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Vertical heterogeneity in physiological characteristics of *Ulva* spp. mats

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Abstract In eutrophic areas, green macroalgae are frequently and for long periods arranged in mats, resulting in a steep light gradient. This study investigates the effect of this gradient on physiological characteristics [tissue nitrogen content, maximal photosynthetic efficiency (F_v/F_m), glutathione levels and redox ratio, absorbance and absorption spectra] of the green macroalga *Ulva* spp. Mats were sampled during the build-up (June), stationary (July), and decomposing (September) phases of a macroalgal bloom in the Veerse Meer, a eutrophic brackish (salinity 15–20 psu) lake in the southwest Netherlands. Water samples were taken for nutrient analyses. At all three sampling dates, the mats were composed almost entirely of *Ulva* spp.; in September the mats were in decay and covered with silt and epiphytes. In June and July, total dissolved inorganic nitrogen concentration (DIN) of the water within the mat was significantly higher than outside the mat. Pronounced vertical differences were found in tissue N, F_v/F_m values, total glutathione levels, glutathione redox ratios, and absorbance. In June and July, tissue N decreased from over 2.2% dry weight (DW; N-sufficient) in the bottom layers to around 1% DW (minimum level for survival) in the top layers. Wide-band absorption increased with depth in the mat and throughout the season, probably due to higher Chl *a* and *b* and lutein contents. The shape of the absorption spectrum was similar for all layers. The absorption of the silt/epiphyte film on the top *Ulva* layer was highest; its absorption spectrum (high absorption in the 500–560 nm range) indicates that the film on the top layers of the macroalgal mats mainly consisted of diatoms. In June, F_v/F_m and the glutathione redox ratio of the algae increased with depth in the layer, while total glutathione decreased. Low F_v/F_m values in the bottom and middle layers in September reflect the bad condition of the algae; the mats were largely decaying. It is concluded that multiple growth-limiting gradients occur in macroalgal mats: upper layers suffer from nitrogen limitation and photoinhibition while bottom layers are light limited. The algae in the mat acclimatize to low light conditions by increasing their absorption through increased pigment contents and by higher photosynthetic efficiency during the build-up and stationary period. This study qualifies the glutathione redox ratio as a promising candidate for stress indicator in macroalgae and provides suggestions for its further development.

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Introduction

In shallow coastal areas (lagoons, bays, etc.) subjected to a high nutrient load, mass developments of green macroalgae can frequently be observed (Schramm and Nienhuis 1996). Typical species of macroalgal blooms are *Enteromorpha* spp., *Ulva* spp., *Chaetomorpha* spp., and *Cladophora* spp. (Lowthion et al. 1985; Brown et al. 1990; Lavery et al. 1991; Duarte 1995). These algae often manifest themselves as free-floating thalli, which are frequently arranged in thick mats. In areas exposed to tidal movement and strong waves, the mats are constantly rearranged because of the tidal currents; however in more stagnant areas (viz. lagoons and sheltered bays) the mat structure persists for weeks and sometimes even for an entire season (Vergara et al. 1998). Consequently, the algae in the different layers experience very different light climates for a long time, each with their own constraints for growth: upper

layers may suffer from photoinhibition and oxidative stress due to high light, whereas bottom layers have to cope with very low light levels (Vergara et al. 1997). Moreover, as the algae are not optically neutral and preferably absorb blue and red light, spectral properties change with depth (Salles et al. 1996; Vergara et al. 1997).

Besides this gradient in light, another gradient can be formed, partly connected with the first one. In laboratory experiments with *U. rotundata* mats, Vergara et al. (1998) showed that high growth rates of algae in the upper layers would rapidly dilute internal nutrient pools, resulting in severe nutrient limitation of growth. Algae in lower layers may still have enough supplies to sustain growth. Additionally, microbial breakdown of dead algae and other organic matter in the sediment will cause a flux of inorganic nutrients from the bottom, which will be largely intercepted by the deepest layers (McGlathery et al. 1997). These two processes will cause a gradient of increasing nutrient limitation toward the surface.

A study of the processes that occur in mats and the physiological responses of the algae will lead to a better insight into the dynamics of macroalgal blooms, especially where the shift from a growing mat to a decomposing mat is concerned. Laboratory studies on vertical heterogeneity in macroalgal mats have been carried out in *Ulva* spp. (Vergara et al. 1998) and *Chaetomorpha linum* (e.g. Krause-Jensen et al. 1996, 1999; McGlathery et al. 1997). However, except for the C/N data given by Krause-Jensen et al. (1999), the experimental work of growth-limiting gradients in algal mats and acclimatization of the algae has not been confirmed with field data. This article aims to fill this gap with data on *Ulva* spp. mats from the eutrophic saline lake the "Veerse Meer" (southwest Netherlands). In this study we describe vertical heterogeneity in physiological characteristics of *Ulva* during the build-up, maintenance, and decomposition of a macroalgal mat. The physiological consequences of the existence of vertical gradients in light and nitrogen are tested using a set of indicators such as tissue pigment, carbon and nitrogen content, and light absorption. The degree of photoinhibition of the algae is estimated using maximum (dark-adapted) quantum efficiency (F_v/F_m) of thalli collected from different depths (Baker and Bowyer 1994). The level of oxidative stress experienced by the algae was determined by measuring the glutathione pools and their redox state (De Vos et al. 1992). Glutathione is a very well studied antioxidant, occurring in all organisms (Ishikawa and Sies 1988); however, as far as we know only two studies present data on the glutathione cycle in macroalgae (Nakano et al. 1995; Rijstenbil et al. 1998). The results will be discussed with respect to physiological acclimation of the algae to long-term gradients in nutrients and light and the consequences of these gradients for the algae's growth rates. The suitability of glutathione as indicator for the occurrence of oxidative stress in the field will be evaluated.

Materials and methods

Study site

The field data were collected in the Veerse Meer, a shallow, manmade, brackish lake (salinity 15–20 psu average), situated in the southwestern part of the Netherlands (51°32′N, 3°46′E). The Veerse Meer receives a high nutrient load, mainly originating from agricultural runoff. The resulting eutrophic conditions give rise to a large macroalgal biomass, mainly consisting of different *Ulva* species that are very difficult to separate from one another but mostly the species *U. scandinavica* Bliding and *U. curvata* (Kütz.) De Toni (Malta and Verschuure 1997; Malta et al. 1999). Details on hydrography and hydrochemistry can be found in Malta (2000). Observations were carried out at the site Middelplaten, which is considered representative with respect to macroalgal biomass for most shallow areas in the lake (Malta and Verschuure 1997). Water samples and algal collections for pigment, carbon and nitrogen analysis, and absorption measurements were carried out in 1997 during the build-up phase of the bloom (10 June), during the stationary phase (30 July), and during the degradation phase (23 September; Malta et al. 2002). Additionally, the maximum quantum efficiency (F_v/F_m) of photosynthesis and the glutathione redox ratio of the algae were determined in June and September. Water samples were always taken first, because algal collection most certainly destroys potential gradients in the water column. At the collection site, algal coverage was 90–100% on all dates. Biomass was arranged in several mats, each ranging in size from about 0.5 m^2 to over 2 m^2 (visual estimates). Mats frequently overlapped each other such that the thallus of one (or more) alga belonged to two mats; however, more often mats were arranged in discrete patches, separated by small patches of bare sediment. Generally five to seven layers of algal thalli could be found in a mat; these could easily be counted without disturbing potential gradients in the water because the algae were floating in the water column, except for the last sampling date when counts may have been less precise. On each sampling date a diver swam three times 25–30 m in a straight line from each of three fixed landmarks in the lake and selected the nearest mat consisting of six layers. Thus, each time samples were taken from three mats consisting of six layers each. As biomass sampling destroys a large part of the mat and navigation while diving is difficult, it is very unlikely that the same mat was sampled at different dates.

Water nutrient analyses and light

SCUBA divers collected water samples between 12.00 and 14.00 hours, using syringes mounted with perforated tubings. Samples were taken from the bottom (±0.8 m depth, under six layers of *Ulva*), the middle (±0.6 m depth, under three layers of *Ulva*), below and above the top layer (±0.2 m depth) of algal mats, and at four depths (0.2, 0.4, 0.6, and 0.8 m) in a water column overlying bare sediment. In September mats were lying flat on the sediment, hence water samples from the mats in that period were taken more or less from the same depth (but from under an equal amount of layers). One sample per layer and depth was taken from each of three different algal mats and water columns. The water was filtered through 0.2-μm disposable filters (Whatman) and stored frozen until analysis. Nitrate, nitrite, and ammonium were analyzed on a Skalar 5100 autoanalyzer.

Irradiance data were obtained from a continuously measuring LI-190 SA (LiCor) quantum meter equipped with a 2π PAR (400–700 nm) sensor that was mounted on top of the institute in Yerseke, approximately 30 km southeast of the field site. The quantum meter was connected to a LI-1000 datalogger (LiCor).

Tissue carbon, nitrogen, and pigment content

Ulva spp. were sampled from the top layer, the third layer (i.e. middle layer, counted from the top), and the sixth layer (i.e. bottom layer) of each of three mats (approximately 500 g wet

weight of algae per layer per mat). Parts of the algae were transported on ice to the laboratory. Samples intended for pigment analysis were stored in tin foil at −80°C. For the analysis, samples were ground and extracted in 95% methanol, buffered with 5% ammonium acetate. Pigments were analyzed by reversed phase HPLC after Wright et al. (1991). The samples were injected through a Waters 171 Plus autosampler into an Alltech column (Econosphere C18). The signal was detected at 436 and 658 nm with a Waters absorbance detector (Barranguet et al. 1998). The remaining material was dried for 48 h at 60°C and ground using a bullet mixer. The carbon and nitrogen contents of the algae were determined on a Carlo-Erba NA 1500 CHN-analyzer (Nieuwenhuize et al. 1994).

Absorbance and fluorescence

The in vivo absorption spectra and total wide-band (400–700 nm) absorbance of the single layers from different depths in the mat were determined immediately after collection by placing the algae on a cosine sensor connected to a MACAM SR9910/PC spectroradiometer, using natural sunlight. A sunlight spectrum was determined just before each measurement, and absorption was calculated with respect to that measurement. Algae were carefully wiped clean with tissue before the measurements. In September, the absorption of the epiphyte/silt layer on the *Ulva* thalli was measured by determining the absorption spectra before and after cleaning of the *Ulva* tissue.

Maximum (dark-adapted) quantum efficiency (F_v/F_m) can be used as an estimate for the degree of photoinhibition of algae (Baker and Bowyer 1994), which happens when a plant is exposed to high light (Henley et al. 1991a, Hanelt et al. 1992). It has also been shown to be sensitive to other stresses such as nutrient limitation (including carbon) and high temperatures (Henley et al. 1991b; Magnusson 1997). Chl *a* fluorescence parameters F_0 and F_m were measured in June and September after 15 min dark acclimation, using a pulse amplitude modulated (PAM) fluorometer (PAM 100–103, Walz). From these parameters the variable fluorescence, $F_v = F_m - F_0$, and F_v/F_m were calculated. In June, mats were sampled at noon (12.00 hours) and in the afternoon (15.00 hours), to detect variation due to diurnal patterns (Hanelt et al. 1993). In September, mats were sampled only at noon. Due to technical problems no measurements could be made in July.

Glutathione redox ratios

Glutathione, the tripeptide γ-glutamyl-cysteinyl-glycine, is one of the major antioxidant molecules in many organisms. One of its functions is therefore to protect plant cells against lipid peroxidation by active oxygen (De Vos et al. 1992). Active oxygen species are formed because of electron leakage from the photosynthetic and respiratory electron transport chains. Reduced glutathione (GSH) is thereby oxidized to GSSG, both via a spontaneous chemical reaction and through enzymatic (peroxidase) reactions. Oxidative agents like the transition metal copper and ultraviolet radiation produce active oxygen (superoxide anion and hydroxyl radicals, hydrogen peroxide). Because such radicals oxidize part of the GSH pool, the redox state of this thiol expressed as GSH:(GSH + 0.5GSSG) can be a useful indicator of oxidative stress in algae living in a steep vertical gradient of sunlight. For this reason, we have measured, using a modified HPLC method (after Fahey and Newton 1987; Rüegsegger and Brunold 1992; Rijstenbil and Wijnholds 1996), the glutathione pools and their redox state of *Ulva* spp. at different positions in the mat. *Ulva* thalli were stored in liquid nitrogen immediately after sampling. For each sample, run in triplicate, 200 mg wet weight of plant material was ground with pestle and mortar under addition of small portions of liquid nitrogen. After determining the initial weight of the cold powder, 1 ml of a mixture of 0.12 *M* HCl and 5 m*M* DTPA (diethylene triamine penta acetic acid) was added. To obtain a cell-free extract this homogenate was sonicated on ice (Soniprep MSA; 14-μm amplitude; 3 min; 0°C). The suspension was centrifuged (Centrikon T-324 Kontron; 15,000 rpm; 20 min; 4°C). The detailed procedures for this precolumn derivatization and the subsequent reversed-phase HPLC runs are described in Rijstenbil and Wijnholds (1996). The supernatant was derivative in a HEPPS-DPTA buffer with monobromobimane (MBrB, Molecular Probes). Separate HPLC runs without and with dithiothreitol (DTT) as reductor were performed to analyze reduced glutathione (GSH) and total glutathione (GSH + 0.5GSSG), respectively. The concentrations of GSH and its redox state were calculated from the peak areas, using GSH as a standard.

Data analysis

Differences between sampling dates and the existence of a gradient in the algal mat of tissue C, tissue N, absorbance, and pigment content were tested for significance by a two-way analysis of variance (ANOVA; Sokal and Rohlf 1995). Multiple post hoc comparisons were done by a Tukey honest significant difference test for unequal sample sizes (Spjøtvoll and Stoline 1973). DIN concentrations were tested for differences between samples taken from the inside and outside of a mat and for vertical differences using a two-way ANOVA, followed by a Tukey test for unequal sample sizes. Significance of seasonal differences in DIN and significance of differences between ammonia and nitrate concentrations were both tested using the nonparametric Kruskal–Wallis test. A two-way ANOVA was used to detect significant differences in sampling time or layer in the mat of F_v/F_m data collected in June. The September measurements of F_v/F_m and the glutathione data were tested for significant differences between layers using a one-way ANOVA, followed by a Tukey test in case of a significant ANOVA result. Data were tested for heteroscedasticity with Bartlett's test for homogeneity. The variables that scored significant (DIN, F_v/F_m, Chl *a*/*b*, and β-carotene content) were transformed using the Box–Cox procedure ($x' = x^{\lambda} + 1$; Box and Cox 1964), which removed the heterogeneity. The values for λ were estimated in an iterative procedure by the Statistica 5.1 software package (StatSoft 1997).

Results

Seasonal dynamics in mat appearance and light

At all three sampling dates, the mats were composed almost entirely of *Ulva* spp. (> 99% of total macroalgal biomass, data not shown) with a bottom coverage of 90–100%. Typically, the mats consisted of five to seven layers, although both higher and lower numbers of layers were counted frequently. In June and July, the upper and middle layers of the mat were floating in the water column. In this period, the upper *Ulva* layer was partly, and the middle and bottom layers were completely covered with a thin brown film. Microscopical inspection of the thalli revealed that a diatom film had settled on thalli from the top and, to a lesser extent, the middle layer. Bottom layers were covered with debris and silt. Cleaned thalli of the upper and middle layers looked bright green; thalli from the bottom layer usually had a dark brownish-green color. In September, the algae were decaying and lying flat on the sediment. The algae were completely covered with a film of silt and epiphytes. Massive amounts of barnacles and tunicates had settled on the thalli of the bottom layers and, to a

Table 1 Total daily irradiance (TDI, 400–700 nm, mol photons m^{-2}) on three sampling days (TDI_{sample}), average TDI (TDI_{avg}, ±SD) during a period from 4 days before to 4 days after the sampling date, and the maximum measured TDI during that same period (TDI_{max}) measured in 1997 in the southwest Netherlands

Date	TDI_{sample}	TDI_{avg}	TDI_{max}
10 June	51.8	37.3 ± 11.9	51.9
30 July	26.6	30.2 ± 13.5	47.4
23 September	23.4	28.1 ± 11.0	40.8

lesser extent, also the middle and top layers. The color of the algae was dark green to brownish; some parts of the bottom layer were black.

In June, weather conditions were fine: generally clear skies with some sparse hazy clouds in the morning. On 30 July, long sunny periods were alternated with short periods with heavy clouding in the morning and more clouds and some rainfall even in the afternoon. In September, skies were covered with hazy clouds. Weather conditions are clearly reflected in the total daily irradiances on the sampling days (TDI_{sample}, Table 1). Average TDI during a period consisting of 4 days before to 4 days after the sampling day shows the normal seasonal decrease in light intensity from June to September. TDI during sampling in June was close to the maximum TDI measured during the period, whereas TDI on the other 2 days was below average TDI (Table 1).

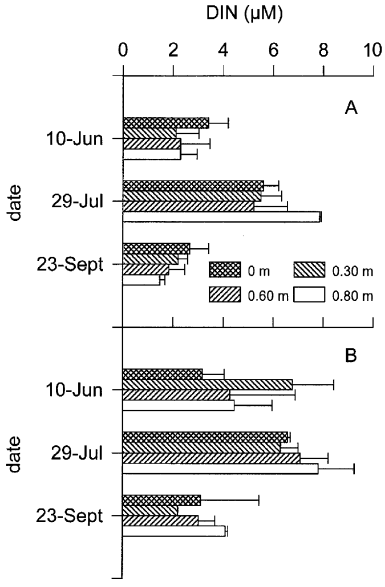

Fig. 1 Vertical profile of dissolved inorganic nitrogen (DIN) in the Veerse Meer (SW Netherlands) on three dates (**A**) at different depths in a water column overlying bare sediment and (**B**) at different depths in the water in an *Ulva* spp. mat. Samples were taken from the surface water (just above the first layer of *Ulva* thallus), from 0.30 m depth (below the first *Ulva* layer), at 0.60 m (below the third layer), and at 0.80 m (below the bottom layer). "Depths" for September samples only refer to place in the mat as the algae lay flat on the sediment (see text). Values are means; *error bars* represent 1 SD ($n = 4$ for June and $n = 3$ for July and September)

Nutrients in the water and in algal tissue

Monthly average (for all layers) DIN was highest in July, both inside and outside the algal mats ($P < 0.01$, Fig. 1A, B). Vertical differences in DIN concentration within the mat could only be determined in July where DIN in the three upper layers was significantly lower than in the bottom layer ($P < 0.05$; Fig. 1A, B). In June (except for the top layer) and July (except for the bottom layer) and for the bottom layer in September DIN concentrations in the mat were significantly higher than those outside the mat ($P < 0.05$ for all cases). In all layers, algal tissue C was slightly higher in September than in June (significant only for top and middle layers, $P < 0.01$); however, no vertical differences were observed (Table 2). Tissue N values in the top layer were threefold higher in September compared to June and July ($P < 0.001$). The middle layer showed an equal pattern with intermediate values for July, whereas in July tissue N levels in the bottom layer were significantly higher

Table 2 Carbon (C) and nitrogen (N) content (% dry weight), absorbance (A), pigment content of different pigments (µg pigment g^{-1} *Ulva* dry weight), and chlorophyll a/b ratio (*Chl a*/*b*) of *Ulva* spp. at three depths (average ± SD, for C, N, and A, $n = 3$; for pigments $n = 2$ for June and $n = 3$ for July and September) in mats collected from the Veerse Meer (SW Netherlands) at three sampling dates and calculated absorbance of the silt/epiphyte film (no replicate measurements) on *Ulva* spp. thalli at three depths in September (see text for explanation). *b.d.* below detection limit

Date	Layer	C	N	A	Chl a	Chl b	β-carotene	Lutein	Chl a/b
10 June	Top	26.4 ± 1.3	0.9 ± 0.2	0.28 ± 0.01	125.4 ± 87.8	45.1 ± 24.9	b.d.	1.3 ± 1.8	2.65 ± 0.49
	Middle	25.0 ± 3.5	0.9 ± 0.1	0.38 ± 0.07	238.8 ± 195.3	99.3 ± 0.7	b.d.	1.3 ± 1.8	2.40 ± 1.95
	Bottom	26.1 ± 1.5	2.1 ± 0.2	0.47 ± 0.02	914.4 ± 528.9	717.1 ± 385.7	b.d.	13.9 ± 19.6	1.26 ± 0.06
30 July	Top	30.7 ± 1.5	1.1 ± 0.2	0.36 ± 0.07	94.5 ± 93.2	107.2 ± 59.2	0.8 ± 1.2	12.3 ± 11.3	0.76 ± 0.45
	Middle	31.1 ± 1.6	2.0 ± 0.3	0.59 ± 0.02	552.6 ± 399.4	444.5 ± 243.0	2.0 ± 2.4	40.7 ± 26.7	1.13 ± 0.34
	Bottom	28.1 ± 0.7	3.0 ± 0.2	0.68 ± 0.11	1621.5 ± 206.3	1308.8 ± 190.5	4.9 ± 4.3	133.0 ± 19.5	1.24 ± 0.12
23 September	Top	32.9 ± 1.1	2.7 ± 0.4	0.82 ± 0.02	455.4 ± 227.5	460.4 ± 135.0	0.6 ± 0.9	25.9 ± 10.7	0.96 ± 0.21
	Silt/epiphytes	–	–	0.39	–	–	–	–	–
	Middle	29.9 ± 2.8	2.6 ± 0.1	0.90 ± 0.17	1190.6 ± 420.9	1073.1 ± 243.0	2.1 ± 3.0	94.7 ± 38.4	1.09 ± 0.14
	Silt/epiphytes	–	–	0.25	–	–	–	–	–
	Bottom	28.4 ± 2.2	3.2 ± 0.3	0.99 ± 0.03	1427.4 ± 438.4	1224.4 ± 293.9	5.4 ± 2.1	127.3 ± 34.6	1.16 ± 0.08
	Silt/epiphytes	–		0.32	–	–	–	–	–

than in June ($P < 0.001$) and remained at that level in September. Tissue N levels showed very clear and significant vertical patterns in June and July. In June no difference was observed between top and middle layers whereas the tissue N in the bottom layer was more than twice as high ($P < 0.001$). In July tissue N gradually increased with mat depth ($P < 0.001$ for all differences). In September the differences were much smaller. The bottom layer still had the highest values; however, only the middle layer values were significantly lower ($P < 0.05$).

Absorbance, pigments, and quantum efficiency

Wide-band (400–700 nm) absorbance of all layers increased significantly during the season ($P < 0.05$ for all layers between June and July and $P < 0.001$ for all layers between July and September, Table 2) to a maximum value of 0.99 in the bottom layer in September. Absorbance showed a clear vertical pattern at all sampling dates (Table 2). Absorbance of top layers was significantly lower than middle and bottom layers in July ($P < 0.01$ and $P < 0.001$, respectively) and September ($P < 0.01$ for both layers). In June, only the difference between the top and the bottom layer was significant ($P < 0.01$). Calculated absorbance values (by extracting absorbance of cleaned thalli from absorbance of untreated thalli) for the epiphyte/silt film on the *Ulva* thalli in September were highest for the upper layer and lowest for the middle layer but could not be tested for significance due to lack of replicates (Table 2). The shape of the absorbance spectrum measured through an increasing number of layers of the *Ulva* thalli was similar at all dates (Fig. 2A–C), showing the typical chlorophyll absorption peaks in the blue and red parts of the spectrum. The spectra for the epiphyte/silt film, however, did show considerable differences along the vertical gradient (Fig. 2D). The spectrum for the top layer was comparable with the *Ulva* spectrum but showed more absorbance in the 500–625 nm region, indicating the presence of epiphytic algae. Judging from the absence of absorption peaks in the epiphyte/silt film from the bottom and middle layers, fewer photosynthetically active epiphytes were present on these layers.

All pigments showed an increasing trend both in time and with mat depth (Table 2). Due to high variability, most differences were not significant, however. For Chl *a* significant differences were found between middle layers in June and September ($P < 0.05$) and for bottom layers between June and July ($P < 0.05$). Significant vertical differences were found between top and bottom and middle and bottom layers in July ($P < 0.01$ and $P < 0.05$, respectively). Chl *b* was significantly higher in the middle layer in June than in September ($P < 0.05$) and in bottom and middle layers compared to top layers in July and September ($P < 0.01$). Although none of the differences in β-carotene contents were significant due to the low values and the high variability, they show the same seasonal and vertical trends as the other pigments.

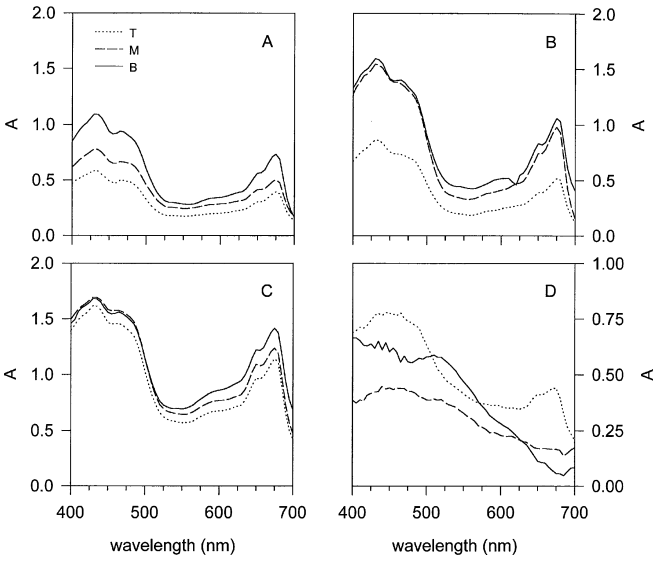

Fig. 2 Absorption spectra of thalli of *Ulva* spp. from the top (*T*), middle (*M*), and bottom (*B*) layer of an algal mat in the Veerse Meer (SW Netherlands), in June (**A**), July (**B**), and September (**C**) and the absorption of the silt/epiphyte film on top of *Ulva* thalli (**D**, note different scale), measured in September

A significant increase from June to July and July to September in lutein contents was found in the middle ($P < 0.05$) and bottom layers ($P < 0.01$). Significant differences in the mat were found in July between the bottom layer and the middle and top layers ($P < 0.01$) and in September between the top and the bottom layer ($P < 0.05$). Chl *a*/*b* ratio in the top layer was significantly higher in June than in July and September ($P < 0.05$); differences between layers were not significant.

Maximum (dark-adapted) quantum efficiency (F_v/F_m) showed a distinct but opposite vertical pattern in June and September (Fig. 3). In June, algae from top layers showed a significant reduction in F_v/F_m compared to algae from bottom layers ($P < 0.001$) with intermediate values for the middle layers. Algae from both the top and bottom layers showed some recovery later that afternoon ($P < 0.001$ for the top and $P < 0.05$ for the

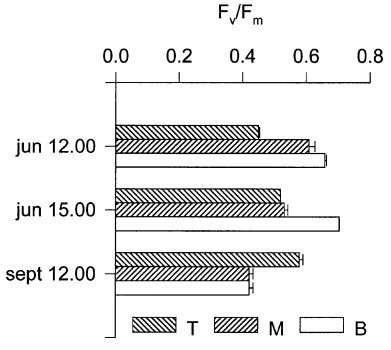

Fig. 3 Maximum quantum efficiency (F_v/F_m) of *Ulva* spp. thalli from the top (*T*), middle (*M*), and bottom (*B*) layer of an algal mat in the Veerse Meer (SW Netherlands), measured at noon and afternoon in June and at noon in September. Values are means; *error bars* represent 1 SD ($n = 3$)

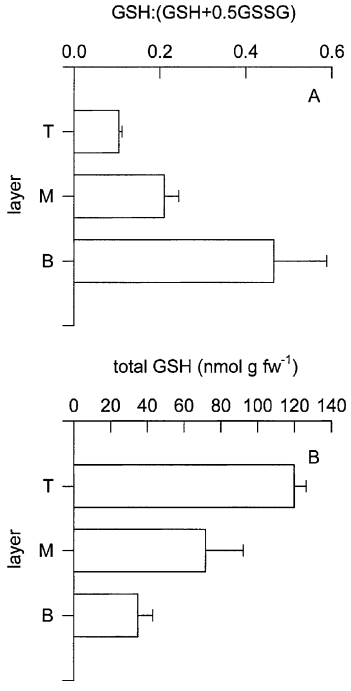

Fig. 4 Glutathione (GSH) redox ratio (**A**) and total reduced and oxidised glutathione pool (**B**) of *Ulva* spp. from the top (*T*), middle (*M*), and bottom (*B*) layer of an algal mat in the Veerse Meer (SW Netherlands) measured in June. Values are means; *error bars* represent 1 SD ($n = 3$)

bottom layer). In September, algae from the bottom and middle layer were more inhibited compared to the top layer algae ($P < 0.001$).

Glutathione redox ratio

Both glutathione redox ratios and total glutathione showed a clear vertical pattern (Fig. 4A, B). Algae from all layers contained more oxidized than reduced glutathione (redox ratios were all lower than 0.5). The ratio in the top layer was significantly lower than that in the middle ($P < 0.05$) and bottom layer ($P < 0.001$); the difference between the middle and bottom layer was also significant ($P < 0.01$). Total glutathione was significantly highest in the top layer and lowest in the bottom layer ($P < 0.01$) with the middle layer having an intermediate value ($P < 0.05$ for both top and bottom layer). No reliable glutathione data could be obtained from the algae collected in September, due to products of decaying algae interfering with the HPLC analysis.

Discussion and conclusions

This study demonstrates the existence in the field of vertical differences in physiological characteristics in mats of *Ulva* spp. Tissue nitrogen levels, F_v/F_m values, total glutathione levels, glutathione redox ratios, and absorbance showed pronounced vertical heterogeneity throughout the year. Except for β-carotene, all pigments showed increasing concentration with depth in the layer; however due to the high variability this trend was rarely significant.

Nitrogen limitation

Vertical gradients in tissue N levels were found in laboratory experiments of Krause-Jensen et al. (1996) and Vergara et al. (1998). This study confirms the existence of such a gradient in nature. The existence of such a gradient in the field is a very important aspect in macroalgal ecology. In most field studies, tissue N in algae is determined in order to be able to assess whether algae are nitrogen limited during certain parts of the season or if other factors are limiting growth (see, for instance, Soulsby et al. 1985; Fujita et al. 1989; Sfriso et al. 1992; Wheeler and Bjornsater 1992; Malta and Verschuure 1997). Considering a minimal tissue N level of 1.0% dry weight (DW) and a critical level (i.e. the level at which growth becomes nitrogen limited) for *Ulva* spp. between 2.0 and 2.4% DW (Fujita et al. 1989; Lavery and McComb 1991b; Pedersen 1994), this means that in the studied *Ulva* spp. mat a gradient in tissue N exists that ranges between levels saturating for maximum growth (bottom layers) and levels that are at the edge of minimum levels required for survival (top layers). Conclusions about potential nitrogen limitations are thus very much dependent on the depth and position in the layer from which the sample is taken.

Two independent processes can lead to the existence of such a vertical gradient: (1) light limitation of growth in the lower layers causes a slower dilution of internal nitrogen resources compared to fast-growing top layers (Malta and Verschuure 1997). This process established the vertical gradient observed by Vergara et al. (1998). (2) Decomposition and subsequent mineralization of bottom layer and sediment organic material and short- or long-term anoxia at the bottom of the mat may cause an upward nutrient flux (Lavery and McComb 1991a; Bartoli et al. 1996) that can be utilized by the viable algae in the mat. As these fluxes come from the sediment, the bottom layers are the first to benefit from these nutrients (Thybo-Christensen et al. 1993; Krause-Jensen et al. 1996). The finding that DIN concentrations were higher in the mat than outside the mat and the tendency to vertical heterogeneity in July support this explanation. However, the detection of these microstratifications requires highly precise and careful sampling methods and more data over a longer period are needed to test this fully. In the field, most probably, both processes occur simultaneously, although the second process will become more important later in the season when the layer of decomposing algae is thicker and higher temperatures enhance microbial activities.

In September, no differences in *Ulva* tissue N were observed between layers and there is no vertical gradient in DIN concentrations. Combined with the visual

observation that the algae are lying flat on the bottom, this led us to conclude that the mats are no longer vertically structured at this time. Mixture due to autumn winds may have destroyed the vertical structure. High tissue N values of the decomposing layers may have been caused by leaching and microbial growth on dissolved organic carbon and nitrogen, resulting in the attachment of nitrogen-rich bacteria (Hanisak 1993; Kristensen 1994).

Photoinhibition and oxidative stress

Ulva spp. and other green algae typically show maximum F_v/F_m values ranging from 0.78 to 0.83 (Magnusson 1997). The values found for the algae from the bottom layer in June are close to these maxima. In contrast, the lower values of the algae from the middle and especially from the top layer indicate photoinhibition or photodamage. The small recovery found in F_v/F_m in the afternoon measurements for the top layer suggests that photoinhibition is partly reversible. Most likely the high irradiance in June is the cause of this reversible part, while severe nitrogen limitation of the algae is probably responsible for the irreversible part. These findings are well in agreement with the recent study of Bischof et al. (2002) on artificial canopies of *Ulva rotundata* in southwest Spain; they also found partial recovery in F_v/F_m under high light and strong photoinhibition in the upper layers in August. Integrating the hourly irradiances of Bischof et al. (2002) we estimate that TDI was between 52 and 57 mol photons m^{-2} day^{-1} during their experiment, which is close to the 50 we found for the June sampling, supporting our hypothesis that photoinhibition was at least partly caused by high light. In September (degradation phase), most algae are dead or decomposing including their chloroplasts and PSII reaction centers, hence the low F_v/F_m values (< 0.4) of the bottom and middle layers during this period. The values for the top layer algae were higher, indicating that the algae in this layer still show some activity.

All glutathione redox ratios we found were on average lower than 0.5. We have no references for an optimal or maximal GSH redox ratio as indicator for the least level of stress in *Ulva*; for the closely related *Enteromorpha prolifera* from the N-repleted Scheldt estuary, however, Rijstenbil et al. (1998) found maximal ratios of approximately 0.6. We assume this to be close to the optimum for *Ulva* as well; hence we conclude that *Ulva* thalli suffered mild (bottom layers) to considerable (middle and top layers) oxidative stress. The almost fourfold higher total glutathione concentration in the top layers compared to the bottom layers further indicates the existence of severe oxidative stress in the top layers. Oxidative stress stimulates the production of reduced GSH (Ishikawa and Sies 1988; Alscher 1989; Agrawal 1992), probably via induction through high GSSG concentrations (Agrawal 1992). What causes the oxidative stress in the bottom layer is unclear; maybe active oxygen is formed in the respiratory pathways (Rijstenbil et al. 1998). The low glutathione redox (0.10 ± 0.01) in the top layer should be attributed to high light stress (Agrawal 1992). When considering the glutathione levels in connection with the results of the fluorescence measurements, we can conclude that the photoinhibition in the top layer is largely due to oxidative stress, caused by high light intensities combined with very low nitrogen levels.

It is interesting to note that this antioxidant mechanism is still highly active under nitrogen limitation in the upper layer. The amount of nitrogen maximally allocated to the GSH pool in our results [120 nmol g^{-1} fresh weight (FW) total GSH, June, top layer] was only 0.56% of the total tissue N pool, given that each GSH molecule contains three N atoms, tissue N = 0.9% DW, and assuming DW:FW = 0.1. Glutathione pools found in this study are not exceptionally small compared to those found in other green algae and plants (Table 3). We conclude that it is a low-energy investment mechanism in terms of nitrogen metabolism, which can still be active when the algae suffer nitrogen shortage.

The glutathione cycle is a very well studied antioxidative mechanism in organisms of almost all major taxonomical groups, such as mammals (including humans; Ishikawa and Sies 1988), higher plants, bacteria, and diatoms (Rijstenbil and Wijnholds 1996). For macroalgae glutathione data are rare (see Nakano et al. 1995; Rijstenbil et al. 1998). In a review, Davison and Pearson (1996) concluded that there is a strong need to develop markers to be able to determine specific environmental stresses or groups of stresses in seaweeds. Considering our results, glutathione could be a good candidate for such a stress indicator. HPLC analyses of GSH and GSSG as used by Rijstenbil and Wijnholds (1996) are quite sensitive, requiring small (150–200 mg FW) amounts of sample. The method might seem complicated; however, once developed, extraction, DTT reduction, MBrB labeling, and GSH analyses can be carried out quickly, on a routine basis, on large numbers of samples. Very recently Davey et al. (2003) published an interesting modification of the technique using the newly developed "Rocket" columns, which reduce analysis time to only 6 min per run, that is, 12 per

Table 3 Published reduced glutathione (GSH) concentrations (nmol g^{-1} fresh weight) in a green microalga, two green macroalgae, and three higher plant species

Species	GSH range	Source
Chlorococcus infusionum	65–384	Agrawal 1992
Enteromorpha prolifera	150–200	Rijstenbil et al. 1998
Ulva sp.	35–120	This study
Brassica napus	800–2500	Ruiz and Blumwald 2002
Lycopersicon peruvianum	180–360	Brüggemann et al. 1999
Zea mays	5–550	Meuwly and Rauser 1992; Rüegsegger and Brunold 1992; Kocsy et al. 2001

sample (for both GSH and GSSG analysis). Further steps in the development of GSH as stress indicator should include analyzing total GSH levels and ratios in algae subjected to various stress conditions (nutrient limitation, high light, suboptimal temperature, etc.) under controlled circumstances and comparing these with other parameters of algal performance, preferably growth rate, to define both critical and optimal GSH levels and redox ratios.

Acclimatization to changes in the light climate

Considering the increases in absorbance, we conclude that the *Ulva* spp. mainly acclimatize to the spectral and intensity changes in a mat by increasing their absorption capacity. Experimental absorption measurements, using green filters (Malta 2000), support this conclusion. The "deep-water" (10 m) alga *U. japonica*, is able to absorb light in the so-called green window (roughly the 500–560 nm region), using the carotenoid siphonaxanthin (Kageyama et al. 1977; Yokohama 1981; Fork and Larkum 1989). Neither siphonaxanthin, nor its esters siphonein A and B could be detected in the Veerse Meer *Ulva* spp. Although the light climate in the lower layers of a mat is very similar to that of deep water (Mercado et al. 1996; Salles et al. 1996), apparently, our *Ulva* spp. do not possess the capacity to use siphonaxanthin for absorption in the 500–560 nm region.

The increase in absorbance is consistent with both the increase in total pigment content and the relatively larger increase in light-harvesting-complex (LHC) pigments as shown by the decreases in Chl *a*/*b* ratio with mat depth. This increase in LHC pigments is a typical response to low light for most algae and plants, regardless of taxonomic affiliation (Ramus 1983; Henley and Ramus 1989a, 1989b; Markager and Sand-Jensen 1994). The seasonal patterns in absorbance, total pigment levels, and Chl *a*/*b* ratios should also be explained as a response to decreasing light levels as normally occur in the northern hemisphere between June and September. Plants can partly compensate for the lower light levels by increasing absorbance through higher total pigment and LHC pigment levels, as has been observed in *Ulva* spp. from various sites (including our own) in Europe (Geertz-Hansen and Sand-Jensen 1992; Vergara et al. 1997; Malta et al. 2002). Additionally, it is known that pigment content strongly correlates with the nitrogen status of a macroalga (Lapointe and Tenore 1981; Ramus 1983; Krause-Jensen et al. 1996) and increases with total N (Markager and Sand-Jensen 1994; Naldi and Wheeler 1999), which may contribute to the vertical trend. The latter also explains the high pigment content of bottom and middle layers found in September. Given the low photosynthetic efficiency as expressed by the low F_v/F_m values and the generally low growth rates during this period (<0.03 day^{-1}; Malta et al. 2002), these pigments are hardly operational. It is unlikely that they act as nitrogen reserve as only between 0.1 and 0.6% of total N is allocated as chlorophyll-N; however, the membranes associated with the pigments are rich in nitrogen. According to Markager and Sand-Jensen (1994) these membrane proteins cause the correlation between total N and pigments.

By measuring absorption on different sediment dilutions, Vergara et al. (1997) showed that a mud film on the algae could contribute significantly to the light absorption. Our September data show that (1) absorbance by a "mud" film is considerable but less than that of an *Ulva* thallus (25–45% of the absorbance of an *Ulva* layer), and (2) the "mud" film is not optically neutral. The latter conclusion is especially obvious in the absorption spectrum of the film obtained from the upper layers of the mat. This film shows an absorption spectrum that differs from the *Ulva* spp. by the higher absorption in the 500–560 nm region, typical for diatoms (see, among others, Huisman 1997). Considering the spectra of the films on the middle and bottom layers and microscopical observations, it can be concluded that the diatoms are mainly present in the top layers of the macroalgal mat. The amount of silt and diatoms seems to increase throughout the season (E. Malta, personal observation); thus frequent measurements are needed to quantify the absorbance of the epiphyte/silt film throughout the season.

Concluding remarks

In this article we tested the hypothesis that multiple growth-limiting gradients in mats of *Ulva* spp. occur in the field. We conclude that (1) during the phase of fast growth, there is progressive nitrogen limitation toward the surface of the mat; (2) surface layers suffer from photoinhibition and oxidative stress caused by high sunlight intensities as is expressed by low quantum efficiencies and glutathione redox ratios; and (3) progressive light limitation with depth leads to an increase in absorption and a higher quantum efficiency (F_v/F_m); furthermore there is a trend of increasing pigment content with depth. This information should be used to refine models estimating primary production and biomass development of systems dominated by free-floating macroalgae. Additionally we found the measurement of glutathione redox ratios a helpful tool to study oxidative stress in *Ulva* spp. and conclude that this parameter deserves further development to serve as a stress indicator in macroalgae.

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