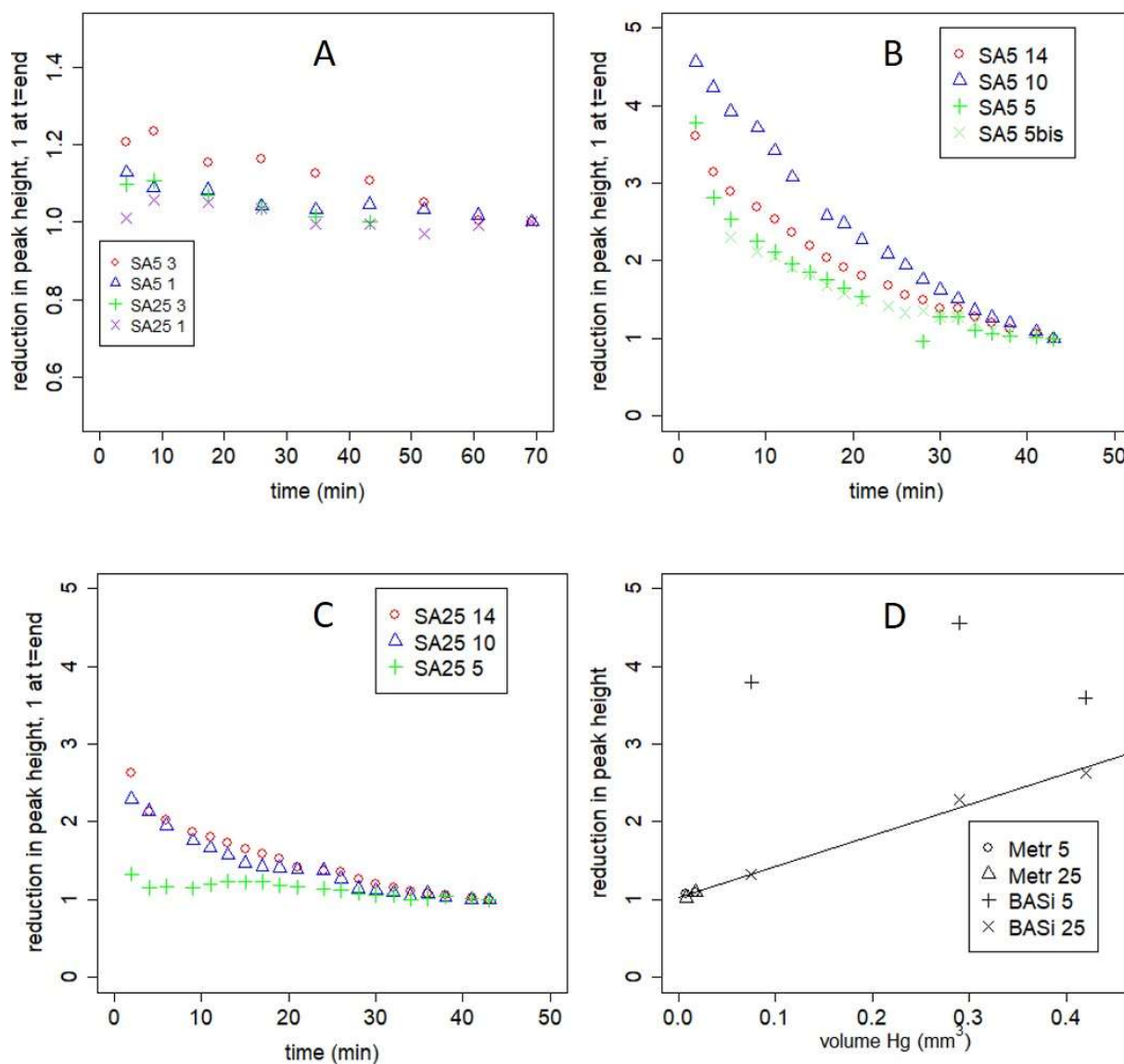


Figure S3: The effect of drop size and accumulation of drops at the bottom of the measuring cup. The reduction in peak size over time might be due to adsorption of the electro active Fe complex on the puddle of dispensed mercury formed in time on the bottom of the cell. S2A,B,C: Peak height versus time for different drop sizes in UV irradiated seawater with 6 nM added Fe. A: Metrohm, SA5 and SA25, drop sizes 1 and 3 (Table S3), B and C: BASi drop sizes 5,10 and 14 (Table S3), B gives SA5 data, C gives SA25 data. The last recorded peak t=end per experiments is set as 1, the other peak heights are related by division through the peak at t=end. T=end is approximately 1 hour for the Metrohm, and 43 minutes for the BASi equipment. Experiment SA5 with drop size 5 at BASi was done in duplicate. S2D: peak height reduction in 43 minutes, versus the volume of dispensed mercury at the bottom of the cell at t=43. Metrohm hardly showed any difference between SA5 and SA25

We tested whether SA adsorbed reversibly on the mercury drops by transferring mercury from SA5 and SA25 applications into seawater containing no SA. If SA adsorbs reversibly, a FeSA signal should be present upon analysis. We used both BASi and Metrohm setups and first measured a sample containing 6nM DFe and buffer but

without SA. Then SA was added (5 and 25 μM in two experiments). A measurement was executed to get a signal, then 20 Hg drops drop size 10 collected from the BASi electrode were added for in the samples for both Metrohm and BASi. The mixtures were left for one hour after which the seawater was discarded. A remnant of seawater was removed by adsorption on the point of a tissue. The remaining mercury was transferred in a seawater sample with the same amount of DFe and buffer as usual but without SA and was subsequently measured.

1.4 In cell and bottle experiments

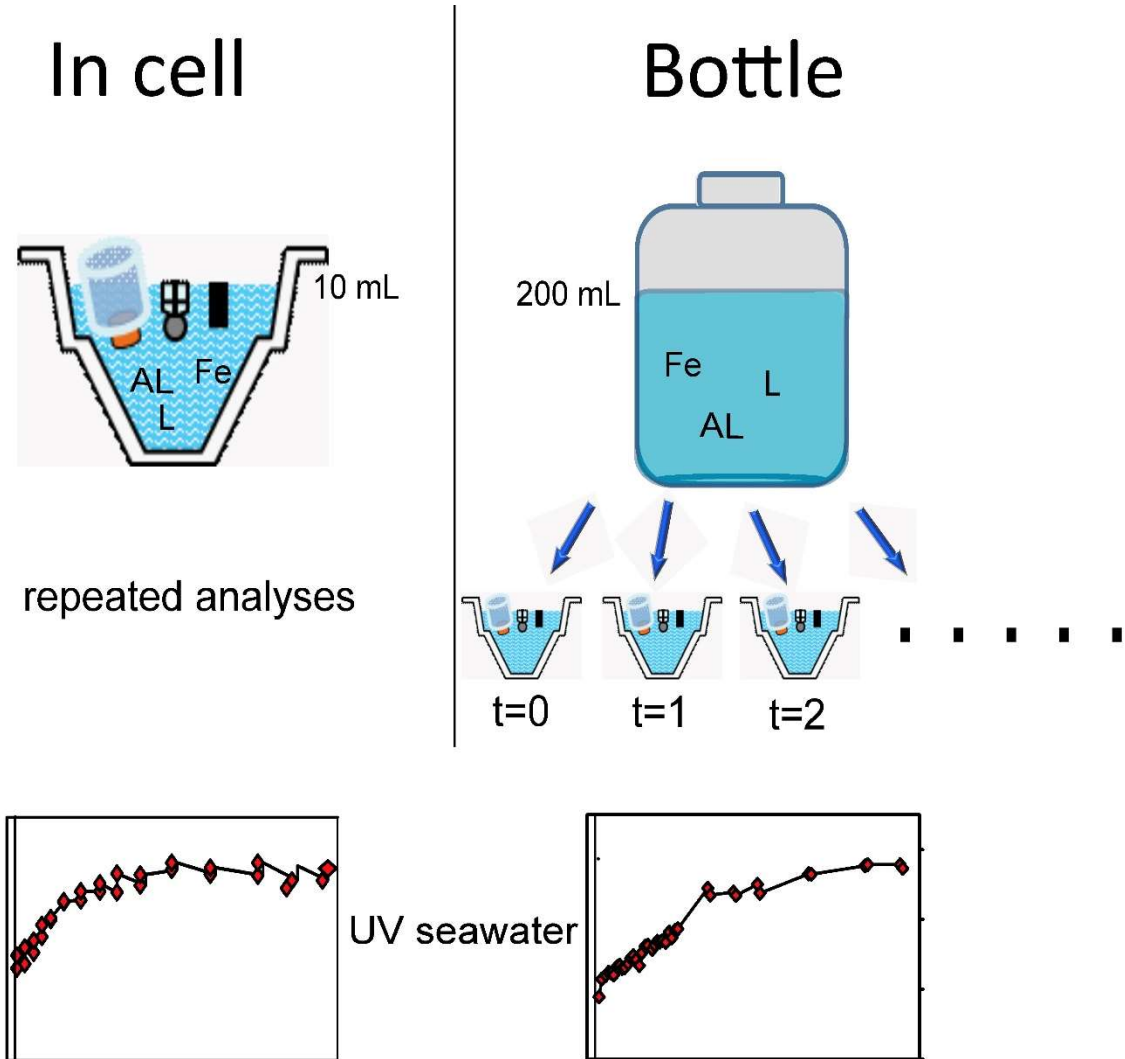


Figure S4: Scheme of the kinetic experiments with at the left the ‘in cell’ procedure and at the right the procedure for the bottle experiments. Below an example of the results in UV irradiated seawater without natural ligands (L) is shown (see figure 6).

1.5 SA concentration

We tested the influence of the SA-concentration on the stability of the measurements over time. We used concentrations between 2.5 and 25 μM , used the Metrohm setup with a small mercury drop (size 1), with regular air purging. By using Metrohm with the smallest drop size and regular air purging we excluded potential interferences due to decreasing oxygen and adsorption on dispensed mercury on the cell bottom.

1.6 Dissociation experiments

In order to check whether dissociation of FeSA2 was possible we did two experiments, one adding a competing ligand to trigger dissociation and one to dilute the SA concentration from 25 to 5 μM SA. The first experiment was done with a Metrohm electrode, small drop size and with regular purging in 10 ml UV-irradiated seawater. The experiment was done twice, with SA5 and SA25. The sample was measured regularly according to the in-cell kinetics (see section 3.3.1). After 80 minutes, 150 nM DTPA was added and the measurements were continued for another two hours.

2. Calibration

In the calibration the conditional stability constants for the Fe-complexes, K^{cond} and β^{cond} values, are defined by,

$$K_{FeDTPA}^{cond} = \frac{[FeDTPA]}{[Fe^{3+}] \times [DTPA]'}, \quad (1)$$

$$\text{and } [FeDTPA] = K_{FeDTPA}^{cond} \times [Fe^{3+}] \times [DTPA]' = \alpha_{\Sigma FeDTPA, Fe^{3+}} \times [Fe^{3+}], \quad (2)$$

with $\alpha_{\Sigma FeDTPA, Fe^{3+}}$ or side reaction coefficient of DTPA with respect to Fe^{3+} .

$$\beta_{FeTAC}^{cond} = \frac{[FeTAC_2]}{[Fe^{3+}] \times [TAC]^2}, \quad (3)$$

$$\text{and } [FeTAC_2] = \beta_{FeTAC}^{cond} \times [Fe^{3+}] \times [TAC]^2 = \alpha_{Fe(TAC)_2, Fe^{3+}} \times [Fe^{3+}]. \quad (4)$$

$$\beta_{FeSA}^{cond} = \frac{[FeSA_2]}{[Fe^{3+}] \times [SA]^2}, \quad (5)$$

$$\text{and } [FeSA_2] = \beta_{FeSA_2}^{cond} \times [Fe^{3+}] \times [SA]^2 = \alpha_{FeSA_2, Fe^{3+}} \times [Fe^{3+}]. \quad (6)$$

$$K_{FeSA}^{cond} = \frac{[FeSA]}{[Fe^{3+}] \times [SA]}, \quad (7)$$

$$\text{and } [FeSA] = K_{FeSA}^{cond} \times [Fe^{3+}] \times [SA] = \alpha_{FeSA, Fe^{3+}} \times [Fe^{3+}]. \quad (8)$$

Thus, the division of Fe over the species depends on the α -values of the calibration ligand DTPA and the AL; for SA this results in the mass balance,

The reduction in peak height or signal with increasing [DTPA] is calculated as a fraction, X, X = 1 at [DTPA]=0 (Figure S4).

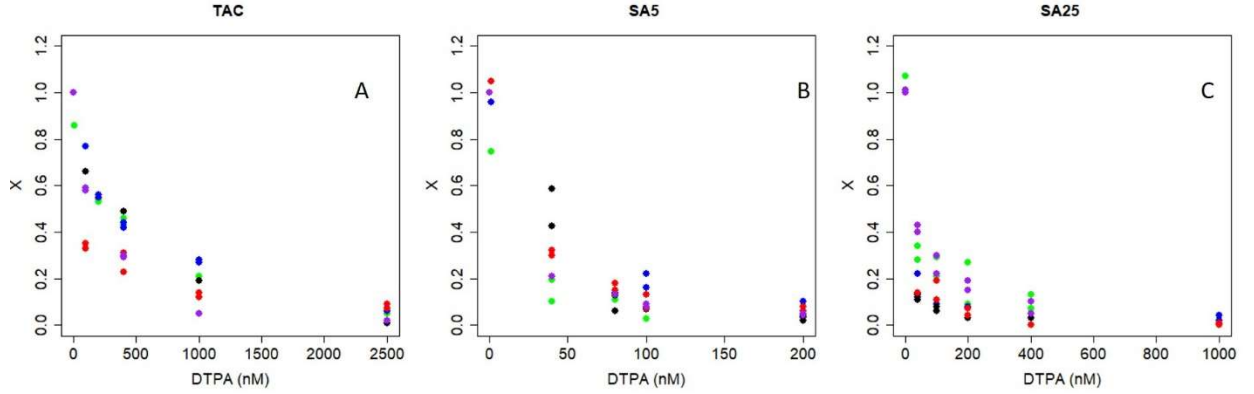


Figure S5: Calibration of the three applications (A, TAC, B, SA5, C, SA25) with DTPA. The calibrations were repeated 4 times indicated by the different colors. X is the peak height divided by the peak height at zero DTPA.

The peak height reduction, X, is directly related to $\alpha_{\Sigma FeDTPA, Fe3+}$,

$$\alpha_{\Sigma FeDTPA, Fe3+} = (1-X) \alpha_{\Sigma FeAL, Fe3+}, \quad (9)$$

which is,

$$K_{DTPA}^{cond} \times [DTPA'] = ((1-X) K_{AL}^{cond} \times [AL']). \quad (10)$$

Using several [DTPA] one can precisely estimate the only unknown parameter K_{AL}^{cond} . The calculation is straightforward for TAC as AL. However, it becomes more complicated when two complexes are formed between Fe and the AL, as is the case for SA, and even more so if one of them is not electro-labile and has to be discounted from the non-labile fraction as proposed by Abualhaija and Van den Berg (2014). They assumed the formation of an electro-active FeSA and a non-electro-active Fe(SA)₂. This last complex causes a reduction in sensitivity at higher [SA], whereas the competing strength, the side-reaction increases.

Then (10) becomes,

$$(K_{DTPA}^{cond} \times [DTPA'] = (1-X)((K_{FeSA}^{cond} \times [SA']) + (\beta_{FeSA_2}^{cond} \times [SA']^2)). \quad (11)$$

When using several SA concentrations, this is mathematically straightforward, assuming that equilibrium is achieved which cannot be guaranteed since SA25 used the short waiting time of 15 min. Still neglecting the assumption of equilibrium, using two SA concentrations we could reasonably estimate $\log K_{FeSA, FeI}^{cond} = 5.48$, but not $\log \beta_{FeSA_2, FeI}^{cond}$. The value of $\log \beta_{FeSA, FeI}^{cond}$ could vary between 6.4 and 9.9 without influencing the quality of the fit to a large extent. The combination of these, only two, concentrations and the difference in protocol between them made it not possible to estimate $\beta'_{Fe(SA)_2}$ in a precise way. We do not need the separate K_{FeSA}^{cond} and β_{FeSA}^{cond} values, because the overall α , $\alpha_{\Sigma FeSAx, Fe3+}$ is sufficient (Table 2) (Hudson et al., 2003; Gledhill and Buck 2012; Gledhill and Gerringa, 2017; Gerringa et al., 2014). It is indeed necessary to use α 's since for SA25 Fe(SA)₂ is formed next to SA5 FeSA. Here the higher [SA] increases competition with the natural ligands, represented by $\alpha_{\Sigma FeSAx, Fe3+}$,

whereas the sensitivity decreases due to a decrease in the exclusive electro labile species FeSA due to formation of $(SA)_2$ (Buck et al., 2007; Abualhaija and Van den Berg, 2014). Only for the ease of recognition and comparison with the literature, we calculated besides $\log\alpha_{inorg}$ also the values of β^{cond} assuming that only Fe(SA)₂ is formed and is electro-active and K^{cond} assuming only FeSA is formed and electro-active (Table 2).

3 Ligand concentrations

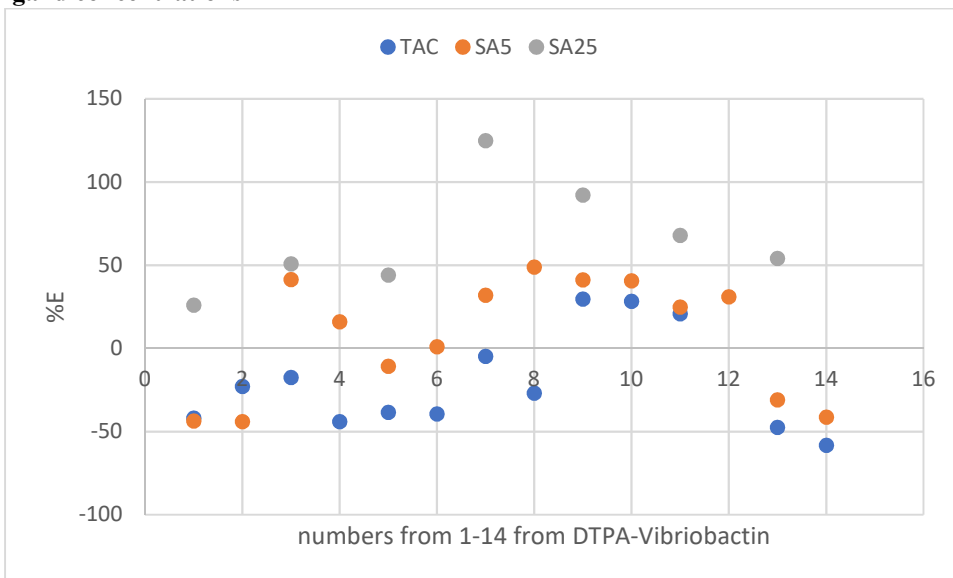


Figure S6: The percentage error (E%) of the estimated ligand concentration per AL [LAL] compared to the added concentration [I]: $E(\%) = ((LAL - [I]) / [I]) \times 100$ for each synthetic model A ligand.

1,2=DTPA; 3,4=phytic acid; 5,6= desferrioxamine B, 7,8=Ferrichrome, 9,10=2nM Ferrioxamine E; 11,12=4nM Ferrioxamine E; 13,14=vibriobactin.

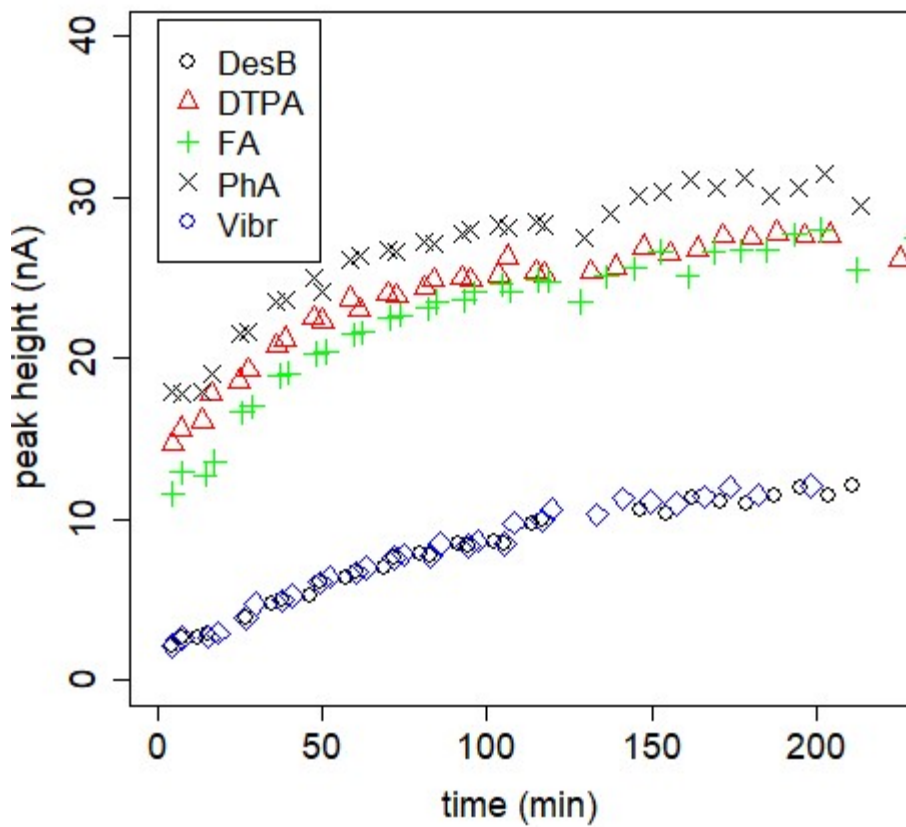


Figure S7: In cell kinetic experiments with the TAC application for different model ligands added at 2nM for the model A ligands and 0.2 mg for the model B ligand (FA).