Fast Detection of Nutrient Limitation in Macroalgae and Seagrass with Nutrient-Induced Fluorescence

Joost den Haan¹, Jef Huisman¹, Friso Dekker¹, Jacomina L. ten Brinke², Amanda K. Ford¹, Jan van Ooijen³, Fleur C. van Duyl³, Mark J. A. Vermeij¹,⁴, Petra M. Visser¹*  
¹Aquatic Microbiology, Institute for Biodiversity and Ecosystem Dynamics, University of Amsterdam, Amsterdam, The Netherlands, ²Aquaculture and Fisheries Group, Wageningen University, Wageningen, The Netherlands, ³Royal Netherlands Institute for Sea Research (NIOZ), Den Burg, Texel, The Netherlands, ⁴CARMABI Foundation, Willemstad, Curaçao

Abstract

**Background:** Rapid determination of which nutrients limit the primary production of macroalgae and seagrasses is vital for understanding the impacts of eutrophication on marine and freshwater ecosystems. However, current methods to assess nutrient limitation are often cumbersome and time consuming. For phytoplankton, a rapid method has been described based on short-term changes in chlorophyll fluorescence upon nutrient addition, also known as Nutrient-Induced Fluorescence Transients (NIFTs). Thus far, though, the NIFT technique was not well suited for macroalgae and seagrasses.

**Methodology & Principal Findings:** We developed a new experimental setup so that the NIFT technique can be used to assess nutrient limitation of benthic macroalgae and seagrasses. We first tested the applicability of the technique on sea lettuce (*Ulva lactuca*) cultured in the laboratory on nutrient-enriched medium without either nitrogen or phosphorus. Addition of the limiting nutrient resulted in a characteristic change in the fluorescence signal, whereas addition of non-limiting nutrients did not yield a response. Next, we applied the NIFT technique to field samples of the encrusting fan-leaf alga *Lobophora variegata*, one of the key algal species often involved in the degradation of coral reef ecosystems. The results pointed at co-limitation of *L. variegata* by phosphorus and nitrogen, although it responded more strongly to phosphate than to nitrate and ammonium addition. For turtle grass (*Thalassia testudinum*) we found the opposite result, with a stronger NIFT response to nitrate and ammonium than to phosphate.

**Conclusions & Significance:** Our extension of the NIFT technique offers an easy and fast method (30–60 min per sample) to determine nutrient limitation of macroalgae and seagrasses. We successfully applied this technique to macroalgae on coral reef ecosystems and to seagrass in a tropical inner bay, and foresee wider application to other aquatic plants, and to other marine and freshwater ecosystems.

Citation: Haan JD, Huisman J, Dekker F, ten Brinke JL, Ford AK, et al. (2013) Fast Detection of Nutrient Limitation in Macroalgae and Seagrass with Nutrient-Induced Fluorescence. PLoS ONE 8(7): e68834. doi:10.1371/journal.pone.0068834

Editor: Heroen Verbruggen, University of Melbourne, Australia

Received April 13, 2013; Accepted June 3, 2013; Published July 5, 2013

Copyright: © 2013 den Haan et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This research was funded by the European Union Seventh Framework Programme (P7/2007–2013) under grant agreement no. 244161 (Future of Reefs in a Changing Environment), and by the Schure-Beijerinck-Popping Fund (SBP/JK/2011-31 KNAW, The Netherlands). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: p.m.visser@uva.nl

Introduction

Eutrophication can lead to highly adverse changes in the structure and functioning of freshwater and marine ecosystems [1–3]. Enrichment with nitrogen (N) and phosphorus (P) often relieves primary producers from nutrient limitation, enhancing the productivity of micro- and macroalgae. This may result in reduced water clarity, development of harmful algal blooms, nighttime oxygen depletion, strong diel fluctuations in pH, and the smothering of coral reefs and other benthic communities [2,4–6]. Therefore, a fast and easy method to identify which nutrients limit the primary production of micro- and macroalgae can be of considerable value to assess potential effects of future nutrient enrichments, and may help to increase the effectiveness of nutrient reduction programs in a wide variety of different water bodies.

Existing methods to assess nutrient limitation in macroalgae and aquatic plants are based on (1) analysis of ambient nutrient concentrations [7,8], (2) element ratio analysis of algal tissue [8–10], and (3) nutrient enrichment assays [8,11–14]. Analysis of ambient nutrient concentrations in the overlying water can be fast, but is not sufficiently informative to determine the nutrient status of benthic organisms. Element ratio analysis of algal tissue and nutrient enrichment assays may take considerable amounts of time to identify nutrient limitation in algae, often lasting several hours or days. Furthermore, especially in nutrient enrichment assays, the organisms are often studied under artificial conditions, possibly complicating the interpretation of results. Hence, there is a need for a fast and informative technique that can be easily applied in situ. For phytoplankton, such a method exists in the form of Nutrient-Induced Fluorescence Transient (NIFT) experiments, where nutrient limitation can be detected within minutes [15].

NIFT experiments are based on the principle that addition of limiting nutrients induces transient changes in chlorophyll a fluorescence, which can be detected with a Pulse Amplitude
Modulation (PAM) fluorometer [15–20]. Enhanced uptake and assimilation of limiting nutrients increases the demand for ATP and/or reductants. This relieves pressure on the photosynthetic electron transport chain, which can alter non-photochemical quenching, the redox state of the plastoquinone pool, state transitions between photosystems I and II, and the relative importance of linear versus cyclic electron transport [21]. These changes affect the fluorescence signal since the processing of absorbed light energy by photochemistry, fluorescence and heat dissipation occurs in competition [22]. Hence, a transient change in fluorescence upon nutrient addition provides direct evidence for a change in algal nutrient status. When a non-limiting nutrient or distilled water is added to a phytoplankton culture, generally no change in fluorescence is observed [9].

Since the photosynthetic apparatus operates essentially in a similar way across all oxygen-producing phototrophic organisms, the NIFT technique should in principle be applicable not only to phytoplankton but also to macroalgae, seagrasses and other aquatic plants. However, a major obstacle for application of the NIFT technique to macroalgae and aquatic plants is that they cannot be homogeneously resuspended in a cuvette, which is standard procedure for microalgae [9,15]. The leaf clips commonly used in PAM fluorometry with macroalgae and seagrasses are not suitable for NIFT studies, because they either cannot hold the sampled leaf at exactly the same position or they interfere with full access of the leaf to the nutrients added during a NIFT experiment. To address this issue, we developed a special set-up that we have called the PAM fluoroscope. This set-up uses a magnetic leaf clip that allows easy and even addition of a nutrient pulse, while keeping the sample in exactly the same position in front of the PAM sensor.

In this study, we tested the applicability of the NIFT technique to macroalgae and sea grasses. We first used laboratory-controlled conditions to ensure that sea lettuce (Ulva lactuca) became either N or P starved, and followed its fluorescence after re-supply of the limiting and non-limiting nutrient to assess its NIFT response. After successful testing of the method, we collected samples of the macroalga Lobophora variegata from a degraded and less degraded coral reef, and assessed by which nutrient it was limited. Similar experiments were conducted with the seagrass Thalassia testudinum, growing in a nearby bay.

Materials and Methods

Research Sites

This study was conducted on the island of Curacao, Southern Caribbean, at research sites ‘Buoy 0’ (12°7’N, 68°58’W), ‘Playa Kalki’ (12°22’N, 69°9’W), ‘Water Factory’ (12°6’N, 68°56’W), and ‘Boka Ascension’ (12°16’N, 69°3’W) (Fig. 1). Buoy 0 and Playa Kalki are both coral reef ecosystems. However, Buoy 0 is a more degraded reef, with a lower cover by hard corals and higher cover by macroalgae and turf algae than Playa Kalki. The site Water Factory is characterized by large beds of sea lettuce in the intertidal zone. Boka Ascension is a shallow inner bay with large beds of turtle grass. Permission to conduct our studies was provided by the Ministry of Health, Environment and Nature (GMN) of the government of Curacao through their permit (#48584) to the Caribbean Marine Biological Institute (CARMABI) at Willemstad.

Laboratory Incubation of Ulva lactuca

Samples (~2 cm²) of leaves of sea lettuce (Ulva lactuca Linnaeus) were manually collected from the intertidal zone at the Water Factory. The sampled leaves were transported to the laboratory facilities of CARMABI, where all NIFT experiments were conducted. During transport from reef to laboratory, samples were kept at a temperature of 27–29°C and shaded using a small cool box with seawater collected at the sampling location.

To test the presence of a NIFT response under controlled laboratory conditions, the collected U. lactuca leaves were starved of either N or P for three weeks. Samples were incubated in 300 ml glass incubators containing filtered seawater (Whatman

![Figure 1. Map of Curacao.](https://example.com/curacao_map.png)
cellulose acetate membrane filters, pore size 0.22 μm, O 25 mm) collected from surface water at Buoy 0. The nutrient concentrations in this seawater were 0.25 μM NO₃⁻, 0.90 μM NH₄⁺, and 0.07 μM PO₄³⁻. Each sample received additional FeCl₃ (0.16 μM) to ensure that iron did not become a limiting factor. To prepare P-limited medium, NO₃⁻ and NH₄⁺ were added to the filtered seawater at final concentrations of 5.1 μM and 18.6 μM, respectively. To prepare N-limited medium, PO₄³⁻ was added at a final concentration of 1.4 μM.

Glass incubators with P-limited and N-limited medium were placed in triplicate inside an aquarium, which was connected to a water pump that provided continuous water flow to keep the samples at a similar temperature of 27–29°C as on the reef. The aquaria were placed outdoors in full sunlight to mimic the natural high-light environment of *U. lactuca*. Water from the aquarium could not mix with the mineral medium in the incubators. Each incubator received continuous aeration using two Sera Precision Air 550R Plus membrane pumps (Sera GmbH, Heinsberg, Germany). Each week, the incubation solution was renewed. The NIFT responses of N-starved and P-starved *U. lactuca* leaves to the addition of NO₃⁻, NH₄⁺ and PO₄³⁻ were determined every other day for 19 days.

### Field Samples of Macroalgae and Seagrass

Individual leaves of the encrusting fan-leaved alga *Lobophora variegata* (J.V. Lamouroux) Womersley ex E.C. Oliveira were collected from 20 m depth on the coral reefs of research sites Buoy 0 and Playa Kalki by means of SCUBA diving. Leaves of turtle grass (*Thalassia testudinum* Banks ex König) were collected from ~1 m depth at Boka Ascencion, and cut into 1 cm² pieces. All sampled leaves were manually cleaned of epiphytes and detritus. The leaves were kept at a temperature of 27–29°C and shaded during transport to the laboratory using a small cool box containing ambient seawater. NIFT measurements on the fresh *L. variegata* and *T. testudinum* samples commenced directly after transportation from the field sites to the laboratory, within 1–2 h after sampling. For *L. variegata*, we used 36 leaves per nutrient treatment from Playa Kalki and 36 leaves per nutrient treatment from Buoy 0. For *T. testudinum*, we measured the NIFT response of 20 leaves.

To interpret possible differences in NIFT response of *L. variegata* sampled from Buoy 0 and Playa Kalki, we briefly compared the environmental growth conditions at these two research sites. At both sites, we placed a 100 m horizontal transect line on the coral reef at 20 m depth. Benthic cover of hard corals and macroalgae was determined from photographs of 60 randomly placed quadrates (1.5 m²) distributed along both sides of this transect line. The photographs were analysed using the computer program Coral Point Count with Excel Extensions (CPCe) [23]. Furthermore, water samples were taken along the horizontal transect at 10 cm above the reef using a 60 ml syringe (n = 14 at Buoy 0, n = 17 at Playa Kalki). Water samples were quickly filtered at the dive site using a 0.22 μm Acrodisc filter and stored in 6 ml polyethylene vials (PerkinElmer, MA, USA) at ~20°C until further analysis. Concentrations of NO₃⁻ [24], NH₄⁺ [25], and PO₄³⁻ [26] were analysed at the Royal Netherlands Institute for Sea Research (NIOZ), the Netherlands, using continuous flow analysis via a Quatro auto-analyzer (Seal Analytical, UK).

### Nutrient-Induced Fluorescence Transient (NIFT) Experiments

Changes in variable chlorophyll *a* fluorescence in response to different nutrient additions were measured with a Diving-PAM/B Underwater Fluorometer (Walz Mess- und Regeltechnik, Effeltrich, Germany) using the experimental set-up shown in Fig. 2. Individual *U. lactuca*, *L. variegata*, and *T. testudinum* leaves were placed between two 2 mm thick ¼ round magnetic rings (see inset in Fig. 2) and attached to a magnetic sensor head to ensure that the samples were situated exactly 2 mm in front of the PAM sensor [27]. The sensor head with the attached sample was then placed inside a Ø 54 mm Petri dish containing 15 ml of either enriched seawater (laboratory incubations of *U. lactuca*) or ambient seawater (field samples of *L. variegata* and *T. testudinum*). The use of the ¼ magnetic rings ensured that the nutrient solution always reached the entire leaf surface of the sample on both sides.

Before each NIFT experiment, samples were incubated in the dark for 10 min. Subsequently, at the start of the NIFT experiment, the weak measuring light of the PAM fluorometer was switched on to determine (1) the initial fluorescence (*F₀*) and (2) maximum fluorescence following a saturating light pulse (*Fₘ*). Thereafter, samples were exposed to actinic light (PAR, 400–700 nm) of 110 μmol photons m⁻² s⁻¹ provided by a LED-56 Microscope Ring Light (AmScope Corp., Irvine, CA), to monitor (3) steady-state fluorescence (*Fₛ*), and (4) maximum fluorescence following a saturating light pulse (*Fₘₛ*). *F₀* and *Fₘₛ* were measured at 30 s intervals (PAM settings: measuring light = 10, gain = 2, SW = 0.4, SI = 4). After 10 min, a 1.5 ml control solution (with the same nutrient composition as in the incubation glass for *U. lactuca* with ambient seawater for *L. variegata* and *T. testudinum*) was added to the Petri dish to check whether the addition itself caused a change in fluorescence. After another 5 min, different nutrient solutions were added at 5-min intervals to assess changes in the fluorescence parameters (*Fᵢ* and *Fₘₛ*) upon nutrient resupply.

**Figure 2. PAM fluoroscope used for NIFT experiments.** PAM fluoroscope, consisting of (1) two ¼ magnetic rings for proper sample placement in front of PAM sensor; (2) magnetic PAM sensor head; (3) PAM sensor; (4) adjustable holder for placement of PAM sensor; (5) adjustable Petri dish holder; (6) LED-light with adjustable light intensity. doi:10.1371/journal.pone.0068834.g002
typical NIFT experiment lasted 30 to 60 min in total (including the 10 min of dark incubation).

Nutrient uptake rates of macroalgae and seagrasses are often enhanced when nutrients are supplied in combination with water movement. However, water movement is not desired during NIFT experiments, as our observations showed that mild movement of the leaves was already sufficient to affect the fluorescence signal. To overcome the limited mass transfer of nutrients across the boundary layer of leaves incubated in stagnant water, we therefore applied relatively high nutrient concentrations in the nutrient additions, ranging from 10 to 250 µM of NO₃⁻, NH₄⁺, and PO₄³⁻. These concentrations are similar to those applied in earlier macroalgal studies [15]. In pilot experiments we measured NH₄ uptake rates and NIFT responses of U. lactuca under controlled laboratory conditions at 10, 100 and 200 µM NH₄ concentrations (unpublished data, J. den Haan), since it is known that NH₄ can have toxic effects at high concentrations. The results did not show any unusual NIFT responses. Furthermore, NH₄ uptake rates were not suppressed at the higher NH₄ levels, and were of similar magnitude as in previous studies with U. lactuca with toxic across this concentration range. Our first NIFT experiments, (Fig. 3):

\[ Q_2 = \frac{(\Delta F_9^{m \text{ nutrient}})}{(\Delta F_2^{m \text{ control}})} \]  

We judged the NIFT response as real, if the response to nutrient addition was at least twice as large as the response to the control solution (i.e., \( Q_2 \geq 2 \) and/or \( Q_2 \geq 2 \)). These criteria are of course somewhat arbitrary. We could have focused on changes in F₉, or \( \Phi_{PSII} \) (instead of \( F_9^{m \text{ nut}} \)), or we could have set the threshold values of \( Q_1 \) and \( Q_2 \) at another value (instead of 2). However, in 95% of the NIFT experiments with L. variegata (n = 108), assessment of the NIFT responses based on these criteria matched our intuitive judgment, which indicated that these criteria provided a useful guideline.

Results

Laboratory Incubations of Nutrient-limited Ulva lactuca

Fig. 4A shows a typical NIFT response to NO₃⁻ addition of an U. lactuca sample that had been N starved for 11 days. \( F_9^{m} \) was at its maximum at the first saturating light pulse (i.e., \( F_m = F_m^{t=0} \), since the sample had previously been dark adapted for 10 minutes. Hence, all PSII reaction centers were ready to carry out photochemistry, while heat dissipation (NPQ) was not yet operational (Eq. 2). After this first light pulse, actinic light was turned on. As a consequence, \( F_9^{m} \) initially decreased while NPQ increased, indicating that the heat dissipation mechanism was operational from the second light pulse [at \( t = 0.5 \) min] onwards. After 20 light pulses \( (t = 10 \) min), a control solution with the same nutrient composition as in the incubation glass was added, which did not result in a change in any of the fluorescence variables \( (F_t, F_m, \Phi_{PSII} \text{ and NPQ}) \). In contrast, after addition of 100 µM NO₃⁻ \( (t = 15 \) min) and 100 µM NO₃⁻ \( (t = 20 \) min), \( F_m \) and \( \Phi_{PSII} \) increased, whereas NPQ decreased. The addition of 250 µM NO₃⁻ after 25 min did not result in a response in any of the variables.

Addition of NH₄⁺ to N-starved U. lactuca led to similar results as NO₃⁻ addition, with an increase of \( F_m \) and reduction of NPQ (Fig. 4B). In contrast, addition of PO₄³⁻ to N-starved U. lactuca did not yield a NIFT response in 90% of the cases (n = 10) (Fig. 4C). Conversely, P-starved U. lactuca did not respond to the addition of NO₃⁻ and NH₄⁺ (n = 8) (Fig. 4D,E), but showed a clear NIFT response to PO₄³⁻ addition (Fig. 4F).

Effect of Starvation Period on the NIFT Response

To assess whether the duration of the starvation period affected the results, we investigated the NIFT response during three different time intervals of nutrient starvation (days 1–5, 6–10, and 11–15). We focused on the NIFT response of N-starved U. lactuca to NO₃⁻ and NH₄⁺ addition, and P-starved U. lactuca to PO₄³⁻ addition, using the same sequence of nutrient additions [10, 100 and 250 µM] as in Fig. 4. In some cases, we did not find a NIFT response at the highest nutrient dosage of 250 µM (see, e.g., Fig. 4A), presumably because the uptake systems were already nutrient-saturated from the earlier addition of 100 µM. Hence, we decided that if the \( F_m \) of U. lactuca responded to at least one of the three nutrient dosages, this was marked as a positive NIFT response, indicating that U. lactuca was indeed N or P limited. Between days 1–5, approximately 50% of the N-starved U. lactuca showed a positive NIFT response to NO₃⁻ and NH₄⁺ addition, while 33% of the P-starved U. lactuca responded to PO₄³⁻ addition. This indicated that the samples were already nutrient limited from the start of the experiments. The percentage of
positive NIFT responses increased up to 60–70% for both N-starved and P-starved leaves of *U. lactuca* after 6–10 days of nutrient starvation. After 11–15 days, the percentage of positive NIFT responses decreased slightly to 47–60%. This coincided with a reduction of *W*<sub>PSII</sub> to 0.2–0.3 after 15 days of nutrient starvation. For comparison, a healthy nutrient-replete *U. lactuca* leaf has a *W*<sub>PSII</sub> of 0.6–0.7.

Field Samples of the Macroalga *Lobophora variegata*

We investigated the NIFT response of *L. variegata* leaves collected from the research sites Playa Kalki and Buoy 0. Playa Kalki is a coral reef ecosystem with ~25% cover by hard corals and ~50% cover by algae (including *L. variegata*) (Table 1). In contrast, Buoy 0 is a more degraded reef ecosystem with only 10% cover by hard corals and almost 60% algal cover. *L. variegata* was nearly twice as abundant at Buoy 0 as at Playa Kalki (Table 1). Concentrations of dissolved NO<sub>3</sub><sup>-</sup> and PO<sub>4</sub><sup>3-</sup> were significantly higher at Buoy 0 than at Playa Kalki, while the NH<sub>4</sub><sup>+</sup> concentration was not significantly different between the two sites (Table 1). The N:P ratio seemed slightly higher at Buoy 0 (16.5:1) than at Playa Kalki (14.4:1), indicating that the growth conditions might be relatively more P limited and less N limited at Buoy 0 than at Playa Kalki, but the difference was not significant (Table 1).

Typical NIFT responses of *L. variegata* to the addition of 100 μM of NO<sub>3</sub><sup>-</sup>, NH<sub>4</sub><sup>+</sup>, and PO<sub>4</sub><sup>3-</sup> are illustrated in Fig. 5A, B and C, respectively. Interestingly, *L. variegata* showed positive NIFT responses to both N and P additions, although a significantly larger percentage of samples responded to PO<sub>4</sub><sup>3-</sup> addition (84%) than to NO<sub>3</sub><sup>-</sup> and NH<sub>4</sub><sup>+</sup> addition (38%) (Fig. 6; Two Proportion Z-test; Z = 4.5, df = 106, P < 0.001). This indicates that *L. variegata* was co-limited by N and P, but with a stronger limitation by P than by N. Moreover, the data suggest that the nutrient limitation pattern was slightly different between the two research sites. That is, although differences between the two sites were only marginally significant, *L. variegata* seemed more strongly limited by PO<sub>4</sub><sup>3-</sup> (Two Proportion Z-test; Z = 1.90; df = 70; P = 0.05) and less strongly limited by NO<sub>3</sub><sup>-</sup> (Two Proportion Z-test; Z = -1.79; df = 70; P = 0.07) at Buoy 0 than at Playa Kalki (Fig. 6).

When combining all positive NIFT responses of *L. variegata*, NO<sub>3</sub><sup>-</sup> addition resulted in an increase in F<sub>m</sub> and decrease of NPQ in 87% of all positive NIFT responses. In 13% of the positive NIFT responses, F<sub>m</sub> decreased while NPQ increased upon NO<sub>3</sub><sup>-</sup> addition. Similar results were obtained for NH<sub>4</sub><sup>+</sup> addition, where 72% of the positive NIFT responses showed an increase in F<sub>m</sub> and 28% a decrease. Interestingly, the NIFT response of *L. variegata* to PO<sub>4</sub><sup>3-</sup> addition showed the opposite pattern, with a decreasing F<sub>m</sub> and increasing NPQ in 94% of all positive NIFT responses. An example is shown in Fig. 5C. Conversely, F<sub>m</sub> increased while NPQ decreased in only 6% of the positive NIFT responses to PO<sub>4</sub><sup>3-</sup> addition.
In a series of extra NIFT experiments we added 100 μM of NO₃⁻, NH₄⁺ and PO₄³⁻ in randomized order at 5 min intervals to the same L. variegata sample. This showed that the first nutrient added did not affect the response to the consecutive addition (P = 0.93; Two Proportion Z-test for data Buoy 0 and Playa Kalki combined, n = 61). This can shorten the duration of NIFT experiments substantially. Earlier we investigated each nutrient separately in NIFT experiments of 20 min per nutrient (Fig. 5).

Figure 4. NIFT responses of nutrient-starved Ulva lactuca. Examples of the NIFT response of a N-starved U. lactuca leaf to (A) NO₃⁻ addition, (B) NH₄⁺ addition, and (C) PO₄³⁻ addition, and a P-starved U. lactuca leaf to (D) NO₃⁻ addition, (E) NH₄⁺ addition, and (F) PO₄³⁻ addition. The graphs show the time courses of steady-state fluorescence, Fₜ (×); maximum fluorescence, F₉₅ (○); the quantum yield of photosystem II, ΦPSII (△); and non-photochemical quenching, NPQ (□). Vertical dashed lines indicate the timing of the control addition (0 μM) and three consecutive nutrient additions (10, 100 and 250 μM).

doi:10.1371/journal.pone.0068834.g004
Each of these experiments was preceded by 10 min of dark adaptation. Hence, studying NO$_3^-$, NH$_4^+$ and PO$_4^{3-}$ in three separate NIFT experiments took at least 90 min. Now all three nutrients can be investigated in one run of 10 min dark adaptation plus 30 min of NIFT measurements, reducing the total duration of the experiment to only 40 min.

Field Samples of the Seagrass *Thalassia testudinum*

Fig. 7 shows a typical NIFT response of *T. testudinum* to the sequential addition of 100 μM PO$_4^{3-}$, NO$_3^-$ and NH$_4^+$ at 5 min intervals. In total, 4 of the 20 *T. testudinum* samples collected from research site Boka Ascencion responded to NO$_3^-$ and/or NH$_4^+$ addition (as in Fig. 7), while 1 sample responded only to PO$_4^{3-}$ addition. In all these cases, $F'_m$ increased while NPQ decreased upon nutrient addition.

**Discussion**

**Evaluation of the NIFT Technique**

Previous studies have shown that nutrient-induced fluorescent transients (NIFTs) provide an easy and fast means to determine which nutrients limit phytoplankton productivity [15–20]. Building upon this existing experience, we aimed to investigate whether NIFT measurements can also assess nutrient limitation in macroalgae and seagrasses. A key ingredient in our approach is the use of a special device that we have called the PAM fluoroscope, which enables exposure of algal thalli and leaves to a series of nutrient additions while keeping these leaves at exactly the same position in front of the PAM sensor. Controlled laboratory experiments with N-starved and P-starved sea lettuce (*U. lactuca*) showed that addition of the limiting nutrient resulted in characteristic changes in chlorophyll a fluorescence ($F'_m$) while addition of a non-limiting nutrient did not affect the fluorescence signal. Furthermore, we showed that the NIFT technique could detect nutrient limitation of the macroalga *L. variegata* and the seagrass *T. testudinum* directly after they were collected from the field. Hence, our results demonstrate that the NIFT technique can be successfully applied to macroalgae and seagrass, important representatives of the benthic primary producers inhabiting many coastal waters, coral reefs and shallow lakes.

Surprisingly, even during controlled nutrient starvation in the laboratory, the percentage of positive NIFT responses in *U. lactuca* never exceeded 70%. That is, even under stringent limitation, approximately one third of the *U. lactuca* leaves did not show a NIFT response. This contrasts with phytoplankton studies, where laboratory experiments have shown positive NIFT responses in up to 100% of the assays [19]. In our field samples, the maximum percentage of positive NIFT responses was 92% for *L. variegata* but only 25% for *T. testudinum*. The low percentage of positive NIFT responses for *T. testudinum* may indicate that either this species is not very responsive to NIFT measurements, or that it was not strongly nutrient limited at its sampling site in the bay of Boka Ascencion. In contrast to macroalgae, seagrasses like *T. testudinum* can also extract nutrients from the sediment through their root system [34,35]. Hence, they may be less subjected to nutrient limitation than macroalgae that acquire their nutrients only from the surrounding water column. Further studies comparing nutrient limitation in macroalgae and seagrasses will be required to investigate this hypothesis in more detail. All in all, these results indicate that studies of nutrient limitation in macroalgae and seagrasses using the NIFT technique should always sample a sufficient number of leaves (say, at least 10–20 leaves) to obtain reliable results.

**The Nature of the NIFT Response**

A somewhat naive but straightforward explanation for nutrient-induced changes in fluorescence would be that enhanced nutrient assimilation increases the demand for ATP and NADPH. This relieves pressure on photosynthetic electron transport, and, hence, one would expect a decrease in chlorophyll fluorescence. However, our results show that fluorescence can either increase or decrease upon nutrient addition, depending on the nutrient being added and the species being studied. For instance, we found that maximum fluorescence ($F'_m$) of *U. lactuca*, *L. variegata*, and *T. testudinum* increased upon NO$_3^-$ addition, while non-photochemical quenching (NPQ) decreased. Similar variation in the NIFT response has also been observed in previous studies with microalgae [15]. An increase in chlorophyll fluorescence and drop in NPQ upon NO$_3^-$ addition was reported for the unicellular green alga *Dunaliella tertiolecta* [36], but another green alga, *Chlorella emersonii*, showed the opposite response [37], NO$_3^-$ uptake and assimilation requires both ATP and NADPH. Shelly et al. [15] therefore hypothesized that the rise in fluorescence and drop in NPQ might be explained by state transitions between PSI and PSII. State transitions are rapid physiological adaptation mechanisms that adjust the way absorbed light is distributed between the two photosystems. A state transition from State 2 to State 1 will increase the contribution of PSII, and hence linear electron transport to produce both ATP and the required reduction equivalents [15]. Since nearly all chlorophyll fluores-
Examples of the NIFT response to (A) NO$_3^-$ addition of a $L.$ variegata leaf collected at Playa Kalki, (B) NH$_4^+$ addition of a $L.$ variegata leaf collected at Buoy 0, and (C) PO$_4^{3-}$ addition of a $L.$ variegata leaf collected from Playa Kalki.

Figure 5. NIFT responses of $L.$ variegata collected from the reef. Examples of the NIFT response to (A) NO$_3^-$ addition of a $L.$ variegata leaf collected at Playa Kalki, (B) NH$_4^+$ addition of a $L.$ variegata leaf collected at Buoy 0, and (C) PO$_4^{3-}$ addition of a $L.$ variegata leaf collected from Playa Kalki. The graphs show the time courses of steady-state fluorescence, $F_0$ (+); maximum fluorescence, $F_m$ (×); the quantum yield of photosystem II, $\Phi_{PSII}$ (○); and non-photochemical quenching, NPQ (□). Vertical dashed lines indicate the timing of the control addition (0 µM) and different nutrient additions (all at 100 µM). doi:10.1371/journal.pone.0068834.g005

The NIFT response increases with the severity of nutrient limitation [19,36]. For instance, Holland et al. [19] sampled natural phytoplankton populations from several Australian waters, and did not observe any positive NIFT responses on the day of collection. Positive NIFT responses appeared only after the samples had been exposed to several days of nutrient starvation under controlled laboratory conditions. This contrasts with our findings, where $U.$ lactuca, $L.$ variegata and to a somewhat lesser extent also $T.$ testudinum all showed positive NIFT responses on the day of collection. Moreover, freshly collected $U.$ lactuca showed relatively mild changes in the percentage of positive NIFT responses during the subsequent two weeks of nutrient starvation in controlled laboratory incubations. This indicates that $U.$ lactuca, and probably also the other two species that we investigated, were already strongly nutrient limited prior to sampling, i.e., in their natural habitat.

Co-limitation by Nitrogen and Phosphorus

Our results indicate that at least part of the natural population of $L.$ variegata was co-limited by nitrogen and phosphorus. Previous NIFT studies with phytoplankton grown under controlled nutrient conditions have shown that addition of the limiting nutrient produces a positive NIFT response, whereas addition of non-limiting nutrients generally does not cause a change in fluorescence [9]. The same pattern was observed in our laboratory incubations with the macroalga $U.$ lactuca, where addition of nitrogen to N-starved leaves and addition of phosphorus to P-starved leaves resulted in a positive NIFT response, while addition of non-limiting nutrients did not affect the fluorescence signal. Hence, the observation that freshly collected leaves of $L.$ variegata showed positive NIFT responses to both nitrogen and phosphorus addition points at co-limitation by these two nutrients. Co-limitation by N and P is consistent with the low concentrations of dissolved inorganic nitrogen and phosphorus, at a N:P ratio close to the Redfield ratio of 16:1, measured in ambient seawater at both research stations Playa Kalki and Buoy 0 (Table 1). Interestingly, the NIFT data even picked up a subtle difference in N:P ratios between the two research sites, as $L.$ variegata was somewhat more P limited and less N limited at Buoy 0 than at Playa Kalki.
Co-limitation by N and P has also been observed for several macroalgal species of the Great Barrier Reef, Australia, including *Sargassum baccularia*, *Hydroclathrus clathratus*, *Turbinaria ornata*, and *Padina tenuis* [38–40], where the addition of short-term N and P pulses resulted in increased primary production and/or incorporation of these nutrients into their thalli as temporary storage to sustain growth during periods of low nutrient availability. Since co-limitation has not been investigated in earlier NIFT studies [15], our study seems to be the first to demonstrate that co-limitation by two nutrients can be detected with NIFT measurements.

**Figure 6. Nutrient limitation of Lobophora variegata at two different research sites.** Percentage of positive NIFT responses of *L. variegata* leaves, collected from Playa Kalki and Buoy 0, to addition of 100 μM of NO$_3^-$, NH$_4^+$ and PO$_4^{3-}$. Differences between the two research sites were tested with the Two Proportion Z-test. NS is not significant at P≥0.10; n = 36 per research site and nutrient treatment. doi:10.1371/journal.pone.0068834.g006

**Figure 7. Typical NIFT response of Thalassia testudinum.** Example of the NIFT response of a *T. testudinum* leaf collected at Boka Ascension. The graph shows the time courses of steady-state fluorescence, $F_s$ (+); maximum fluorescence, $F_{\text{m}}$ (×); the quantum yield of photosystem II, $\Phi_{\text{PSII}}$; and non-photochemical quenching, NPQ (○). Vertical dashed lines indicate the timing of the control addition (0 μM) and the sequential addition of different nutrients (all at 100 μM). doi:10.1371/journal.pone.0068834.g007
Perspectives for Application

Our results show that the NIFT technique can be successfully applied to macroalgae and seagrass. The method is relatively fast and straightforward, and provides important information on the nutrients limiting the photosynthetic rates of primary producers. For instance, the macroalga *L. variegata* is one of the key algal species involved in large-scale shifts from coral to macroalgal dominance in coral reef ecosystems across the globe, including the Caribbean Sea [41,42] and the Great Barrier Reef [43,44]. Our finding that *L. variegata* is co-limited by nitrogen and phosphorus on the coral reefs of Curacao, and reaches higher abundances in more nutrient-rich waters near urbanized areas (Table 1), indicates that eutrophication of these coastal waters is likely to enhance the capacity of this algal species to overgrow coral reefs. Further expansion of *L. variegata* and other algal species involved in the degradation of coral reef ecosystems may be curtailed by reductions in nitrogen and phosphorus loads from terrestrial sources, for instance by more extensive wastewater treatment. These results illustrate that use of the NIFT response to assess the nutrient status of primary producers can serve as a valuable tool in coastal management. While we worked on macroalga and seagrass in tropical marine ecosystems, we foresee a wider application of this method to other benthic algae and submersed aquatic plants in other marine and freshwater habitats.

Acknowledgments

We are most grateful to Mattij Baeker for his help with the PAM setup and Figure 2. We thank Anna Simeon, Laura Weiand, Katy Davis, and Hannah Brooke for their help with the NIFT experiments and biological surveys. We would like to thank Prof. Dr. John Beardall and an anonymous reviewer for their constructive comments on the manuscript.

Author Contributions

Conceived and designed the experiments: JdH PMV MJAV. Performed the experiments: JdH JD LiB AKF. Analyzed the data: JdH FD LiB AKF. Contributed reagents/materials/analysis tools: JdH JD JdO FCD MJAV PMV. Wrote the paper: JdH JD MJAV PMV.

References

1. Smith VH, Tilman GD, Necka JC (1999) Eutrophication: impacts of excess nutrient inputs on freshwater, marine, and terrestrial ecosystems. Environ Pollut 100: 179–196.
2. Howarth R, Chan F, Conley DJ, Garnier J, Doney SC, et al. (2011) Coupled biogeochemical cycles: eutrophication and hypoxia in temperate estuarine and coastal marine ecosystems. Front Ecol Environ 9: 10–26.
3. Brauer VS, Stomp M, Huismann J (2012) The nutrient-load hypothesis: patterns of resource limitation and community structure driven by competition for nutrients and light. Am Nat 179: 721–740.
4. Paerl HW (1980) Nutrient phyttoplankton blooms in coastal, estuarine, and inland waters. Limnol Oceanogr 35: 823–847.
5. Bell PRF (1992) Eutrophication and coral reefs - some examples in the Great Barrier Reef Lagoon. Water Res 26: 553–568.
6. Smith VH, Joyce SR, Howarth RW (2006) Eutrophication of freshwater and marine ecosystems. Limnol Oceanogr 51: 351–355.
7. Fong P, Donohoe RM, Zedler JB (1994) Nutrient concentration in tissue of the marine algae Enteromorpha as a function of nutrient history: an experimental evaluation using field microcosms. Mar Ecol-Prog Ser 106: 273–291.
8. Lepointe BE (1997) Nutrient thresholds for bottom-up control of macroalgal blooms on coral reefs in Jamaica and southeast Florida. Limnol Oceanogr 42: 1119–1131.
9. Beadnell J, Young E, Roberts S (2001) Approaches for determining phytoplankton nutrient limitation. Aquat Sci 63: 44–69.
10. Townsend SA, Schult JH, Douglas MM, Skinner S (2008) Does the Redfield ratio infer nutrient limitation in the macroalga, *Sargassum fluitans*? Freshw Biol 53: 509–520.
11. Larned ST (1998) Nitrogen- versus phosphorus-limited growth and sources of nutrients for coral reef macroalgae. Mar Ecol 132: 409–421.
12. Armitage AR, Frankovich TA, Heck KL, Fourqurean JW (2005) Experimental nutrient enrichment causes complex changes in seagrass, macroalgae, and macroalgal community structure in Florida Bay. Estuaries 28: 422–434.
13. Teichberg M, Fox SE, Aguila C, Olsen VS, Valiela I (2008) Macroalgal responses to experimental nutrient enrichment in shallow coastal waters: growth, internal nutrient pools, and isotopic signatures. Mar Ecol-Prog Ser 368: 117–126.
14. Ba Nacht R, Hay ME (2009) Nutrient versus herbivore control of macroalgal community development and coral growth on a Caribbean reef. Mar Ecol-Prog Ser 389: 71–84.
15. Shelly K, Holland D, Beadnell J (2010) Assessing nutrient status of macroalgal using chlorophyll *a* fluorescence. In: Sugget DJ, Borowitzka MA, Pratil O, editors. Chlorophyll *a* fluorescence in aquatic sciences: methods and applications. Heidelberg: Springer. 223–235.
16. Turpin DH, Weger HG (1980) Steady-state chlorophyll *a* fluorescence transients during ammonium assimilation by the N-limited green alga *Selenastrum minutum*. Plant Physiol 88: 97–101.
17. Wood MD, Oliver RL (1995) Fluorescence transients in response to nutrient enrichment of nitrogen- and phosphorus-limited *Monodus subterraneus* cultures and natural phytoplankton populations: a measure of nutrient limitation. Aust J Plant Physiol 22: 331–340.
18. Beadnell J, Berman T, Hrada P, Omo Kadiri M, Light BR, et al. (2001) A comparison of methods for detection of phosphate limitation in macroalgae. Aquat Sci 63: 197–212.
19. Holland D, Roberts S, Beadnell J (2004) Assessment of the nutrient status of phytoplankton: a comparison between conventional bioassays and nutrient-induced fluorescence transients (NIFTs). Ecol Indic 4: 149–159.
20. Pettke D, Robol MA, Smith RA, Ralph PJ, Shelly K, et al. (2008) State transitions and nonphotochemical quenching during a nutrient-induced fluorescence transient in phosphorus-starved *Dunaliella tertiolecta*. J Phycol 44: 1204–1211.
21. Hsu Y, Babin M (2010) Overview of fluorescence protocols: theory, basic concepts, and practice. In: Sugget DJ, Borowitzka MA, Pratil O, editors. Chlorophyll *a* fluorescence in aquatic sciences: methods and applications. Heidelberg: Springer. 31–74.
22. Maxwell K, Johnson GN (2000) Chlorophyll fluorescence - a practical guide. J Exp Bot 51: 659–668.
23. Kelder KE, Gill SM (2006) Coral Point Count with Excel extensions (CPCe): a Visual Basic program for the determination of coral and substrate coverage using point random count methodology. Comput Geosci 32: 1259–1269.
24. Grasshoff K, Ehrhardt M, Kremling K, editors (1983) Methods of seawater analysis, second edition. Weinheim, Verslag Chemie. 419 p.
25. Helder W, De Vries RTP (1979) An automatic phenol-hypochlorite method for the determination of ammonia in sea- and brackish waters. Neth J Sea Res 13: 154–160.
26. Murphy J, Riley JP (1962) A modified single solution method for the determination of phosphate in natural waters. Anal Chim Acta 27: 31–36.
27. Kronkamp J, Barranguet C, Peene J (1998) Determination of microphytobenthos PSII quantum efficiency and photosynthetic activity by means of variable chlorophyll *a* fluorescence. Mar Ecol-Prog Ser 162: 43–53.
28. Fujita RM (1985) The role of nitrogen status in regulating transient ammonium uptake and nitrogen storage by macroalgae. J Exp Mar Biol Ecol 92: 293–301.
29. Luo MB, Liu F, Xu ZL (2012) Growth and nutrient uptake capacity of two co-occurring species, *Ulua pedifolia* and *Ulua lanza*. Aquat Bot 100: 18–24.
30. Gentry B, Brianian JM, Baker NR (1989) The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. Biochim Biophys Acta 990: 97–92.
31. Geider RJ, La Roche J, Greene RM, Olizama M (1993) Response of the photosynthetic apparatus of *Phaeodactylum tricornutum* (Bacillariophyceae) to nitrate, phosphate or iron starvation. J Phycol 29: 755–766.
32. Lippencott S, Hintze R, Vanselow KH, Hartig P, Colijn F (2001) In-line recording of PAM fluorescence of phytoplankton cultures as a new tool for studying effects of fluctuating nutrient supply on photosynthesis. Eur J Phycol 36: 89–100.
33. Enríquez S, Borowitzka MA (2010) The use of the fluorescence signal in studies of seagrasses and macroalgae. In: Sugget DJ, Borowitzka MA, Pratil O, editors. Chlorophyll *a* fluorescence in aquatic sciences: methods and applications. Heidelberg: Springer. 167–208.
34. Patriquin D (1972) The origin of nitrogen and phosphorus for growth of the marine angiosperm *Thalassia testudinum*. Mar Biol 15: 35–46.
35. Touchette BW, Burkhart JD (2000) Review of nitrogen and phosphorus metabolism in seagrasses. J Exp Mar Biol Ecol 250: 133–167.
36. Young EB, Beardall J (2003) Rapid ammonium- and nitrate-induced perturbations to chl a fluorescence in nitrogen-stressed *Dunaliella tertiolecta* (Chlorophyta). J Phycol 39: 332–342.
37. Shelly K, Higgins T, Beadnell J, Wood B, McNaboughton D, et al. (2007) Characterising nutrient-induced fluorescence transients (NIFTs) in nitrogen-stressed *Chlorella emersonii* (Chlorophyta). Phycolgy 46: 303–312.
38. Schaffelke B, Khumpp DW (1996) Nutrient-limited growth of the coral reef macroalga *Sargassum sachalinense* and experimental growth enhancement by nutrient addition in continuous flow culture. Mar Ecol-Prog Ser 164: 199–211.
39. Schaffelke B, Klumpp DW (1998) Short-term nutrient pulses enhance growth and photosynthesis of the coral reef macroalga Sargassum bacillare. Mar Ecol-Prog Ser 170: 95–105.
40. Schaffelke B (1999) Short-term nutrient pulses as tools to assess responses of coral reef macroalgae to enhanced nutrient availability. Mar Ecol-Prog Ser 182: 305–310.
41. Mumby PJ, Foster NL, Fahy EAG (2005) Patch dynamics of coral reef macroalgae under chronic and acute disturbance. Coral Reefs 24: 681–692.
42. Nugues M, Bak R (2008) Long-term dynamics of the brown macroalga Lobophora variagata on deep reefs in Curacao. Coral Reefs 27: 389–393.
43. Diaz-Pulido G, McCook LJ, Dove S, Berkelmans R, Roff G, et al. (2009) Doom and boom on a resilient reef: climate change, algal overgrowth and coral recovery. BioS ONE 4: e5229.
44. Cheal AJ, MacNeil MA, Cripps E, Emslie MJ, Jonker M, et al. (2010) Coral macroalgal phase shifts or reef resilience: links with diversity and functional roles of herbivorous fishes on the Great Barrier Reef. Coral Reefs 29: 1005–1015.