

# Growth and biomass development of the introduced red alga *Gracilaria vermiculophylla* is unaffected by nutrient limitation and grazing

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**ABSTRACT:** The invasive red alga *Gracilaria vermiculophylla* was first recorded in Denmark and Sweden in 2003 and has since been reported from several sites in Denmark, Sweden and northern Germany. The abundance of *G. vermiculophylla* is typically high at more eutrophic sites, whereas it remains relatively low at more oligotrophic sites. We recorded seasonal variations in growth and biomass development at 2 sites with contrasting nutrient status (eutrophic versus oligotrophic) to investigate whether nutrient limitation of growth and loss of biomass due to grazing from invertebrate herbivores could explain observed variations in biomass. The biomass of *G. vermiculophylla* at the eutrophic site (Holckenhavn Fjord) was ca. 300-fold larger than at the oligotrophic site (Fyns Hoved). Growth rates ranged from almost 0 in early spring to ca. 0.08 d<sup>-1</sup> in mid-summer and did not vary among sites. The seasonal pattern of growth was correlated to insolation and water temperature, suggesting that nutrient availability played a minor role in controlling growth. Experimental nutrient enrichment confirmed these findings; nutrient enrichment enhanced the level of tissue nutrients, but only had a marginal effect on growth. Grazing losses were insignificant throughout the entire study period in both systems. Hence, site-specific variations in biomass could not be explained by differences in grazing pressure. Given the lack of support for nutrient limitation or herbivory to explain the observed variations of *G. vermiculophylla* biomass, we suggest that physical exposure caused by wind-driven waves may be the factor that controls biomass of *G. vermiculophylla* in these shallow estuaries.

**KEY WORDS:** Macroalgae · Invasive species · Nutrient limitation · Grazing

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## INTRODUCTION

The red alga *Gracilaria vermiculophylla* (Ohmi) Papenfuss originates from Asia (Tseng & Xia 1999) but has recently spread to North America and Europe (Bellorin et al. 2004, Rueness 2005, Freshwater et al. 2006, Thomsen et al. 2006, 2007). It was introduced to northern France with transplanted oysters intended for aquaculture (Mollet et al. 1998) and has since spread to Portugal in the south and Scandinavia in the north (Rueness 2005). *G. vermiculophylla* was first observed in Scandinavia in 2003 (Horsens Fjord, Denmark; Thomsen et al. 2006, Göteborg archipelago, Sweden; Nyberg et al. 2009) and has subsequently spread to several sites in Denmark, on the Swedish west coast

and to the Baltic coast of northern Germany (Rueness 2005, Thomsen et al. 2007, Weinberger et al. 2008). *G. vermiculophylla* is commonly found in shallow, sheltered estuaries with soft sediment and relatively high nutrient levels (Thomsen et al. 2006). The abundance of *G. vermiculophylla* can be high, and it has become the dominant macroalga in several Danish estuaries where it has replaced fast-growing algae such as *Ulva* (including previous species of *Enteromorpha*) and *Chaetomorpha* (M. F. Pedersen pers. obs.), while in other cases, it has replaced more slow-growing benthic algae such as *Fucus vesiculosus* L. (e.g. Kiel Bight, Germany; Weinberger et al. 2008).

The fast spread of *Gracilaria vermiculophylla* and its potential effect on community composition in recipient

systems has stimulated research on its ecological performance. A number of studies have investigated how environmental factors such as salinity, light and water temperature affect growth under laboratory conditions (e.g. Yokoya et al. 1999, Raikar et al. 2001). Yet, *in situ* growth and biomass development as well as the regulation of these parameters have received less attention (but see Thomsen & McGlathery 2007, Weinberger et al. 2008, Thomsen et al. 2009)

*Gracilaria vermiculophylla* grows fast under optimal culture conditions (i.e.  $0.1 \text{ d}^{-1}$ , Yokoya et al. 1999), but little is known about the seasonal variations in growth or the factors controlling that growth under field conditions. Weinberger et al. (2008) found that seasonal changes in the growth of *G. vermiculophylla* in Kiel Bight were closely correlated to insolation, water depth and temperature, but not to salinity, although the salinity in Kiel Bight is relatively low ( $<16$ ). These authors did not study the potential effect of nutrient availability on seasonality in growth, but fast-growing algae are typically more affected by nutrient limitation than more slow-growing species (Pedersen 1995, Pedersen & Borum 1996, 1997, Pedersen et al. 2010). It is therefore possible that nutrient limitation may restrict growth and thus slow down the accumulation of *G. vermiculophylla* biomass at more nutrient-poor sites and/or during periods of low nutrient availability.

*Gracilaria vermiculophylla* has spread to several new sites in Denmark since it was first observed in Horsens Fjord in 2003. Yet, the subsequent development of biomass differs substantially among sites. In eutrophic sites, such as Holckehavn Fjord, *G. vermiculophylla* has become very abundant, forming dense, drifting mats that cover large areas. At more nutrient-poor sites, it remains much less abundant. These site-specific variations in nutrient availability may affect growth and, hence, the accumulation of biomass. Similarly, site-specific variations in grazing pressure may affect the rate at which standing biomass of the alga is lost from a site. Fast-growing algae are more susceptible to grazing losses than more slow-growing species (Cebrián & Duarte 1994), because they tend to have a high nutritional value, less structural tissues and few morphological and chemical defences (Mattson 1980, Nicotri 1980, Duffy & Hay 1990). Herbivory can control the abundance of fast-growing algae and affect algal community structure (e.g. Lubchenco 1978, Geertz-Hansen et al. 1993, Hauxwell et al. 1998), but the abundance of grazers and the potential grazing pressure may vary substantially among sites due to site-specific variations in salinity and level of eutrophication. Low salinity, like that found in the inner portions of many estuaries, may lower the abundance of grazers, and eutrophication may have a similar effect. Hauxwell et al. (1998) showed that eutrophic sites

dominated by drift algae had fewer invertebrate grazers and lower grazing pressure on macroalgae than more oligotrophic sites with a more diverse plant assemblage, likely because mats of drift algae create hypoxic conditions which affect the benthic fauna negatively (e.g. Norkko & Bonsdorff 1996, Tagliapietra et al. 1998). Low biomass of *G. vermiculophylla* at oligotrophic sites may thus result from a combination of nutrient-limited growth and high grazing pressure, processes that are assumed to be less important at more eutrophic sites.

The aim of the present study was 2-fold: (1) to study seasonal patterns in *in situ* growth and biomass development of *Gracilaria vermiculophylla* in 2 contrasting (nutrient rich versus nutrient poor) estuaries to test for nutrient limitation effects on growth, and (2) to test whether site-specific variations in growth rate and invertebrate grazing could explain obvious differences in standing biomass. The study was carried out in 2 small, shallow estuaries on the island of Fynen, Denmark, from March to November 2008. The sites were assumed to differ with respect to nutrient loading and in the abundance of *G. vermiculophylla*. Holckehavn Fjord is a eutrophic estuary with a very high abundance of *G. vermiculophylla*, while Fyns Hoved is a presumed oligotrophic inlet with a low abundance of *G. vermiculophylla*. We hypothesized that nutrient-limited growth and higher grazing pressure can explain the low biomass of *G. vermiculophylla* at the oligotrophic site.

## MATERIALS AND METHODS

**Study sites.** Fyns Hoved is a shallow, semi-closed inlet ( $55^{\circ} 36.9' \text{ N}$ ,  $10^{\circ} 36.7' \text{ E}$ ) with an area of  $1.1 \text{ km}^2$  and a mean depth of less than  $0.5 \text{ m}$ . The inlet connects to the Kattegat Sea through a narrow opening. The catchment is dominated by non-cultivated land, and no freshwater streams enter the inlet. The nutrient input to the inlet is presumed to be very low, being made up mainly of intruding water from the Kattegat. The sediment is made up of coarse sand with a relatively low organic content ( $0.7 \pm 0.1\%$  dry weight, DW). Seagrasses (*Zostera marina* L. and *Ruppia maritima* L.) are found in scattered patches, whereas attached *Gracilaria vermiculophylla* and *Fucus vesiculosus* dominate the macroalgal assemblage.

Holckehavn Fjord is a small estuary ( $55^{\circ} 17.8' \text{ N}$ ,  $10^{\circ} 46.2' \text{ E}$ ) covering  $0.7 \text{ km}^2$  and with a mean depth of  $1.1 \text{ m}$ . Two streams enter the western most end of the estuary, which connects to Nyborg Fjord and the Belt Sea through a very narrow channel at the eastern end. The catchment area ( $221 \text{ km}^2$ ) is dominated by agriculture, and the annual loading of N and P to the fjord is  $452 \text{ t N}$  and  $9 \text{ t P yr}^{-1}$  (Regional Environmental Centre

Fynen unpubl. data). Holckenhavn Fjord is among the most eutrophic estuaries in Denmark. The substrate is dominated by organically rich mud ( $4.3 \pm 0.1\%$  DW). *Gracilaria vermiculophylla* is the dominant macrophyte and is mainly found unattached, forming a dense mat which covers most of the bottom (M. F. Pedersen pers. obs.). Prior to the introduction of *G. vermiculophylla*, Holckenhavn Fjord was dominated by ephemeral macroalgae such as *Chaetomorpha linum* (OF Müller) Kützing and *Ulva* spp. (including species previously included in *Enteromorpha*), which are still found in low abundance.

**Biomass.** The biomass of *Gracilaria vermiculophylla* was surveyed in spring (April), summer (July) and fall (September) 2008. Surveys at Fyns Hoved, where the algal biomass was low and heterogeneously distributed, were conducted by placing 14 to 16 transects (each 40 m long) haphazardly across the inlet. Each transect was surveyed for *G. vermiculophylla* by moving a 1 m<sup>2</sup> frame metre by metre along each transect. *G. vermiculophylla* occurring within the frame were collected and returned to the laboratory. In Holckenhavn Fjord, where the algal biomass was much higher and much more uniformly distributed across the estuary, 5 to 8 transects (each 40 m long) were placed haphazardly across the estuary. Biomass samples were collected for each 5 m along each transect using a smaller 0.25 m<sup>2</sup> frame. Biomass samples were returned to the laboratory, rinsed and then dried to constant weight at 85°C to determine dry weight biomass.

**Growth, nutrient limitation and grazing.** *In situ* growth and the importance of nutrient limitation and grazing were examined monthly in both systems from March to October 2008. Healthy looking *Gracilaria vermiculophylla* was collected and cleaned of sediment, epiphytes and epifauna. Apical parts with several branches were weighted (blotted wet weight) and incubated in cylindrical transparent PVC incubation chambers (10 cm in diameter and 20 cm long) that were closed at both ends with a net (0.5 mm mesh size) that allowed water movement through the cylinders but prevented entry of grazers. The algae were randomly allocated to 1 of the following 3 treatments: (1) control (without added nutrients and with a net to exclude grazers), (2) +NP (with added nutrients and with nets to exclude grazers) and (3) +Grazers (without added nutrients and without a net at one end to allow entry of mobile grazers). Algae were fixed to a horizontally placed plate with silicon strings in each chamber. Nutrients were added to the +NP treatment chambers by leaving a net-tube filled with ca. 30 g slow-release fertiliser (PlataCore Depot 6M, Uranium Agrochem; total N = 14%, total P = 4%) in the chambers. The incubation chambers were finally mounted on a frame (3 chambers, 1 of each treatment, per frame), which was

fixed to the sediment with stainless steel pegs at a water depth of 0.5 to 0.7 m outside existing algal mats. Five replicate frames were placed haphazardly with a distance of about 10 to 15 m between them at each study site. Nutrient addition enhances the concentrations of inorganic nutrients within the incubation chambers, but nutrient enrichment does not affect the nutrient content of algae in neighbouring incubation chambers placed more than 15 to 20 cm away (M. F. Pedersen unpubl.). The algae were left to grow for 5 to 15 d (depending on season) and were then collected, rinsed of sand and debris and weighed (blotted wet weight). Relative growth rates ( $\mu$ ) were calculated assuming exponential growth:

$$\mu = \frac{(\ln FW_t - \ln FW_0)}{t} \quad (1)$$

where  $FW_t$  and  $FW_0$  are fresh weight biomass before and after the incubation, respectively, and  $t$  is the incubation time in days. Grazing rates ( $g$ ) were estimated as the difference between the mean growth rate obtained from the control treatments ( $\mu_C$ ) and growth rates obtained from the +Grazers treatment ( $\mu_G$ ):

$$g = \bar{\mu}_C - \mu_G \quad (2)$$

The incubated algae were finally dried to constant dry weight at 85°C together with 3 initial algal samples and stored for later analyses of tissue nutrients.

**Environmental variables.** Water temperature and light irradiance at the surface of the incubation chambers were recorded every 30 min during each incubation using HOBO loggers (Onset Computer Corporation) that were mounted on the frames carrying the incubation chambers. Readings from the loggers were transformed to photosynthetically active radiation (PAR) by calibration against an irradiance sensor (LiCor Li-190SA).

Light and temperature data were used to calculate daily light irradiance and average water temperature (per day) during each incubation period.

Three replicate water samples (each 250 ml) were collected at each site at the beginning and termination of all incubations. Salinity was measured in each water sample using a refractometer (ATAGO S/Mill) before samples were frozen at -20°C for later analyses of dissolved inorganic nutrients (ammonium, nitrate, phosphorus). All water samples were GF/C-filtered prior to nutrient analysis. The concentrations of ammonium, nitrate and dissolved inorganic phosphorus were determined on duplicate sub-samples from each water sample using a QuickChem FIA 8000 autoanalyser (Zellweger Analysis). Mean concentrations were estimated from the 3 replicate samples.

**Tissue nutrients.** Concentrations of tissue C and N in plants collected at the onset of each incubation (initial content) and in plants from the incubations (final sam-

ples from the control and +NP treatments only) were determined on dried samples using an EA 1110 CHNS elemental analyser (CE instruments). Total tissue-P was determined on the same individuals after wet oxidation with boiling  $\text{H}_2\text{SO}_4$  followed by spectrophotometric analysis (Strickland & Parsons 1968). The concentration of N and P in algae was finally estimated as the mean between initial and final samples.

**Statistical treatment.** The effect of Treatment, Site and time (Month) on environmental variables (light, temperature, salinity and inorganic nutrients) and on *Gracilaria vermiculophylla* growth and tissue nutrients, and the effects of Site and Season (spring, summer, fall) on biomass of *G. vermiculophylla* were analysed using factorial analysis of variance (ANOVA) followed by Tukey's test for pairwise comparisons. Missing values appeared in all data sets (mostly due to lost observations), but especially in the data set for biomass because the number of transects surveyed intentionally differed among sites. Type III sum of squares, which are based on unweighted means and therefore not influenced by the sample size of each cell in the data set, was consequently used for all ANOVA analyses as recommended by Quinn & Keough (2002). The factors treatment and site were considered fixed, while time (month or season) was considered a random factor (Underwood 1997). All data sets were ln-transformed prior to analysis to conform to the requirements for parametric analysis (i.e. normality of data and equal variances).

## RESULTS

### Environmental variables

Light at the surface of the incubation chambers was low in spring, increased during summer and decreased by late fall (Fig. 1, Table 1). Incident light on the chambers was significantly higher at Fyns Hoved than in Holckehavn Fjord. The significant interaction 'Month  $\times$  Site' indicates that the seasonal variation in light differed among sites. Water temperature varied seasonally (Fig. 1, Table 1), with low temperatures (5 to 10°C) occurring in early spring and late fall and the highest temperatures (18 to 19°C) occurring in August. Water temperature did not vary among sites. Salinity varied significantly with time (Fig. 1, Table 1), but the variations did not follow a clear seasonal pattern. The mean salinity at Fyns Hoved ( $20 \pm 3$ , range 16 to 25) was significantly higher than that observed in Holckehavn Fjord ( $14 \pm 3$ , range 10 to 16).

The concentration of dissolved inorganic nitrogen (DIN) varied seasonally and was higher in Holckehavn Fjord than at Fyns Hoved, in spring and fall

(Fig. 1, Table 1). The mean concentration of DIN at Fyns Hoved was  $2.3 \pm 2.2 \mu\text{M}$ , with the highest concentrations occurring in March ( $3.4 \mu\text{M}$ ) and October ( $7.2 \mu\text{M}$ ). Mean concentrations of DIN in Holckehavn Fjord ( $31.0 \pm 42.8 \mu\text{M}$ ) were higher than at Fyns Hoved and varied from  $<3 \mu\text{M}$  in summer to  $109.2 \mu\text{M}$  in April. The significant interaction Month  $\times$  Site shows that site-specific variations in DIN depended on time. The concentration of dissolved inorganic phosphorus (DIP) varied seasonally at both sites (Fig. 1, Table 1) and tended to increase during late summer and early fall. The mean concentration of DIP in Holckehavn Fjord ( $1.1 \pm 0.6 \mu\text{M}$ ) was slightly higher than at Fyns Hoved ( $0.6 \pm 0.5 \mu\text{M}$ ), albeit not significantly so. The ratio between DIN and DIP varied seasonally at both sites (Fig. 1, Table 1). The mean DIN:DIP ratio in Holckehavn Fjord ( $74.8 \pm 86.9$ ) was higher than that at Fyns Hoved ( $14.9 \pm 11.7$ ).

### *Gracilaria* biomass

The biomass of *Gracilaria vermiculophylla* (Table 2) at Fyns Hoved was small, ranging from  $0.4 \pm 0.7 \text{ g DW m}^{-2}$  in spring to  $1.6 \pm 1.4 \text{ g DW m}^{-2}$  in summer. The biomass in Holckehavn Fjord was significantly higher than at Fyns Hoved (Tables 2 & 3). The biomass in Holckehavn Fjord peaked in spring ( $464.4 \pm 426.3 \text{ g DW m}^{-2}$ ) and reached the lowest level in fall ( $176.4 \pm 65.4 \text{ g DW m}^{-2}$ ). No significant seasonal (spring, summer, fall) difference was detected, nor a significant Season  $\times$  Site interaction.

### Growth of *Gracilaria*

Growth of *Gracilaria vermiculophylla* varied seasonally, with treatment, but not among sites (Fig. 2, Table 3). The significant interaction Month  $\times$  Site suggests that the seasonal variation in growth differed between the 2 sites. Low rates were observed in March ( $0.004 \pm 0.003 \text{ d}^{-1}$  at Fyns Hoved and  $0.009 \pm 0.002 \text{ d}^{-1}$  in Holckehavn Fjord), while fast growth was observed at Fyns Hoved in June ( $0.079 \pm 0.001 \text{ d}^{-1}$ ) and in Holckehavn Fjord in July ( $0.083 \pm 0.016 \text{ d}^{-1}$ ). Growth slowed down at both sites in fall.

Growth rate was affected by nutrient treatment, but not by grazing when compared across all sampling events (Table 3, Tukey's test: control vs. +NP,  $p = 0.025$ , control vs. +Grazing,  $p = 0.736$ ). When averaged across all sampling events, nutrient-enriched algae grew 15 and 18% faster than those from the control treatment at Fyns Hoved and in Holckehavn Fjord, respectively, but no differences were significant when compared at individual sampling dates (Tukey's test, all  $p > 0.05$ ).

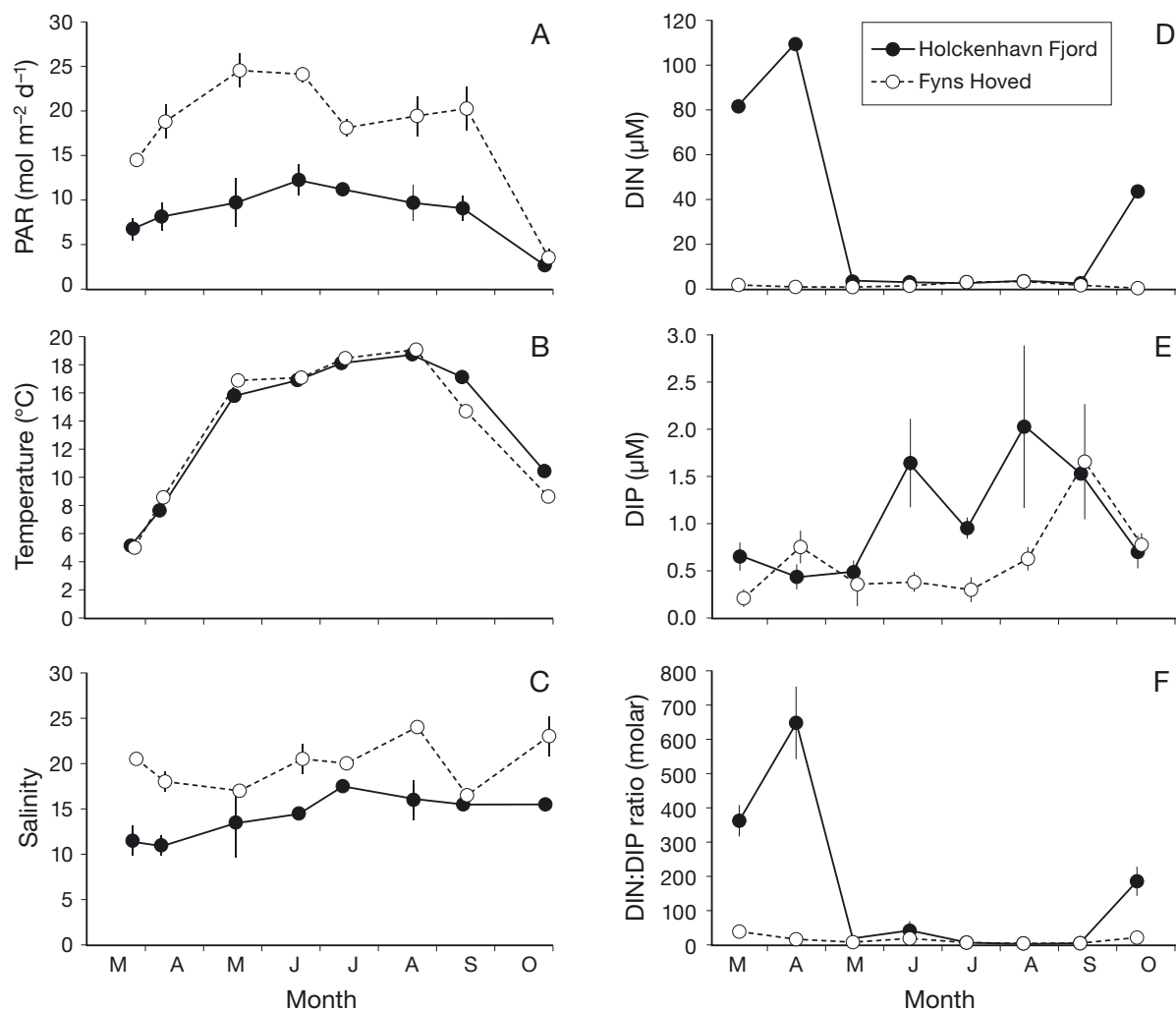


Fig. 1. Seasonal variation (mean  $\pm$  SD) in average daily (A) light availability (photosynthetically active radiation; PAR), (B) water temperature, (C) salinity, (D) concentrations of dissolved inorganic nitrogen (DIN =  $\text{NH}_4^+$  +  $\text{NO}_3^-$ ), (E) dissolved inorganic phosphorus (DIP) and (F) the molar ratio between DIN and DIP at Fyns Hoved (O) and in Holckehavn Fjord ( $\bullet$ ) from March to October 2008

We were unable to document any site-specific variation in the response to nutrient addition (Treatment  $\times$  Site:  $F = 1.58$ ,  $p = 0.241$ ). Growth rates of algae exposed to grazers ( $\mu_G$ ) were never significantly different from those of algae in the control treatment ( $\mu_C$ ), and estimated grazing rates ( $g$ ) were never significantly different from 0 (Student's  $t$ -test, all  $p > 0.05$ ). Grazing thus had no effect on growth rates.

#### Tissue nutrients

The C content of *Gracilaria vermiculophylla* averaged  $32.6 \pm 1.9\%$  C of DW (across all treatments, sites and months) and was neither affected by the +NP treatment nor by site (data not shown). Month had a significant effect on C content ( $F = 39.5$ ,  $p < 0.001$ ), but

this effect was caused by a single sample (September) where plants had a slightly lower content of C than average ( $29.2 \pm 0.7\%$  C of DW). Tissue N varied seasonally (Fig. 3, Table 4) and was higher in spring and late fall (3 to 3.5% N of DW) than in summer (ca. 1.5% N of DW). This pattern was more pronounced in Holckehavn Fjord than at Fyns Hoved as indicated by the significant interaction Month  $\times$  Site. Algae from the control treatment in Holckehavn Fjord were richer in N than those from Fyns Hoved when averaged across all sampling events ( $2.18 \pm 0.51$  versus  $1.80 \pm 0.48\%$  N in DW,  $F = 22.7$ ,  $p = 0.002$ ). Algae from Holckehavn Fjord were richer in N than those from Fyns Hoved in April, May, August, September and October (Tukey's test,  $p < 0.05$ ). Nutrient enrichment caused a significant increase in tissue N (across all sampling events, Table 4), and algae from the +NP treatment were 11%



Table 1. ANOVA for the effect of Site and Month on environmental variables (salinity, incident light on the incubation chambers, water temperature and dissolved inorganic nutrients) at Fyns Hoved and in Holckenhavn Fjord from March to October 2008. DIN, DIP: dissolved inorganic nitrogen and phosphorus, respectively

Response	Factors	df	MS	F	p
Salinity	Site	1	258.78	36.24	0.001
	Month	7	17.07	4.88	0.004
	Site × Month	7	7.14	2.04	0.113
	Error	16	3.50		
Light	Site	1	1408.03	37.68	<0.001
	Month	7	207.23	86.69	<0.001
	Site × Month	7	37.37	15.63	<0.001
	Error	54	2.39		
Water temperature	Site	1	0.68	0.18	0.686
	Month	7	250.39	17897.30	<0.001
	Site × Month	7	3.82	273.17	<0.001
	Error	59	0.01		
DIN	Site	1	21.00	5.50	0.057
	Month	6	8.44	15.08	<0.001
	Site × Month	6	3.82	6.82	<0.001
	Error	54	0.60		
DIP	Site	1	0.86	3.60	0.107
	Month	6	0.41	3.48	0.004
	Site × Month	6	0.24	2.10	0.075
	Error	72	0.12		
DIN:DIP	Site	1	20.80	3.25	0.121
	Month	6	11.59	8.07	<0.001
	Site × Month	6	6.40	4.46	0.001
	Error	51	1.44		

Table 2. *Gracilaria vermiculophylla*. Dry weight biomass (mean ± SD and range) at Fyns Hoved and in Holckenhavn Fjord across season. (n = no. of transects surveyed). DW: dry weight

Site	Census	n	Mean biomass (g DW m <sup>-2</sup> )	Range (g DW m <sup>-2</sup> )
Fyns Hoved	Spring	15	0.4 ± 0.7	0 – 2.1
	Summer	14	1.6 ± 1.4	0 – 3.4
	Fall	15	0.9 ± 1.3	0 – 5.1
Holckenhavn Fjord	Spring	6	464.4 ± 426.3	87.4 – 1075.7
	Summer	8	299.8 ± 156.6	83.5 – 574.4
	Fall	5	176.4 ± 65.4	98.1 – 233.7

Table 3. ANOVA for the effect of Site and Season on biomass and the effects of experimental treatment (control, +NP and +Grazers), Site and Month on growth of *Gracilaria vermiculophylla* at Fyns Hoved and in Holckenhavn Fjord from March to October 2008

Response	Factors	df	MS	F	p
Biomass	Site	1	317.28	355.69	0.003
	Season	2	0.58	1.82	0.172
	Site × Season	2	0.89	2.78	0.071
	Error	57	0.33		
Growth rate	Treatment	2	0.00047	4.80	0.026
	Site	1	0.00033	0.16	0.699
	Month	7	0.00779	56.00	<0.001
	Treatment × Site	2	0.00007	1.58	0.241
	Treatment × Month	14	0.00010	0.70	0.768
	Month × Site	7	0.00201	14.47	<0.001
	Treatment × Month × Site	14	0.00005	0.33	0.989
	Error	161	0.00014		

(Holckenhavn Fjord) and 7% (Fyns Hoved) richer in N than those from the control treatment. Differences were not significant when compared across treatment at individual sampling dates (Tukey's test, all  $p > 0.05$ ). The P content in algae from Holckenhavn Fjord varied seasonally with high values in spring and late fall (0.3% P in DW) and low in summer (ca 0.16% P in DW). In contrast, the P content in algae from Fyns Hoved remained almost constant at 0.2% P in DW throughout the study period (Fig. 3, Table 4). Algae from the control treatment in Holckenhavn Fjord were richer in P (mean =  $0.24 \pm 0.05\%$  P in DW) than those from Fyns Hoved ( $0.18 \pm 0.01\%$  P in DW) when compared across all sampling events. Time-specific differences occurred in March, April, September and October (Tukey's test,  $p < 0.05$ ). Nutrient enrichment had a significant effect on the P content of *G. vermiculophylla* when compared across all sampling events ( $F = 39.3$ ,  $p = 0.006$ ). Plants from the +NP treatment contained 7 to 8% more P than those from the control treatment at both sites, but no differences were significant at individual sampling dates (Tukey's test, all  $p > 0.05$ ). Tissue N:P ratios in plants from Fyns Hoved varied seasonally and were higher in spring (ca. 50) than in summer and fall (ca. 20). In contrast, the N:P ratio in algae from Holckenhavn Fjord remained almost constant (ca. 21) throughout the study period. The overall mean N:P ratio in plants from the control treatment at Fyns Hoved ( $27.1 \pm 11.0$ ) was higher than in control algae from Holckenhavn Fjord ( $20.6 \pm 3.4$ ), and N:P ratios were unaffected by nutrient treatment ( $26.5 \pm 10.3$  and  $20.6 \pm 3.0$  at Fyns Hoved and in Holckenhavn Fjord, respectively).

## DISCUSSION

This study shows that the biomass of *Gracilaria vermiculophylla* was ca. 300-fold higher in eutrophic Holckenhavn Fjord than in the more nutrient-poor Fyns Hoved, although *G. vermicu-*

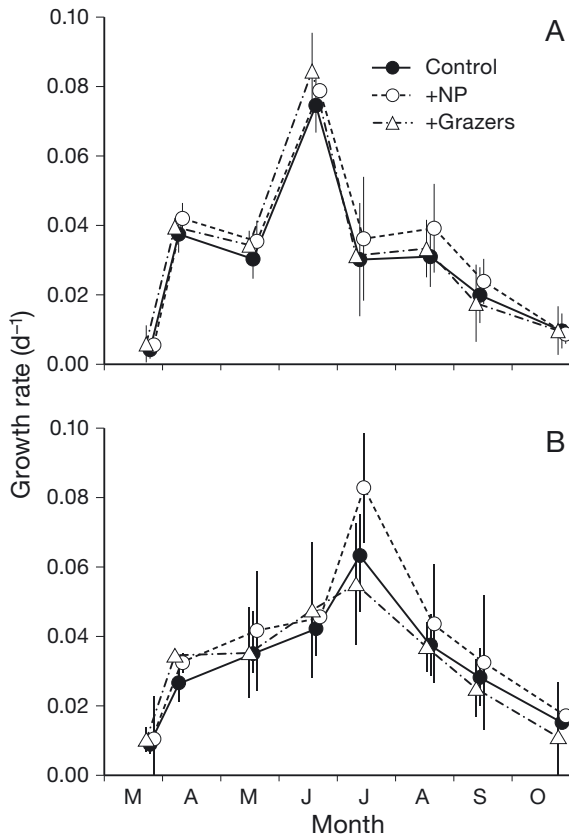


Fig. 2. *Gracilaria vermiculophylla*. Growth rates of plants from the 3 experimental treatments: control, +NP and +Grazers (A) at Fyns Hoved and (B) in Holckenhavn Fjord from March to October 2008. Data are mean  $\pm$  SD ( $n = 4$  or  $5$ )

*lophylla* arrived in these systems at approximately the same time. The marked difference in biomass corresponds well to the general observation that *G. vermiculophylla* in Denmark are more abundant in relatively nutrient-rich sites. A similar distributional pattern has been reported for *G. tikvahiae* McLachlan in Waquoit Bay, Massachusetts, USA (Fox et al. 2008), suggesting that nutrient richness may affect the abundance of *Gracilaria* spp. Relatively fast-growing algae, such as *G. vermiculophylla*, should not only be more susceptible to nutrient limitation but also to grazing, than more slow-growing species (Cebrián & Duarte 1994, Pedersen & Borum 1996, Pedersen et al. 2010), so we expected that site-specific variations in both nutrient availability and grazing pressure could explain the obvious difference in biomass between the 2 study sites.

*Gracilaria vermiculophylla* grew fast during summer ( $0.07$ – $0.08$   $d^{-1}$ , corresponding to a doubling time of 8 to 10 d), which is equal to *in situ* rates reported from Kiel Bight, Germany (Weinberger et al. 2008), and from Hog Island Bay, Virginia, USA (Thomsen & McGlathery 2007). Growth rates were similar in Holckenhavn Fjord and Fyns Hoved, and the seasonal patterns of

growth followed temporal changes in incident light and water temperature rather than variations in nutrient concentrations. This would either suggest that nutrients were equally available in the 2 systems (i.e. no difference in the degree and extent of nutrient limitation) or, alternatively, that the availability of nutrients differed among systems but was still high enough to ensure sufficient nutrient uptake and fast growth (i.e. no nutrient limitation) of *G. vermiculophylla* in both.

Nutrient richness was presumed to differ considerably between the sites, although we only had data for nutrient loading for one site (Holckenhavn Fjord). The catchment of Holckenhavn Fjord is relatively large and is dominated by agricultural areas. Nutrient loading is high, and Holckenhavn Fjord is one of the most eutrophic estuaries in Denmark. In contrast, the catchment of Fyns Hoved is small and dominated by uncultivated land and recreational areas. The contention that nutrient richness differed between the 2 systems was supported by much higher concentrations of inorganic nutrients, especially of DIN, in Holckenhavn Fjord during winter, spring and fall, whereas the concentrations in summer were equally low in both systems. Concentrations of inorganic nutrients may not necessarily be a good indicator of nutrient richness, as they reflect what is not taken up by the primary producers (Dodds 2003). However, the mean concentrations of DIN and DIP in Holckenhavn Fjord were 10 and 2 times higher, respectively, than at Fyns Hoved, while at the same time, the biomass of the dominant primary producer (*Gracilaria vermiculophylla*) was ca. 300 times higher in the former system. The presumed difference in nutrient richness between the 2 systems was further reflected by the fact that the N and P contents of algae from Holckenhavn Fjord were ca. 30% higher than those of algae from Fyns Hoved. The site-specific variation in standing stock of inorganic nutrients and the marked difference in algal N and P content together provide strong evidence that Holckenhavn Fjord is richer in nutrients than Fyns Hoved.

Low availability of nutrients (permanently at Fyns Hoved, periodically in both systems) should lead to nutrient-limited growth among faster-growing algae with high nutrient requirements and relatively low storage capacity (Pedersen & Borum 1996, 1997, Pedersen et al. 2010). However, *Gracilaria vermiculophylla* grew at the same rate in the 2 systems, although the availability of inorganic nutrients differed substantially, and one would expect growth to be limited by nutrient shortage for a relatively long period at Fyns Hoved. This suggests that nutrient limitation was not at work, a suggestion that was supported by the fact that algae from both systems contained relatively high levels of tissue N and P throughout the summer. N and P concentrations in the algal tissues always remained

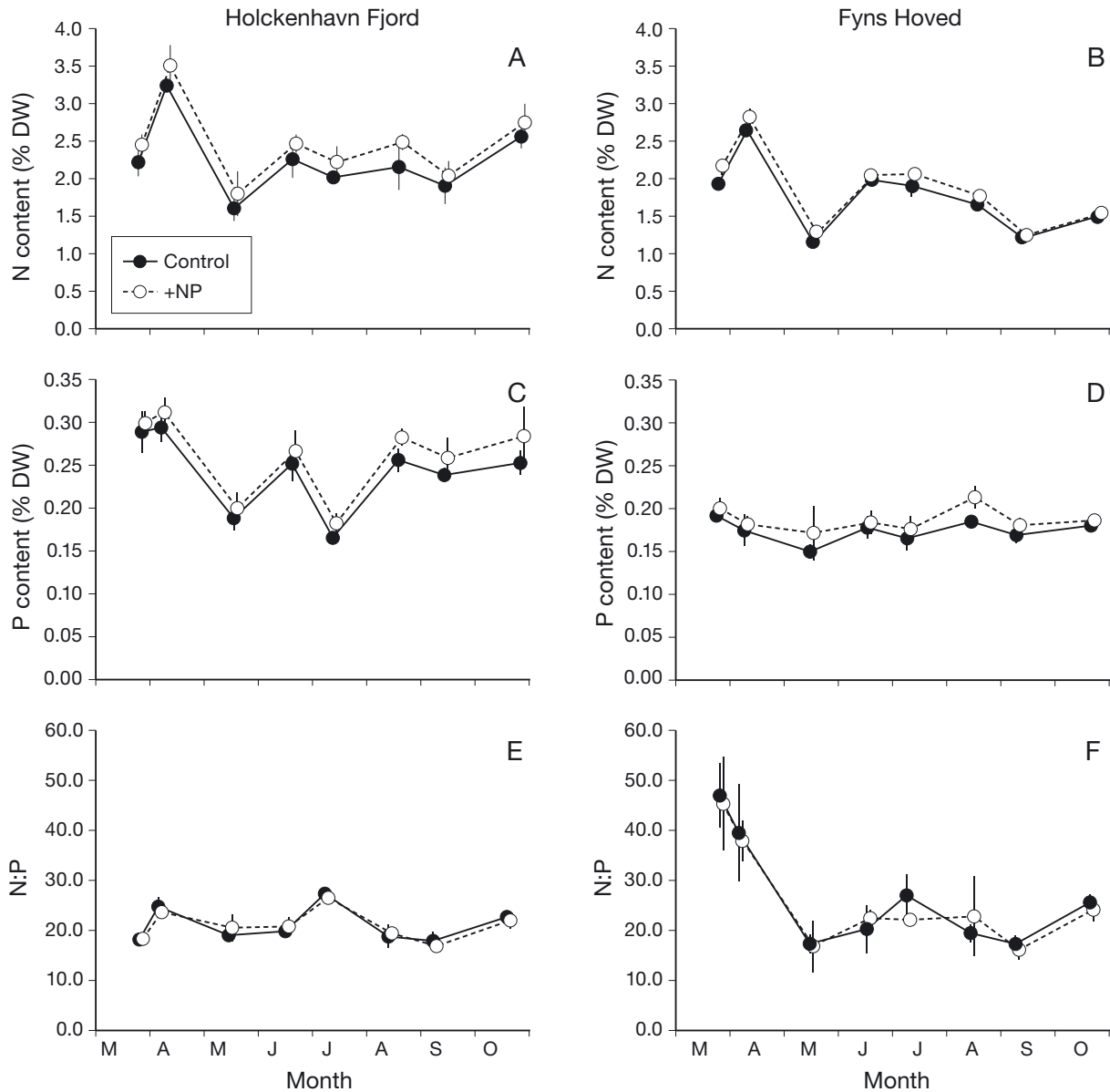


Fig. 3. *Gracilaria vermiculophylla*. (A,B) N content, (C,D) P content and (E,F) molar N:P ratios in algae from the control treatment and the +NP treatment at Fyns Hoved and in Holckenhavn Fjord from March to October 2008. Data are mean  $\pm$  SD (n = 4 or 5)

above levels expected to limit macroalgal growth (ca. 1.6 to 2.2% N in DW and 0.16 to 0.2% P in DW; Duarte 1992, Pedersen & Borum 1996, Pedersen et al. 2010). The low importance of nutrient limitation was further supported by the fact that experimental nutrient enrichment led to higher nutrient levels in the algae, while it only had a marginal effect on growth. Nutrient enrichment only raised the N and P content in algae by 7 to 11%, but the absolute increase was much larger since the enriched algae also grew (15 to 18%) faster than algae from the control treatment. The absolute enrichment was thus larger than indicated from differences in the relative content of N and P (i.e. % of DW).

We therefore conclude that nutrient limitation had a marginal effect on the growth of *G. vermiculophylla* at both sites. Other studies have shown that the growth of *Gracilaria* is little affected by nutrient limitation; nutrient enrichment did not stimulate growth of *G. vermiculophylla* in Hog Island Bay, Virginia, in summer (Thomsen & McGlathery 2007), and Teichberg et al. (2008) were unable to document nutrient limited growth of *G. tikvahiae* in Childs River and Sage Lot Pond, Massachusetts. *In situ* conditions and nutrient availability may well vary among these studies, but the results nevertheless indicate that members of the Gracilariales seem able to gain sufficient nutrients



Table 4. Analysis of variance results for the effect of experimental treatment (control and +NP), site and month on the content of nitrogen, phosphorus and tissue N:P (molar) ratio in *Gracilaria vermiculophylla* at Fyns Hoved and in Holckenhavn Fjord from March to October 2008

Response	Factors	df	MS	F	p
N content	Treatment	1	1.24	123.96	<0.001
	Site	1	9.52	22.67	0.002
	Month	7	4.06	170.91	<0.001
	Treatment × Site	1	0.13	13.23	0.008
	Treatment × Month	7	0.01	0.41	0.896
	Site × Month	7	0.42	17.71	<0.001
	Treatment × Site × Month	7	0.01	0.44	0.876
	Error	112	0.02		
P content	Treatment	1	0.0079	39.28	<0.001
	Site	1	0.2919	14.74	0.006
	Month	7	0.0120	15.56	<0.001
	Treatment × Site	1	0.0007	2.29	0.174
	Treatment × Month	7	0.0002	0.28	0.961
	Site × Month	7	0.0198	25.59	<0.001
	Treatment × Site × Month	7	0.0003	0.52	0.818
	Error	121	0.0008		
N:P	Treatment	1	0.08	0.01	0.930
	Site	1	1104.10	2.09	0.191
	Month	7	565.74	39.36	<0.001
	Treatment × Site	1	2.7	0.83	0.393
	Treatment × Month	7	9.75	0.68	0.690
	Site × Month	7	527.13	36.68	<0.001
	Treatment × Site × Month	7	3.34	0.23	0.977
	Error	107	14.37		

even at low substrate concentrations. *Gracilaria* may thus have a high affinity for inorganic nutrients, organic nutrients may serve as additional nutrient sources (Tyler et al. 2005), and/or it may have a large storage capacity for nutrients.

The storage capacity of *Gracilaria vermiculophylla* should potentially be larger than that of true fast-growing algae (i.e. uniseriate filamentous or foliose species being 1 to 2 cell layers thick) because *G. vermiculophylla* is thicker and grows at lower rates. On the other hand, the growth rate of *G. vermiculophylla* reached 0.08 d<sup>-1</sup> in mid-summer, corresponding to a doubling time of ca. 7.5 d. This means that the tissue concentrations of N and P would be reduced by 50% and get below the threshold considered limiting for algal growth within 1 wk if the concentrations of dissolved nutrients were low enough to prevent *G. vermiculophylla* to acquire any external nutrients. A storage capacity of 7.5 d is still rather short compared to that of other algae (Pedersen & Borum 1996, Pedersen et al. 2010), and cannot explain why *G. vermiculophylla* remained unaffected by nutrient limitation throughout summer. Whatever the reason, nutrient limitation had very little impact on *in situ* growth of *G. vermiculophylla*, and the observed

variation in biomass across sites could therefore not be explained by site-specific variations in growth caused by nutrient limitation.

Standing biomass is not only determined by the rate at which new biomass is produced, but also by the rate at which biomass is lost. Herbivory can constitute an important loss factor for plants, and invertebrate grazers can reduce algal biomass and affect the composition of algal assemblages (e.g. Lubchenco 1978, Geertz-Hansen et al. 1993, Hauxwell et al. 1998, Duffy & Hay 2000). We were nevertheless unable to document any significant grazing losses on *Gracilaria vermiculophylla* in the present investigation. We initially expected that the abundance of grazers, and thus the grazing pressure, would be low in eutrophic Holckenhavn Fjord because the abundance of grazers often correlates negatively to nutrient richness (Hauxwell et al. 1998) due to a higher frequency of hypoxia at more eutrophic sites (e.g. Norkko & Bonsdorff 1996, Fox et al. 2009). The abundance of grazers was not quantified in this study, but many grazers (amphipods, isopods and snails) were found hiding in the algae

sampled for the biomass survey and in the open cages used for the grazer treatment at both sites (see also Nyberg et al. 2009). Typical grazers were thus abundant at both sites, but grazing rates were always small and never significantly different from 0. Weinberger et al. (2008) found that common grazers from Kiel Bight consumed little *G. vermiculophylla* in no-choice trials and preferred *Fucus vesiculosus* to *G. vermiculophylla* in 2-choice feeding trials. Vinzent (2009) obtained similar results in laboratory feeding studies where *Idotea baltica*, *Gammarus* sp. and *Littorina littorea* preferred fast-growing macroalgae (*Ulva* sp. and *Ceramium virgatulum* Roth, formerly *C. rubrum*) to *G. vermiculophylla* and *F. vesiculosus* when given a choice, although these herbivores would consume and survive on *G. vermiculophylla* when they did not have a choice. However, feeding rates were low when *G. vermiculophylla* was offered alone, and the herbivores grew less well than when fed with other algae (Vinzent 2009). The low preference for *G. vermiculophylla* observed in these culture and field studies may be due to morphological, nutritional or chemical cues that prevent or reduce herbivory (Duffy & Hay 1990), although the exact mechanisms in this case remain unknown.

The marked difference in *Gracilaria* biomass between the 2 study sites could not be explained by site-specific variations in growth, nor by differences in grazer-mediated biomass losses. This result was quite surprising, but it may nevertheless add to explain why *G. vermiculophylla* is such a strong invader. Proliferation of fast-growing macroalgae is often restricted by a combination of nutrient limitation and grazing (e.g. Geertz-Hansen et al. 1993), but *G. vermiculophylla* seemed completely unaffected by these processes even when nutrient concentrations were low and the potential grazing pressure was high (i.e. at Fyns Hoved).

Which mechanisms are then causing the observed site-specific variations in biomass of *Gracilaria vermiculophylla* if nutrient richness and grazing pressure are unimportant? Holckenhavn Fjord and Fyns Hoved do not only differ in nutrient richness, but also with respect to exposure to winds and mean depth. Holckenhavn Fjord is surrounded by hills to the north, west and south and is therefore relatively protected against winds. In contrast, Fyns Hoved is surrounded by low land (a few metres above sea level), making it much more susceptible to wind exposure than Holckenhavn Fjord. Further, the mean water depth at Fyns Hoved is only half that in Holckenhavn Fjord. The effect of higher wind exposure combined with more shallow waters at Fyns Hoved leave benthic organisms subject to higher levels of wave exposure and water movement than in Holckenhavn Fjord. Water movement may have a dramatic effect on the biomass of drift macroalgae, which may be exported to deeper waters or become washed upon the shore (Flindt et al. 1997, Salomonsen et al. 1997). For example, Salomonsen et al. (1999) showed that advective transport of *Ulva lactuca* due to currents and wind-driven water movement caused a higher loss of biomass than grazing, which is otherwise considered a significant loss process for *Ulva* in many shallow systems. We find it very likely that the physical stress imposed by wind and waves at Fyns Hoved is strong enough to detach attached specimens of *G. vermiculophylla* and remove them from the system and that site-specific variations in physical stress therefore may explain observed variations in biomass of *G. vermiculophylla*. Variations in wind regime and morphometry may, to some extent, also explain the observed variation in the abundance of *G. vermiculophylla* among other Danish estuaries. Systems with a high biomass of *Gracilaria* are not only richer in nutrients than systems of low biomass, but they also tend to be more sheltered (having a lower fetch and higher depth) and have more narrow connections to the neighbouring sea (i.e. lower water exchange) than areas with a relatively low abundance. At present, we have no estimates of the amount of *G. vermiculophylla* that are exported and/or washed up on the shores in

different systems, and future studies should be conducted to test this possibility and quantify its potential importance.

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