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Fertilizer-derived N in opportunistic macroalgae after flooding of agricultural land

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ABSTRACT: Managed realignment by deliberate flooding of coastal areas is an adaptation to sea level rise but may risk enriching the coastal zone with nutrients when seawater floods agricultural soil. This study focuses on the early development of macroalgae and their sources of nitrogen (N) in Gyldensteen Coastal Lagoon, Denmark. The lagoon was claimed for agricultural purposes in 1871 and reflooded by managed realignment 143 yr later (2014). Our hypotheses were: (1) that nutrients of agricultural origin from the newly flooded soil initiate opportunistic macroalgal blooms; and (2) that the isotopic composition of green algae rapidly reflects the origin of nutrient sources. We monitored macroalgal cover and conducted stable isotope ($\delta^{15}N$) analyses to assess the origin of N sources. Intense green macroalgal blooms occurred during the first summer after flooding and diminished in the 2 following years as a result of rapid water exchange. Low $\delta^{15}N$ in macroalgae in the first year (mean ± SE, 4.2 ± 0.3‰) increased significantly in the next year (8.0 ± 0.1‰). A laboratory experiment tested the $\delta^{15}N$ response of opportunistic green macroalgae (*Ulva* spp.) exposed to organic manure and synthetic inorganic fertilizers. Higher $\delta^{15}N$ (11.1 ± 0.1‰) characterized manure-treated algae compared to fertilizer-treated algae (2.7 ± 0.2‰). Based on these field and laboratory results, we accept both hypotheses and conclude that the major N source supporting macroalgal growth in 2014 was derived from synthetic fertilizers; however, rapid tidal flushing during the following years resulted in nutrient limitation and lower macroalgal growth.

KEY WORDS: Nitrogen · Macroalgal bloom · Nutrient sources · Sea level rise · Coastal realignment · Gyldensteen Coastal Lagoon

1. INTRODUCTION

Sea level is expected to rise by up to 16 mm yr^{-1} as a result of climate change, increasing to a global average of 0.85 m within the 21st century (IPCC 2014), and low-lying coastal areas are consequently at risk of flooding. Many of these areas in Europe, including Denmark, were shallow marine areas claimed for agricultural purposes that are now protected from the sea by dikes (Peirup 2006, Rupp-Armstrong & Nicholls 2007). In the future, the protection of such areas may become economically unsustainable (Hazelden & Boorman 2001). Instead, managed coastal realignment can be applied as an adaptation strategy to control inland flooding (Esteves 2014). By constructing reinforced dikes along the inland perimeter and surrendering the outside farmland to the sea by breaching old dikes, managers can form a protective buffer zone reducing storm surge pressure on the dikes along the new coastline (e.g. Esteves 2014). Most studies on managed realignment have focused on hydrology and risk assessment to human residents, beaches/tourism, and other economic issues (Rupp-Armstrong & Nicholls 2007, Rulleau et al. 2017), resulting in limited information on the ecological consequences when seawater floods agricultural lands.

Agricultural soils are typically fertilized with organic (e.g. animal manure) or synthetic fertilizers.

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Flooding of such soil with saline water facilitates rapid nutrient release to the water column as a result of changes in salinity, pH, and redox conditions (Portnoy & Giblin 1997). Ion exchange is particularly important where NH_4^+ is desorbed and released in the presence of Na^+ (Gardner et al. 1991). However, over time, anoxia in the water-saturated soil reduces microbial mineralization of organic-bound nutrients (Sjøgaard et al. 2017, 2018). The initial pulsed release of nutrients to the water column can create eutrophic conditions characterized by excessive growth of phytoplankton and opportunistic green algae (Pedersen & Borum 1996, Sundbäck et al. 2003, Liu et al. 2013). Green algae of the genera *Cladophora* and *Ulva* can rapidly use the high levels of nutrients released from the soil, and may form large blooms (Sundbäck et al. 2003). Numerous studies demonstrate harmful effects of macroalgal blooms, such as benthic oxygen depletion during algal degradation (Flindt 1999, Flindt et al. 2016), shading by resuspension of sediment (Canal-Vergés et al. 2010), and asphyxiation of seagrass beds (Hauxwell et al. 2001, Valdemarsen et al. 2010, Rasmussen et al. 2012), as well as microphytobenthos and benthic fauna (Valiela et al. 1997, Schmidt et al. 2017). Blooms of macroalgae may therefore delay the development of realigned coastal areas into stable ecosystems.

Macroalgae have been used as bioindicators for tracing the origin of N sources in coastal environments (Pedersen & Borum 1996, McClelland & Valiela 1998a,b). Stable nitrogen isotope signatures ($\delta^{15}N$) of macroalgal tissues can indicate the N source (e.g. McClelland & Valiela 1998a, Dailer et al. 2010, Lemesle et al. 2016); green algae of the genus *Ulva* are particularly sensitive indicators (Cohen & Fong 2005, Barr et al. 2013, Orlandi et al. 2014). Given limited fractionation during assimilation of inorganic nutrients in these algae, the $\delta^{15}N$ strongly reflects the origin of their sources (Cohen & Fong 2005, Dudley et al. 2010, Barr et al. 2013). The sediment fluxes of dissolved inorganic nitrogen (DIN = NO_2^- + NO_3^- + NH_4^+) in shallow coastal areas originate from various N sources, such as anthropogenic discharges, agricultural runoff from land, and atmospheric precipitation, each of which has a distinct $\delta^{15}N$ signature.

Danish agriculture typically uses both organic and synthetic fertilizers for optimizing crop yield. Manure-derived organic fertilizer is rich in ^{15}N because of discriminative processes in the digestive system of animals favoring assimilation of ^{14}N over ^{15}N, resulting in $\delta^{15}N$ values in manure typically >+10‰ (Heaton 1986). Production of synthetic fertilizers uses atmospheric N_2 via the Haber-Bosch process to yield $\delta^{15}N$ ~0‰ (Heaton 1986). Past studies used macroalgae primarily as indicators of high $\delta^{15}N$ sources such as organic manure and sewage (Costanzo et al. 2005, Thornber et al. 2008, Teichberg et al. 2010), whereas few have traced low $\delta^{15}N$ originating from synthetic fertilizers (Ochoa-Izaguirre & Soto-Jiménez 2015, Orlandi et al. 2017). However, this tracing approach may be complicated when processes such as nitrification and denitrification influence the true isotopic signal of DIN sources by leaving the heavy $\delta^{15}N$ behind (Kendall 1998, Pardo & Nadelhoffer 2010).

Stable carbon isotope signatures in primary producers vary according to the type of photosynthesis. C_3 primary producers use the enzyme rubisco, which strongly fractionates against ^{13}C, leading to tissues very low in $\delta^{13}C$ (Farquhar et al. 1989, Maberly et al. 1992). Although most aquatic primary producers use the C_3 pathway, their $\delta^{13}C$ signal varies according to their ability to use CO_2 or HCO_3^-. Rapid growth rates induced by either light or nutrients cause CO_2 depletion and thus the need for active uptake of HCO_3^- (Raven et al. 2002, 2011). High C demand may counteract rubisco fractionation because it provides more energy for active HCO_3^- uptake, which is more enriched in ^{13}C than CO_2, resulting in higher tissue $\delta^{13}C$ (Peterson & Fry 1987, Dudley et al. 2010).

Few studies have assessed the persistence of any effect of creating a new lagoon by flooding farmland formerly used for agriculture. Potentially, high levels of nutrient release from the agricultural soil could affect ecological conditions for several years after lagoon creation. The duration of such an impact will depend on the residence time and exchange of water with the outside marine environment. Our study investigated the initial development of green macroalgal growth in Gyldensteen Coastal Lagoon, created after flooding of an agricultural area by managed coastal realignment, and to trace potential agriculturally derived N sources in tissues of the green algae. Our test hypotheses were that: (1) nutrients of agricultural origin released from the newly flooded soil initiate opportunistic macroalgal blooms; and (2) the isotopic composition of green algae rapidly reflects nutrient origin.

2. MATERIALS AND METHODS

2.1. Study site

The Gyldensteen Coastal Lagoon is located on the northern coast of the island of Fyn, Denmark (Fig. 1). It is part of a 616 ha nature reserve acquired by Aage

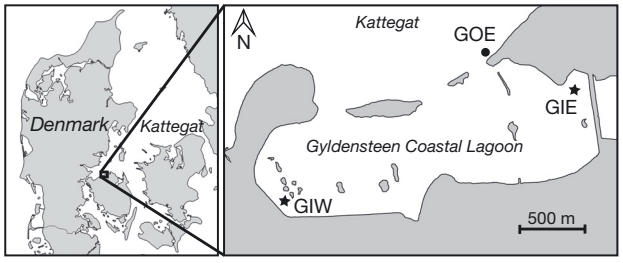

Fig. 1. Study site located at the northern part of Fyn, Denmark. Stars mark the 2 main stations inside the lagoon (east: GIE and west: GIW). A dot marks the outside station to the east (GOE)

V. Jensen Nature Foundation in 2011 to conserve wildlife for educational and recreational purposes. The shallow marine lagoon was drained in 1871 for agricultural use by building a seaward embankment. Managed realignment after establishment of new inland dikes restored a 214 ha shallow coastal lagoon (average depth ~1.0 m). Seawater rapidly flooded the farmland when the sea dikes were breached on 29 March 2014, creating 3 openings to the sea that allowed tidal water exchange. Despite a narrow tidal range of ±20 cm, water level can vary >100 cm depending on wind direction and wind speed, leading to a water residence time of ~24 h. Accordingly, low tides frequently expose large areas of the lagoon. The salinity range resembles the salinity conditions (20–30) in the Kattegat outside the lagoon, and no freshwater discharges into the lagoon, except for occasional and rare overflow from a neighboring freshwater wetland east of the lagoon. Seasonality due to the northern hemisphere location limits the growing season for vegetation from April to October.

The soils received 93 kg N ha^{-1} organic (animal manure) and 132 kg N ha^{-1} synthetic inorganic fertilizers during cultivation of barley and seed grass in 2011. However, the cultivated area and application of fertilizers was reduced and limited to synthetic fertilizers in 2012 (139 kg N ha^{-1}) and 2013 (151 kg N ha^{-1} on 35% of the total area) (Gyldensteen Manor pers. comm.) in an attempt to minimize the excess fertilizer pools in the soils prior to flooding. We established 2 sampling stations inside the lagoon, Stn East (GIE: 55° 34.67′ N, 10° 9.48′ E) and Stn West (GIW: 55° 34.19′ N, 10° 7.22′ E), as well as 1 control station outside the lagoon (GOE: 55° 34.823′ N, 10° 8.773′ E). Prior to flooding, the soil to the east (GIE) was an undisturbed stubble field from seed grass crops in 2013, whereas soil to the west (GIW) was bare and disturbed by tracks from heavy machinery used to construct inland dikes. Most of the aboveground plant stubbles (especially at GIE) were degraded or washed away by the end of 2015.

2.2. Field survey: monitoring of macroalgae and nutrients

We monitored green macroalgal cover at monthly intervals at Stns GIE and GIW from April 2014 to June 2016 using a semi-quantitative approach that identified 5 levels of macroalgal cover (according to the method adopted by the Danish Environmental Protection Agency, Fyns Amt 2001): Level 0 = 0%, Level 1 = 1–25%, Level 2 = 26–50%, Level 3 = 51–75%, Level 4 = 76–100% bottom coverage. We determined cover visually from photos (~1 m height covering 0.25 m^2) taken at 10–16 random locations from the shoreline and 100 m outwards. At the same time, we sampled specimens of the dominant algal species in the intertidal zones while avoiding drifting algae that potentially originated outside the lagoon. We also sampled green macroalgae along the shoreline outside the lagoon (near Stn GOE) to identify the marine N source. Sampled algae were stored in ziplock bags and kept in coolers during transport. In the laboratory, they were rinsed carefully to remove visual debris, epiphytes, etc., washed in distilled water, and dried at 60°C for 24–48 h. The dried material was stored in ziplock bags until further processing for N content and stable isotope ($\delta^{15}N$) analysis.

We collected water samples (n = 3 mo^{-1}) for analyses of DIN every 1–3 mo at Stns GIE, GIW, and GOE. DIN was analyzed on a flow-injection analyzer (QuickChem 8500 Series) using the Lachat instruments, QuickChem method 10-107-041-C + method 10-107-06-3-D. Unfortunately, we could not perform stable isotope analysis for DIN with the available instruments.

2.3. Laboratory experiment: *Ulva* spp. $\delta^{15}N$ and nutrient sources

We also examined the impact of DIN source (organic animal manure or inorganic synthetic fertilizer) on the stable isotope signal of the common opportunistic green macroalgae, *Ulva* spp., in a 13 d controlled laboratory experiment. Macroalgae for the experiment were collected from Stn GIE in September 2015. We used sheet-forming *U. lactuca* and a mixture of tube-forming *U. intestinalis* (66%) and *U. prolifera* (34%) (the 2 latter species were previously assigned to the genus *Enteromorpha*). We treated the mixture

of tube formers here as *U. intestinalis* because of the dominance of this species. Collected macroalgae were rinsed of visual debris and acclimated to experimental conditions (temperature of 15°C and salinity of 21) for 2 d. We cut the sheet-forming *U. lactuca* into 2.5 cm diameter circular disks with a starting biomass of 0.055–0.066 g wet weight (ww), and cut the tube-forming *U. intestinalis* into small pieces (starting biomass of 0.491–0.614 g ww). The disks and pieces of each species were carefully blotted on paper tissue to remove excess water and then weighed before transfer to 15 marked mesh bags (7 × 7 cm, mesh size 1 mm) for each species. Three replicate mesh bags of each species were placed in each of 5 white trays (5.5 × 23 × 29 cm) continuously supplied with aerated seawater from 5 separate reservoirs using a 5-channel peristaltic pump supplying a flow rate of 8 ml min^{-1} for each tray, corresponding to a total water exchange of about 3 times d^{-1}. An LED lamp providing photosynthetically active radiation of 400–500 µmol m^{-2} s^{-1} supplied light in a 12:12 h light:dark cycle.

Organic animal manure (M) from livestock cattle, pigs, and mink was collected as the degassed by-product from a biogas plant (Biogadan in Fangel, Denmark) in September 2015. We prepared stock solution M by centrifuging the manure in 50 ml centrifuge tubes at 1200 × *g*, and then decanting ~100 ml of supernatant from the tubes to prepare a stock solution by mixing with 900 ml filtered seawater from the study site. After filtration (GF/C) the stock solution contained concentrations of 13.1 mM NH_4^+ and 2.4 µM NO_3^-. We prepared synthetic fertilizer stock solution (F) by dissolving ~100 g fertilizer pellets (Vitagro NPK 12-1-13, Bayer Garden) in 1 l of seawater from the study site. The solution was filtered (GF/C) and the DIN concentration was measured as described above. The DIN stock solution for F contained concentrations of 21.7 mM NH_4^+ + 70.5 mM NO_3^-. Samples of raw M and F material were freeze dried for stable isotope analysis.

We established 5 fertilizer treatments in separate trays: (1) Control with untreated seawater (C); (2) high concentration of M (HM), 110–167 µM DIN; (3) low concentration of M (LM), 11–24 µM DIN; (4) high concentration of F (HF), 70–123 µM DIN; and (5) low concentration of F (LF), 7–19 µM DIN. We evaluated growth of macroalgae as change in biomass (g ww) as determined every 2–3 d by blotting and weighing as described above. DIN concentrations in trays and reservoir were monitored regularly during the experiment to maintain stable concentrations. We prepared new DIN reservoirs and changed them every 2–3 d for all treatments.

The experiment lasted for 13 d, but we terminated *U. lactuca* treatments after 8 d because sporulation occurred. At the end of the experiment, the algae were blotted, weighed, and prepared for C-N elemental and stable isotope (tissue-$\delta^{15}N$ and -$\delta^{13}C$) analysis as described for field algae.

2.4. Stable isotope analysis

Dried macroalgae and dried fertilizers (M and F) were ground into powder using a plant mill (Retsch MM301), and subsamples of 1–2.5 mg dry weight were transferred to tin capsules. We analyzed elemental C and N content along with stable isotopes ($\delta^{13}C$ and $\delta^{15}N$) on a Thermo Analytical elemental analyzer, Flash EA 2000 Series coupled via a ConFlo IV interface to a Thermo Delta V Isotope Ratio mass spectrometer. The δ-notation expresses the isotopic ratio as the relative difference between the sample and the conventional standards (atmospheric N_2 for ^{15}N, and Pee Dee belemnite for ^{13}C) using this formula: δY (‰) = $[(R_{sample} - R_{standard})/(R_{standard})]\times10^3$, where Y represents ^{13}C or ^{15}N, and R is $^{13}C/^{12}C$ for carbon and $^{15}N/^{14}N$ for nitrogen.

2.5. Statistical analysis

We grouped tissue-$\delta^{15}N$ values of algae sampled inside the lagoon by sampling month, accepting a single outside $\delta^{15}N$ mean (baseline) after testing for no change over time (*t*-test). We used multiple *t*-tests (or Mann-Whitney tests for comparisons defying normal distribution and homogeneous variance) to test whether $\delta^{15}N$ values in algae sampled inside the lagoon differed significantly from those sampled outside the lagoon. We performed 1- and 2-way ANOVAs followed by post hoc tests (Tukey or Holm-Sidak) to test for significant effects of fertilizer treatments (M or F) and concentrations (H or L) on experimental data (stable isotopes, biomass, growth rates, and elemental C and N content). All statistical analyses were conducted with the software SigmaStat 12 and used an α level of 0.05.

3. RESULTS

3.1. Field observations: green algae and nutrients

The first green macroalgae appeared in late May 2014, and their cover increased rapidly thereafter at

both GIE and GIW from 25–50% in June to 51–75% in July 2014 (Fig. 2). A mass bloom (76–100%) occurred at GIE in August 2014, forming mats up to 30 cm thick (data not shown), and lasting until November. Filamentous *Ulva intestinalis* and *U. prolifera* dominated the initial bloom in early summer 2014, replaced with *Cladophora* spp. during the mass bloom later in the summer. The bloom at GIW only reached 75% cover, dominated by the same algal species as GIE. The macroalgal coverage at GIE and GIW never exceeded 25% during the following years (2015–2016) and was generally <10%.

DIN concentrations followed a seasonal pattern with low levels in summer and high levels in winter, both inside and outside the lagoon (Fig. 3). However, winter concentrations were generally higher inside than outside (up to 204% at GIE and 165% at GIW), particularly during the first year. Thus, DIN inside the lagoon was 30–50% higher in winter 2014/2015 (mean ± SD, January: 52.4 ± 0.5 µM at GIE and 54.6 ± 0.5 µM at GIW) than in the following winter of 2015/2016 (February: 26.6 ± 0.8 µM at GIE and 36.4 ± 0.3 µM at GIW), whereas no differences occurred between years outside the lagoon. DIN concentrations were low during the growth season at all 3 stations and ranged from 0.2–6.2 µM inside (GIW and GIE) and 2.0–4.4 µM outside (GOE) the lagoon.

The $\delta^{15}N$ in macroalgal tissue (i.e. green algae) was significantly lower ($p < 0.05$) inside the lagoon during summer 2014 compared to the baseline of (mean ± SE) 8.7 ± 0.3‰ for macroalgal tissue from outside (Fig. 4). Subsequently, macroalgal $\delta^{15}N$ increased inside the lagoon; mostly during the first year where

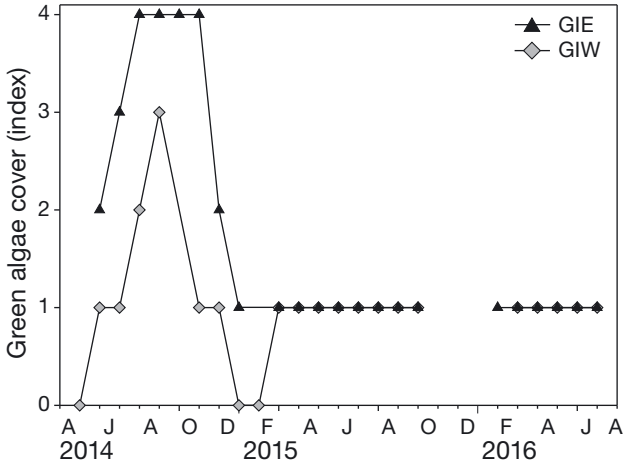

Fig. 2. Opportunistic green macroalgal cover inside the lagoon from April 2014 to July 2016. Index level 0 = 0%, Level 1 = 1–25%, Level 2 = 26–50%, Level 3 = 51–75%, Level 4 = 76–100% cover. GIE and GIW are the sampling stations inside the lagoon (see Fig. 1)

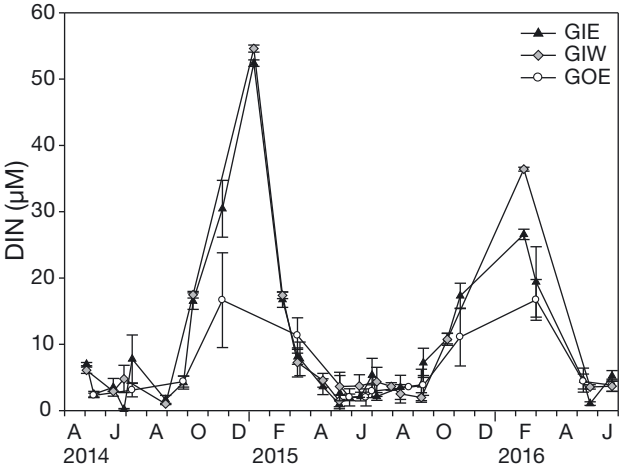

Fig. 3. Mean ± SD dissolved inorganic nitrogen (DIN: NH_4^+ + NO_3^-) concentrations (µM) in water from inside (Stns GIE and GIW) and outside (Stn GOE) Gyldensteen Coastal Lagoon from May 2014 to June 2016 (n = 3–88)

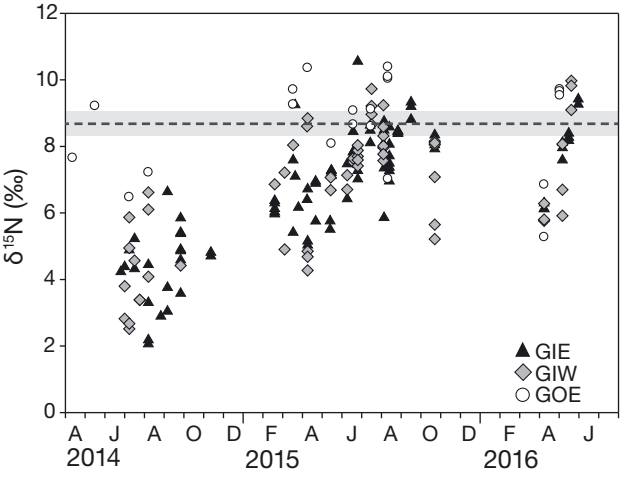

Fig. 4. $\delta^{15}N$ (‰) in green macroalgae from spring 2014 to May 2016. Dashed horizontal line indicates the average $\delta^{15}N$ for algae from GOE. Grey field is ± SE (n = 22)

$\delta^{15}N$ changed from (mean ± SE) 4.2 ± 0.3 to 8.0 ± 0.1‰ at GIE and from 4.2 ± 0.4 to 8.1 ± 0.2‰ at GIW, and thus rapidly approached the outside level. $\delta^{15}N$ of algae from inside remained significantly lower than outside the lagoon in spring 2015 and 2016 ($p < 0.05$), but we detected no significant difference in later seasons of these 2 years.

The N content of macroalgal tissue (tissue-N) varied significantly ($p < 0.05$) over the growing season (Fig. 5). Highest tissue-N occurred in periods of slow growth (fall/winter), and lowest values during spring/summer when competition for N increased. Accordingly, we found a significant correlation between tissue-N and DIN from water inside the lagoon at both GIE and GIW ($p = 0.002$, r^2 0.567, n = 14; and

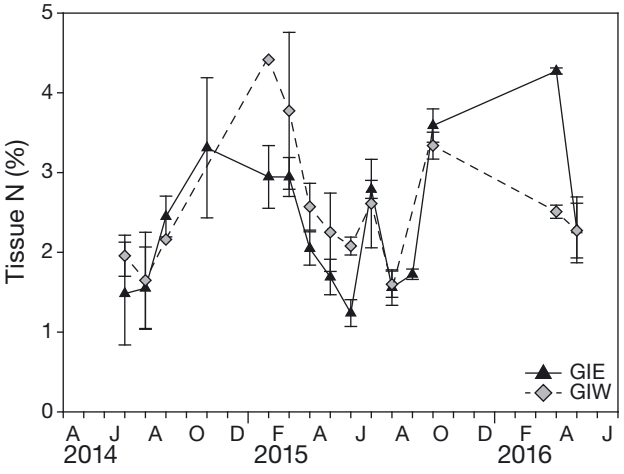

Fig. 5. Mean ± SE monthly tissue-N content (%) in green macroalgae from July 2014 to May 2016 at Stns GIE and GIW within the Gyldensteen Coastal Lagoon (see Fig. 1)

p = 0.001, r^2 = 0.845, n = 9, respectively). Neither tissue-N nor DIN correlated significantly with tissue-$\delta^{15}N$.

3.2. Laboratory assay: fertilizer impact on $\delta^{15}N$ of *Ulva* tissue

All treatments with both *Ulva* species grew well during the experiment as indicated by increased biomasses from start to finish (Fig. 6). The effect of fertilizer in M and F treatments became initially apparent after 5 d of exposure, where relative growth rates accelerated in HM and HF treatments, although the effect was significant only for *U. intestinalis* ($p < 0.05$) (Table 1). The non-significant result for *U. lactuca* likely reflects the great variability within treatments caused by sporulation. HM and HF exposure increased the biomass of *U. lactuca* by 207 and 185% after 7 d, respectively, and *U. intestinalis* by 301 (HM) and 246% (HF) after 13 d. LM and LF exposure resulted in biomass increases of 91 and 75%, respectively, for *U. lactuca* after 7 d and 146 (LM) and 131% (LF) for *U. intestinalis* after 13 d. Growth was slower in control treatments, with biomasses only increasing by 68% for *U. lactuca* and 73% for *U. intestinalis* during the same period (7 and 13 d, respectively), which was slower than during the first 5 d for all treatments and species.

Most of the algae increased their tissue C and N content during the experiment (Table 2). The control algae were nutrient limited, leading to loss of both C and N (1.9 and 39%, respectively) for *U. lactuca* and loss of N (53%) for *U. intestinalis.* Fertilizers signifi-

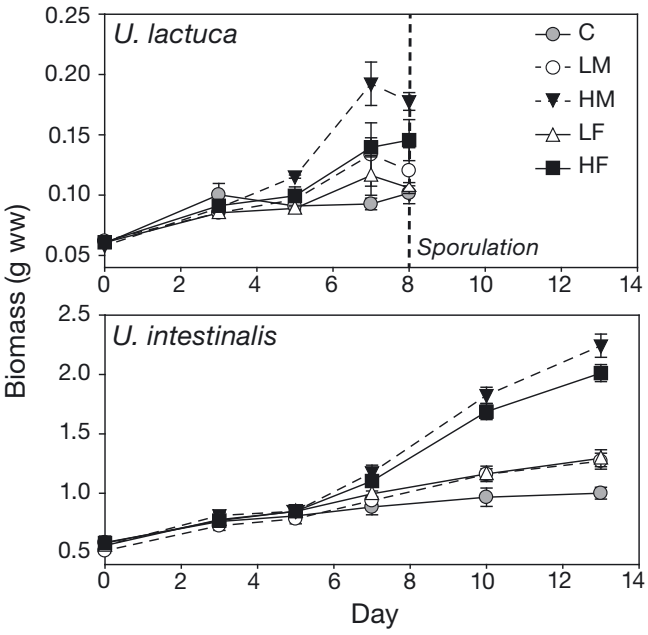

Fig. 6. Biomass development in the experiment with *Ulva lactuca* and *Ulva intestinalis* + *prolifera* (*U. intestinalis*). Note different scale on the biomass (g wet weight, ww) axis. Treatments are C: control; LM: low manure; HM: high manur;, LF: low fertilizer; HF: high fertilizer (mean ± SE, n = 3)

Table 1. Relative growth rates in relation to starting biomass (g wet weight [ww] d^{-1}) in different treatments (C: control, LM: low manure; HM: high manure; LF: low fertilizer; HF: high fertilizer). Rates are calculated from biomass measurements (in Fig. 6) (we consider Days 0–5 to be an acclimatization phase) (n = 3). Due to sporulation of *Ulva lactuca*, we omitted Day 8 from calculations. One-way ANOVA tested treatment effect within each species after determining no interaction between species and treatment. Different superscripts (abc) mark significance between treatments after pairwise multiple comparisons of treatment within each species (Holm-Sidak or Tukey, $\alpha = 0.05$)

C*U. lactuca* Treatment	Relative growth (g ww d^{-1}) Days 5–7	SE
C	0.0085[a]	0.01
LM	0.18[ab]	0.08
HM	0.33[b]	0.07
LF	0.15[ab]	0.09
HF	0.28[ab]	0.03

U. intestinalis Treatment	*+ U. prolifera* Days 5–7	SE	Days 5–13	SE
C	0.05[a]	0.01	0.03[a]	0.01
LM	0.10[a]	0.02	0.08[b]	0.01
HM	0.19[b]	0.01	0.20[c]	0.00
LF	0.09[a]	0.01	0.07[ab]	0.01
HF	0.15[b]	0.02	0.17[c]	0.01

Table 2. Mean and SE tissue content of C and N (%) and C:N ratio in *Ulva lactuca* and *U. intestinalis*. Treatments as in Table 1. Different superscripts indicate significance within species after pairwise multiple comparison (Holm-Sidak or Tukey). *indicates significance between species (*U. lactuca* vs. *U. intestinalis*) (2-way ANOVA, $p < 0.05$)

Treatment	%C	SE	%N	SE	C:N	SE
U. lactuca (8 d)						
Start	30.02^{a}	0.42	1.05^{*a}	0.08	33.9^{ab*}	2.4
Control	29.46^{*ab}	0.16	0.64^{b}	0.05	54.5^{c*}	4.5
LM	30.18^{ab}	0.07	1.13^{a}	0.09	31.5^{a}	2.7
HM	32.92^{*ab}	0.11	3.64^{*c}	0.08	10.6^{d}	0.2
LF	30.72^{ab}	0.88	1.04^{*a}	0.04	34.5^{b*}	2.1
HF	33.93^{*b}	0.10	3.59^{*c}	0.04	11.0^{d}	0.1
U. intestinalis (13 d)						
Start	30.12^{a}	0.07	1.72^{*a}	0.06	20.4^{ab*}	0.8
Control	30.41^{*a}	0.16	0.81^{b}	0.05	44.0^{c*}	2.6
LM	30.97^{a}	0.05	1.28^{c}	0.12	28.7^{a}	2.5
HM	31.27^{*b}	0.20	3.92^{*e}	0.12	9.3^{e*}	0.2
LF	31.02^{a}	0.22	1.76^{*a}	0.01	20.6^{b*}	0.2
HF	31.76^{*b}	0.35	3.25^{*d}	0.08	11.4^{d}	0.4

cantly affected tissue-C (both species) and -N (*U. lactuca*), but only by the HM and HF treatments ($p < 0.05$), with no differential impact of nutrient sources (M or F). The *Ulva* species responded differently to fertilizer treatments, with C and N content of *U. lactuca* increasing more than of *U. intestinalis*, but significantly so only for tissue N.

All fertilizer treatments modified $\delta^{15}N$ of *Ulva* tissue from the start level of (mean ± SD) 8.2 ± 0.9‰ for *U. lactuca* and 9.1 ± 0.2‰ for *U. intestinalis* (Fig. 7). Tissue-$\delta^{15}N$ of both *Ulva* species increased in manure treatments and approached the M-signal ($\delta^{15}N_M$ = 12.6 ± 0.01). HM-treated algae reached the highest tissue-$\delta^{15}N$ of 10.7 ± 0.4‰ in *U. lactuca* and 11.2 ± 0.1 ‰ in *U. intestinalis*, which was significantly higher than tissue-$\delta^{15}N$ of controls (6.6 ± 0.2 and 8.1 ± 0.2‰, respectively, $p < 0.05$) and LM treatment (8.8 ± 0.2 and 10.0 ± 0.2‰, respectively, $p < 0.05$; Table 3). Exposure to synthetic fertilizer (F) resulted in a decreased nitrogen signal to near the F value ($\delta^{15}N_F$ = 1.2 ± 0.1‰). The lowest tissue-$\delta^{15}N$ occurred after exposure to HF (2.5 ± 0.1‰ for *U. lactuca* and 2.7 ± 0.2‰ *U. intestinalis*). The tissue of LF-treated algae was less affected (4.9 ± 0.2 and 4.0 ± 0.2‰, respectively), but nonetheless remained significantly lower than controls ($p < 0.05$). Although the overall tissue-$\delta^{15}N$ trend was similar for the 2 *Ulva* species, differences were evident as indicated by a generally lower tissue-$\delta^{15}N$ for *U. lactuca* than *U. intestinalis* ($p < 0.05$).

Fertilizer treatments also affected tissue-$\delta^{13}C$ of the 2 *Ulva* species ($p < 0.05$). Addition of both M and F led to higher $\delta^{13}C$. Although the DIN level (L and H) had a significant effect ($p < 0.05$) and increased $\delta^{13}C$ in proportion to the concentration, nutrient source (M or F) had no impact (Table 3). Thus, $\delta^{13}C$ for both *Ulva* species was comparable for HM and HF (−8.1 ± 0.1‰ and −7.0 ± 0.4‰) and significantly higher than controls (−16.7 ± 0.7‰).

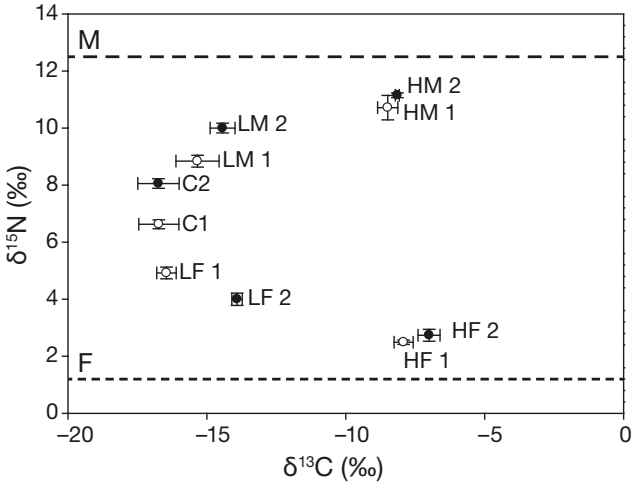

Fig. 7. Mean $\delta^{13}C$ and $\delta^{15}N$ of macroalgae after exposure to different nutrient sources, (‰ ± SE, n = 3). Horizontal dashed lines indicate $\delta^{15}N$ for nutrient sources: M: animal manure; F: synthetic fertilizer. H/L = high/low concentration of M or F; C: control in natural seawater. 1 = *U. lactuca*, 2 = *U. intestinalis* / *U. prolifera*

Table 3. Statistical outcome from 2-way ANOVA on $\delta^{15}N$ and $\delta^{13}C$ data in the experiment followed by post hoc pairwise multiple comparison of treatment (Holm-Sidak, $p < 0.05$); different letters indicate significant treatment effects. Significance: *$p < 0.05$

	df	*F*	p
$\delta^{15}N$			
Treatment	5	41.939	<0.001*
Ulva species	1	4.961	0.036*
Treatment × *Ulva* species	5	1.346	0.279
$\delta^{13}C$			
Treatment	5	129.542	<0.001*
Ulva species	1	4.254	0.050
Treatment × *Ulva* species	5	2.937	0.033*
Post hoc	$\delta^{15}N$ within Treatment	$\delta^{13}C$ Treatment within *U. lactuca*	$\delta^{13}C$ Treatment within *U. intestinalis*
Start	ab	ab	ab
Control	a	ac	a
LM	b	ab	bc
HM	b	d	d
LF	c	bc	c
HF	d	d	d

4. DISCUSSION

4.1. Field algae growth and nutrient dynamics

In 2014, the green macroalgae bloomed in response to the instant nutrient release from the flooded agricultural soils. Rapid initial release of nutrients from soils covered with saline water was to a large extent coupled with Na^+ ion exchange within the soil matrix, leading to substantial NH_4^+ desorption (Rysgaard et al. 1999, Sjøgaard et al. 2018) and with reduced conditions in the waterlogged soil leading to PO_4^{3-} desorption following Fe(III) reduction (Sundby et al. 1992, Sjøgaard et al. 2017). The high availability of nutrients, together with the large area of newly suitable substrata for settling of opportunistic algae, initiated the 2014 bloom of the green algae *Ulva* sp. and *Cladophora* sp. Furthermore, algae were tangled with and trapped by stubble from historical farmland cultivation, particularly at GIE, preventing the tides from washing them out. Similar blooms of green algae typically occur in eutrophic coastal waters (e.g. Valiela et al. 1997, Nelson et al. 2008, de Paula Silva et al. 2012, Lyons et al. 2014) as a result of their rapid assimilation of available nutrients and high growth rates. The limited macroalgal cover in subsequent years (2015–2016) was surprising because we expected that continued microbial nutrient release from the soil could support macroalgal growth for an extended time after flooding.

The low macroalgal cover (<25%) and absence of blooms in 2015 and 2016 resulted from several factors, including DIN limitation, low temperature, light limitation, and disappearance of plant stubble. Although the macroalgal bloom in 2014 indicated high DIN release from the soils, DIN concentrations in the growing seasons of 2014, 2015, and 2016 were low inside and similar to those outside the lagoon. Thus the rapid DIN-uptake by the massive macroalgal cover likely masked the high DIN release from the flooded soils in summer 2014. The relatively low tissue-N of the harvested algae supported the potential for rapid uptake and limitation of DIN during the growing season of that year (Pedersen & Borum 1996, Barr & Rees 2003). Algal DIN uptake is slow during winter, and the algae are nutrient replete as indicated by their high tissue-N. The much higher DIN levels observed during winter 2014/2015 than winter 2015/2016 support our assertion of a decline in the available N pool through time inside the lagoon. This trend resulted from the combination of gradually declining DIN release from the soil and DIN export through rapid water exchange with the outside marine environment. The strong DIN limitation after the first year is further supported by increasing abundance of N-fixing cyanobacteria at the study sites in summer 2016 and 2017 (unpublished data), indicating DIN exhaustion and high P availability in the coastal lagoon. Conversely, biogeochemical and microbial studies demonstrated slow denitrification with little impact on DIN availability at the study site (Sjøgaard et al. 2018). Finally, the gradual disappearance of stubble fields during winter 2014/2015 eliminated the mechanism to entangle algae and thus prevent their tidal export from the lagoon by 2015.

The significantly lower tissue-$\delta^{15}N$ of green algae inside than outside the lagoon in 2014 indicated a marked difference in the origin of N sources. The macroalgal bloom that year was driven by N released from agricultural soils that probably originated from synthetic fertilizers, given that the $\delta^{15}N$ of the green macroalgae in 2014 corresponds to that of similar algae exposed to inorganic fertilizer (Orlandi et al. 2017), Ochoa-Izaguirre & Soto-Jiménez (2015) found similar low $\delta^{15}N$ of *Cladophora* (2.5 ± 1.3‰) and *Ulva* (3.4 ± 1.2‰) in a coastal lagoon and argued that N was primarily derived from synthetic fertilizers. The higher tissue-$\delta^{15}N$ of algae in the lagoon during summer 2015 and 2016 at a level similar to that of algae from outside suggests that the N sources inside and outside after 1 yr approached similar composition. The decline in water column DIN and the generally sparse algal vegetation in 2015 and 2016 supports our interpretation that N derived from the synthetic fertilizers was probably exhausted and flushed out during the first summer and winter. The rapid loss of the initial artificial fertilizer-derived N isotope signal could actually be traced in the surface soil (0–5 mm), in that $\delta^{15}N$ values increased from ~2‰ in 2014 to ~5‰ in 2015 (unpublished data).

The increasing $\delta^{15}N$ signal with time after flooding could be explained by microbial fractionation during mineralization processes producing ^{15}N-enriched NH_4^+, whereas nitrification enriches the remaining N pool in the soil with an even heavier $\delta^{15}N$ signal relative to newly produced NH_4^+ (Heaton 1986, Kendall 1998). However, knowledge of the soil history (former sea bottom cultivated for a long period) as well as fertilizer applications before flooding suggest another explanation. Most of the initial N pool derived from synthetic fertilizers is more mobile and accessible for algae than organic-bound N in manure. Thus, a faster loss of N from soils generally occurs when combining synthetic fertilizers with manure compared to soils treated with only manure (Ren et al. 2014). Interestingly, the low $\delta^{15}N$ of algae

sampled in spring of both 2015 and 2016 suggests that some DIN from fertilizers of synthetic origin continued to accumulate during winter. However, a laboratory study of flooded soil from the study site suggests that most soil-bound N during this period occurred in organic form and DIN was released by microbial degradation (Sjøgaard et al. 2017). Thus, degradation of organic N of synthetic fertilizer origin was responsible for continued release of low $\delta^{15}N$ DIN in the lagoon beyond the first year. A lower $\delta^{15}N$ characterizes soils and crops grown on soil fertilized with synthetic fertilizers compared to manure-fertilized soil and crops (Watzka et al. 2006, Kriszan et al. 2009). However, $\delta^{15}N$ of non-fertilized soils does not always differ from that of synthetic fertilized soils (e.g. Watzka et al. 2006, Kriszan et al. 2009) because fractionation processes (volatilization and denitrification) increase the $\delta^{15}N$ signal of N in the fertilized soil (Pardo & Nadelhoffer 2010). These processes may have affected the $\delta^{15}N$ signal of the soil N at the study site to some extent prior to flooding. Irrespective, we believe that the lower $\delta^{15}N$ signal in tissues of the first algal appearance largely derived from fertilizer N because (1) the soil at the study site was continuously used as farm land for 150 yr, prior to which it was seabed with minimal input of terrestrial organic matter; (2) the lagoon receives no groundwater or stream delivery of terrestrial derived N; (3) flooding the soils with seawater induces a rapid release of adsorbed fertilizer NH_4^+ from the top layer of the soil through ion exchange with Na^+; and (4) DIN derived from stored organic matter in the flooded soil derived from plant material grown with fertilizers.

4.2. Nutrient control of *Ulva* growth and stable isotope fractionation

Both laboratory-tested *Ulva* species grew well in the presence of manure and synthetic fertilizer with limited effect of the nutrient source (M or F). Green algae assimilate NH_4^+ faster and with less energetic cost than NO_3^- because organisms can readily use DIN in the reduced NH_4^+ form for direct syntheses of amino acids (Pedersen & Borum 1997, Naldi & Wheeler 2002, Ale et al. 2011). Accordingly, the similarity in growth rates of both algal species when treated with the 2 fertilizer types is caused by the high NH_4^+ availability in both treatments. High tissue-N in C:N ratios as low as 10 at high concentrations of M or F directly resulted from luxury uptake of fertilizer-derived N. Orlandi et al. (2017) found similar low C:N ratios of *Ulva* sp. treated with synthetic fertilizer. Thus, *Ulva* spp. strongly benefit from both manure and synthetic fertilizers delivered as water extracts, whereas the greater accessibility of synthetic N source compared to manure when administering both fertilizers through the soil matrix explains the 2014 macroalgal bloom in Gyldensteen Coastal Lagoon.

The short-term laboratory treatment with manure and synthetic fertilizer markedly increased and decreased, respectively, the tissue-$\delta^{15}N$ of both *Ulva* species. Other studies reported similar rapid responses of macroalgal $\delta^{15}N$ to the N source (Costanzo et al. 2005, Orlandi et al. 2017). The minor, but significant, difference in $\delta^{15}N$ between the 2 *Ulva* species may result from undetected changes in $\delta^{15}N$ of the M and F sources during the incubation (e.g. denitrification) or may reflect a consequence of sporulation in *U. lactuca*. Accordingly, Marconi et al. (2011) observed no taxonomic effect on tissue-$\delta^{15}N$ in closely related macroalgae assimilating similar N sources. Therefore, our experiment confirms that $\delta^{15}N$ of macroalgae clearly reflects the availability of agricultural fertilizer N sources in their growth environment.

Our study found higher tissue $\delta^{15}N$ of manure-treated *Ulva* spp. than reported by Orlandi et al. (2017) for *U. lactuca* treated with fresh cow manure. Our manure contained a mixture of degassed cow and pig manure from a biogas plant where enrichment of ^{15}N will occur as a result of preferential assimilation of ^{14}N during microbial growth (Heaton 1986, Kendall 1998). Accordingly, a higher $\delta^{15}N$ value characterized the manure in our study compared to the fresh manure of Orlandi et al. (2017), whereas tissue $\delta^{15}N$ for HF- and LF-treated *Ulva* species were comparable to those reported by Orlandi et al. (2017) for *U. lactuca* exposed to synthetic fertilizer (NH_4NO_3).

The apparent dependence of tissue-$\delta^{15}N$ on concentration of manure and synthetic fertilizer is puzzling, and contrasts Cohen & Fong (2005), who reported no relationship between tissue-$\delta^{15}N$ of algae and N concentration. Our *Ulva* species should therefore similarly reflect the source $\delta^{15}N$, irrespective of DIN concentration. Moreover, the applied concentrations apparently saturated the algae in all fertilizer treatments as indicated by tissue-N > 1% (Barr et al. 2013). Thus, we expected similar $\delta^{15}N$ in the new tissue produced by L- and H-treated algae. That we determined the final tissue-$\delta^{15}N$ on homogenized samples of whole algae from the experiment likely explains the observed discrepancy. These samples obviously contained a mixed signature of the old and newly produced tissue. The true $\delta^{15}N$ of new tissue

grown on the 2 types of fertilizers therefore requires correction for $\delta^{15}N$ of the old tissue following a 2-source mixing formula (Fry 2003):

$$F_{old} \times \delta^{15}N_{old} + F_{new} \times \delta^{15}N_{new} = \delta^{15}N_{mix} \quad (1)$$

where F_{old} and F_{new} denote the fraction of old and new algal tissue (calculated from the total biomass increase at the end of the experiment), $\delta^{15}N_{old}$ and $\delta^{15}N_{new}$ are the isotope signatures of the old initial and new tissue, and $\delta^{15}N_{mix}$ defines the overall isotope signature of the final mixed algal tissue. The $\delta^{15}N_{new}$ can then be calculated by solving this equation using the measured parameters (Table 4). Interestingly, we calculated almost identical $\delta^{15}N_{new}$ for both algal species, irrespective of fertilizer concentration. The resulting $\delta^{15}N_{new}$ values of 9.5 to 11.9‰ for manure-treated algae and −0.6 to 0.5‰ for synthetic fertilizer-treated algae approximate the $\delta^{15}N$ for the 2 fertilizer types. Such similarity between $\delta^{15}N_{new}$ and the DIN sources requires relatively limited isotope fractionation (Cohen & Fong 2005, Dudley et al. 2010, Barr et al. 2013), as occurs here, with only slight ^{15}N depletion (−0.7 to −3.1‰). Other studies have reported similar low fractionation for green algae during N assimilation (Cohen & Fong 2005, Dudley et al. 2010, Barr et al. 2013), whereas others reported slight fractionation (Orlandi et al. 2017). However, N metabolism and release may fractionate against ^{14}N in brown algae, resulting in fractionation of +2 to +4‰ (Viana & Bode 2015). In any case, the results obtained here support the contention that $\delta^{15}N$ in green algae is a strong indicator of the DIN source, with limited fractionation irrespective of DIN concentration.

Table 4. Input parameters (where F_{old} is the fraction of old algal tissue, and $\delta^{15}N_{mix}$ defines the overall isotope signature of the final mixed algal tissue) and output (isotope signature of the new tissue: $\delta^{15}N_{new}$) of the applied mixing model. $\delta^{15}N_{mix}$ for the initial algal tissue is equivalent to $\delta^{15}N_{old}$. F_{old} was calculated from biomass increases (Fig. 6); see Section 4.2 for further details. Treatments as in Table 1

	Initial	LM	HM	LF	HF	C
F_{old}						
U. lactuca	1	0.524	0.326	0.571	0.351	0.595
U. intestinalis	1	0.406	0.249	0.433	0.289	0.578
$\delta^{15}N_{mix}$ (‰)						
U. lactuca	8.2	8.8	10.7	4.9	2.5	6.6
U. intestinalis	9.1	10	11.2	4	2.7	8.1
$\delta^{15}N_{new}$ (‰)						
U. lactuca	–	9.46	11.91	0.51	−0.58	4.25
U. intestinalis	–	10.62	11.90	0.11	0.10	6.73

The high demand for dissolved inorganic carbon to keep up with the enhanced growth likely contributed to the increase in $\delta^{13}C$ with DIN concentration, irrespective of fertilizer type. Rapid-growing macroalgae rapidly exhaust CO_2 and use HCO_3^- as a carbon source, which leads to $\delta^{13}C$ values more positive than −10‰ (Raven et al. 2002), whereas slow-growing species primarily rely on CO_2 diffusion to rubisco where strong fractionation against ^{13}C occurs (Raven 1997, Raven et al. 2002). The 2 *Ulva* species in our L treatment apparently assimilated CO_2 to the same extent as the control algae, resulting in stronger fractionation than those grown in the H treatment in which HCO_3^- likely provided the primary carbon source. Previous experiments on *Ulva* species report similar growth-dependent changes in tissue $\delta^{13}C$ when exposed to increasing irradiance (Cornelisen et al. 2007). Furthermore, incubation studies on kelp species report higher $\delta^{13}C$ when conditions increase photosynthesis and growth rates (Carvalho et al. 2009a,b, 2010). We exposed identical algae in our experimental enrichments to similar environmental conditions (light, temperature, etc.), which suggests a correlation between relative ^{13}C enrichment and DIN concentration. However, fully understanding the correlation between DIN concentration and relative ^{13}C enrichment in green macroalgal tissue requires further investigation.

The overall outcome of the fertilizer experiment confirms that agriculturally derived N from manure and synthetic fertilizer stimulate green algal growth and that the tissue-$\delta^{15}N$ signal reflected the N source. Furthermore, the low tissue-$\delta^{15}N$ from the F treatments corresponds to the level in green algae sampled in the lagoon during the 2014 bloom, suggesting that algae at that time derived N primarily from synthetic fertilizer in the agricultural soil. The tissue-$\delta^{15}N$ from the 2015–2016 harvested field algae was higher than observed in 2014, but did not reach the level of the manure, likely because of the slow release and rapid dilution of organic bound N in manure (Anwar et al. 2005). However, tissue-$\delta^{15}N$ of green algae clearly reflected the average $\delta^{15}N$ value of the surrounding waters, and this $\delta^{15}N$ can represent a mixture of various sources, complicating the interpretation of tissue $\delta^{15}N$ of field-harvested algae (Ochoa-Izaguirre & Soto-Jiménez 2015). The similarity in tissue-$\delta^{15}N$ of field algae from inside the lagoon in 2015 and 2016 to values obtained in algae from outside could be coincidental. Despite the decrease in DIN concentration between the first and the second winters, concentrations remained higher inside than outside the lagoon despite rapid water exchange,

suggesting continued release of DIN from the former agricultural soils. Thus, the tissue-$\delta^{15}N$ of algae harvested in 2015 and 2016 may therefore reflect a mixture of the gradually exhausted manure- and fertilizer-derived N pools, thereby obscuring the true origin. This uncertainty about the various origins of N sources and the lack of specific growth measurement negates application of the mixed $\delta^{15}N$ model to the field algae. Irrespective, given slow green algal growth during winter and spring in temperate waters (Pedersen & Borum 1996), the lower tissue-$\delta^{15}N$ measured in spring algae of 2015 and 2016 must reflect a longer history than summer algae and suggests the high DIN levels in winter continued to reflect the influence of fertilizer-derived N.

5. CONCLUSIONS

The bloom of opportunistic green algae in Gyldensteen Coastal Lagoon during 2014 was apparently driven by DIN sources with light $\delta^{15}N$ that differed from those in the outside marine environment. The low tissue-$\delta^{15}N$ of the algae inside the newly flooded area confirms our first hypothesis, that release of nutrients from the previous agricultural fertilization may drive opportunistic algal growth just after flooding. We acknowledge that the laboratory experiment did not fully replicate natural conditions. Nevertheless, the findings support our assertion that tissue-$\delta^{15}N$ in green algae (*Ulva lactuca* and *U. intestinalis*) reflects the N source, which confirms our second hypothesis. Together these findings suggest that N derived from synthetic fertilizers released rapidly after flooding primarily drove the 2014 algal bloom. However, algae quickly exhausted this N source, and tissue-$\delta^{15}N$ from algae inside the lagoon already resembled that of algae outside by 2015. Furthermore, sparse macroalgal growth in 2015–2016 resulted from low N availability (from fertilizers), rapid water exchange, and loss of appropriate substrata for attachment once stubble fields degraded. The lagoon can benefit from rapidly decreasing impact of fertilizer N and algal cover as it develops into a balanced ecosystem with high species diversity, although newly developed cyanobacteria blooms still prevent full recovery of the ecosystem.

Future managed realignment projects that flood agricultural land with seawater should consider soil nutrient levels and water exchange when predicting ecological development. Rapid release of nutrients from the soil may result in massive blooms of green macroalgae that may persist over time when water exchange is limited. Thus, systems with reduced water exchange, longer residence time, and fresh water contributing to nutrient loading may be influenced by their agricultural past for a long time before entering into a well-functioning ecosystem status.

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