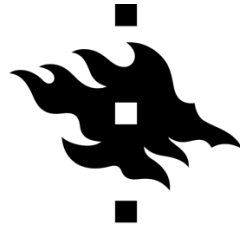


# **The importance of atmospheric and anthropogenic iron for the macroalgae overgrowing coral reefs in the Caribbean Sea**

**MASTER'S THESIS**

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| Tiivistelmä – Referat – Abstract<br>Iron is a trace element but indispensable for all photosynthesizing organisms. It is unevenly distributed in the world's oceans, limiting production in offshore high nitrogen low chlorophyll (HNLC) seas. The Caribbean Sea periodically receives high amounts of iron-carrying aeolian dust originating in the African desert. This aerosol Fe is estimated to contribute three times as much as riverine input to the total iron in the seawater, a considerable fraction of it being soluble ferrous Fe(II) due to photochemical reactions. It has been hypothesized that the excess iron in the Caribbean Sea is one of the reasons why the Caribbean coral reefs are less resilient to degradation. The algae that are not limited by iron have the potential to efficiently utilize the macronutrients from e.g. anthropogenic sources and overgrow the corals.<br>In this study Fe, N & P enrichment experiments were conducted <i>in situ</i> in Guadeloupe to find out if iron limitation can be detected and to contemplate the role of atmospheric iron and the anthropogenic impact. <i>Sargassum polyceratum</i> and <i>Dictyota</i> spp. (Phaeophyceae) were collected from four locations that had degraded coral reefs with macroalgae growing on them. The samples' fluorescence was measured using Pulse Amplitude Modulator (PAM) fluoroscope to detect nutrient-induced fluorescence transients (NIFTs), rapid changes in chlorophyll fluorescence caused by nutrient assimilation in the algal specimen.<br>Iron limitation was detected in all of the study locations but it was weak, which gives limited support to the hypothesis about iron deteriorating the Caribbean reefs' chances against disturbance. Comparison of the locations did not result in differences in iron limitation according to the anthropogenic impact level. The difference was statistically significant in P limitation, the algae from high impact sites expressing greater demand. Ammonium and nitrate enrichments did not result in significant differences, but NH <sub>4</sub> limitation did occur, as well as co-limitation of N & P. Iron has an important role in the phosphorus flux in the sediments and high Fe availability benefits N-fixing cyanobacteria. Redox conditions in the sediment control both Fe and P availability in the water column. Nutrient leaching does affect the local nutrient dynamics but the effects of eutrophication depend on both the species and the community. Notable differences in the NIFT responses were detected between the species that may indeed exert differing nutritional strategies. Coral reef ecosystem complexity emphasizes the importance of timing as well as consistence in quantification of the environmental parameters. The applicability of NIFT results would improve if they were combined with nutrient concentrations data. The fluorescence method appears to be useful in studying iron limitation but more research on iron-induced NIFTs is needed. |                                           |                                                  |
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| Tiivistelmä – Referat – Abstract                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                        |                                                  |                                                         |
| <p>Kaikki yhteyttävät organismit tarvitsevat rautaa. Se rajoittaa tuotantoa laajoilla alueilla avomerta, jossa muita ravinteita olisi riittävästi. Sen jakautuminen on kuitenkin epätasaista. Rautapitoista pölyä kulkeutuu jaksoittaisesti Afrikan aavikolta tuulien mukana Atlantin yli Karibianmerelle. Siitä merkittävä osa on valoreaktioiden johdosta pelkistynyttä ferrorautaa (<math>Fe^{2+}</math>), joka liukenee veteen ja on siten levien saatavilla. Meriin kulkeutuu rautaa ilmakehässä kolme kertaa niin paljon kuin jokivesissä. Karibianmeren koralliriutat selviävät häiriöistä suhteessa heikommin kuin Indo-Tyynenmeren riutat, ja yhdeksi syyksi tähän on arveltu raudan ylimäärää. Kun rauta ei rajoita levien kasvua, ne ovat valmiita hyödyntämään esimerkiksi ihmistoiminnasta seuraavia ravinnepestöjä ja voivat tukahduttaa korallit kasvamalla niiden päälle.</p> <p>Tässä tutkimuksessa tarkastellaan ilmakehävälitteisen raudan merkitystä Karibian riuttaekosysteemin ravinnetasapainon kannalta sekä ihmistoiminnan vaikutusta tähän. Rauta-, typpi- ja fosforirikastuskokeet tehtiin neljältä Guadeloupen tilaltaan heikentyneeltä riutalta kerätyille ruskolevälajeille <i>in situ</i>. Lajit olivat alueella yleiset <i>Sargassum polyceratum</i>, <i>Dictyota menstrualis</i> ja <i>Dictyota pinnatifida</i>. Ravinteen rajoittavuutta selvitettiin mittaamalla levien klorofyllifluoresenssia Pulse Amplitude Modulator (PAM) -fluorometrillä. Näytteistä havainnoitiin nutrient-induced fluorescence transient (NIFT) -vasteita, jotka osoittavat leväyksilön aktiivisesti assimiloivan tutkittavaa ravinnetta.</p> <p>Raudan rajoittavuutta havaittiin 10 % näytteistä. Alhainen määrä antaa osin tukea hypoteesille raudan kumuloitumisen heikentävästä vaikutuksesta Karibiassa. Ihmistoiminnalla ei ollut vaikutusta levien raudantarpeeseen, mutta sen sijaan fosfaatti rajoitti voimakkaammin ihmistoiminnan läheisyydessä. Typen osalta nitraattivasteessa ei ollut merkittävää eroa, mutta ammoniumin rajoittavuutta osoittavia NIFT:a havaittiin. Typen ja fosforin yhteisrajoittavuutta esiintyi myös.</p> <p>Sedimentin sitoman fosforin ja raudan liukoisuudet riippuvat raudan redox-potentiaalista. Raudan saatavuus hyödyntää myös tyyppiä sitovia bakteereja, jotka kilpailevat levien kanssa. Ravinnepestöjen vaikutukset riippuvat sekä kyseessä olevasta yhteisöstä että lajista: eri lajien väliset erot ravinteiden hyödyntämisen strategioissa - ja siten myös NIFT-vasteissa - tulivat esiin. Koralliriuttaekosysteemi on monen tekijän yhteispeli, ja sitä tutkittaessa tulee ottaa kvantitatiivisesti huomioon sekä ympäristötekijät että epäyhtenäinen ajallinen taso. NIFT-kokeiden sovellettavuus parantuisi myös, jos käytössä olisi tietoa ympäristön ravinnepestöisyyksistä. Menetelmä vaikuttaisi soveltuvan raudan rajoittavuuden tarkasteluun, mutta lisää tutkimusta sen tueksi tarvitaan.</p> |                                                  |                                                         |
| Avainsanat – Nyckelord – Keywords<br><b>Rauta, Karibianmeri, ravinne, NIFT, PAM, Sargassum, Dictyota, koralliriutta</b>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                 |                                                  |                                                         |
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## 1. Introduction

Nutrient enrichment plays a role in the worldwide demise of coral reefs (Lapointe, 1997). The Caribbean Sea periodically receives high amounts of aeolian dust that carries iron, among other elements. Iron is a trace element but crucial for all photosynthesizing organisms. This iron of African desert origin is in ferrous Fe(II) form due to light reactions during the transportation and so it is readily bioavailable to the macroalgae growing on the reefs. The algae are then not limited by iron and thus capable to utilize nitrogen and phosphorus that become available from e.g. anthropogenic sources. Roff & Mumby (2012) list six reasons to hypothesize why the coral reefs in the Caribbean seem to be less resilient than Indo-Pacific ones to degrading factors, one of the six being iron-carrying dust. When the macroalgae become more abundant in the reefs they have the potential to overgrow and smother the corals and cause bioerosion (Hallock and Petersburg, 2013). This leads to degradation and finally to a phase shift from coral to algal dominance (McManus and Polsenberg, 2004). The dust storms are likely to increase with global climate change and human land use causing the African deserts to expand (Prospero 2003). Human disturbance can cause damage in environments that are poorly buffered against change.

Guadeloupe is located in the Lesser Antilles, Caribbean Sea. Continuous input of iron falling from the sky surely has an effect on the nutrient dynamics of the reefs surrounding it. Coral cover has declined since the 1950's and algae such as *Dictyota* spp. and *Sargassum* spp. have proliferated in the degraded Guadeloupian reefs (Kopp *et al.*, 2010). The balance of the coral reef ecosystem is fragile and the nutrient dynamics are locally variable with several factors like species assemblage and differing responses between species making the situation more complex (Cullen, Yang and MacIntyre, 1992; Clausing and Fong, 2016). Local nutrient limitation can be detected *in situ* by measuring changes in chlorophyll fluorescence after nutrient enrichment (NIFT) in an algal specimen collected from the field. *In situ* studies provide locally more detailed knowledge of the reef's balance to be

used in environmental management and in choosing the mitigation measures to tackle eutrophication.

### 1.1. Iron requirements of algae

Iron is a trace element for algae, so it is required in minute amounts. Nevertheless it is an indispensable element for all photosynthetic organisms (Price, 1968) and iron is needed in various compounds. One major use for iron in algae is the photosynthetic apparatus, where iron is required in cytochrome complex and ferredoxin (Figure 1) that are parts of the electron transport chain (Spiller and Norman, 1980). Iron deficiency lowers photosynthetic efficiency, causing insufficient amount of chloroplasts – chlorosis – and the replacement of iron-containing proteins with photosynthetically less efficient electron-transfer compounds like flavodoxin (Entsch, Sim and Hatcher, 1983; Sandmann and Malkin, 1983).

### 1.2. Iron in the world ocean

Iron is distributed unevenly in the world ocean. Sea surface iron concentration varies from ca. 15 pM to 4000 pM (Moore *et al.*, 2002). The concentrations are higher at <50 km distance from the coast on the continental shelves and drop in open-ocean areas (Johnson, Gordon and Coale, 1997). The open-ocean concentrations are generally notably higher in the northern hemisphere that has more land area (Moore *et al.*, 2002). Iron has a nutrient-like distribution pattern considering its vertical distribution: Fe concentrations rise towards thermohaline layer and become stable in deeper layers (Johnson, Gordon and Coale, 1997). However, in contrast to nutrients like phosphorus, iron has a turnover rate less than the ocean turnover time (1000 yrs.) (Johnson, Gordon and Coale, 1997). The southern hemisphere has vast areas with low iron concentrations. These are high nitrogen low chlorophyll (HNLC) seas, where primary production is limited by iron rather than other nutrients (Martin, 1990). An experimental iron addition in the Southern Ocean was followed by elevated diatom production rates (Frew *et al.*, 2001). Overall, iron concentrations in the seawater are

very low, especially compared to the Earth's crust, where it is an abundant element (Achterberg *et al.*, 2001).

#### 1.2.1. Atmospheric transport of iron

Most of the dissolved (<0,4  $\mu\text{m}$ ) iron is transported in aerosols originating from continental regions. Aeolian dust is estimated to contribute three times as much iron to the ocean surface waters as the riverine input, the dust flux being largest when rainfall and high dust concentration coincide. The North Atlantic Ocean, The Caribbean Sea as a part of it, is estimated to receive  $7.7 \times 10^{12} \text{ g yr}^{-1}$  with approximately 10 per cent of it being soluble (Duce and Tindale, 1991). Land-derived dust particles carry several other metals, like aluminum, as well as potential pathogens (Garrison *et al.*, 2003). Nitrogen limits the primary production in more than half of the world ocean, but nitrogen fixation is strongly correlated with the availability of atmospheric iron (Moore *et al.*, 2002).

#### 1.2.3. Solubility and bioavailability of iron in seawater

The solubility of iron is strongly negatively correlated with pH and temperature, thus making it considerably less soluble in warm ocean surface waters (Liu and Millero, 2002). However, the overall solubility of iron is a more complex combination of factors, like ligand characteristics, total dissolved iron, aerosol source and size and biological influences (Baker and Croot, 2010). The soluble iron that is available for macrophytes is in bivalent ferrous form Fe(II). In oxic conditions iron is rapidly oxidized into Fe(III) which is adsorbed to e.g. phosphate and settled in the sediment. Iron in the sediment can be reduced again into Fe(II) if the redox conditions change (Millero, Sotolongo and Izaguirre, 1987). Reduction in the reef sediments can be induced by organisms like benthic bacteria through their anaerobic metabolism (Weber, Achenbach and Coates, 2006). The iron transported in the aeolian dust can be highly soluble due to photochemical reduction, but how large the bioavailable fraction is, is not agreed (Baker and Croot, 2010).

### 1.3. Nutrient enrichment in coral reefs

Balanced nutrient availability in a coral reef ecosystem is a narrow band between depletion and excess (Corredor *et al.*, 1999). Nevertheless, nutrient enrichment seems rarely the sole reason for coral reef decline but rather an additional stressor to the reef's community facing a more obvious problem, e.g. disease or over-fishing (Szmant, 2016). Nutrient enrichment may also enhance the severity and prevalence of the reef-forming scleractinian coral disease and bleaching (Vega Thurber *et al.*, 2014). The combination of several stressors - that may indeed originate from very different sources - is what makes the coral reefs in the Caribbean more vulnerable (Roff and Mumby, 2012). The availability of nutrients is essential when the reef shifts from coral to algal dominance (Larned, 1998; Kuffner and Paul, 2001). The limiting nutrient cannot necessarily be identified only by measuring the nutrient concentrations of the surrounding seawater or the algal tissue, mainly due to varying relationships of different environmental factors. The main limiting nutrient in the ecosystems close to the coastline is generally phosphorus, whereas nitrogen limits production in offshore planktonic ecosystems (Corredor *et al.*, 1999). Anthropogenic impact may change these dynamics. Water turbidity or organic matter, for example, can make nutrient assimilation and availability relations more complicated (Desmond Wooda and Oliverab, 1995). Holland, Roberts and Beardall (2004), on the other hand, demonstrate that NIFT studies give more reliable understanding of the actual *in situ* nutrient state of the algae whereas more traditional growth assays tell about potential (*Liebig*) nutrient limitation when other environmental conditions are excluded. Liebig's law of the minimum addresses the nutrient to most likely limit production, i.e. that is the most scarce with respect to the requirements of the algae, like iron in the HNLC seas (Cullen, Yang and MacIntyre, 1992). If we want to understand the local environmental conditions, like the nutrient relations and limitations, we need more detailed data considering the location or habitat and the species.



### 1.3.1. Anthropogenic impact: nutrient leaching

Terrestrial human activities cause various sources of nutrient load in the coastal waters of the Caribbean. Poorly or not at all treated sewage, agriculture and industry leaches carry iron as well as nitrogen and phosphorus (Fernandez, Singh and Jaffé, 2007). Shipwrecks have also been identified as sources of iron pollution in the naturally iron-poor remote atolls in the Pacific Ocean (Kelly *et al.*, 2012). The reefs subjected to iron enrichment rapidly shifted from coral to algal dominance. They also suffered from iron-associated microbe infestations that kill coral. The Caribbean Sea is under the influence of the Amazon discharge plume that carries nutrients of both natural and anthropogenic origin (Martinelli *et al.*, 2012; Wynne SP, 2017).

### 1.4. Nutrient-induced fluorescence transients (NIFTs)

To understand the underlying mechanisms of nutrient-induced fluorescence transients, how they can be measured and what they may tell us, we first need to look at what happens in photosynthesis and what creates fluorescence. Measuring chlorophyll fluorescence can be used to observe photosynthetic efficiency and the different processes that influence it.

#### 1.4.1. Photosynthetic electron transport chain (ETR)

Henriques (2009) describes the linear photosynthetic electron transport chain (ETR) and the main components in chlorophyll fluorescence.

Photosynthesis happens in the chloroplasts of autotrophic cells and includes two photosystems, photosystem II (PSII) and photosystem I (PSI), which are located on the thylakoid membrane (Figure 1). A photon reaches first the light-harvesting antennae, protein complexes containing pigments, of PSII. This excites an electron in the pigment to a higher energy level and the excitation energy is further transferred to a neighboring pigment. The linear rise and fall of the electron's energy level reaches finally the reaction center complex and the central chlorophyll- $\alpha$  pair, P680. The main electron donor is named after the far-red light that is its typical excitation wavelength. P680 transfers its electron to pheophytin and becomes P680<sup>+</sup>, a very powerful

oxidizer that is capable to take an electron from a water molecule. The splitting is catalyzed by the manganese complex enzyme with  $Mn_4Ca$ , where water is bound. From pheophytin, the intermediate electron acceptor, the electron is transferred to the primary electron acceptor plastoquinone, then via the cytochrome complex and plastocyanin to PSI. While transferred, the electron provides energy that is then used to pump protons from water molecules to the thylakoid lumen. The proton gradient over the thylakoid membrane provides energy to synthesize ATP via chemiosmosis. The leftover oxygen is merely a side product in this process, although it might end up in use elsewhere.

Very similarly to PSII functions PSI. It harvests photons and transfers their energy with the use of pigments to the central pigment pair, P700. After transferring an electron to ferredoxin, P700<sup>+</sup> receives a new one from plastocyanin. The NADP<sup>+</sup> reductase enzyme catalyzes transferring the electron from ferredoxin to NADP<sup>+</sup>. After receiving two electrons and removing one proton from the thylakoid lumen, NADPH is created. The ATP and NADPH from these processes are used in the Calvin cycle, synthesizing glyseraldehyde-3-phosphate (G3P) and ribulose phosphate (RuBP) from carbon dioxide.

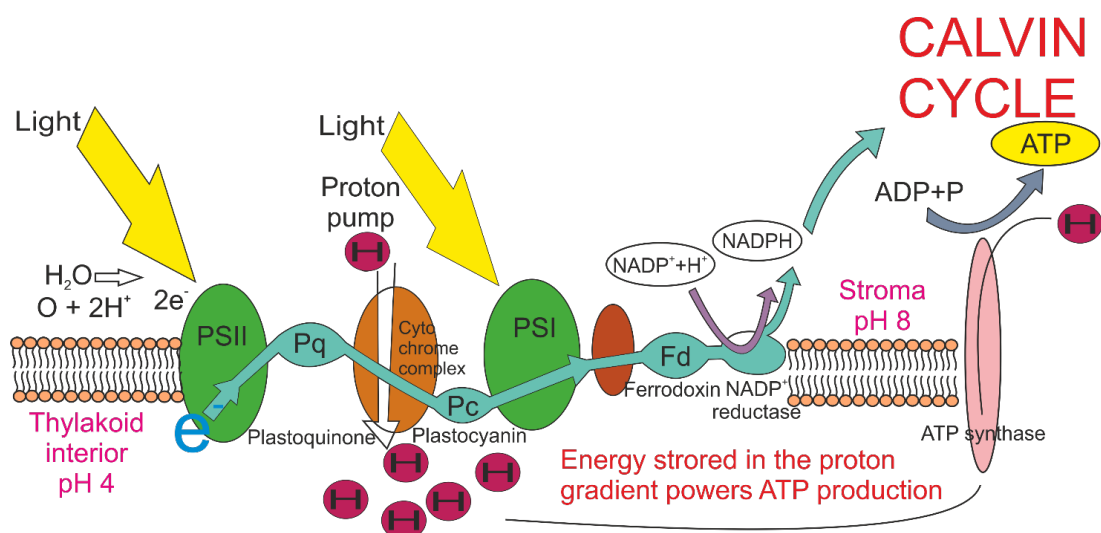


Figure 1. Electron transport chain on the thylakoid membrane. Adapted from Reece & Campbell (2011).

#### 1.4.2. The process behind chlorophyll fluorescence

The light energy absorbed by a chloroplast can end up used in photochemical reactions (photosynthesis), it can be quenched in non-photochemical reactions or it can be re-emitted as light (fluorescence) (Maxwell and Johnson, 2000). Fluorescence is created when an electron falls from a higher energy level to the ground level. The wavelength of the occurring fluorescence is equivalent to the energy the electron loses. Most of the chloroplast's fluorescence originates in PSII (Henriques, 2009).

Photosynthesis, non-photochemical quenching and fluorescence are competitive processes in terms of light energy (Maxwell and Johnson, 2000; den Haan *et al.*, 2013). When P680 has negative charge it can carry out the change in charge needed in the photochemical reaction and is thus considered to be "open". Reversely, when it has donated the electron it is "closed". P680<sup>+</sup> cannot utilize light energy in photosynthesis and so the energy has to be quenched non-photochemically or photochemically – as fluorescence (Henriques, 2009). This happens typically in the light-harvesting antennae of PSII where the charge returns when P680<sup>+</sup> is closed. Light intensity and temperature determine how big proportion of the PSII reaction centers are open (Murchie and Lawson, 2013).

#### 1.4.3. Measuring fluorescence

Only 1-2 % of the light absorbed by a chloroplast is re-emitted as fluorescence. Fortunately measuring it is relatively easy: fluorescence is mostly red or far-red light with long wavelength and the measuring can be targeted at this partition of the spectrum (Maxwell and Johnson, 2000). The majority of the fluorescence originates in PSII but PSI can affect the measurement (Henriques, 2009) and some light energy being lost is inevitable. Different algal species and even specimens have their own typical fluorescence values. It is for these reasons that fluorescence measurements serve best in studies that compare relative figures.

#### 1.4.3.1. Pulse amplitude modulator (PAM)

Murchie and Lawson (2013) describe the principles of the device widely used in measuring fluorescence. Pulse amplitude modulator (PAM) was first introduced in 1986 and since it has been in common use in studying photosynthesis (Huot and Babin, 2010). First, the alga is placed in front of a sensor that measures fluorescence using long red light wavelength and short (ca. 10  $\mu$ s) saturating light impulses. The studied specimen is dark adapted in the beginning to relax photosystems' electron transport chain and to open the reaction centers. Background fluorescence  $F_0$  can be measured in dark using the weak measuring light with enough intensity to excite the electron in PSII but not enough to initiate the electron transport chain. After this the alga is exposed to a series of saturating light impulses that close all the reaction centers. This allows measuring maximum fluorescence  $F_m$  that is not yet effected by non-photochemical quenching (NPQ). The difference of background fluorescence and maximum fluorescence is yield  $\Phi_{PSII}=(F_m-F_0)/F_m$ . Yield is a figure of the photosynthetic efficiency of PSII with theoretical maximum value of 0,83. After dark adaptation a light with enough intensity to sustain photosynthesis is turned on. Saturating measurements are made in regular intervals, for example 30 seconds. This allows the researcher to follow changes in maximum fluorescence in light  $F'_m$ , yield  $\Phi_{PSII}$  and NPQ. The value of  $F'_m$  is usually close to  $F_m$  in the beginning but it comes down when photosynthesis and NPQ compete with it. When an equilibrium is reached the measured value of  $F'_m$  is relatively stable. That allows adding control and nutrient solutions to the alga and observe the possible following transient change in fluorescence in response to the addition.

#### 1.4.4. How NIFTs are created

Measuring fluorescence with different applications has been used mostly in studying microscopic algae but it has also contributed in adding knowledge about macroalgae (Enrquez and Borowitzka, 2010). Algae need the main nutrients, carbon, nitrogen and phosphorus, as well as trace elements, like iron, to grow. The absolute or relative availability of any of the nutrients can thus limit the production (Shelly, Holland and Beardall, 2010).

The physiological mechanism behind the nutrient-induced fluorescence transient (NIFT) response is not known in detail. The direction of the response – whether the fluorescence value rises (positive) or falls (negative) – is also dependent on the species and the nutrient in question (den Haan *et al.*, 2013). Shelly *et al.* (2007) studied the NIFT responses of nitrogen-starved *Chlorella emersonii* cultured in laboratory. This species responded with a very sharp rise in  $F'_m$  after an ammonium addition and a gradient fall after a nitrate addition. Turpin and Weger (1988) observed a similar ammonium-induced response in *Selenastrum minutum*. Petrou *et al.*, (2008) studied *Dunaliella tertiolecta* which expressed a negative NIFT response after a phosphate addition but the fluorescence recovered later. Den Haan *et al.* (2013) studied both phosphate-starved *Ulva lactuca* cultured in laboratory and *Lobophora variegata* collected from its natural environment. The former showed a positive NIFT response after phosphate addition, as well as the latter after nitrate and ammonium enrichments, but its phosphate-induced NIFT was negative.

The transient change in fluorescence after nutrient assimilation is likely caused by allocating the use of light energy between the photosystems when the demand for ATP and NADHP rises (Shelly *et al.*, 2007). Almost all of the chloroplast's fluorescence originates in PSII and when its energy status changes and ETR accelerates or relaxes, a change in fluorescence could be expected (Huot and Babin, 2010). Perhaps a situation where assimilating a certain nutrient requires, for example, extra NADPH, would move the pressure more on PSI and thereby relax PSII. NPQ and helping pigments, like xanthophyll, also affect the ETR of the cell assimilating the nutrient (Petrou *et al.*, 2008).

NIFT studies have often been carried out using also another protocol in addition to PAM and the results have then been compared (Holland, Roberts and Beardall, 2004). These data indicate that even though the physiology is not completely understood, the PAM fluorometry protocol can be used in studying nutrient limitation.

## 1.5. Aims of the study

The aims of this study are to find out

- 1) Can iron limitation be detected in the algae growing on the reefs of Guadeloupe by the means of measuring NIFTs?
- 2) What does these *in situ* data indicate and how they can be used?
- 3) What is the importance of iron in the nutrient dynamics of the studied locations?
- 4) Does local anthropogenic impact have an effect on iron limitation?

## 2. Materials and methods

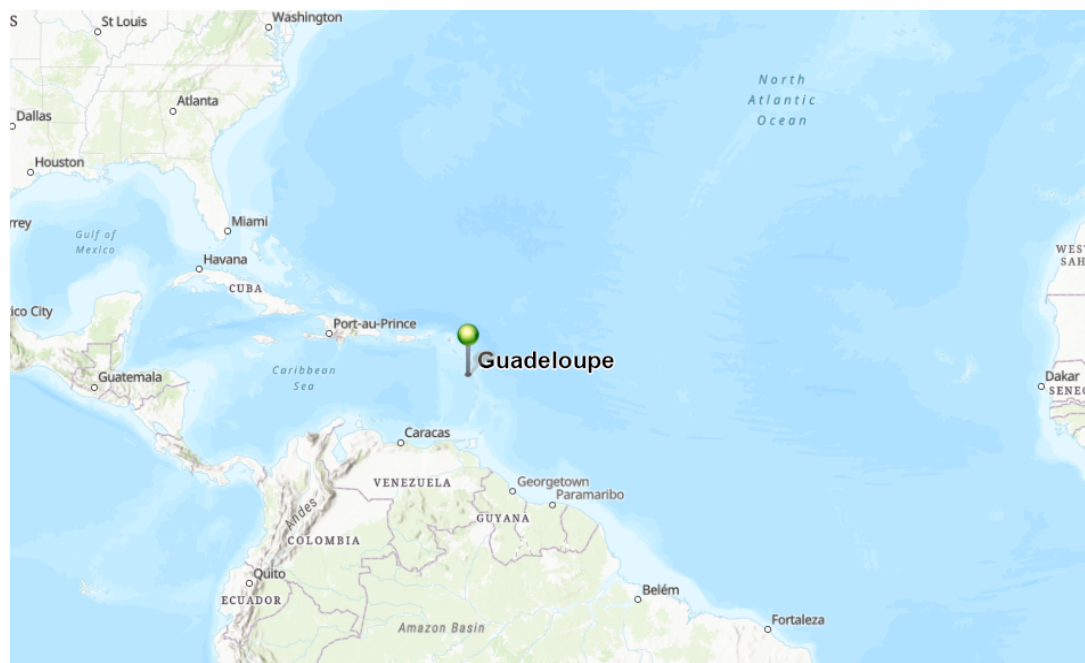


Figure 2. Map showing the location of Guadeloupe in the Caribbean Sea (done with ArcGis Online, 2020)

### 2.1. Study sites and timing

The NIFT experiments were conducted in French Guadeloupe from July to September 2016. The group of islands is located in the eastern Caribbean Sea (Figure 2) and consists of six inhabited islands. The two main islands are Grande-Terre with calcareous terrain in the east and volcanic Basse-Terre in the west. The studied algae were collected from four locations: Anse des Salines ( $16^{\circ}14'59.42''\text{N}$ ,  $61^{\circ}10'50.45''\text{W}$ ), Îlet à Fajou ( $16^{\circ}21'11.43''\text{N}$ ,

61°34'49.49"W), Îlet à Cochons (16°12'53.50"N, 61°32'39.22"W) and Îlets Pigeon (16°10'0.37"N, 61°47'18.49"W) (Figure 3). The study sites were observed visually by snorkeling to declare the presence of degraded coral reefs and the associated algae. The locations were categorized into two levels of anthropogenic impact based on inspection of the map. Îlet à Cochons (IAC), a high anthropogenic impact site, had a wastewater outlet in its vicinity as well as an active harbor. The water column in IAC was visibly turbid and vast bacterial mats were present on the sediment. Îlet à Fajou (IAF) is located further from the coast, close to a protected area, but the prevailing current from the east and the relative vicinity of a city caused the location to be categorized as a high anthropogenic impact site. One low anthropogenic impact site, Anse des Salines (ADS), is located in the easternmost end of the island and constantly receives current water from the Atlantic. Another low impact site in Basse-Terre, Îlets Pigeon (IP), is situated in a national park and does not have a city or similar human disturbance potential in the vicinity.

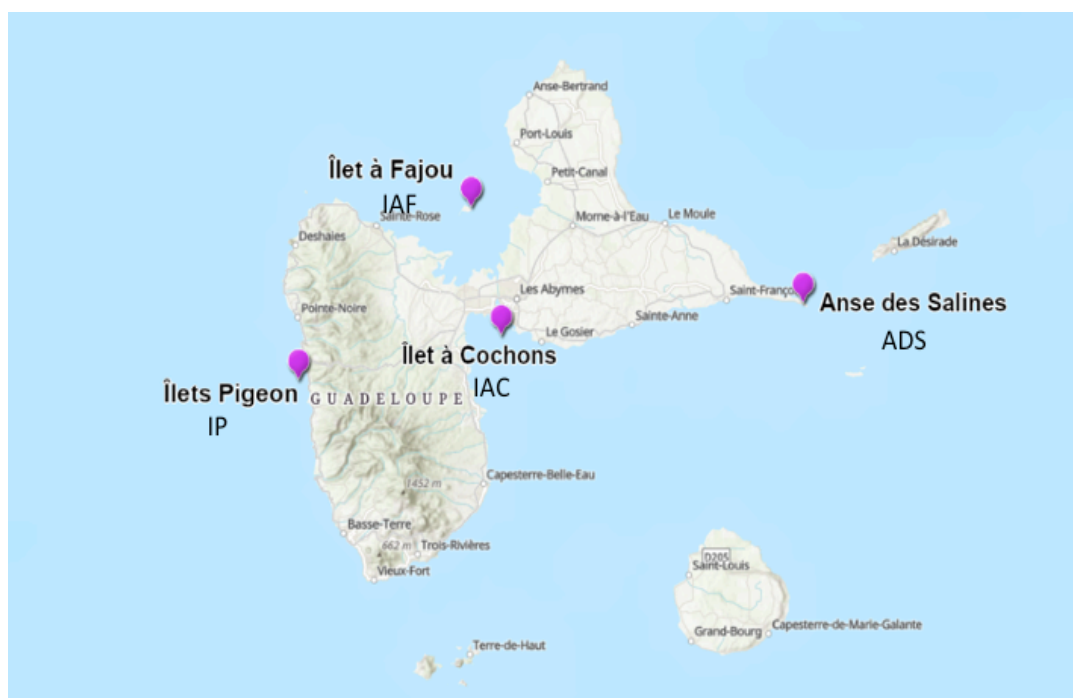


Figure 3. Map of the study locations in Guadeloupe (done with ArcGis Online, 2020). They were categorized according to the anthropogenic impact level in two classes, high and low. IAC and IAF were considered to experience high impact due to the vicinity of a city and the prevailing current direction from east to west. Low impact sites were ADS and IP because they are farther from any city. Ocean currents bring water to ADS from the Atlantic in the east and



IP is located in a national park with no inhabitants and a limited amount of visitors.

## 2.2. Sampling

Four brown algal species, *Sargassum polyceratium* (Montagne), *Dictyota pinnatifida* (Kützing) and *Dictyota menstrualis* (Hoyt), were studied. These species belong to the class Phaeophyceae and are abundant on the reefs of Guadeloupe. Species belonging to the genera *Dictyota* and *Sargassum* are widespread in the world's oceans. *Sargassum* spp. are especially interesting since two free-floating species have raised great concern in the Caribbean due to the vast outbreaks of floating high density algal mats (Louime, Fortune and Gervais, 2017).



Figure 4. *Dictyota pinnatifida* patch in Îlet Fajou.

The algal samples were collected by hand in each reef snorkeling or SCUBA diving from depths between 1 and 7 meters. They were placed in shaded plastic containers to avoid exposing them to direct sunlight when brought to the surface. Specimens were cut from the top parts of the individuals and shaken gently to expel any epiphytes or other material on the surface. The samples were collected evenly from the reef area and in excess to have a heterogeneous representation of the specimens. Ambient water was also collected to be used in the laboratory. The water was collected in glass



bottles of one liter volume from the same location where the sampled algae were growing, towards the prevailing current and without any obvious boats etc. to bring impurities. Both the water containers and the boxes with algae were placed in a cool box and taken into the laboratory within 1–2 hours.

### 2.3. NIFT experiments

The nutrient enrichment experiments were conducted 240 times with 120 samples. The laboratory windows were shaded to protect the samples and to have steady light conditions. The algae were kept in ambient water. The water samples that were used in nutrient stock solutions and incubation were filtered with GE Healthcare Life Sciences grade GF/F 0.22  $\mu\text{m}$  glass microfiber filter.

The algal thallus blade of ca. 1-2 cm in diameter was placed in front of the PAM sensor between magnet rings in a petri dish with 12 ml of filtered ambient seawater. An aeration pump's tube was also placed inside the petri dish to mix the water, placed behind the sensor to avoid any interference with the fluorescence signal or contact with the pipette. After removing the possibly occurring gas bubble between the alga and sensor the LED light was turned off and the setup was enclosed in a box (Figures 5 & 6) for a 10-minute dark adaptation. After the dark incubation the  $F_m$  was measured and then the LED light of 125  $\mu\text{E}$  in intensity as well as the aeration pump were turned on. The alga was kept in light for ten minutes to allow the  $F'_m$  to become steady. The PAM fluoroscope was programmed to measure every 30 seconds. After ten minutes 1 ml of control solution – filtered ambient seawater – was added in the petri dish with the alga. The addition always happened in between the measurements to avoid any interference with the fluorescence signal. Five minutes (ten PAM measurements) later the iron stock solution was applied. In N and P enrichment experiments the phosphate stock was applied five minutes after control, then nitrate and ammonium, five minutes between each.

### 2.3.1. Stock solutions

The enrichment stock solutions had the following concentrations: 0,5  $\mu\text{M}$  iron based on ferrous sulfate chelate solution  $\text{FeSO}_4$ , 1  $\mu\text{M}$  phosphate based on  $\text{KH}_2\text{PO}_4$ , 10  $\mu\text{M}$  nitrate based on  $\text{NaNO}_3$  and 10  $\mu\text{M}$  ammonium based  $\text{NH}_4\text{Cl}$ . The concentrations of the solutions followed actual measured eutrophication events by Kuffner and Paul (2001) for iron and by den Haan (2015) for phosphate and nitrate. They were prepared in glass bottles using filtered ambient seawater that was collected at the study site at the same day.

### 2.3.2. PAM fluoroscope setup

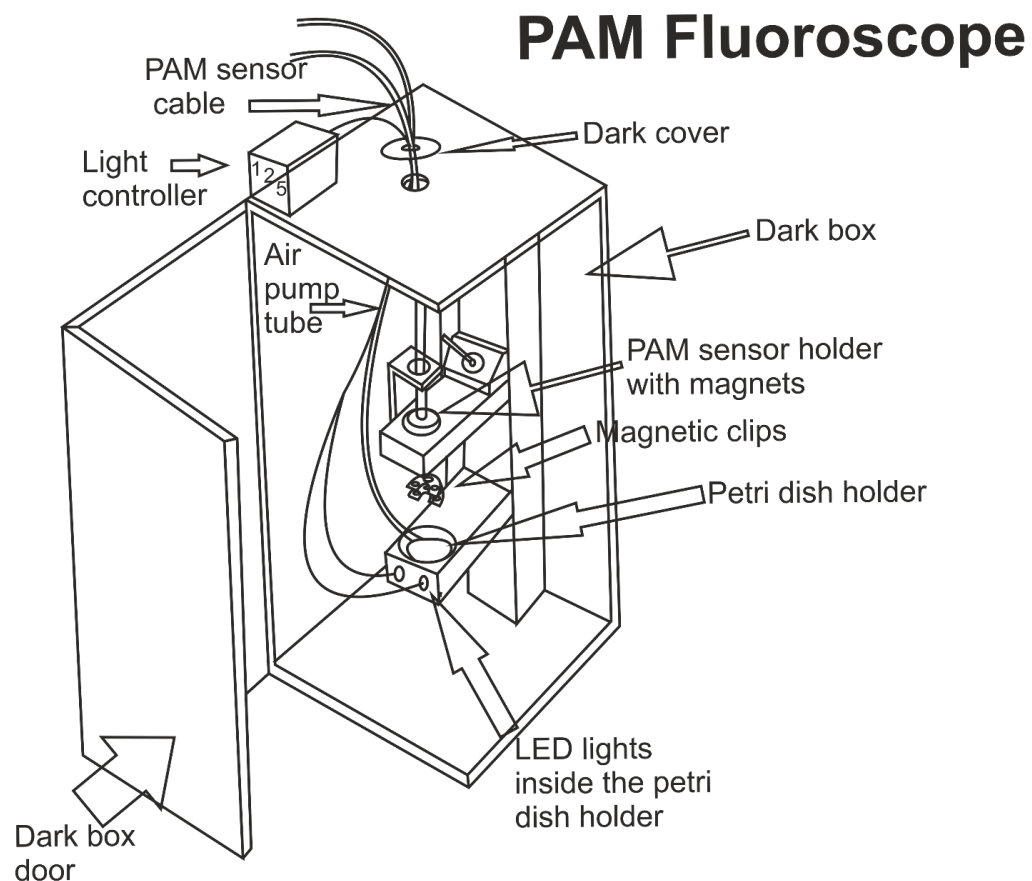


Figure 5. PAM fluoroscope setup, adapted from Haan *et al.*, (2013) and figure 6.

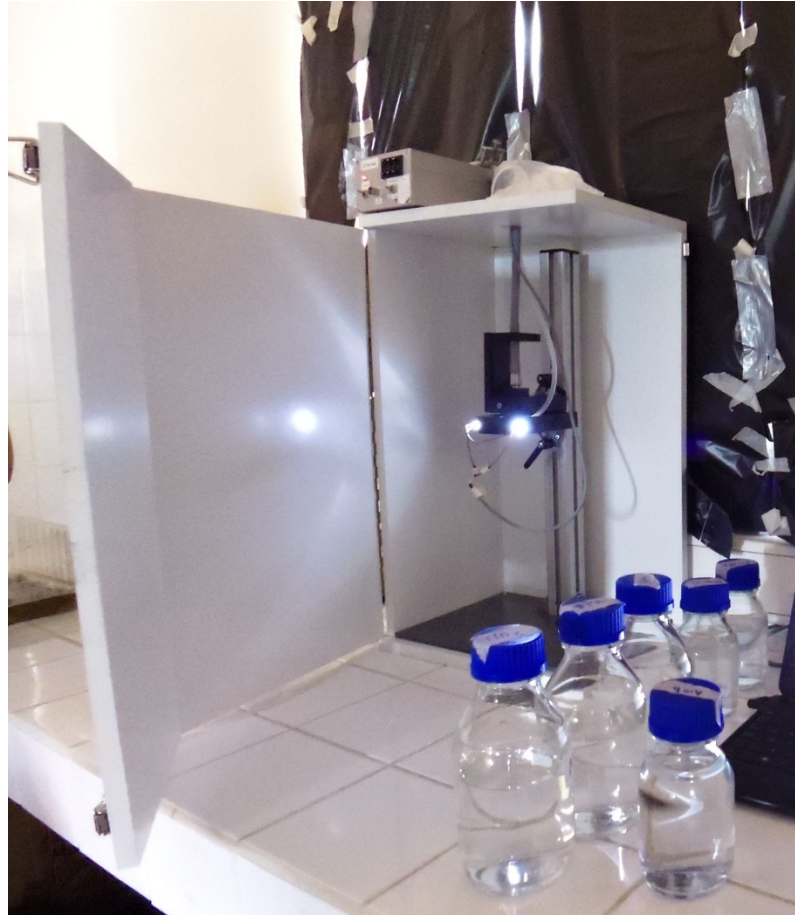


Figure 6. PAM fluoroscope in the laboratory. The laboratory windows were covered with black plastic to protect the samples from direct sunlight. The dark box's door was opened and the LED light of  $125 \mu\text{E}$  intensity inside the petri dish holder was turned on after 10 minute dark incubation of the sample. The algal thallus blade was placed in a petri dish with 12 ml of filtered ambient seawater between magnetic clips to hold it in place in front of the PAM sensor. PAM measurements were programmed every 30 seconds. 1 ml of control (filtered ambient seawater) and the nutrient stock solutions were added with a pipette in five minute intervals, the addition not coinciding with the measurements.

## 2.4. Data analysis

### 2.4.1. NIFT response

Three parameters were measured with the PAM fluoroscope: maximum fluorescence  $F'_m$ , yield  $\Phi_{\text{PSII}}$  and steady-state fluorescence  $F_t$ . A response was interpreted to occur when there was a relatively rapid change in either  $F'_m$  or  $\Phi_{\text{PSII}}$  that was at least twice as large after nutrient addition as the change after adding the control solution, using the following formula:

$$response = \frac{F'm \text{ or } \Phi PSII/dt \text{ NUTRIENT}}{F'm \text{ or } \Phi PSII/dt \text{ CONTROL}} \geq 2$$

This equation is also used by den Haan *et al.* (2013) in detecting NIFT responses.

#### 2.4.2. Statistical analysis

High (IAC and IAF) and low (IP & ADS) anthropogenic impact site NIFT response results were compared and analyzed with two-proportionate z-test. The test was done with each nutrient using R software. Sample size was n = 30 in each test and confidence interval 95%. This test was used in comparing proportions in nutrient limitation between locations in a NIFT study conducted in Curacáo (den Haan *et al.*, 2013).

### 3. Results

A strong enough rapid change in fluorescence for that to be interpreted as a sign of limitation occurred in 30/240 of the experiments. NIFT responses were detected in all of the studied algal species. Figure 7 shows first the smaller drop in F'm after control addition at ten minutes and then bigger rise after iron stock solution addition at fifteen minutes.

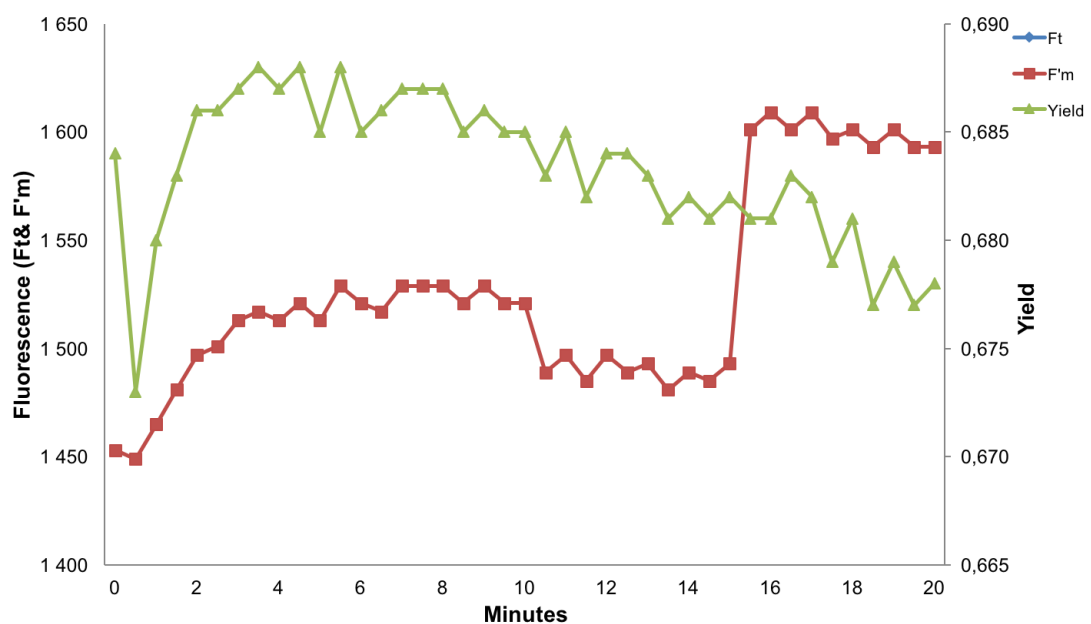


Figure 7. A NIFT response to iron addition at 15 minutes in *Sargassum polyceratum* collected from ADS.

NIFT responses were detected in all the species from each location except for *Dictyota menstrualis* from ADS (Figure 8). NIFTs were measured upon iron enrichment (Figure 7) as well as N and P enrichments (Figure 14).

Comparison of combined results from high and low anthropogenic impact sites shows that anthropogenic impact had the greatest effect in phosphate limitation (Figure 9). Second largest difference was in ammonium limitation, but with 95% confidence interval and  $n = 30$  the difference was not significant ( $p$ -value = 0.317). The demand for both phosphate and ammonium was higher in high anthropogenic impact sites. There was no difference in either iron or nitrate limitation between the high and the low anthropogenic impact sites ( $p$ -value = 1).

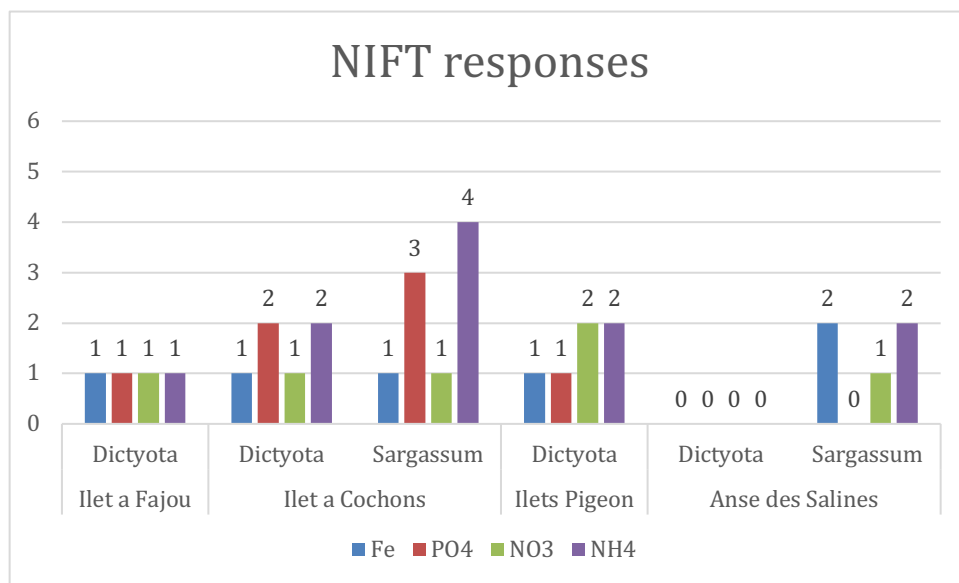


Figure 8. NIFT responses to iron, phosphate, nitrate and ammonium in *Dictyota* spp. and *Sargassum polyceratum* samples from the four study sites. N=10 in each experiment.

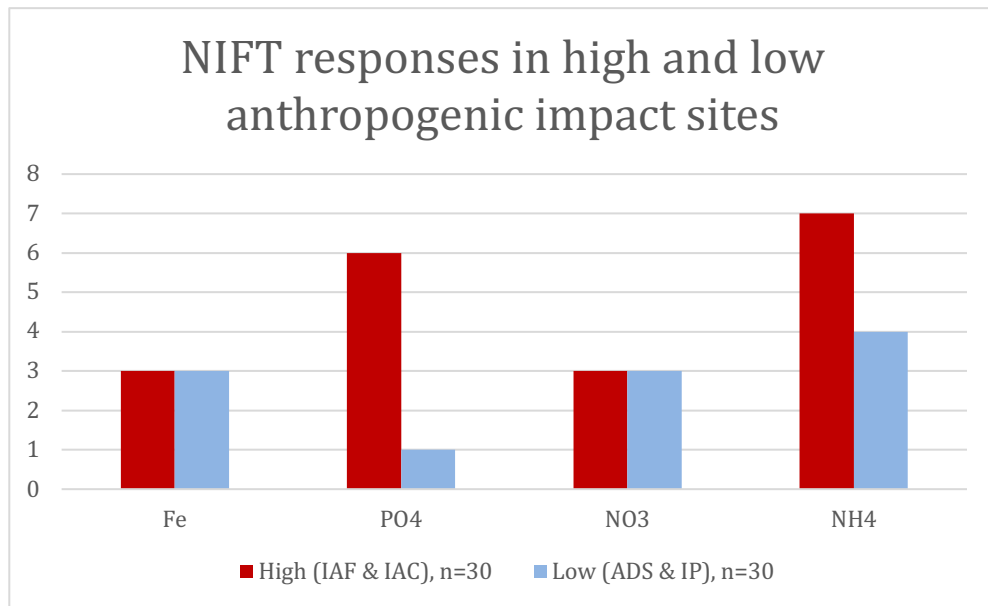


Figure 9. Combined NIFT response results in high anthropogenic impact (IAF & IAC) and low anthropogenic impact (ADS & IP) locations. N=30 in each experiment.. Difference is significant in PO<sub>4</sub> limitation (two-proportionate z-test, 95% confidence interval,  $p = 0,04$ ,  $z = 2,010753$ ).

### 3.1. Iron

Weak iron limitation was detected in all of the study sites. Six out of 60 iron enrichment experiments resulted in a NIFT response. The average response to iron was the same (10 per cent) in all locations. There was a notable difference in between the genera: *Sargassum polyceratum* in ADS showed iron limitation in 2/10 iron enrichment experiments (Figure 11), whereas *Dictyota menstrualis* 0/10 (figure 10). However, this difference was not statistically significant (two-proportionate z-test,  $p=0,136$ ,  $z=-1.491$ ).

Anthropogenic impact did not have an effect on iron limitation: iron enrichment resulted in NIFT response in 3/30 experiments both in high and low anthropogenic impact sites. The difference in iron limitation was thus greater between species than between sites.

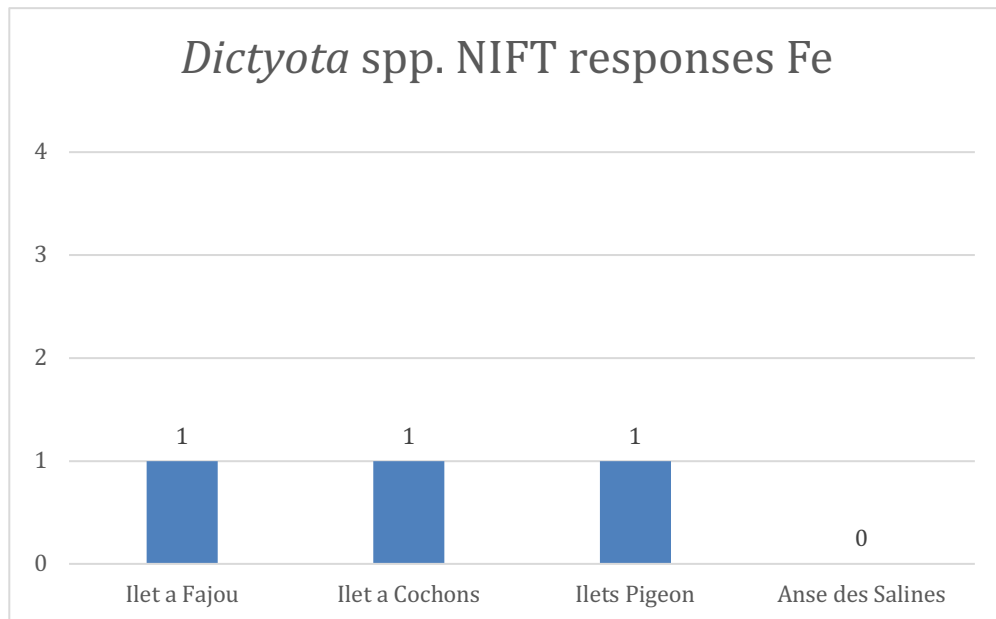


Figure 10. *Dictyota* spp. NIFT responses to iron enrichment experiments. N = 10 in each location.

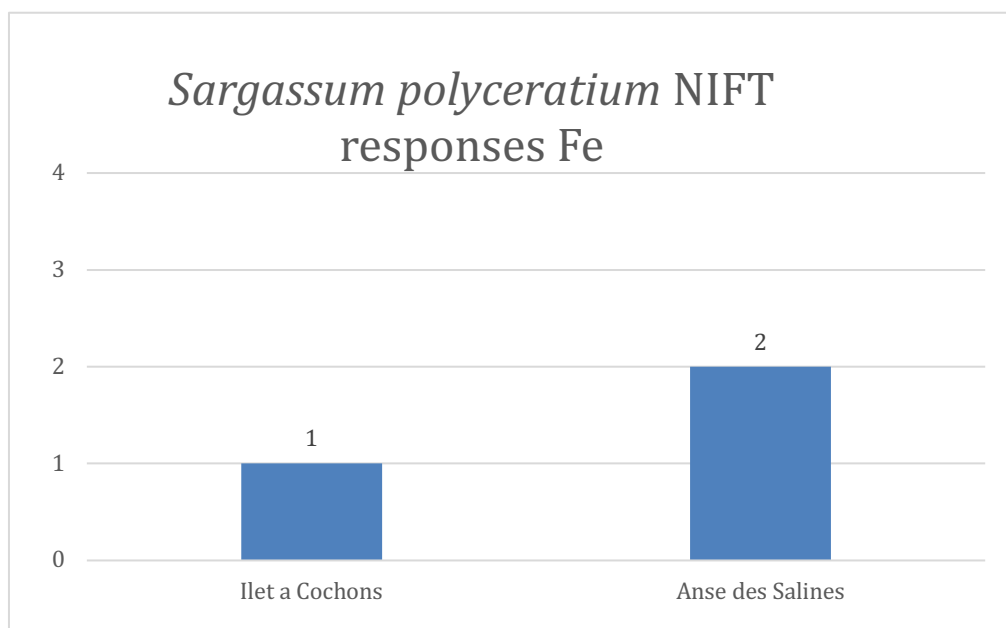


Figure 11. *Sargassum polyceratum* NIFT responses to iron enrichment experiments. N = 10 in both locations.

### 3.2. Phosphorus and nitrogen

NIFT responses to N and P were detected in all of the study sites. Seven out of 60 phosphate, 6/60 nitrate and 11/60 ammonium enrichment experiments resulted in a response (Figure 8). Phosphate and ammonium were the most limiting in IAC, the location with a wastewater outlet and an active harbor.

*Sargassum polyceratum* showed stronger limitation than *Dictyota* spp. in both IAC and ADS (Figures 12 & 13).

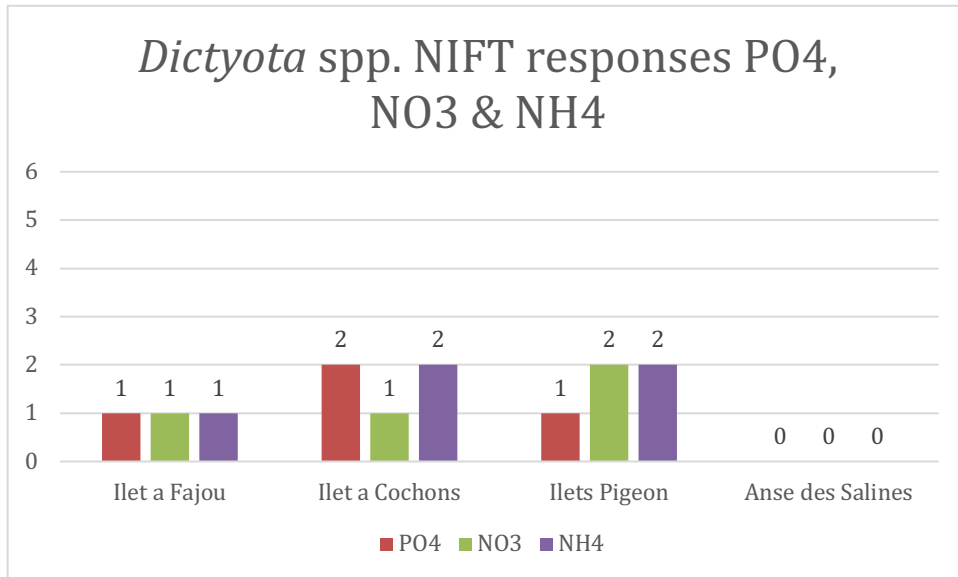


Figure 12. *Dictyota* spp. NIFT responses to phosphate, nitrate and ammonium additions in the four study sites. N = 10 in each nutrient enrichment and study site.

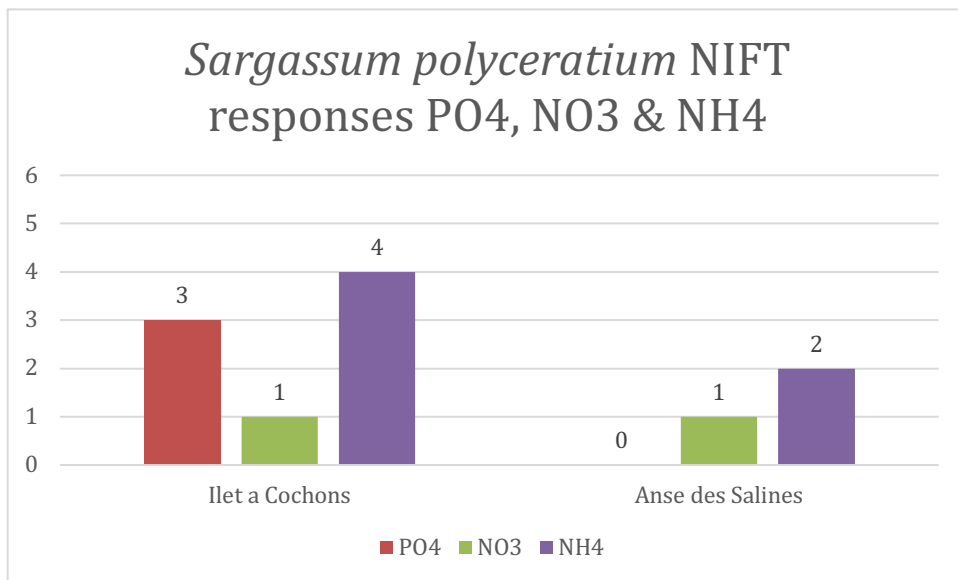


Figure 13. *Sargassum polyceratum* NIFT responses to phosphate, nitrate and ammonium additions in IAC and ADS. N = 10 in each nutrient enrichment and site.



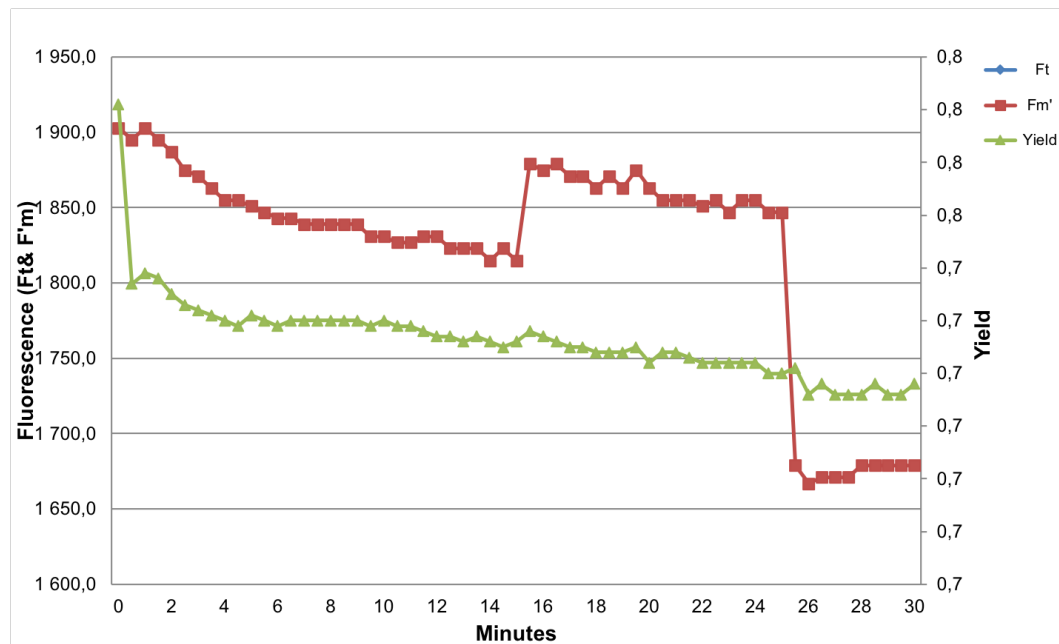


Figure 14. Phosphate and ammonium are co-limiting a *Dictyota menstrualis* specimen collected from IAC. NIFTs occur when F'm rises as a response to PO<sub>4</sub>-addition at 15 minutes and drops at 25 minutes after NH<sub>4</sub>-addition.

#### 4. Discussion

Before this study it was unclear if NIFT experiments are suitable for studying iron *in situ* because this was the first time the PAM fluorometer was used in the purpose. NIFTs occurred in response to iron enrichments, which suggests that this method can be used in iron studies. The observed rapid changes in the samples' fluorescence occurred in a similar pattern than in the NIFTs detected in the well-established studies on macronutrient limitation. Active assimilation of iron by an alga indicates iron limitation in the specimen. Another question is whether the equation developed by den Haan *et al.* (2013) sets the threshold of a NIFT response correctly. In the absence of a theoretical consensus on the physiology behind the response the results of this study are generated using this rather arbitrary value (a nutrient addition causing at least twice the size of a change in fluorescence compared to a control).

These results give limited support to the hypothesis of Roff & Mumby (2012) about the lowered resilience of the Caribbean reefs that are not limited by iron. In this study the iron limitation was weak or non-existent and there was no significant difference between the high and the low anthropogenic impact

sites ( $p=1.00$ ). The aeolian dust deposit is thus probably more important than anthropogenic impact and iron is not the main element to limit the productivity of the algae in these Guadeloupian coral reefs. Roff & Mumby state that the iron reserve that has accumulated over time in the Caribbean sediments would benefit the macroalgae that can overgrow the corals when required macronutrients are available. Aeolian dust is estimated to contribute three times as much iron in the ocean as riverine input. Iron is generally more abundant in the sediments on the continental shelf closer than 50 km to the shoreline, where most of the fringing and barrier reefs are located. Hannah Earp (unpublished data) conducted an *in situ* NIFT study in Moorea, French Polynesia, which is an area that does not receive aeolian dust but the study sites were closer than 50 kilometers from the shore, i.e. on the continental shelf. Iron limitation was weak in Earp's data: the most limited *Sargassum pacificum* expressed NIFT responses in 30% of the samples from a high anthropogenic impact site. Iron enrichment has caused severe changes in remote atolls naturally deplete of it (Kelly *et al.*, 2012). Considering that iron is present in the sediments in all areas close to the shore, these results do not provide enough information to determine whether the Caribbean reefs would be more vulnerable due to the aeolian iron but do back up the notion of the Caribbean Sea not being limited by iron.

The locations of this study were categorized according to the anthropogenic impact they experience. Nevertheless, the anthropogenic impact parameters were not quantified and thus one has to be careful in interpreting the results. Here was found that the nutrient load of anthropogenic origin does not affect localized iron limitation, but it has a significant effect on phosphorus limitation. The sediment's ability to bind and immobilize phosphorus depends greatly on the Fe(III) concentration of the sediment (Slomp *et al.*, 2013). In aerobic conditions the marine sediments with pore water Fe:P ratio of at least 2 have a high potential of binding phosphorus (Lehtoranta, Ekholm and Pitkänen, 2009). The study site with the greatest  $PO_4$  demand had a wastewater outlet. Sewage is a major source of organic matter and anthropogenic nutrient pollution in the Caribbean (DeGeorges, Goreau and

Reilly, 2010). Organic matter decomposition consumes oxygen in the sediment and causes reduction of Fe(III) into Fe(II), releasing P (Lehtoranta, Ekholm and Pitkänen, 2009). This process is largely controlled by Fe(III)-reducing bacteria (Lovley, Holmes and Nevin, 1991). Sewage nitrogen likely contributes to the molar N:P ratio of the water column, making it higher than the Redfield ratio of 16:1 that is generally considered optimal for algal growth (Redfield, 1958). In localities like this the anthropogenic impact might eventually result in hypoxia, changing of redox conditions and the arising elevated availability of dissolved inorganic phosphorus (Rozaan *et al.*, 2002). This eutrophication-induced process happens first in a smooth continuous manner but, after the critical threshold level of oxygen consumption has been reached and respiration exceeds the O<sub>2</sub> delivery to near-bottom water, results in rapid depletion of O<sub>2</sub> and consequent state transition (Lehtoranta, Ekholm and Pitkänen, 2009). On the other hand, if the redox conditions do not change for the worse, is it not good for the coastal P-limited reefs to receive iron to bind phosphorus and take it off from cycling? Fe(III) availability controls the P-flux in the sediment, making iron redox chemistry the key to the availability of inorganic phosphorus (Rozaan *et al.*, 2002).

While considering the roles of nitrogen and phosphorus, a common feature in ecosystems is co-limitation of N and P, which highlights the simplistic nature of the Liebig's law of the minimum (Harpole *et al.*, 2011). Ammonium, as well as phosphate, enrichments were followed by NIFT responses, in some cases in the same specimen (Figure 14). Macroalgae take up ammonium more readily than nitrate because the first is the reduced form of the latter and requires less energy to be assimilated (D'Elia and DeBoer, 1978; Teichberg *et al.*, 2008). Complementary use of different forms of N in multispecies ecosystems has been reported (Bracken and Stachowicz, 2006). Algae need nitrogen for protein synthesis and the strategies for uptake and storage are diverse. Iron is needed by N-fixating (diazotrophic) cyanobacteria in the nitrogenase enzyme (Kustka *et al.*, 2003). This underlines the complexity of the interactions in an ecosystem. The inorganic nutrients are a subject of

competition between different groups of autotrophs. Cyanobacteria are shown to exhibit direct competition with macroalgae and their abundance affects the nutrient dynamics of the reef (Kuffner and Paul, 2001). Perhaps the microalgae production in the most nutrient limited site Îlet à Cochons is more important than in Anse des Salines or Îlets Pigeon. Since iron in aerobic conditions is bioavailable in the water column only for a short period of time, it might be that the benthic macroalgae benefit from iron availability in sediment-water interface but lose in competition for soluble nitrogen and phosphorus that are more readily available to planktic producers. Nitrogen-fixing cyanobacteria might be more effective in the uptake of phosphorus and thus could cause a more severe P-limitation in macroalgae and consequently more dramatic impact of anthropogenic P-leaching. Microalgae and microbial mats were visibly present in Îlet à Cochons, where ammonium and phosphorus were the most important limiting nutrients (Figure 8).

The complexity of the coral reef ecosystem and the fragility of the oligotrophic balance call for vigorous quantification of the factors contributing to the studied question. Reef algal or coral cover was not quantified in this study. This might be problematic when interpreting the results since the nutrient flux and the reef's ability to recover from eutrophication event is dependent on the benthic community, i.e. species assemblage and relative abundance (Miyajima *et al.*, 2007). The NIFT response seems to be highly dependent on the studied species. Tropical marine macroalgal nutrient limitation has proved to be species and ecosystem-specific (Lapointe, Littler and Littler, 1987). *Sargassum polyceratium* appeared more "eager" than *Dictyota* spp. to assimilate nutrients; could this be more of sign of a different nutrient uptake strategy than an actual nutrient limitation in the reef? The life history of *S. polyceratium* is flexible and it is capable of plasticity in surviving disturbance events (Engelen *et al.*, 2005). Floating *Sargassum* spp. have caused nuisance blooms and thus seem to be effective in utilizing the available nutrients. *S. natans* and *S. fluitans* blooms in the Caribbean are discussed to be due to Amazon-originating nutrients while the import of dust aerosols is not so

important (Djakouré *et al.*, 2017). *S. baccularia* is highly responsive to nutrient pulses (Schaffelke and Klumpp, 1998). The NIFT study results of Earp (unpublished) show that differences in iron NIFT responses are greater between species than between locations. In Earp's data *Dictyota* spp. exhibited least nutrient limitation. Species of this genera are connected to nutrient pollution in the Caribbean Sea (DeGeorges, Goreau and Reilly, 2010). Beach, Walters and Borgeas (2006) demonstrate that responses in *Dictyota menstrualis* to nutrient enrichments vary both spatially and temporally but express generally very good adaptation ability. *Dictyota* spp. were present in most of the potential study sites in Guadeloupe during the inventory for this study. *D. bartayresiana* is characterized as a fast growing opportunist with a nutrient storage (Clausing and Fong, 2016). Perhaps the strategy of *Dictyota* spp. is to store nutrients in oligotrophic environments with episodic supply whereas *Sargassum* spp. invest in rapid uptake and growth when the nutrients are available. This emphasizes caution one should take with interpreting results gained studying only one species for a limited time. Information about algal tissue nutrient concentrations would also be beneficial to better understand the species-specific nutrient strategy and the importance of the individual's internal nutrient storage.

Indeed, one very important aspect to be considered is the temporal scale: since the dust storms are uneven in time, the timing of the sampling is crucial, especially with iron. Precipitation events can rapidly change the nutrient composition of a water column (Mendoza *et al.*, 2009) and thus affect the nutritional status of an alga. Episodic rainfall may contribute a large percentage of dust input in a particular location (Duce and Tindale, 1991). The enrichment experiments of this study were partially conducted during the so-called hurricane season with strong winds and rainfall, which might have affected the results. The necessity of frequent sampling in coral reef nutrient dynamics studies is underlined in Rouzé *et al.*'s (2015) research on the eutrophication effects on fringing reefs. Rapid uptake of iron by an alga happens in a similar pattern to that of macronutrients. If the input of bioavailable iron is continuous, it will likely affect iron limitation and iron-

associated nutrient dynamics in the areas receiving it. Atmospheric Fe(II) is bioavailable as a nutrient from seconds to minutes when entering ocean surface before settling to deeper water (Duce and Tindale, 1991). Organic ligands, especially bacterial siderophores, can enhance iron solubility considerably (Baker and Croot, 2010). The iron concentrations of ambient water or sediment interstitial water were not known in this study, so it was not possible to combine NIFT results with these data. More studies, e.g. iron starvation experiments combined with NIFT studies, are needed to apprehend both the nature of the NIFT response and the iron requirements of algae in a larger scale. Starvation studies performed in laboratory conditions are the backbone of the testimony power of the fluorescence-based method. NIFT experiments are a useful tool in building a more detailed understanding of the locality-specific nutritional state of the reef-degrading algae and thus the most effective mitigation actions, but best used combined with other necessary environmental data and adequately frequent sampling. Coral reef ecosystems are shown to be able to recover if ample measures are taken (Diaz-Pulido *et al.*, 2009).

## 5. References

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