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Iron chelating agents and their effects on the growth of *Pseudokirchneriella subcapitata, Chlorella vulgaris, Phaeodactylum tricornutum* and *Spirulina platensis* in comparison to Fe- EDTA

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Abstract
The industry standard iron source for algae culture is in most cases Ethylenediaminetetraacetic acid ferric sodium salt (Fe-EDTA). During algae production pH and light intensity are not favorable for maintaining the integrity of the Fe-EDTA complex. As a consequence, iron availability becomes the limiting factor for growth. A series of growth studies and biomass determinations with a selection of alternative iron chelating agents were conducted under controlled conditions. Fe-EDTA was used as the control iron source to determine if any potential advantages of the test chelates could be identified. Results demonstrated slower initial growth in short term tests for chelating agents with higher stability constants than Fe-EDTA and similar results for iron chelating agents with a similar or lower stability constant. Considerable long term improvements in algae growth during extended studies (>7 days) with *Chlorella vulgaris* and *Spirulina platensis* were observed for higher stability chelates. Optimization of the iron source with chelating agents that have better pH and light stability can delay the onset of iron limiting conditions. Advantages to commercial batch processes such as increasing the growth between cleanouts, elevating lipid and biomass production and possible savings of raw materials are potentially achievable with optimized iron chelation.

Keywords: *Spirulina platensis; Chlorella vulgaris;* Iron chelation; Algae micronutrients; pH; Algae growth; Biomass.

Introduction

Iron Nutrition

Iron is an essential micronutrient for plant (and algae) growth. Despite being one of the most abundant elements on the planet it is often the first element to become growth limiting for algae due to its physical chemical properties. Ferric oxide (Fe_2O_3) is the most prevalent form of iron and has extremely low solubility and is often present in a hydrated or phosphate form ($Fe_2O_3 \cdot H_2O$ / $FePO_4$). At higher pH the hydroxide ($Fe(OH)_X$) forms prevail (Hochmuth 2011). Iron hydroxide is even less soluble than the hydrate or phosphate forms resulting in a tendency for iron deficiency under such conditions. At lower pH's the solubility of iron increases making iron exchange possible via humic and fulvic acids (natural chelators) in the soil and hence iron deficiency at lower pH is generally less of a problem.

Iron is required in the algae cell for chlorophyll production although not being a part of the pigment itself. It is also present in enzyme cofactors for nitrogenase, hydrogenase enzymes. Iron is an integral part of the electron transport systems cytochromes found in chloroplasts and mitochondria as well as the iron containing protein ferredoxin that assist the electron transport reactions. (Purves et al 1996;Lewandoska et al 2004; Kelley J.C.O1974). Evidence exists that higher iron concentrations (in particular in the exponential growth phase) stimulate lipid production and result in increased biomass (Liu et al 2007). Iron availability may therefore not only be important for growth alone but also for the efficiency of the oil production process in the cell with the obvious commercial benefits thereof.

It is evident that iron is essential for algae growth and is often a limiting factor to proliferation of algae under natural conditions. Under culture conditions the same logic applies and hence solutions to the problem of biological availability of iron were already

sought right from the earliest days of commercial algae culture and are still of relevance today in terms of improving commercial algae culture processes.

Algae culture

Evidence exists that as far back as 2000 years ago algae was used in China as a food source during famine periods (Priyadarshani et al 2012).Some of the first documented algae cultures for research purposes dates back to the late 19th century (Granum et al 2002). Growth of algae for commercial processes dates back to the late 1940's with some of the earliest up scaling of pilot plants being documented by (Burlew 1953). The introduction of synthetic chelating agents during the 1950's made commercial algae production a possibility with Ethylenediaminetetraacetic acid (EDTA) replacing soil extract in the existing media recipes of the time and later being used as a metal ion buffer in seawater media (Sunda et al 2005). EDTA is today still the most widely used iron chelating agent used in the vast majority of fresh and salt water algae culture media.

Commercial culture of both marine and freshwater algae species for proteins, lipids, carbohydrates, carotenoids, vitamins, food and feed additives and cosmetics is well documented (Priyadarshani et al 2012). More recently attention has intensified further still with many seeing mass algae culture as one of the main alternatives to fossil fuels as well as being thought of as an important future addition to the human diet (Bekker 2007).

There are a plethora of studies and guidelines and other information sources detailing suitable or optimized media for every imaginable algae species. Sticking to the relevant species investigated during this study (Zarrouk 1966; Kong et al 2012; M.Sostaric et al 2009; Thurumala M 2012) and guidelines (OECD 2006; NPR65031980; ISO102531995) all describe standard algae growth media. These media form the basis for the reported investigations. The iron source being the only variable tested with Fe – EDTA used as the control chelate. There are undoubtedly variations in commercial media for the tested species not covered in the scope of these references. However the reoccurring take home message from the literature is the total dominance of Fe-EDTA as an iron source in almost all algae culture media whether it be for research or commercial purposes.

Higher stability chelates, (that is chelates with a stronger ligand metal ion interaction see Table 1) have been investigated to some extent by (Weger et al 2009.) The focus of their research being on the negative effects higher stability chelates (N,N'-di[2-hydroxybenzyl]ethylenediamine-N,N-diacetic acid) or [HBED] have on iron acquisition mechanisms of *Chlorella kessleri* under the tested conditions. Not the potential benefits higher stability chelates may have commercially under other conditions. In this sense commercial algae culture could be considered to be lagging behind more traditional agricultural sectors such as fruit farming that have developed customized feeding solutions to micronutrient deficiencies (Alvarez-Fernandez et al 2005). This is not limited to just the chelation of iron but also other micronutrients such as zinc, copper and manganese in both soil and soil less culture (AkzoNobel 2014). Furthermore the use of alternative iron chelating agents with a low stability constants such as N,N-Bis(Carboxymethyl) -L- Glutamic Acid Ferric sodium complex (Fe-GLDA) for example may also be beneficial should biodegradability be considered an important parameter for a particular use.

Iron deficiency

Micronutrient deficiency depends largely on the susceptibility of the crop being farmed and the conditions of the soil (Tindal et al 1996). Furthermore they describe the conditions under which iron deficiency can arise, indicated as pH conditions above 7.0 and the presence of free lime, ($CaCO_3$) that lowers the already poor solubility of ferric iron resulting in very limited availability to the plant. One effective method for preventing and to a lesser extent treating iron chlorosis under such conditions is by the application of synthetic iron chelates, ethylenediamine di(*o*-hydroxyphenylacetic) acid (EDDHA) and ethylenediamine di(*2*-hydroxy-4-methylphenylacetic) acid (EDDHMA) (Tindal et al 1996; Alvarez-Fernandez et al 2005). Iron deficiency is considered one of the most widespread cultivation problems to the fruit industry worldwide with regards to yield reduction. Lucena (2006) discusses in great detail iron nutrition in fruit crops and potential treatments. Hansen et al (2006) confirm that the application of synthetic chelates is one of the most effective solutions for these iron deficiency problems.

The use of higher stability chelates to address iron shortages under higher pH conditions in soil based crops is therefore well documented but appears to have been barely researched for possible applications in algae culture. In addition, the pH effectiveness of the currently available iron chelates is also well documented. The maximum effective working range of Fe-EDTA being pH 6.5, see Figure 1 and Table 1. The improved photo stability of higher stability constant chelates (AkzoNobel 2012) in comparison to Fe-EDTA (Fidler et al 2004) could be another factor that contributes to Fe-EDTA not performing optimally under the conditions required for commercial algae culture.

Considering that the majority of commercial algae species (in batch culture) and marine algae species (sea water pH 7.5-8.4) have an optimal pH outside the working range of Fe-EDTA, as well as the relatively high light intensities at which culture takes place. Further investigation of the growth of algae with various higher stability iron chelates seems more than justified. Delaying the onset of iron limitation without the need for iron re-dosing may be beneficial in terms of labor, raw material savings and productivity. Alkaliphilic species with desirable properties would also undoubtedly benefit from an iron source that is stable under the conditions required for their optimal growth.

Fig-1 The working pH range of some common iron chelating agents (AkzoNobel 2014).

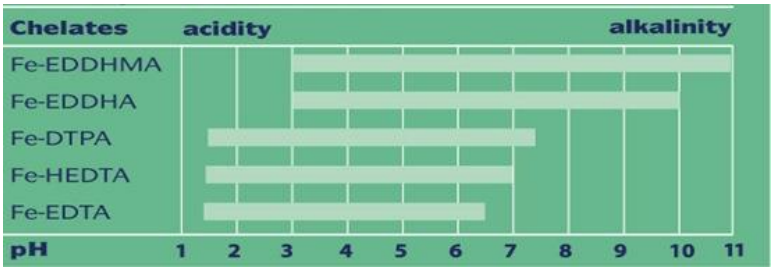


Table-1 The stability constants of some common iron chelating agents.

Ionic Strength	Stability Constant (LogK)		Reference
Fe-EDTA	**25.1**	0.1 M KNO3	Martell et al (2004)
Fe-GLDA	**15.3**	0.1 M KNO3	Begum et al (2012)
Fe-DTPA	**27.7**	0.1 M KNO3	Martell et al (2004
Fe-o,o-EDDHA	35.09	0.1 M NaCl	Yunta (2003)
	33.91	0.1 M KNO3	L'Eplattenier (1967)
Fe-o,o-EDDHA racemic	35.86	0.1 M NaCl	Yunta (2003)
	35.54	0.1 M KCl	Bannochie (1989)
Fe-o,o-EDDHA meso	34.15	0.1 M NaCl	Yunta (2003)
	33.28	0.1 M KCl	Bannochie (1989)
Fe-o,o-EDDHMA	34.44	0.1 M NaCl	Yunta (2003)
Fe-o,o-EDDHMA racemic	33.75	0.1 M NaCl	Yunta (2003)
	(37.9)	1 M NaCl	Ahrland (1990)
Fe-o,o-EDDHMA meso	35.54	0.1 M NaCl	Yunta (2003)
	(39.0)	1 M NaCl	Ahrland (1990)
Fe-o,o-HBED	39.01	0.1 M KCl	Ma (1994)

Note: Values in brackets indicate measurements on substances with an unknown purity

Overview of conducted studies

The series of tests conducted in this paper were aimed at establishing initially if 4 commercially farmed algae species: *P. subcapitata, C.vulgaris, S. platensis and P. tricornutum* could utilize more strongly chelated iron in comparison to the industrial standard Fe-EDTA used as a control. The *S. platensis* study was then extended to investigate behavior under iron limiting conditions at pH levels outside the effective working range of Fe-EDTA.

Secondly, the culture duration was extended and volume up scaled for *S. platensis and C. vulgaris* in the relevant media to encourage iron limiting conditions. A semi static medium replenishment (without iron component) and a biomass harvest step were introduced. Effects on growth were observed under these extended conditions further exploring the iron availability under these conditions.

Finally, the extended test was repeated for *S. platensis* with the best performing iron chelate in order to confirm our findings. As an introduction to further work varying ratios of Fe-HBED : Fe-EDTA were then tested in the same manner to investigate the possibilities of a chelated iron mixture.

A series of small supplementary tests were conducted throughout testing to establish the cause of the halted growth in the control replicates and to confirm the stimulated growth in best performing test replicates. These included microscopic examination and photographs, Fe-EDTA analysis, and re-addition of Fe-EDTA to the exhausted control replicates.

Materials

Chemicals

Ethylenediaminetetraacetic (Na-EDTA) from Acros Organics was used in combination with iron (III) chloride hexahydrate ($FeCl_3.6H_20$) from Sigma-Aldrich to generate Fe-EDTA for use in *P. tricornutum, C. vulgaris and P. subcapitata* control media. For *S. platensis* media, EDTA (as above) was used in combination with Iron (II) sulfate heptahydrate FeS04.7H20 from Fluka chemicals.

The following test (Fe) chelates were obtained from AkzoNobel Functional Chemicals:

Diethylenetriamine Penta Acetic Acid , Ferric – sodium complex (Fe-DTPA)

Ethylene diamine-N,N bis (2hydroxyphenylacetic acid) Ferric sodium complex (Fe-EDDHA)

Ethylenediaminetetraacetic Ferric sodium complex Fe-EDTA

Ethylene diamine-N,N bis(2hydroxyphenylacetic acid) Ferric sodium complex (Fe-EDDHMA)

N,N-Bis(Carboxymethyl) -L- Glutamic Acid Ferric sodium complex (Fe-GLDA)

Bis (2-Hydroxy benzyl ethylenediamine diacetic acid ferric potassium complex (Fe-HBED)

Fe-DTPA + Mn/Zn/Cu-EDTA + Boron and Molybdenum (non chelated) (Micromix APN)

All chemicals used were of reagent grade. The de-ionized water used contained not more than 0.01 mg/L of copper, had a Total organic carbon (TOC)-content of not more than 2.0 mg/L and a conductivity of less than 5 μS/cm. This water was produced from tap water in a water purification system.

Test media

Adapted (OECD) algae medium according to (OECD 2006) was used for *P. subcapitata* culture and for short term culture of *C. vulgaris.* Marine culture medium (MCM) according to (ISO 10253 1995) was used for *P. tricornutum* culture. Adapted Chlorella medium (ACM) according to (NPR 6503 1980) was used for longer term *C. vulgaris* culture. Chlorella medium was adapted with $FeCl_3.6H_20$ (10-13 mg/L) and Na-EDTA (12.7 mg/L) as opposed to the Fe (III) citrate 3-H_2O (10 mg/L) recommended in the original NPR 6503 guideline. OECD medium was adapted with 150 mg/L $NaHCO_3$ instead of 50mg/L for improved pH stability. For *S. platensis*, culture medium as detailed by Zarrouk (1966) was used.

The test media as detailed above formed the control groups for all of the tests conducted for the relevant species. The experimental media was prepared identically to that of the control in all cases except for the iron component, which was replaced in each case with a test chelate as indicated in the chemicals section. Thus the iron component was the only variable in each of the conducted tests. Each experimental chelate was added at an appropriate concentration from a concentrated stock solution so as to result in the same concentration of iron (by weight) as was present in the control media. Information regarding the composition by weight of iron for each of the test chelates was provided by AkzoNobel Micronutrients with each test chelate. The iron concentration per media type is detailed in Table 2 below.

Table-2 The iron concentration in each of the standard media used.

Media	**Fe (mg/L)**
OECD- adapted *(P. subcapitata and C. vulgaris)*	0.01
MCM (for *P. tricornutum*)	0.15
ACM-adapted (*C. vulgaris*)	2.38
Zarrouk (*S. platensis*)	2.01

Test flasks

Test were performed in sterile 100 mL Erlenmeyer flasks with cotton wool stops for the short term tests and in 500 mL Erlenmeyer flasks for the up scaled longer term tests.

Culturing cabinet and test conditions

The tests were carried out in temperature-controlled illuminated orbital incubators (Gallenkamp or New Brunswick) in which the temperature was set at the optimal for the species being tested. Temperature was maintained at ± 2°C during the test periods. Uniform Illumination was provided in the spectral range of 400 to 700 nm by using fluorescent lamps at a distance of about 0.36 ± 0.02 m from the algal cultures. The light intensity was in the range of 60 to 120 $\mu E \cdot m^{-2} \cdot s$. The test vessels were agitated continuously at a speed sufficient to prevent sedimentation of the algae (100 rpm approx).

Other Apparatus

The pH was determined with a microcomputer pH meter. The temperature was measured with a temperature sensor and data recorder. The light intensity was measured with a light intensity meter. The cell counts for the inoculation calibration curve as were all other cell observations were conducted using a Zeiss microscope with integrated digital camera and counting chamber. Absorbance at 436nM was measured using a Shimadzu UV-1800 spectrophotometer. Filtration of algae biomass was carried out using Whatman® pre-pleated qualitative filter paper of 150µm thickness and 4-7µm pore size and plastic funnels. Drying of biomass was carried out using an electric stove set at 100ºC. Algae centrifugation was carried out with Sorvall evolution centrifuge. All weighing of test chelates, media ingredients and algal biomass was carried out using an analytical balance. All chelate stock solutions were stored in temperature monitored refrigerators in the dark. All chelate stock solutions were wrapped in aluminum foil to protect against light degradation when outside the refrigerator. All pipetting was conducted using Gilson pipettes checked twice yearly for their accuracy. Chemical analysis of the EDTA was conducted using Separations Spark Holland apparatus, a Midas auto sampler (with built in column oven), smart line 2500 UV detector, smart line gradient manager 5000 and smart line 1000 pump integration software (Chromeleon V 6.80).

Source and maintenance of algae

Growth tests were carried out with 4 different algae species. *P. subcapitata (*CCAP 278/4), *P. tricornutum* (CCAP1052/1A) and *C. vulgaris* (CCAP 211/11B) were obtained from the Culture Collection of Algae and Protozoa, Dunstaffnage Marine Laboratory, Oban, Argyll, Scotland, UK. After purchasing, this strain was cultured in the relevant media and maintained according to laboratory standard operating procedures. Cultures on sloped agar tubes containing the relevant growth media were stored at 4°C until required.

Exponentially growing cultures were maintained at the recommended temperature for the relevant species in a temperature-controlled illuminated orbital incubator and were re-cultured under sterile conditions weekly to keep the algae in this growth phase. *S. platensis (TISTR8222)* was sourced from the Thailand institute of Scientific and Technological research Department of Bioscience. The culture was maintained and grown as detailed above.

Methods

Short term tests

P. subcapitata, C. vulgaris and P. tricornutum were tested in short term growth tests. The OECD / MCM stock solutions were diluted in an appropriate vessel with de-ionized water according (OECD 2006) and (ISO 10253 1995) with the adaptions indicated in the materials section. The resulting media was then sterilized by filter sterilization. These media were used directly for the control replicates. For each of the test chelates medium was prepared in exactly the same manner but without Fe-EDTA. The test chelate was then added instead as iron source. The concentration of test chelate added was adjusted so as to contain an identical iron concentration (by weight) as that present in the control as indicated in Table 2.

Controls were prepared with four replicates. Test chelates were prepared in duplicate. Test vessels were then inoculated and incubated for 8 days at 23ºC with a light intensity of 103 $\mu E \cdot m^{-2} \cdot s$. Absorption measurements were made on days 0, 2, 5 ,7 and 8 at 436 nm in a cuvette with 4 cm light path. After each measurement the test vessels were replaced in a different location in the incubator. 40 ml of test medium was prepared per vessel.

For the *P. tricornutum* test slightly different methodology was applied. Six control replicates were used and test chelates were prepared in triplicate. Test vessels were incubated for 5 days at 18ºC with a light intensity of 112 $\mu E \cdot m^{-2} \cdot s$. Absorbtion measurements were made on days 0, 1, 2, 3, 4 and 6 days at 436 nm in a cuvette with 4 cm light path. After each measurement the test vessels were replaced in a different location.

Inoculation

Cell density of the inoculum (cell culture in exponential phase) was firstly determined spectrophotometrically. Measurements were carried out at 436 nm (a wavelength at which chlorophyll absorbs well) in a cuvette with a light path of 4 cm. Using a calibration curve (generated historically) for the relevant species relating cell/mL to absorbance at 436nm, the inoculum volume was calculated. All vessels were inoculated with an identical volume so as to result in approximately $1x10^4$ cells per ml in the test vessel as indicated in (OECD 2006) at the start of the test. Due to the red color of some of the chelate treatments all measurements were corrected for background absorbance of the media only to ensure that changes in absorbance were due to algae growth.

Simple extended test

S. platensis was tested during this test. The stock solutions for the test medium were diluted in an appropriate vessel with de-ionized water according to according to (Zarrouk 1966). The resulting medium was then sterilized by filter sterilization. This medium was used directly for the control replicates. Test chelates were then added as the iron source instead of Fe-EDTA in the same manner as described for the short term tests.

Controls were prepared in triplicate. Test chelates were also prepared in triplicate. Test vessels were then inoculated and incubated for 10 days at 28ºC with a light intensity of approximately 100 $\mu E \cdot m^{-2} \cdot s$. Absorption measurements were made on days 0,1,2,3,7,8 and on day 10. Due to the red color of some of the chelate treatments all measurements were corrected for background absorbance of the media only to ensure that changes in absorbance were due to algae growth. Corresponding pH measurements were also made on these days. Absorbance was measured at 436 nm in a cuvette with 4 cm light path. 40 ml of test medium was prepared per vessel. After each measurement the test vessels were replaced in a different location in the incubator. Only Fe-EDTA, Fe-EDDHA, Fe-EDDHMA and Fe-HBED were continued after 8 days. The other treatments were stopped at day 8.

Inoculation

For *S. platensis* no historical data was present with which to inoculate with a set number of cells per ml. Identical inoculation volumes from the same pre-culture were therefore used in each test for each treatment. The pre-culture was continually agitated to ensure homogeneous distribution of cells.

Extended tests with medium replenishment and harvest step

C. vulgaris and *S. platensis* were tested. The ACM / Zarrouk stock solutions were diluted according to (NPR 6503 1980) and (Zarrouk 1966) in an appropriate vessel with de-ionized water with adaptions as indicated in the materials section. The resulting media was then sterilized by filter sterilization. These media were used directly for the control replicates. Test chelates were then added as the iron source instead of Fe-EDTA in the same manner as described for previous tests.

All controls were prepared in triplicate. Test chelates were also prepared in triplicate. Test vessels were inoculated and incubated for a total of 14 days (*S. platensis*) and 28 days (*C. vulgaris*) at
28 ºC and 18ºC respectively at a light intensity of 100 $\mu E \cdot m^{-2} \cdot s$. Regular absorbance measurements were made at 436 nm in a cuvette with a 4 cm light path (see results). Due to the red color of some of the chelate treatments all measurements were corrected for background absorbance of the media only to ensure that changes in absorbance were due to algae growth. Corresponding pH measurements were also carried out. 300 ml of test medium was prepared per vessel. After each measurement the test vessels were replaced in a different location in the incubator. At regular intervals throughout the (*C. vulgaris*) test and once in the (*S. platensis*) test algae biomass was centrifuged for 10 minutes at 20º at 800 rpm from all three replicates to separate biomass. Biomass was subsequently dried for 24 hours at approximately 100ºC. All traces of algae remaining in the supernatant were also filtered and then dried in the same manner. The filters and test vessels were pre weighed and hence total algae biomass could be determined. The algae free filtrate was then re-dosed up to 400 ml with all nutrients at the corresponding original concentrations with the exception of the iron component. No additional iron was therefore added. The replenished media were then re-inoculated and returned to the incubator in different positions. This step was intended to represent a harvest step in a semi static batch process. All replicates were then allowed to re-grow under identical conditions to those previously described.
The extended (*S. platensis)* test was repeated with best performing chelate using identical methodology. Firstly with the Fe-FBED only and again with varying ratios of Fe-EDTA: Fe-HBED.

Innoculation

Initial inoculation was carried out in the same manner as with previous tests. All vessels were inoculated with an identical volume so as to result in approximately $1x10^4$ cells per ml in the test vessel for *Chlorella vulgaris.* For *S. platensis* no historical data was present with which to inoculate with a set number of cells per ml. Identical inoculation volumes from the same pre-culture were therefore used for each treatment. The pre-culture was continually agitated to ensure homogeneous distribution of cells. After filtration re-inoculation was carried out using 2 ml of the corresponding cell suspension prior to filtration not from a separately growing inoculum. Cells used for re-inoculation had therefore been previously exposed to the test chelate.

Confirmatory tests

Iron-EDTA was re-dosed at the original concentration to the exhausted control replicates of the extended test with *S. platensis* to establish if growth could be restarted and confirm iron deficiency as the cause of the lack of growth. An Fe-EDTA analysis using the method detailed below was also conducted in exhausted media. Vessels were also observed for any signs of iron precipitation. Cell observations were conducted in the counting chamber to confirm presence and assess the density of the desired species.

Analytical method

EDTA in the exhausted control medium from the extended tests was analyzed using high performance liquid chromatography with the settings as detailed below. The responses of the 2 mg/L and 15 mg/L control standards from and the duplicate analysis of the exhausted control medium are presented in Figure 16.

Table-3 Summary of the analytical method.

Column:	anion exchange column: Dionex Ionpac AS7 with AG7 guard
Mobile phase:	50 mM Nitrate + 50 mM acetate; dissolve 4.1 g of NaCH3COO in 800 ml demineralized water add 6.95 ml of nitric acid (7.2 M) and fill to 1 liter with demineralized water. Adjust pH to 2.75 +/- 0.2 with H2S04 (98%). Before use filter over 0.45 pm filter.
Reagents:	Dissolve 5 g of Fe(N03)3.9H20 in 800 ml demineralized water add 21 ml nitric acid (7.2 M) dilute to 1 liter.
Flow rate:	0.5 ml/min
Injection volume:	50µl
Detection:	UV 330 nm
Sample preparation:	4 ml sample + 1 ml Fe-N03 solution (stand 15 minutes in the dark). Filter before use over 0.45 pm filter

Results & Discussion

Short term tests

Fig-2 ***P.subcapitata*** **growth in OECD medium with different chelated iron sources.**

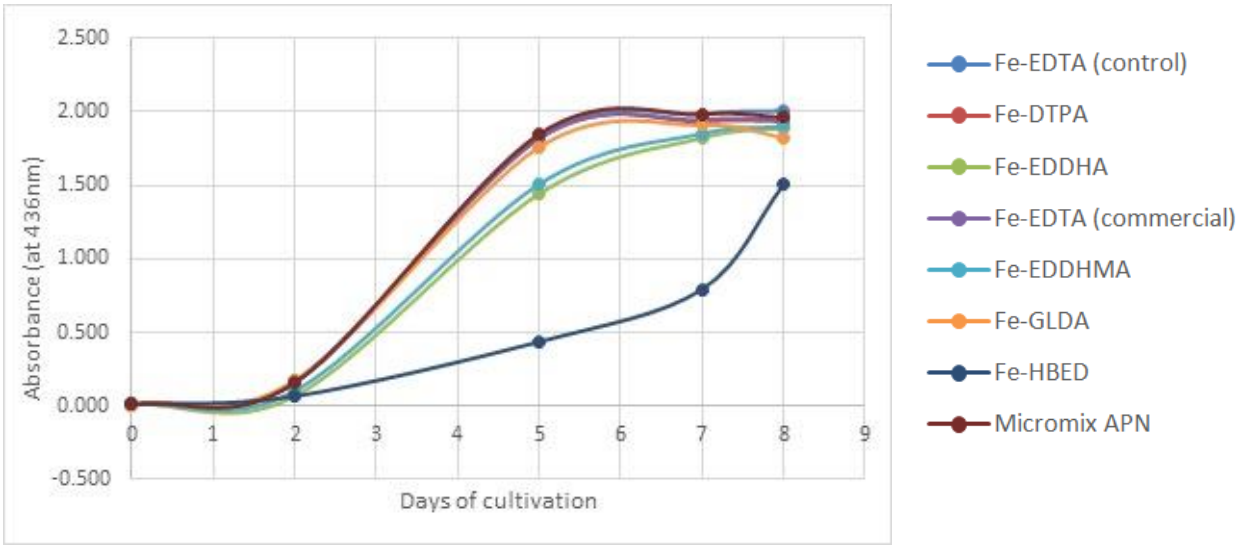


Fig-3 ***C.vulgaris*** **growth in OECD medium with different chelated iron sources.**

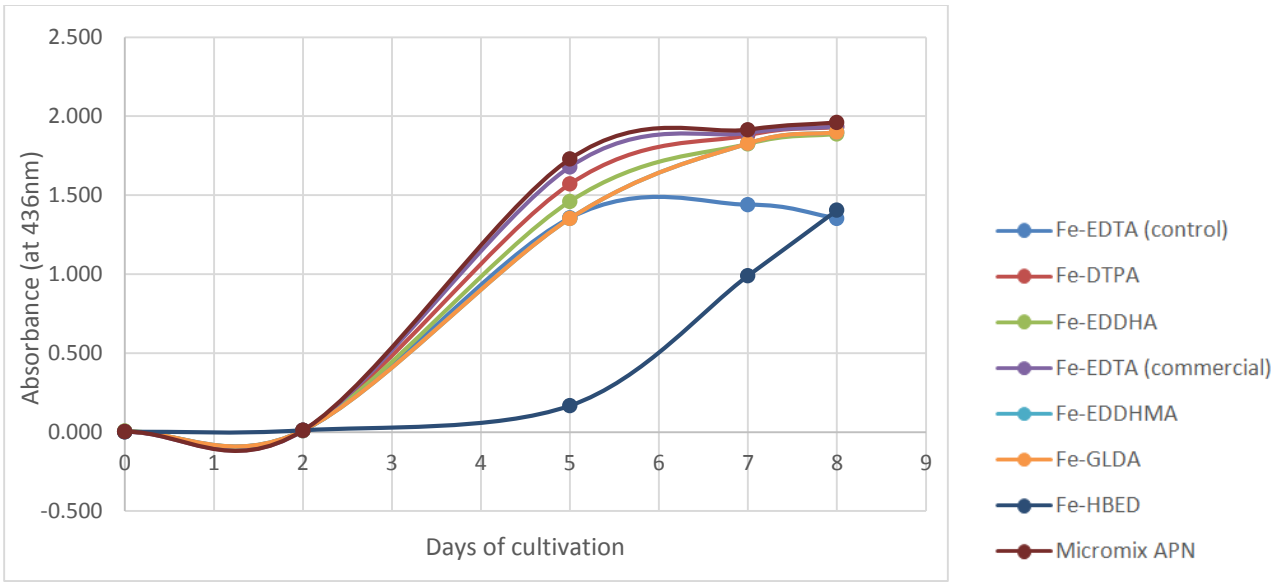
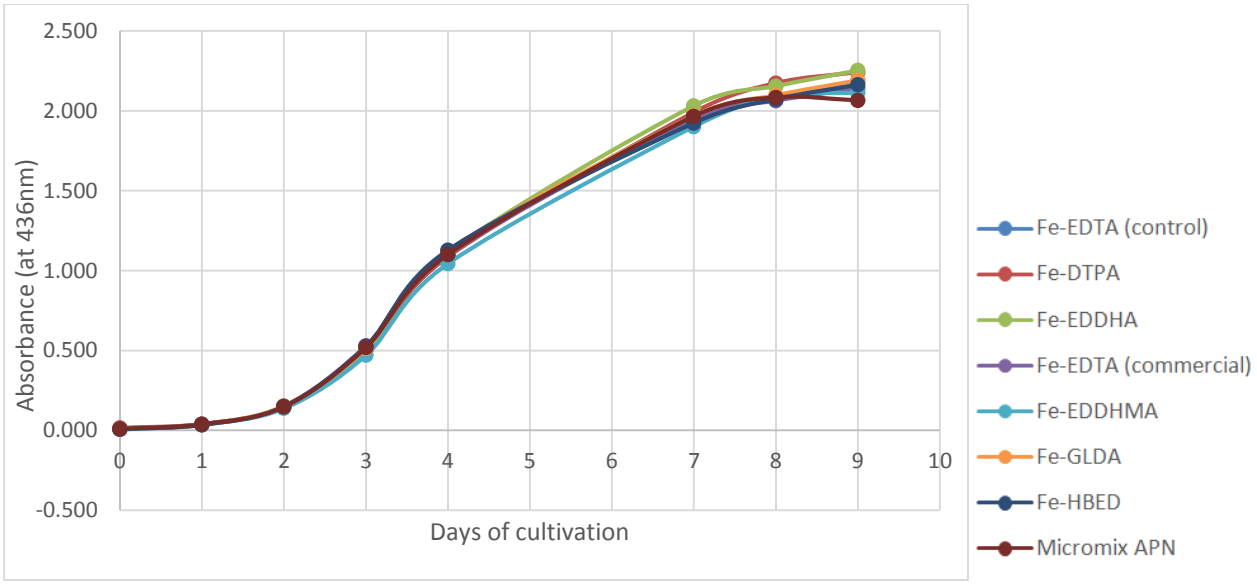


Fig- 4 ***P.tricornutum*** **growth in MCM medium with different chelated iron sources.**



Discussion short term tests (Fig 2-4)

Initial results as displayed in Figures 2 and 3 support the existing of findings of Weger et al (2009) in that the chelates with the higher stability, in particular HBED results in poorer growth than the other test chelates. It is also clear particularly in Figure 2 that Fe-EDDHA and Fe-EDDHMA also result in poorer growth than the control and other chelates with lower stability constants. Weger et al (2009) concluded this to be caused by a lower rate of reduction from higher stability chelates, direct inhibition of ferric reductase and competition of free chelate for Fe^{2+} with the ferrous transport system in organisms with a "Strategy I" iron uptake system.

A reduction of growth was not observed in Figure 4 for any of the test chelates. Indicating that although *P. tricornutum* aquires its iron by ferric reductase and is tolerant to particularly low iron levels (Morrissey &Bower 2012), it is apparently unaffected by chelates with a higher stability constant perhaps due to the components salt water medium mitigating the mechanisms detailed by Weger et al (2009) or the relatively high iron concentration of the MCM medium in comparison to OECD medium. This difference is more likely explained by the different iron uptake mechanism of this organism. Two ferric reductases have been identified in *P. tricornutum* as well as its ability to utilize siderophore complexes during the iron acquisition process (Morrissey & Bower, 2012). It is possible that *P. tricornutum* is therefore less susceptible to the negative short term effects observed in *C. vulgaris* and *P. subcapitata*. After day 6 (Figures 1 and 2) it is clear that growth with the HBED chelated iron begins to accelerate as the plateau of growth is reached for the other chelating agents. Worthy to note in Figure 2 is the earlier exhaustion of the control replicate and the start of death phase on day 7- 8. In addition, Fe-DTPA and Fe-GLDA performs equally as well as the Fe-EDTA in these tests.

Simple extended test

Fig-5 ***S.platensis*** **growth in an extended test in Zarrouk medium with different chelated iron sources**

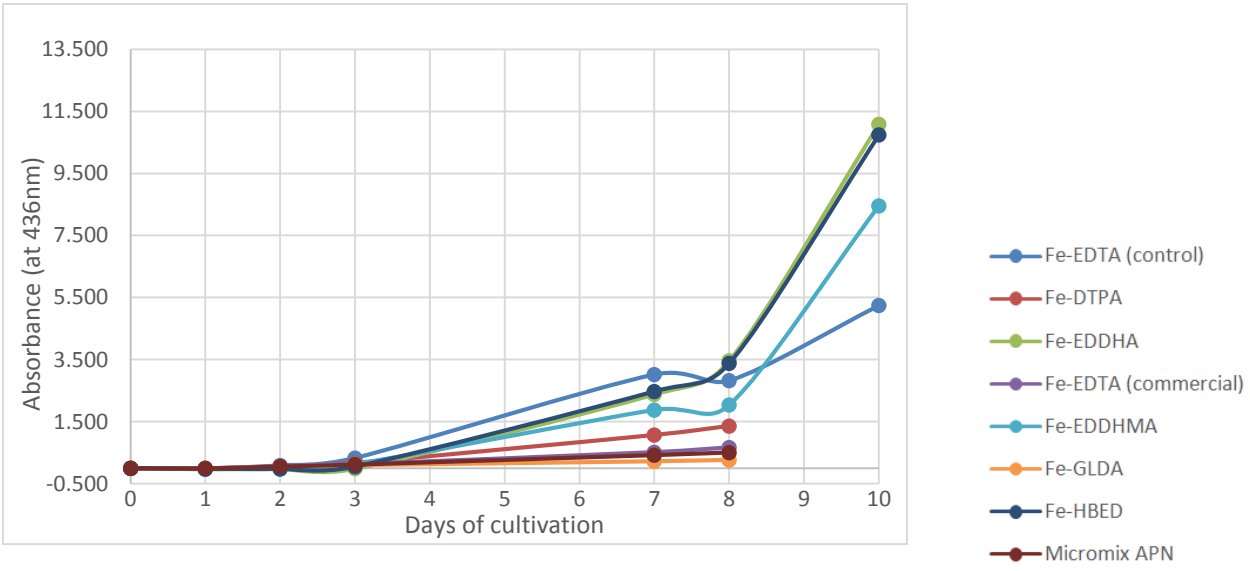
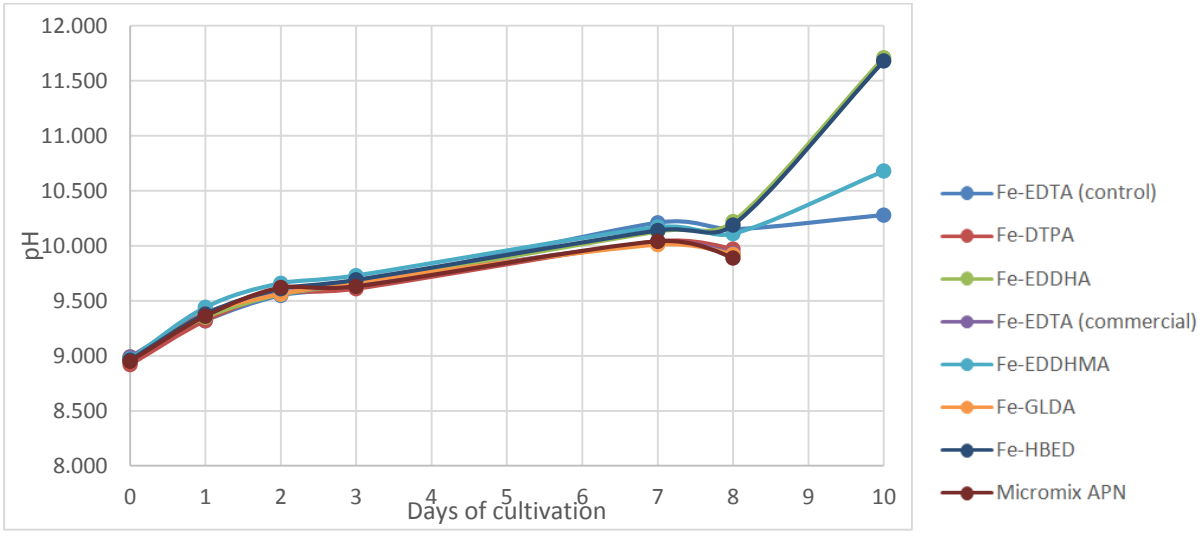


Fig-6 **pH measurements from** ***S.platensis*** **extended test depicted in figure 5.**



Discussion simple extended test (Fig 5-6)

From Figure 5 it is evident that growth in the control exceeds that of all test chelates until day 7 further supporting the trends observed in Figures 2&3 and supporting the general findings of Weger et al (2009).

After day 7 growth in the control faltered and then proceeded to grow at a lower rate until day 10. After day 8 a large increase in growth rate of the Fe-EDDHA and Fe-HBED replicates through today 10 was observed. The cell density for these test chelates was significantly higher than that observed in the control when the test was terminated. The corresponding pH measurements from Figure 5 show a pH of approximately 11.5 for the best performing test chelates and 10.2 in the control. Measurement of growth in the Fe-GLDA, Fe-DTPA and the MicroMix APN replicates was stopped on day 8 due to very poor growth. Results appear to indicate an increase of biologically available iron after day 8 for test chelates Fe-EDDHA, Fe-EDDHMA and Fe-HBED despite the slower start. Results also demonstrate that the iron component of test medium is indeed the first to become limiting as was indicated in the literature.

Extended tests *S. platensis*

Fig-7 ***S. platensis*** **growth in an extended test in Zarrouk medium with high stability iron chelates and harvest/replenishment at day 6. Iron component was excluded from the medium for medium replenishment.**

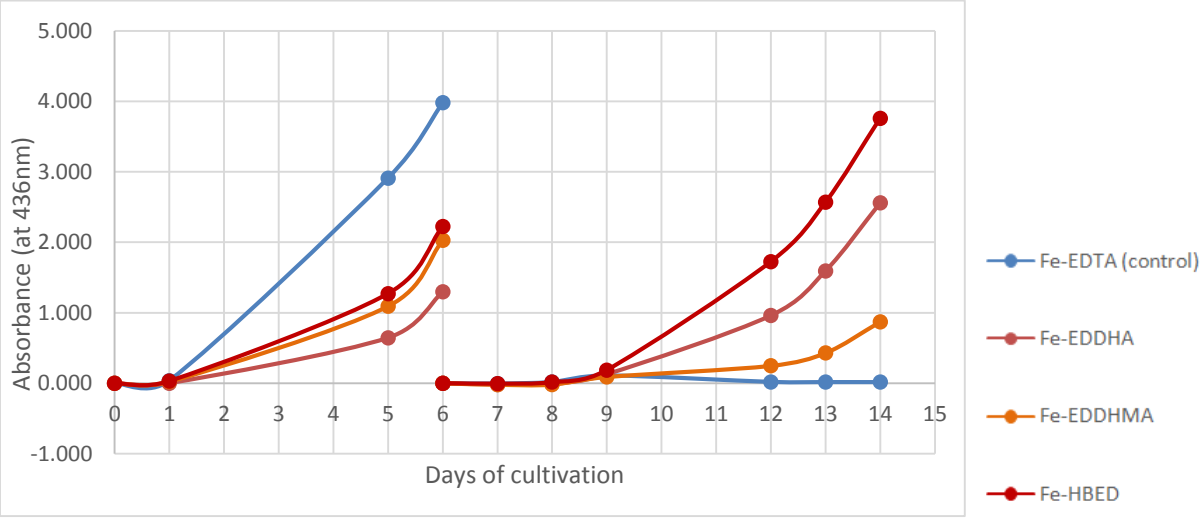


Fig-8 pH measurements from the *S. platensis* extended test depicted in figure 7.

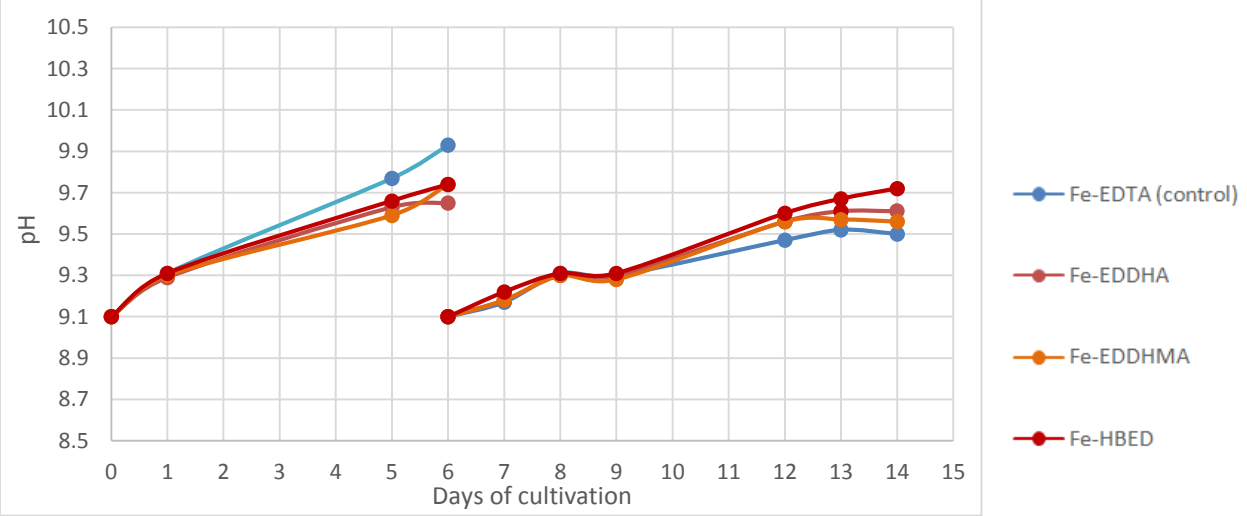


Fig-9 Total biomass from the *S.platensis* extended test depicted in figure 7.

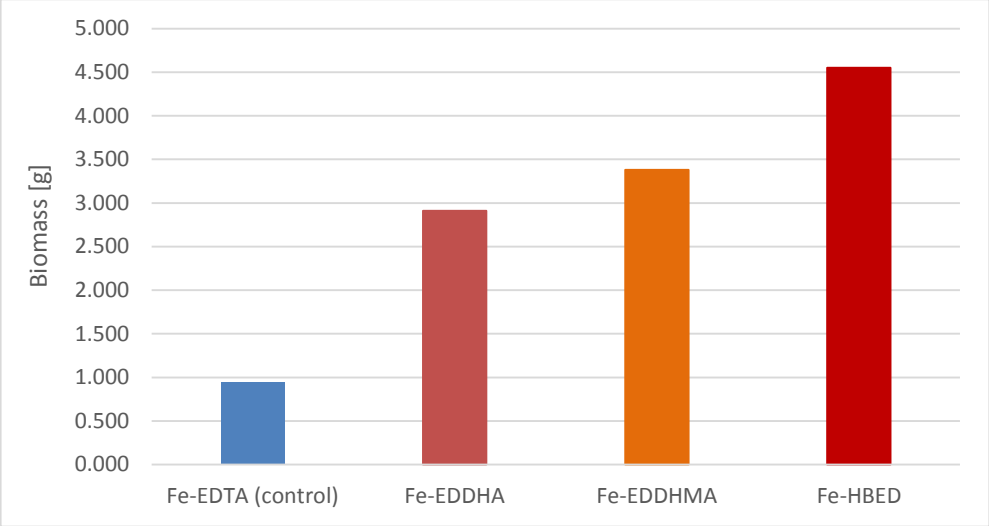


Fig-10 Confirmation of *S. platensis* growth in an extended test in Zarrouk medium with Fe-HBED and harvest/replenishment at day 7. Iron component was excluded from the medium for medium replenishment.

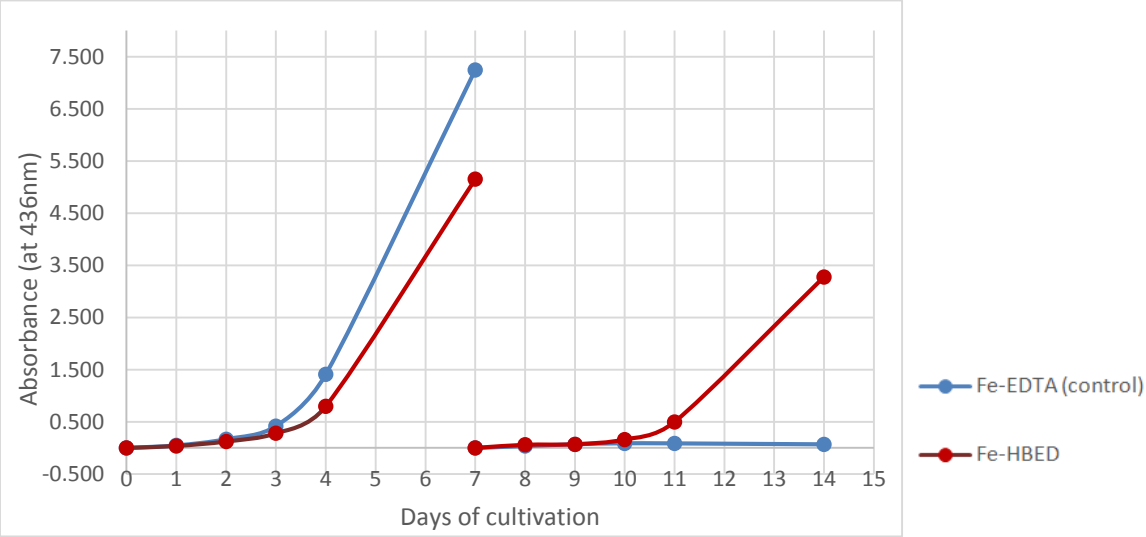


Fig-11 *C. vulgaris* growth in an extended test in ACM medium with Fe-HBED and a Fe-HBED:Fe-EDTA mixture as iron sources. Harvest/replenishment steps at day 7, 14, 22 and 28. Iron component was excluded from the medium for medium replenishment.

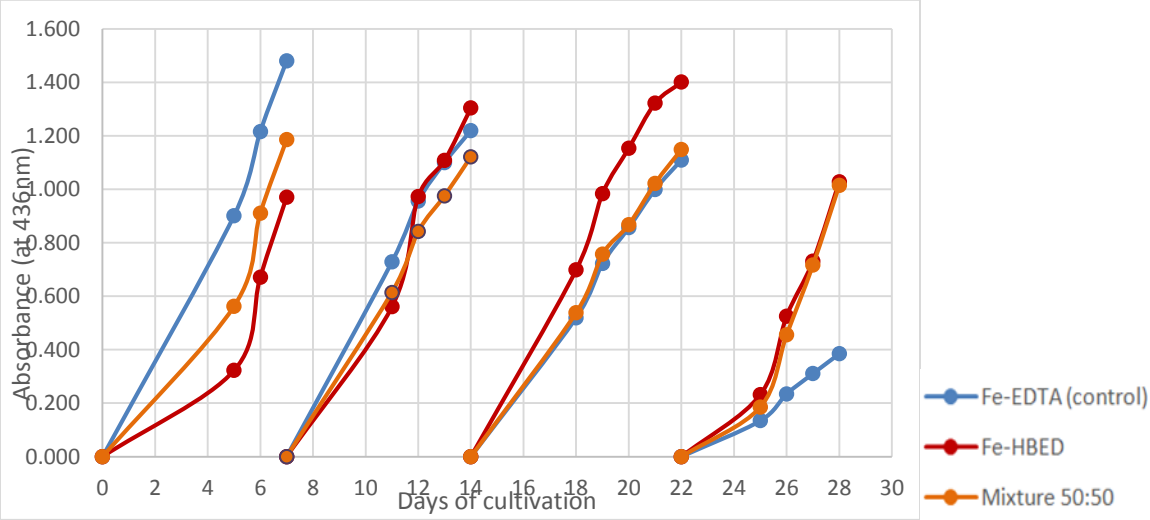
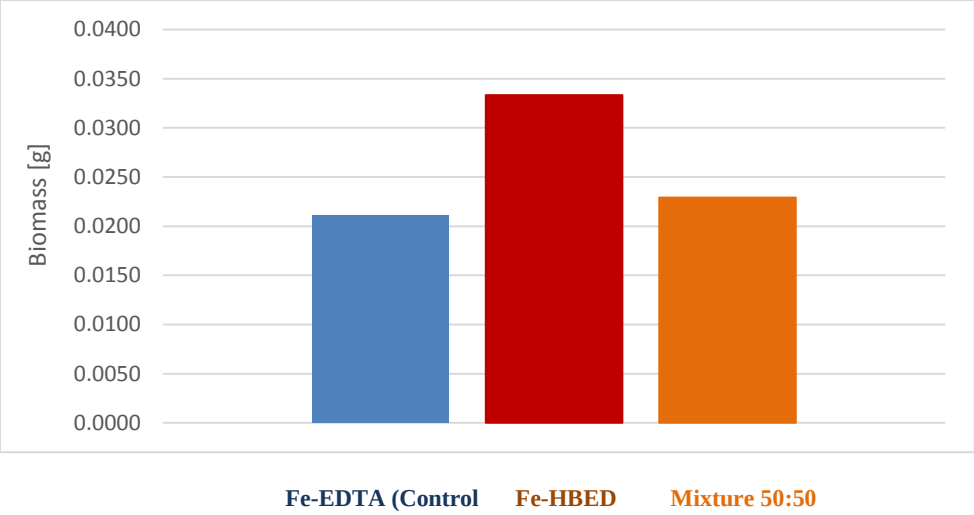


Fig-12 The biomass from the third harvest of the *C. vulgaris* extended test depicted in figure 11.



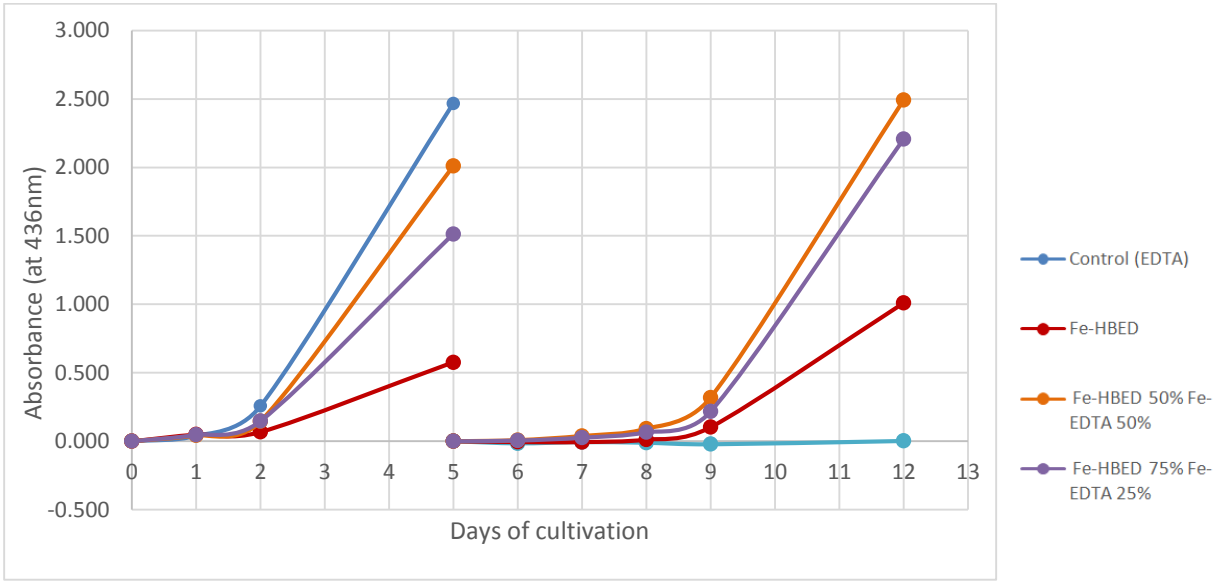
Discussion Extended tests (Figures 7-12)

The control group (Figure 7) grew substantially faster than all test chelates until day 6 as was observed in the previous short term tests. The pH in the control reached approximately 10 on day 6. Following filtration and re-dosing of nutrients (without iron) and re-inoculation, growth could not be restarted in the control. Growth of algae in Fe-EDDHA and Fe-HBED containing replicates continued strongly until day 14. Algae in Fe-EDDHMA replicates also continued to grow but to a lesser extent. Figure 9 indicates the clear differences in biomass generated over the total test period with the same initial dose of iron. A repeat test was carried out for confirmation of results with the best performing chelate Fe-HBED. The results are displayed in Figure 10 and support the trend observed in Figure 7. This test was repeated again with *C.vulgaris* (Figure 11) which gave less pronounced

results likely due to the medium itself containing much higher levels of Fe-EDTA. The general trend was however the same although delayed. Elevated levels of biomass in comparison to the control were observed in the latter part of the test (Figure 12).

A 50:50 mixture was also tested here. See Figure 13 for further mixture results. Fe-HBED and the other high stability constant chelates continue to support growth under conditions at which Fe-EDTA is no longer effective. A mixture of Fe-EDTA and Fe-HBED allow for increased growth in the first few days of the test when comparing to Fe-HBED alone and also continues to support growth well at later stages in the test at a similar rate to 100% Fe-HBED indicating that significantly lower dosing of Fe-HBED should be investigated for efficacy.

Fig-13 *S. platensis* growth in an extended test in Zarrouk medium with chelated iron mixtures and harvest/replenishment step at day 5. Iron component was excluded from the medium for medium replenishment.



Discussion Extended test Figure 13

Figure 13 demonstrates that a mixture of high and lower stability chelates can eliminate the initial growth lag observed with Fe-HBED only in previous extended tests. Following filtration and addition of nutrients (without iron) the mixtures contain sufficient available iron (mostly via HBED) to maintain rapid growth. Growth in the control could not be maintained as with previous extended studies. Growth in the 100% Fe-HBED replicates is significantly lower both before and after filtration and re-dosing than the replicates containing mixtures of Fe-HBED and Fe-EDTA. The reason for this is unclear. It was proposed by Weger et al (2009) that Fe-HBED actually inhibits iron reductase and competes for free $Fe2^{+}$. Due to the mixtures containing a lower concentration of Fe-HBED and likely less free HBED it is feasible that less inhibition via the mechanisms discussed by Weger et al (2009) takes place. An optimized chelate mixture may therefore be the answer to minimizing the inhibitory effects Fe-HBED may have on iron uptake in the short term while allowing a significant delay of iron limitation under conditions that are unfavorable to the Fe-EDTA complex in extended cultures. Literature suggests that optimal iron availability during the exponential phase increases both biomass and lipid production (Liu et al 2007). Further work on optimal dosing of chelates with higher stability constants such as HBED as well as the applicability of the findings for other species, field conditions and potentially also other essential micronutrients is desirable. Furthermore relevance to commercial processes would also be advantageous areas for further research as the studies conducted for this paper are by no means an accurate representation of the conditions that occur during all commercial algae culture processes.

Confirmatory tests

Fig-14 Growth of *S. platensis* after 14 days left (Fe-HBED) right (Fe-EDTA).

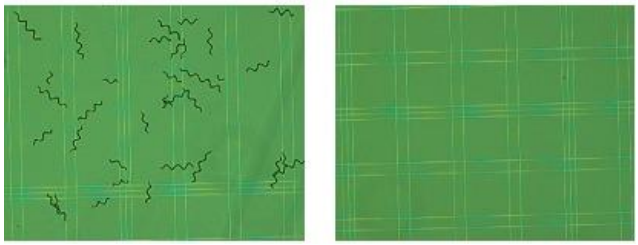
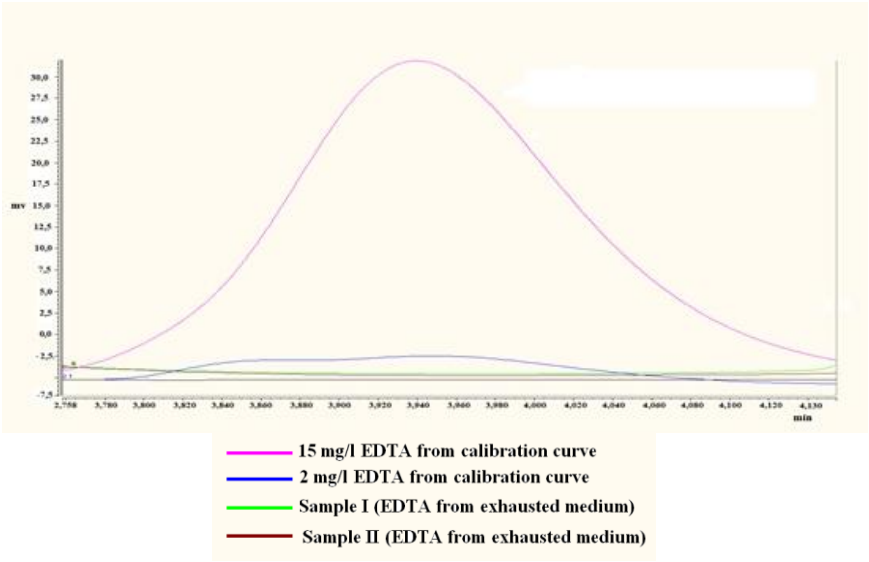


Fig-15 Exhausted control replicates from the extended test with *S.platensis* after re-incubation. (Left + Fe-EDTA, Middle + $FeCl_3$, Right No Addition)

Discussion confirmatory tests

Addition of new Fe-EDTA and re-incubation restored growth to the exhausted control replicates (Figure 15). A simple EDTA analysis (Figure 16) demonstrated that only < 2 mg/L of the original 15 mg/L remained in the exhausted control medium. Furthermore a pinky orange precipitate in the control replicates was observed after centrifugation. An analytical method for Fe-HBED was not available at the time of the investigation. Future analytical confirmation of the improved stability of Fe-HBED over Fe-EDTA under the test culture conditions would therefore be beneficial. However all evidence indicates that a lack of available iron was preventing regrowth in the control replicates chelated with Fe-EDTA.

Fig-16 Measurement of the Fe-EDTA concentration in exhausted Zarrouk media from an extended test with *S.platensis.* (Freshly prepared Zarrouk medium contains 15 mg/L EDTA).



Conclusions

Short term tests suggest that some algae species appear to be able to make easier use of iron chelated by high stability chelating agents than others. Probably due to the differences in iron acquisition mechanisms. In general however it can be concluded that no beneficial effects of higher stability chelates in comparison to Fe-EDTA were observed below pH 10 or before 8 days. Fe-GLDA and Fe-DTPA gave similar if not identical results to the Fe-EDTA control. Should biodegradability be important to the selection of the chelating agent Fe-GLDA could be favored over the other lower stability chelates (Ginkel et al 2008).

Simple extended tests revealed that above pH 10, Fe-EDTA is no longer capable of chelating iron to support growth of *S. platensis*. Fe-HBED, Fe-EDDHA and Fe-EDDHMA support growth and delay the onset of iron limitation caused by pH effects. It cannot be excluded that the better light stability of Fe-HBED, Fe-EDDHA and Fe-EDDHMA also contributes to the better performance. The growth boost observed at pH levels above 10 suggest iron is more readily released or taken up under these conditions. Sufficient iron remains biologically available longer and at higher pH when chelated with Fe-HBED, Fe-EDDHA or Fe-EDDHMA.

Re-dosing tests proved that Fe-EDTA loses the ability to support growth in extended studies following the first harvest and re-dosing step. Higher stability chelates such as Fe-HBED, Fe-EDDHA and Fe-EDDHMA continue to allow extended growth and allow better biomass yields for the same initial iron concentration without re-dosing iron to the medium.

The drawbacks i.e. slow start observed for Fe-HBED in short term tests can be overcome by a chelate mixture allowing a quick start and an extended availability of iron.

Confirmatory tests have shown that in extended tests it is the lack of Fe-EDTA stability which limits iron availability resulting in poor performance.

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