

Excessive epibios growth influenced the use of red macroalgae as polyculture organisms around fish farms in inner Danish waters

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Abstract

Three commercial red macroalgae were investigated as candidates for polyculture at two fish farms in inner Danish waters in the period 2004 to 2006. Growth of the investigated macroalgae species expressed decreasing specific growth rate (SGR) through duration of deployment in field due to exponentially growth of epibios. Almost 50 % of the variation in SGR of *Chondrus crispus* was explained by temperature (23 %) and feed usage of fish production (4 %) with positive correlation and negatively correlation with epibios per investigated macroalgal biomass (16 %) and distance (6 %) to fish farm. However, growth of *Gracilaria vermiculophylla* was stimulated by epibios due to the nutrient enrichment by the fouling mussels. In experiments with the least epibiotic growth SGR was $0.018 \pm 0.006 \text{ d}^{-1}$, $0.011 \pm 0.004 \text{ d}^{-1}$ and $0.039 \pm 0.007 \text{ d}^{-1}$ for *C. crispus*, *Furcellaria lumbricalis* and *G. vermiculophylla*, respectively. The slow growth and/or the high degree of epibios coverage, seen in these experiments, rejects the three red macroalgae as potential candidates for polyculture near Danish fish farms, because the macroalgae biomass will be impossible to put on market for utilization of the gelling agent content.

Key words: *Chondrus crispus*, epibios, *Furcellaria lumbricalis*, *Gracilaria vermiculophylla*, polyculture, specific growth rate

1. Introduction

There is a considerable potential for polyculture using seaweeds for the bioremediation of nutrient-rich waters from fish farms. Excess nutrient from fish farms can be assimilated by seaweeds, and in this way converted to biomass harvested and sold as product. This is well known in studies from land-based fish farms with fish such as sea bream, salmon, trout or sea bass where seaweeds species *Chondrus crispus*, *Gracilaria bursa pastoris*, *Palmaria palmata*, *Ulva lactuca*, *Saccharina latissima*, *Asparagopsis armata* act as biofilter, and furthermore from open water fish farms with salmon where *Gracilaria chilensis*, *Saccharina latissima* or *Porphyra* sp. are grown as polyculture giving rise to increased production of the seaweed and reduced nutrient load to environment from the fish farm (Subandar et al. 1993; Krom et al. 1995; Neori 1996; Troell et al. 1997; Chopin et al. 1999b; Matos et al. 2006; Ridler et al. 2006).

Since 2003 legislations for marine fish production in Denmark have recommended research on polyculture with either mussel or seaweeds near fish farms (Havbrugsudvalget 2003), and in 2006 establishment of biofilters became mandatory if fish production was to be increased in the largest Danish fish farm. In Denmark polyculture is still in the experimental phase and in regard to seaweeds the best species (robust and fast growing with valuable by-product(s)) have yet to be found for this purpose.

The three valuable red macroalgae, *Chondrus crispus* Stackhouse, *Furcellaria lumbricalis* (Hudson) Lamouroux and *Gracilaria vermiculophylla* (Ohmi) Papenfuss were tested as potential candidates for polyculture near two off-shore fish farms. *Chondrus crispus* and *F. lumbricalis* are species native to Europe and can tolerate the reduction in salinities down to 10 ‰ in the inner Danish waters towards the Baltic Sea. *Chondrus crispus* contains the valuable polysaccharide and stabilising agent carrageenan used in a wide range of products such as soft ice, chocolate, marmalade, toothpaste (Guist 1990; Bixler 1996), and scientists worldwide have investigated the growth, tolerance to different parameters etc. of this commercially interesting species (Bird et al. 1979; Amat and Braud 1990; Chopin and Wagey 1999; Chopin et al. 1999a; Zertuche-González et al. 2001; Matos et al. 2006). Natural populations of *F. lumbricalis* have previously (in the 70ties) been harvested in Denmark to exploit the polysaccharide and gelling agent furcellaran (“Danish agar”) somewhat similar to carrageenan (Guist 1990). *Gracilaria vermiculophylla* is a newly introduced species in Scandinavia and has so far been observed at some locations in Danish waters (Rueness 2005; Nielsen 2007;

Thomsen et al. 2007). *Gracilaria vermiculophylla* also contain a polysaccharide, agar, that similar to carrageenan and furcellaran is used as a thickener in jellies, ice cream etc. (Guist 1990; Rueness 2005).

In this work *C. crispus*, *F. lumbricalis* and *G. vermiculophylla* have been investigated as candidates for polyculture production in two off-shore rainbow trout (*Oncorhynchus mykiss*) fish farms in the inner Danish waters. Specific growth rates and production of the macroalgae were estimated at different distances from the fish farms together with epibios biomass in three seasons of fish production. Furthermore depth experiments were carried out as an attempt to examine if epiphytes on the macroalgae of interest could be reduced. Tissue content of carbon, nitrogen and sulphur, the latter to quantify carrageenan content, was also investigated in *C. crispus* experiments.

2. Materials and methods

2.1. Environmental setting

Algal growth experiments were carried out near two fish farms, Musholm Lax A/S located in the Great Belt (55°29.1' N, 11°04.0' E) and Snaptun Fisk Export A/S in Horsens Fjord (55°50.1' N, 10°01.4' E; see Figure 1).

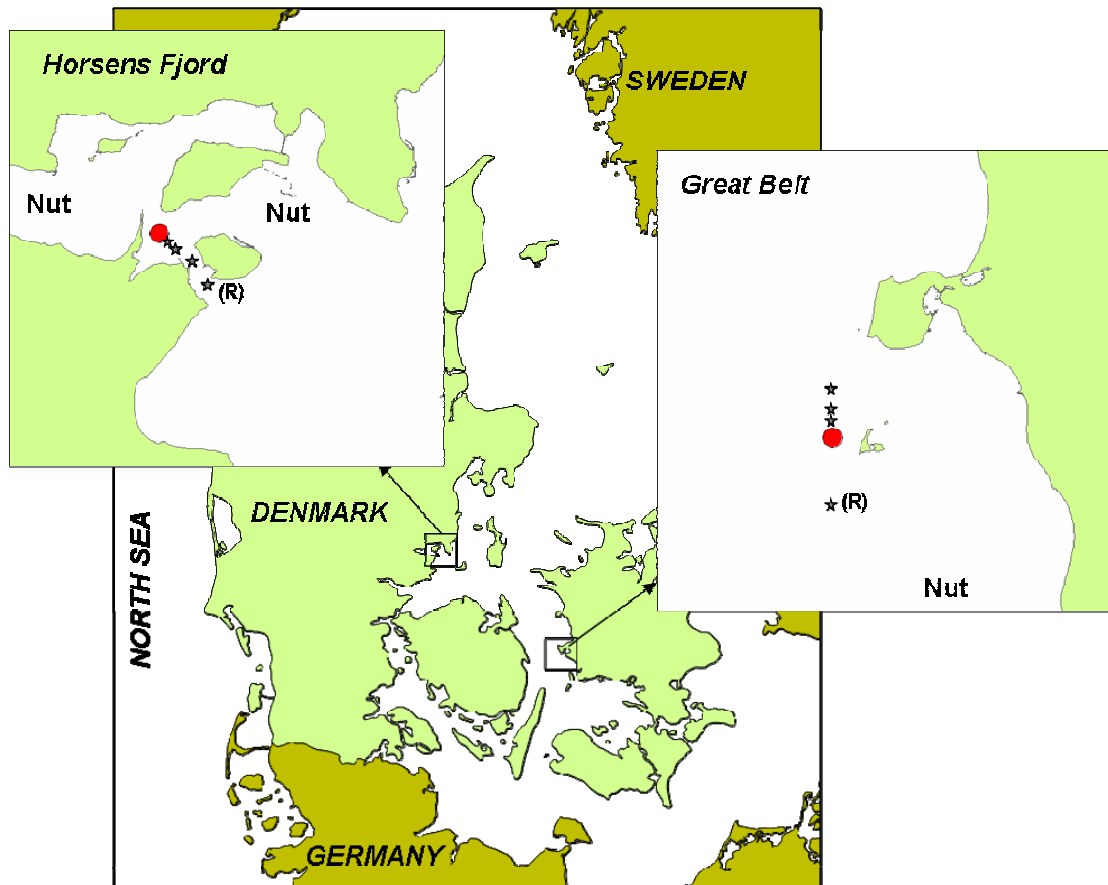


Fig. 1 Map of inner Danish waters showing fish farm locations (●) in Horsens Fjord and Great Belt, deployment stations for growth experiment with red algae (✱) and sampling stations for “background” nutrient concentrations (Nut)

The Great Belt constitutes the main strait connecting the Baltic Sea with the North Sea and the dominating northerly surface (0-10 m) current averages 0.25 m s^{-1} driven by the freshwater surplus in the Baltic catchment area. Surface salinity averages 16 ‰ but varies between 11 and 28 ‰ according to current direction and air pressure variation. In comparison, surface currents are primarily wind-driven and on average much lower in Horsens Fjord at ca. 0.05 m s^{-1} . Salinity in the outer Horsens Fjord averages 21 ‰. The average surface water temperature during the experimental period in the Great Belt in 2005 and in Horsens Fjord in 2006 was $17.1 \pm 1.3 \text{ }^\circ\text{C}$ and $19.0 \pm 2.9 \text{ }^\circ\text{C}$, respectively (DHI 2008).

Mean irradiances at 1 m interval depths from 1 to 5 m at the two field experiment locations were calculated from two sources, 1) surface insolation (extract from Waterforecast; (DHI 2008) and 2) averages of Secchi depth at nearby stations extracted from the national database MADS operated by National Environmental Research Institute (NERI 2008; Table 1 Appendix 1).

The formula used was:

$$I_z = I_0 C \exp^{-kz}$$

With I as irradiance at depth z or surface 0 (surface insolation), C as correction factor for the difference in sunlight hours per month between the data retrieved from 2004 and the experimental periods in 2005 and 2006 and k as the light extinction calculated by:

$$K_d = 2.1 / \text{Secchi depth}$$

With the constant 2.1 chosen based on empirical values ranging between 1.5 and 3.6 in the Danish waters (Markager 2004).

The effect of the fish farm, and therefore possible extra seston, on the water turbidity was not taken into account. The daily usage of feed for the fish production at Musholm Lax A/S in summer 2005 was provided by the fish farm (Jensen 2007).

2.2. Experimental designs

During the summer 2004 two pilot growth experiments with *Chondrus crispus* and *Furcellaria lumbricalis* were carried out using non-fertile individuals of *C. crispus* in size class II and III (4-10 cm; (Pringle and Mathieson 1986) (n= 7-10) and *F. lumbricalis* size 5-7 cm (n=7-10) collected at Koster Fjord, Sweden (eastern Skagerrak, 58°52.4' N, 11°07.4' E). Stipes of *C. crispus* and *F. lumbricalis* were placed in-between strands of 6 mm polyester rope at intervals of 0.2 m and strands were kept together with small cable ties next to thalli. Ropes with thalli were placed vertically (0-2 m depth) beneath buoys deployed where current direction was mainly downstream (50 m, 200 m and 500 m) from the fish farms and ropes with thalli upstream (500 m in Snaptun and 1000 m in the Musholm experiments) served as control. The duration of the experiments at Musholm was 30 days (August, Experiment 1) and 28 days (September, Experiment 2) and at Snaptun 43 days (August–October; Table 2) and

specific growth rate was estimated for each replicate of thalli, whereas epibios biomass was calculated as average of all replicates on each rope.

Table 2. Schematic table of experiments (Exp) and duration of experiments performed in 2004-2006 with three macroalgae species *Chondrus crispus*, *Furcellaria lumbricalis* and *Gracilaria vermiculophylla* at two Danish fish farms, Musholm Lax A/S (Musholm) and Snaptun Fish Export A/S (Snaptun) located in Great Belt and Horsens Fjord, respectively. *G. vermiculophylla* Snaptun Exp 3 did not succeed (dashed line).

Summer	Species	Experiment	Month [days of experiment]				
			June	July	Aug	Sept	Oct
2004	<i>C. crispus</i> and <i>F. lumbricalis</i>	Musholm Exp 1			[30]		
		Musholm Exp 2				[28]	
		Snaptun				[43]	
2005	<i>Chondrus</i> <i>crispus</i>	Musholm Exp 3		[21]			
		Musholm Exp 4		[46]			
		Musholm Exp 5		[74]			
		Musholm Exp 6			[23]		
	<i>Furcellaria</i> <i>lumbricalis</i>	Musholm Exp 7			[51]		
		Musholm Exp 3		[31]			
		Musholm Exp 4		[54]			
2006	<i>Gracilaria</i> <i>vermiculophylla</i>	Snaptun Exp 1	[34]				
		Snaptun Exp 2	[76]				
		Snaptun Exp 3	[-----131-----]				
		Snaptun Exp 4		[42]			
		Snaptun Exp 5				[55]	

Mesoscale experiments were furthermore conducted at the Musholm Lax A/S with *C. crispus* and *F. lumbricalis* during summer in 2005 with algae collected at Vellerup Vig (Roskilde Fjord, Denmark; 55°44.0' N, 11°51.2' E) and Gilleleje (Denmark; 56°07.5'N, 12°18.3E), respectively. Thalli (n=8) were attached to polyester ropes like in the previous experiment. These ropes were tied transversely on to aluminium rings (0.7 m in diameter) like spokes in a bicycle wheel. This deployment system in field was scaled down and modified from the patented off-shore ring structure (Buck and Buchholz 2005).

The “wheels” were suspended in four coated 3 mm steel wires connected to the “wheel”, both up and downward (giving each “wheel” system a vertical length of 1 m). “Wheels” were stacked and wires connected with shackles with top “wheel” connected to a trawl float and the bottom “wheel” in the vertical row to a 5 kg weight. To compensate for eventual losses, five horizontally oriented wheels were distributed on two buoys (1 m interval from 1-5 m) deployed at 100 m, 300 m and 700 m mainly downstream and 1000 m mainly upstream serving as control near the Musholm fish farm. Ropes with *C. crispus* and *F. lumbricalis* were deployed in the field twice and once in 2006, respectively, and ropes from each position and depth were collected and

algal biomass measured after different durations in field. This resulted in Experiment 3 (July, 21 days) to Experiment 7 (August–October, 51 days) for *C. crispus* and Experiment 3 (July–August, 31 days) to Experiment 5 (July–October, 82 days) for *F. lumbricalis* (see Table 2 for further details) and specific growth rate and epibios biomass was estimated for each replicate.

Few buoys with experimental ropes were lost, and a few thalli either fell of the rope or had a negative growth rate, the latter was not taken into account in averages.

Gracilaria vermiculophylla was collected at low water sandy sediment in Horsens Fjord (Denmark; 55°50.1' N, 10°01.4' E) and fragments, 15–20 cm in length, were attached to polyester ropes in between strands (n=6 per rope) and three ropes placed on “wheels” as previously mentioned for *C. crispus*. Two “wheels” were placed beneath each other in 1 and 2 m depth and attached to trawl floats and buoys deployed 100 m, 300 m, 700 m and 1000 m from Snaptun fish farm. Ropes were deployed in field three times and ropes from each position and depth were collected and algae measured after different duration of deployment in field.

This resulted in Experiment 1 (June–July, 34 days) to Experiment 5 (August–October, 55 days; see Table 2 for further details). Experiment 3 failed as biomass was lost. Two replicates of the six *Gracilaria vermiculophylla* thalli per rope at 1 m and 2 m depth were estimated in Experiment 1, two replicates per rope at 1 m depth and one replicate per rope in 2 m depth in Experiment 2 and 4. For Experiment 5 only one replicate from ropes at 1 m depth was measured.

Control positions upstream the fish farms in experiments in 2005 and 2006 are noted as 500 m and 1000 m distance (downstream) in analysis to represent nutrient concentration gradient for Snaptun and Musholm experiments, respectively.

The stocking density was for *C. crispus*, *F. lumbricalis* and *G. vermiculophylla* 190 gWW m⁻², 100 gWW m⁻² and 750 gWW m⁻², the latter extrapolated to production in 5 depths (1–5 m) as for the other two investigated species for comparison. These stocking densities were used for calculations for production size.

2.3. Specific growth rate

Specific growth rates (SGR) of every replicate of *C. crispus*, *F. lumbricalis* and *G. vermiculophylla* were calculated from equation 1, with blotted wet weight (WW) initially (t_0) and at end (t_1) of experiments measured gravimetrically.

$$\text{Specific growth rate} = SGR = \frac{\ln(WW_{t_1} - WW_{t_0})}{t_1 - t_0} \quad (1)$$

Epibios biomass was estimated by the blotted wet weight of the investigated thallus measured gravimetrically before and after removal of epibios.

Experiments were separated into smaller experimental periods in order to identify the growth from one experiment was terminated to the next (Table 2 Appendix 1). These separated SGR's were calculated from the average weight of the 8–16 replicates from the prior experiment (WW_{t_0}) and the just terminated experiment as (WW_{t_1}). Epibios biomass was also averaged between replicates of each depth and distance.

2.4. Elemental and areal analyses

Chondrus crispus thalli from Experiment 3 and 5 (depth 1 m, 3 m and 5 m) were dried at 105°C for 24 hours and kept in freezer for later biomass composition analysis of carbon (C), nitrogen (N) and sulphur (S) by a CHNS – Automatic Elemental Analyzer (EA 1110 CHNS, CE Instruments, Milan, Italy). Sulphur content was estimated because carrageenan is a sulphated polysaccharide (McCandless and Craigie 1979; Roberts and Quemener 1999), and sulphur content gives an indication of tissue carrageenan content, as the cysteine and methionine amino acid content containing sulphur bridges is expected to remain unchanged during experiments (Tveter-Gallagher et al. 1984).

The surface area of *C. crispus* thalli ($n=40$; Experiment 3) were estimated using Adobe Photoshop Elements 4.0. From photographs of thalli, the number of dark pixels representing the thallus on a white background was estimated from the total pixels of the photograph.

2.5. Statistics

F-Test Two Sample was performed to test if the variances of two populations were equal, followed by the Two Sample t-Test to test for equality of the population means underlying each sample (Microsoft Excel Add-In). Factorial ANOVA and regression

analyses on experiment, distance, irradiance and SGR were performed in STATISTICA 6. To correct for Type I errors in the regression analyses, in which repeatedly analyses decreases the significance ($p > 0.05$), a modified Bonferroni correction was applied taking account of the average correlation in 18 (SGR and epibios vs. distance) and 12 (SGR and epibios vs. irradiance) related tests (Sankoh et al. 1997).

PLS regression was used to quantify the relationship between algal growth rate (Y-variable) and a suite of potential predictors (X-variables) including calculated light availability, temperature, distance from fish net pens, feed use in fish production and weight of epibios per algal biomass. PLS is a bilinear modelling method where information in the matrix X is projected onto a small number of underlying (“latent”) variables called PLS components, referred to as PCs (Møhlenberg et al. 2007). The matrix Y is simultaneously used in estimating the “latent” variables in X that is the most relevant for predicting the Y variables. PLS models were built based on maximum 1-2 PCs as adding additional PCs increased $R^2(Y)$ with less than 2-3%. The optimal number of PCs and the identity of significant predictors, e.g. average surface temperature were assessed by leave-one-out jack-knifing and minimizing the root square error of prediction. The scaled regression coefficients in the final PLS models were taken as measures of the relative importance of predictors. All averages of replicates are presented as average \pm standard deviation.

3. Results

All field experiments with the three seaweed species were carried out successfully with vegetative thalli attached to ropes hanging from buoys or attached on “wheels” hanging from buoys with minor thallus losses. Some buoys with attached “wheels” were lost in the late Musholm experiments 2005 probably due to strong currents and waves. However, growth of the three investigated macroalgae suffered severely from growth of epibios, and biomass loss of *Gracilaria vermiculophylla* thalli in Experiment 3 in 2006 was most likely due to increase in epibios (mussel) biomass resulting in thallus break off. Hence, this experiment was rejected.

3.2. Specific growth rates

SGR was unrelated to the initial weight of *C. crispus* in Experiment 3, 4 and 5, and therefore comparable regardless of thallus size and experiments. There was no regression between SGR and irradiance and neither between distances of deployment from the fish farm in either of the investigated species in 2004-2006. The ANOVA analyses showed significant difference between experiments in *C. crispus* Exp 3-7, *F. lumbricalis* Exp 3-5 and in all *G. vermiculophylla* experiments ($p < 0.001$).

Due to the experimental design in 2005 and 2006 with a large number of replicates deployed initially and then harvested after different duration in the field, it was possible to see changes in SGR with duration of experiment. None of the investigated species showed exponential growth during experiment, however SGR of the three investigated species showed significant negative regression with increased duration of deployment in 2005 and 2006 (Figure 2; *C. crispus*: $F_{1,741}=299.6$, $p < 0.00001$, *F. lumbricalis*: $F_{1,324}=55.09$, $p < 0.00001$, and *G. vermiculophylla*: $F_{1,43}=31.29$, $p < 0.00001$). The highest SGR as average of experiments (SGR at shortest duration of deployment) was for *C. crispus*, *F. lumbricalis* and *G. vermiculophylla* $0.018 \pm 0.006 \text{ d}^{-1}$, $0.011 \pm 0.004 \text{ d}^{-1}$ and $0.039 \pm 0.007 \text{ d}^{-1}$, respectively.

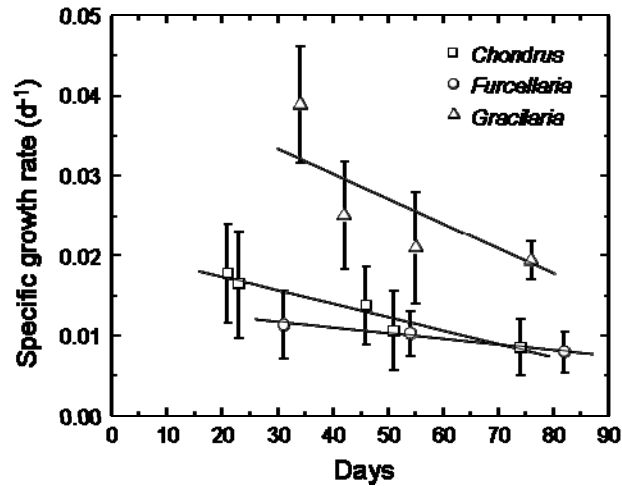


Fig. 2 Regression between specific growth rate (SGR) and the duration of *Chondrus crispus* (Exp 3 ($n=201$), 4 ($n=136$), 5 ($n=142$), 6 ($n=116$) and 7 (126), *Furcellaria lumbricalis* (Exp 3 ($n=121$), 4 ($n=96$) and 5 ($n=99$)) and *Gracilaria vermiculophylla* (Exp 1 ($n=15$), 2 ($n=11$), 4 ($n=11$) and 5 ($n=7$)) deployed in the field in 2005 or 2006. SGR of *C. crispus* and *F. lumbricalis* exhibit significant negative regression (represented by lines) with duration in the field ($R^2=0.2880$, $y=0.021-0.2*10^{-3}x$; $R^2=0.1453$, $y=0.0135-0.7*10^{-4}x$, respectively, $p<0.001$). Y-bars represent standard deviations

The PLS model for *C. crispus* was able to explain approx 50 % of the observed variation in SGR separated for each period (Figure 3). Surface temperature stimulated SGR and contributed with 23 % of the SGR variation, high biomass of epibios per *C. crispus* suppressed SGR and variation in epibios explained 16 % of observed variation, while distance from fish cages and feed use were less important contributing with 6 % and 4 %, respectively to SGR variation. Decrease of SGR with distance and increase with feed use underline the importance of the nutrient source originating from the fish farm for algal growth.

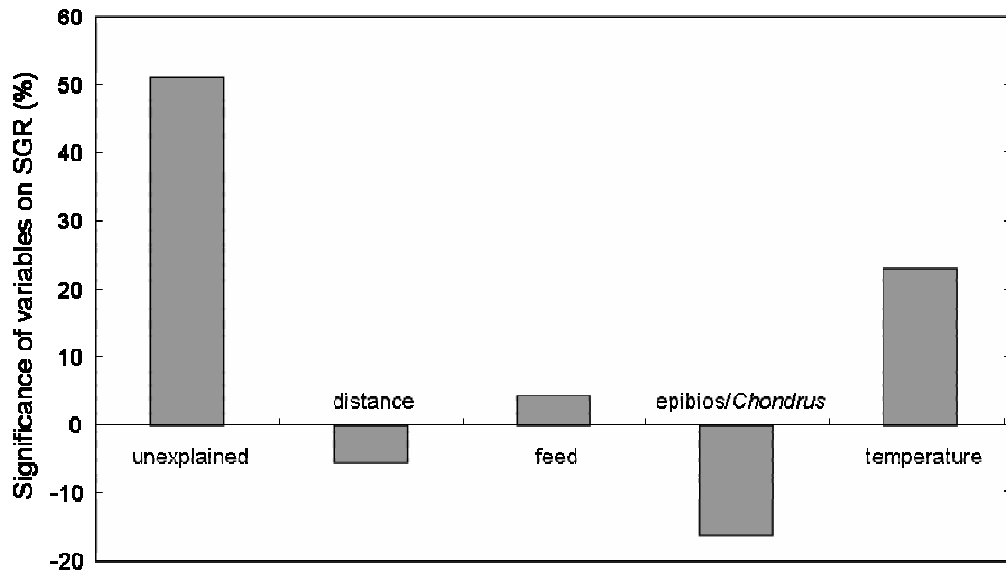


Fig. 3 The significance of the variables on specific growth rate (SGR) of *Chondrus crispus* was identified through PLS statistics, with 51 % unexplained and with temperature and epibios per *Chondrus* explaining 23 % and 16 %, respectively the latter with negative correlation with SGR.

In contrast to *C. crispus*, PLS modelling was less successful to identify independent variables affecting SGR in the *F. lumbricalis* and *G. vermiculophylla* growth experiments. Interestingly, epibios biomass on thalli stimulated SGR in *G. vermiculophylla* (Figure 4).

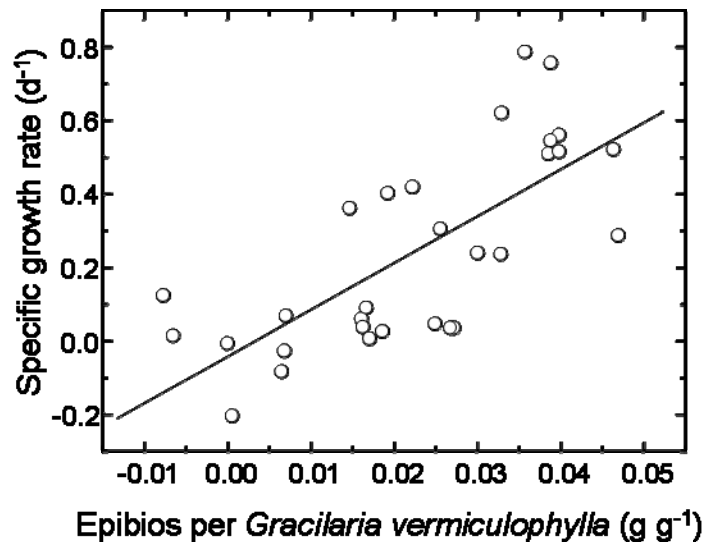


Fig. 4 Regression between epibios per *Gracilaria vermiculophylla* (g WW g WW^{-1}) and specific growth rate of *G. vermiculophylla* (d^{-1}). Trend line; $R^2=0.5330$, $y=-0.0415+12.723x$, $p<0.05$.

3.3. Epibios species and biomass

Epibios on *Chondrus crispus* and *Furcellaria lumbricalis* in 2004 experiments were dominated by *Enteromorpha* sp., *Cladophora* sp., *Ectocarpus* sp., *Ceramium* sp. and some blue mussels (*Mytilus edulis*) at algae deployed at Musholm and *Cladophora* sp., *hydrozoa* sp. and *Ceramium* sp. at Snaptun fish farm.

In the 2005 experiments blue mussels (*Mytilus edulis*), *Enteromorpha* sp., *Cladophora* sp., *Ectocarpus* sp., *Ceramium* sp., *Polysiphonia* sp. and barnacles dominated the epibios on *C. crispus* and *F. lumbricalis*.

Thalli of *Gracilaria vermiculophylla* in experiments at Snaptun fish farm in 2006 became totally covered by small blue mussels (*Mytilus edulis*) during Experiment 1 and later also some *Ceramium* sp. and *Dasya baillouviana* were present as epibios.

The epibios biomass per biomass of *C. crispus* in Experiment 3–5 and *F. lumbricalis* in Experiment 1–4 exhibited exponential growth during deployment in the field. For *G. vermiculophylla* the epibios biomass seemingly leveled off with the duration of experiments (Figure 5).

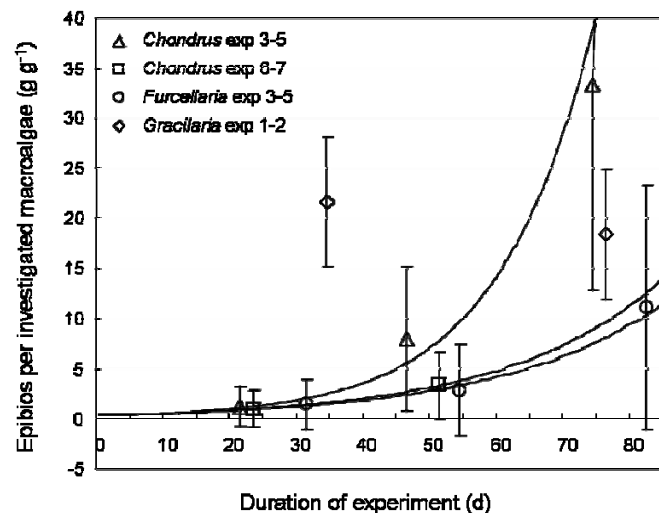


Fig. 5 Epibios biomass per investigated macroalgae (*Chondrus crispus*, *Furcellaria lumbricalis* and *Gracilaria vermiculophylla*) biomass (g WW g WW^{-1}) deployed near Musholm Lax fish farm in 2005 versus days of deployment. Epibios on *Gracilaria vermiculophylla* are from experiments performed at Snaptun fish farm in 2006. Lines represent exponential fit for *Chondrus* and *Furcellaria* experiments (*Chondrus* exp 3-5 $R^2 = 0.9887$; *Chondrus* exp 6-7 $R^2 = 1$ and *Furcellaria* exp 3-5 $R^2 = 0.9849$). Y-bars represent standard deviations.

Experiments with *C. crispus* and *F. lumbricalis* in 2004 showed negative regressions between epibios biomass per thallus and increase in distance from the fish farm ($p<0.05$), although a few were not significant. Significant relations were both positive and negative between epibios per thallus and irradiance for *C. crispus* in 2005 (data not shown).

3.4. Area and weight compared with epibios

Due to the morphology of *Chondrus crispus* it was possible to measure the area of the thalli and the area and weight showed a positive linear correlation ($R^2=0.86$, final weight (gWW)=0.055*area (cm²)).

Positive regressions were found for epibios biomass per thallus (Log₁₀) and the final weight of *C. crispus* (Log₁₀) in Experiment 3, 4, 5, 6 and 7 (example given for Experiment 5, $R^2=0.27$, $\text{Log}_{10}(y)=1.296+0.521 \text{Log}_{10}(x)$, $F_{1,141}=53.06$, $p<0.00001$).

There were negative regressions between SGR and epibios per thallus area in all five experiments with *C. crispus* in 2005, and these were significant in Experiment 5 ($F_{1,141}=35.31$, $p<0.00001$), and 7 ($F_{1,127}=4.41$, $p<0.04$) (see regression of Experiment 5 in Figure 6).

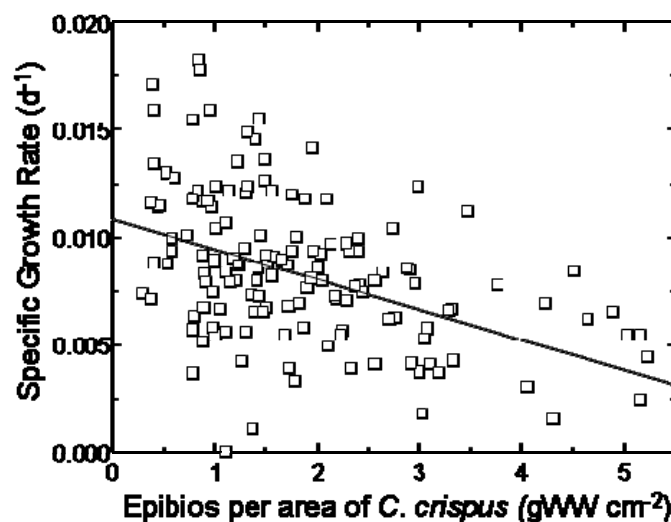


Fig. 6 Regression between specific growth rate (d⁻¹) of *Chondrus crispus* and epibios per area of thalli from Experiment 5 in 2005 (gWW cm⁻²). Line represent significant linear negative regression ($R^2=0.20$, $F_{1,141}=35.31$, $y= 0.0111-0.00137x$, $p<0.00001$).

3.5. Tissue analysis, ambient DIN and N-loss from fish farm

Carbon, nitrogen and sulphur analysis were performed on initial *C. crispus* biomass and at the end of Experiment 3 and 5 in 2005. No consistency in carbon content was seen between initial tissue and different distances and average was 30.3 ± 2.9 % C gDW⁻¹ for initial tissue, 31.2 ± 0.8 % C gDW⁻¹ for Experiment 3 and 31.9 ± 1.2 % C gDW⁻¹ for Experiment 5. There was a negative correlation between N and S content of *C. crispus* and generally with higher N:S content for Experiment 5 compared to Experiment 3 (Figure 7).

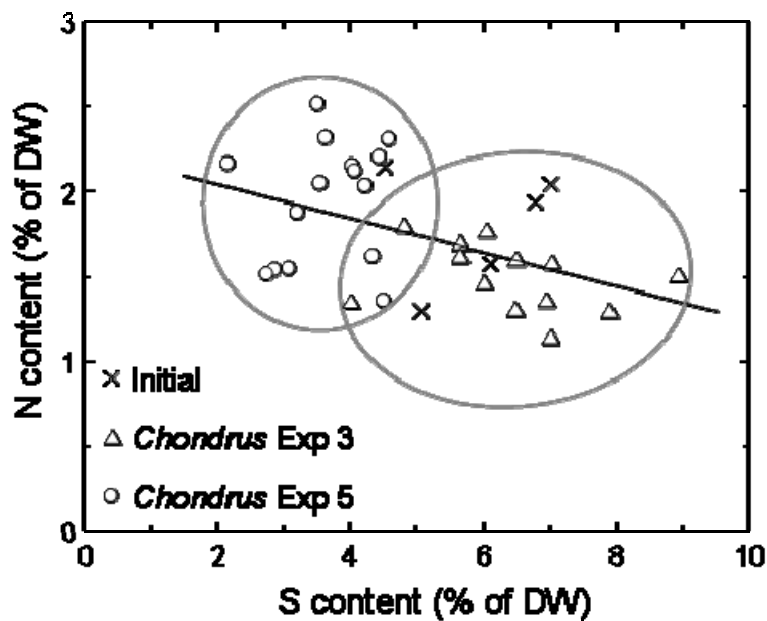


Fig. 7 Correlation between nitrogen (N) and sulphur (S) content (% of DW) of initial biomass of *Chondrus crispus* and thalli deployed near Musholm Lax A/S fish farm in Great Belt for 21 days (Exp 3) and 74 days (Exp 5) in summer 2005. Line represent the trend of the data points ($p < 0.01$) and large circles represent groupings of correlations in Exp 3 and 5.

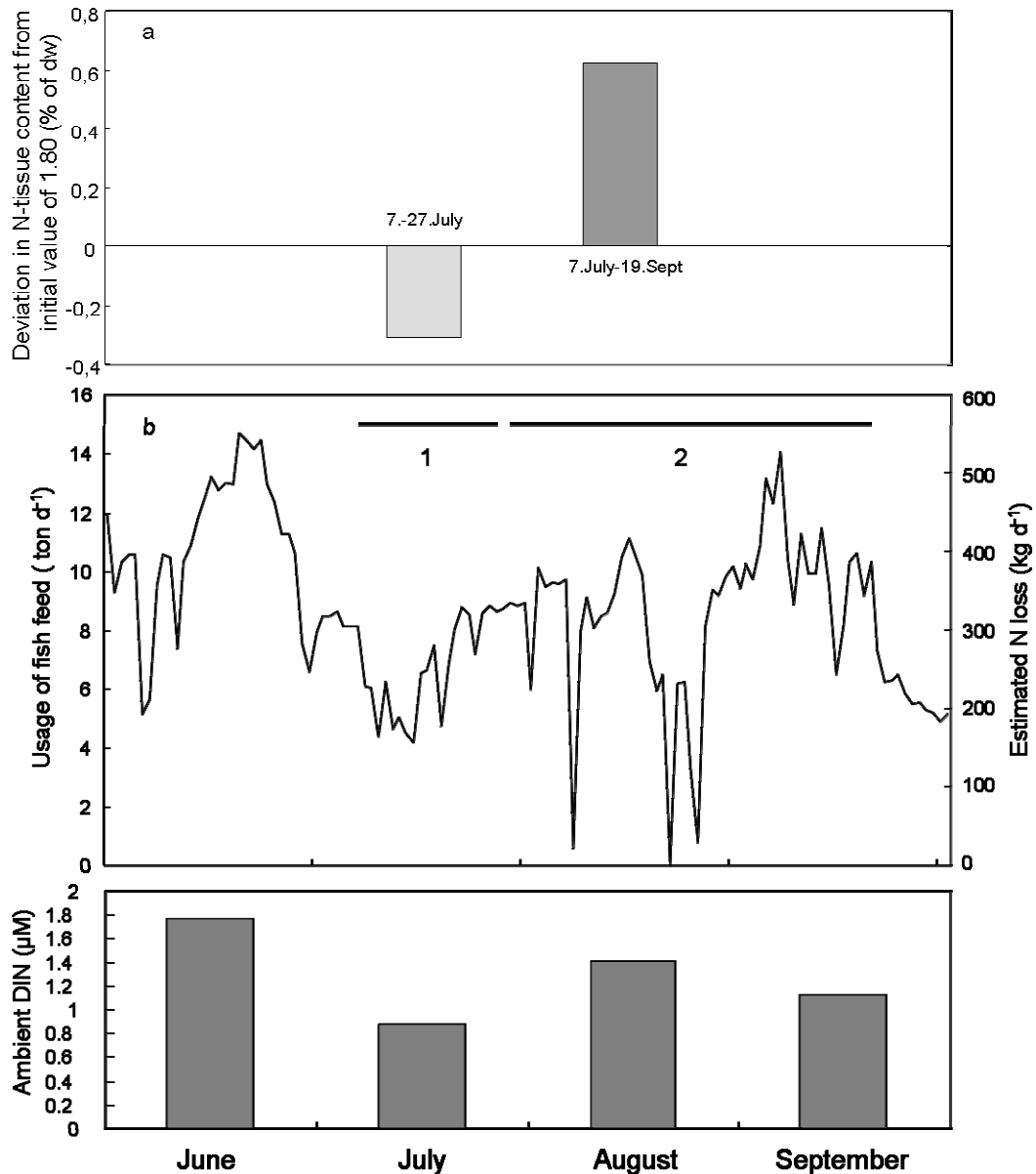


Fig. 8 (a) Deviation in N-tissue content in *Chondrus crispus* in regard to initial value (1.80 % of DW) in Experiment 3 (7-27 July) and Experiment 5 (7 July-19 September). (b) Daily amount of feed used (metric tonnes d⁻¹) and estimated nitrogen loss (kg d⁻¹) in summer 2005 at the Musholm Lax A/S fish farm located in the Great Belt, Denmark. Horizontal lines indicate Period 1 (7-27 July) and 2 (28 July-19 September; Jensen 2007). (c) Ambient dissolved inorganic nitrogen (DIN; μM) in Great Belt near the fish farm (data from June–September extracted from Figure 9 Appendix 1 (NERI 2008).

The tissue N-content of *C. crispus* was in Experiment 3 lower than the initial and in this experimental Period 1 the average estimated N loss was 270±60 kg N d⁻¹ and somewhat lower than for Period 2 with 350±111 kg N d⁻¹. The tissue N-content at end of Experiment 5 was higher than initially and that of Experiment 3, and estimated N-loss was simultaneous higher in Period 2, the last 56 days of Experiment 5. The averaged ambient concentrations of DIN in these periods were 0.88 μM DIN in July and 1.40 μM DIN in August (Figure 8).

4. Discussion

The three investigated macroalgae did not express exponential growth as expected, however, the SGR decreased with increasing duration of experiment. The physio-chemical variables such as light, temperature, epibios coverage and feed usage from fish farm are discussed as reasons for this and estimated N-loss from fish farm and averages of ambient summer DIN concentrations are compared to the macroalgae tissue N-content.

Temperature was the main explanation for the variation in SGR of *Chondrus crispus* with highest SGR when temperature was highest in Experiment 3 (18.3 ± 0.6 °C). Temperatures were in the optimal range (14-20 °C) in the entire experimental period according to what has been found in other studies for both *C. crispus* and *Furcellaria lumbricalis* (Bird et al. 1979; Simpson and Shacklock 1979; Juanes and McLachlan 1992). However, for *Gracilaria vermiculophylla* gametophytes the optimum temperature was found to be 20-30 °C (Yokoya et al. 1999).

No relations were found between SGR and irradiance for any of the three investigated species. The irradiance data are not actual measurements at experimental site from the periods, but data retrieved from databases of nearby sample stations in 2004, and corrected for sunlight hours per month to use the data for the following years. Furthermore, it is most likely that increased turbidity was present in the water column, due to seston from the fish farm, but this was not taken into account in the irradiance values presented. Irradiance values are therefore probably over estimated.

Outdoor tank studies show that *C. crispus* is saturated for photosynthesis at irradiance of $100 \mu\text{mol E m}^{-2} \text{ s}^{-1}$, and in laboratory experiments with *C. crispus*, *F. lumbricalis* and *Gracilaria tikvahiae* were saturated at $65 \mu\text{mol E m}^{-2} \text{ s}^{-1}$ (Bird et al. 1979; Cabello-Pasini et al. 2000). According to our estimation of light, these levels of irradiances were present in all experiments at least down to 3 or 4 m depths. Single thallus of *C. crispus* has high light absorption with relatively high thallus specific carbon (Markager and Sand-Jensen 1992) and irradiance was above this species light compensation point ($0.44 \mu\text{mol E m}^{-2} \text{ s}^{-1}$) in all experimental depths in 2005. This means that *C. crispus* should not have been inhibited in growth due to light and we expected an advantage of *C. crispus* growing at greater depths compared to epiphytes. This was not evident in this investigation, with a possible explanation that epibios measurements were both expressed in epiphytes and mussels (as the primary epifauna) with the latter being independent of light. For *C. crispus* the growth was not directly

suppressed by decreasing light availability, however indirectly by the epibios as this coverage (epibios per thalli) had negative effect on growth and explained 16 % of the variation of SGR. Epibios were present in such large abundance and size that shading of the investigated macroalgae was considerable high (Pringle and Mathieson 1986; Kemp et al. 2000). The size and thereby the surface of *C. crispus* determined the abundance of epibios with positive regression.

The epibios is not just expected to have negative effect on the macroalgal host. While epiphytes reduce light and compete for other resources such as inorganic nutrients and carbon dioxide (Sand-Jensen et al. 2007), mussels increase the ammonium concentration significantly (Asmus and Asmus 1991; Retamales and Buschmann 1996) and provide the macroalgal host considerable quantities due to excretion, up to 0.3 % gDIN (gWW mussel)⁻¹d⁻¹ (pers. comm. Møhlenberg, 2008). This would in Experiment 1 have provided each *Gracilaria vermiculophylla* thallus (replicate) 0.06 g DIN in addition. This positive effect of mussels complies with our finding for *G. vermiculophylla* with mussels as the main epibios, because in these experiments SGR showed positive regression with epibios per thalli. This means that *G. vermiculophylla* tested in 1 and 2 m depth was enhanced in growth by fouling mussels, despite almost total coverage.

Another recognized positive effect on SGR of *C. crispus* was the feed and thereby N-loss from the fish farm, and with greater SGR closest to the fish farm, due to excess nutrients. A nutrient gradient with highest concentrations of nitrogen closest to the fish farm and with decreasing concentrations towards North and no nutrient impact by the fish farm at 1000 m South (reference) is furthermore indicated by earlier environmental impact assessments (EIA) (Birklund and Petersen 2005). These EIA's were performed with net nitrogen uptake in *Ulva lactuca* deployed in ventilated tubes for 14 days around the fish farm ((Lyngby et al. 1999; Birklund and Petersen 2005). This negative regression between SGR and distance was also seen from other studies with *Saccharina latissima* and *G. chilensis* deployed near fish farms (Troell et al. 1997; Ridler et al. 2006). Furthermore, the nitrogen tissue content of *C. crispus* did fluctuate with the N-loss from the fish farm and/or ambient concentrations. Background concentrations (average of 6 years) of inorganic nutrients show low dissolved inorganic nitrogen (DIN) in July but somewhat increased in August (Figure 9 Appendix 1; NERI 2008). Data from the Musholm fish farm at the period of experiments showed that there was greater feeding of fish in the farm in the period of Experiment 5 (August and September) after

Experiment 3 (July), compared to the 21 days of deployment of Experiment 3 (Jensen 2007). This means that in Experiment 3 the low ambient DIN concentration and low feeding of fish is reflected in the tissue nitrogen content of *C. crispus* which is lower than the initial tissue content and critical level of nitrogen ($2\% \text{ N gDW}^{-1}$), resulting in decrease of macroalgal growth as seen in other macroalgal species (Bird et al. 1982; Hanisak 1987). However, nitrogen tissue content increased to a level above critical ($2.12 \pm 0.30\% \text{ N gDW}^{-1}$) in Experiment 5 in the last period after Experiment 3, probably due to excess nutrient from the increased feeding of fish and the somewhat increased ambient DIN concentrations. Calculations based on K_m and V_{max} of ammonium uptake by *C. crispus* show that during the average ambient summer DIN concentration the increase in tissue N-content between Experiment 3 and 5 (56 days) would have taken 35 days (Rees 2003). The available ambient DIN and that irradiance saturated for photosynthesis in at least the 1 m and 2 m depth should have resulted in suitable growth conditions for *C. crispus* in August and September. However, the growth of the algae was inhibited which is partly explained by the considerably amount of epibios, and furthermore supported by the negative regression between SGR and epibios biomass per area of *C. crispus* in this experiment.

As for *C. crispus* the epibios biomass showed negative regression with distance to fish farm in 2004 and 2005 for experiments with both *C. crispus* and *F. lumbricalis*. It is debated whether the increased seston from the fish farm increases growth of nearby mussels, and therefore the increased feed particles and faeces near the fish farm could have stimulated to the increased epibios (Stirling and Okumus 1995; Mazzola and Sarà 2001; Cheshuk et al. 2003). Furthermore, the increase of epiphytes near the fish farm, where also DIN concentrations are expected to be higher, can also be explained by growth of opportunistic, ephemeral, fast growing, filamentous epiphytic macroalgae with high nitrogen V_{max}/K_s ratios which are favored and stimulated in high nutrient concentrations (Borum 1985; Pedersen and Borum 1996a; Pedersen and Borum 1996b; Raven and Taylor 2003; Sand-Jensen et al. 2007).

Carbon content for *C. crispus* thalli from initial tissue averaged $31.2 \pm 1.56\% \text{ C gDW}^{-1}$, and was comparable to concentrations found in other studies (Duarte 1992). Content of sulphur, as an indirect measurement of carrageenan, was lower in Experiment 5 with thalli deployed in the period with higher feeding of the fish (period 2) and higher average ambient DIN-concentration compared to Experiment 3. The inverse relation between sulphur content (carrageenan content) and nitrogen content

is in support of the “Neish-effect”. “Neish-effect” has been observed in growth of *C. crispus* in laboratory and greenhouse studies where carrageenan content decreases with increased nitrogen supply (Neish et al. 1977; Simpson and Shacklock 1979; Chopin et al. 1995).

In our experiments SGR's of *C. crispus* and *G. vermiculophylla* were $0.018 \pm 0.0062 \text{ d}^{-1}$ and $0.039 \pm 0.0073 \text{ d}^{-1}$, respectively and somewhat lower to what is found in literature for field experiments. In peak growth periods SGR's of $0.01\text{--}0.02 \text{ d}^{-1}$ and $0.03\text{--}0.04 \text{ d}^{-1}$ were found for *C. crispus* in field (Chopin et al. 1999a; Zertuche-González et al. 2001), *G. chilensis* grown close (10 m) to fish farm exhibited SGR of 0.07 d^{-1} (40 % higher than further away from fish farm) and 0.065 d^{-1} was found in field studies with *Laminaria digitata* (Troell et al. 1997; Chopin and Wagey 1999; Makarov et al. 1999; Chopin et al. 1999a; Kemp et al. 2000). *Gracilaria lemaneiformis* growth in polyculture area of bivalve and kelp reached 0.082 d^{-1} and in that study not only functioned as biofilter for nutrient, but also increased the dissolved oxygen in the experimental area by the photosynthetic activity of the algae (Yang et al. 2005). The SGR for *F. lumbricalis* was in this study $0.011 \pm 0.004 \text{ d}^{-1}$, however, values of up to 0.03 d^{-1} have been found under laboratory conditions by other scientist (Bird et al. 1979).

Seaweed productions of *Chondrus crispus*, *Furcellaria lumbricalis* and *Gracilaria vermiculophylla* in these experiments before epibios dominated were $4 \text{ gWW m}^{-2} \text{ d}^{-1}$, $2 \text{ gWW m}^{-2} \text{ d}^{-1}$ and $60 \text{ gWW m}^{-2} \text{ d}^{-1}$ respectively. Only the production of *G. vermiculophylla* complies with extrapolated field productions documented for another *Gracilaria* species.. In these studies productions of *G. chilensis* were $70 \text{ gWW m}^{-2} \text{ d}^{-1}$ in both field experiments with stock density of either 450 gWW m^{-2} germlings generated from spores or 1.7 kgWW m^{-2} from bundles of vegetative thalli (Troell et al. 1997; Alveal et al. 1997; Zertuche-González et al. 2001). Seaweed production is density dependent and stocking density could most likely be increased compared to this setup without noticeable self-shading. Nevertheless, production of *C. crispus* in this investigation would be equivalent to a N-removal/assimilation of $0.08 \text{ gN m}^{-2} \text{ d}^{-1}$ and does not make this species a candidate as biofilter. Not even with higher densities of initial biomass or compensation for extremely high carrageenan yield for slow growth of *C. crispus* compared to other carrageenan containing cultivated species like *Eucheuma* and *Kappaphycus alvarezii* with higher specific growth rates (Chopin et al. 1999a). These investigations also exclude *F. lumbricalis* with even lower

production, as candidate for polyculture and acting as biofilter near fish farms, whereas *G. vermiculophylla* might stand a chance with similar production as other studies. Nevertheless, all three investigated red macroalgae are rejected as candidate species for polyculture in the inner Danish waters, because biomass cannot be sold and utilized for gelling agent content, due to the high degree of epibios growth.

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Appendix 1

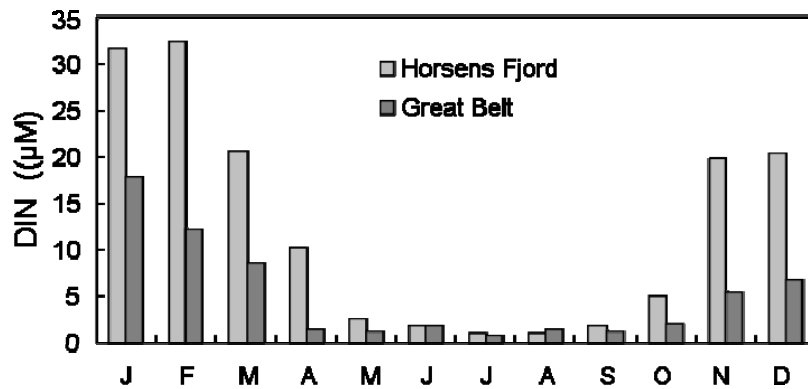


Fig. 9 Monthly averages of dissolved inorganic nitrogen (DIN) in the Great Belt and Horsens Fjord sampled at stations (Nut) shown in Figure 1. Based on 6 years sampling at monthly intervals (Horsens: 2001-2006; Great Belt: 1991-1997); data from the MADS database (NERI 2008).

Table 1 Mean irradiance ($\mu E m^{-1} s^{-1}$) in depths, locations and experiments with the three investigated macroalgae. Experimental periods are separated into smaller periods compared to some of the original durations of experiment (see Chapter 2.3 Specific growth rate for explanation). Irradiance calculated from surface insolation (extract from Waterforecast; (DHI 2008) and weekly-to-biweekly measurements of light extinction (Explained in 2.1 Environmental setting; NERI 2008))

Summer	Species	Experiment	Irradiance ($\mu E m^{-1} s^{-1}$)				
			Depth				
			1 m	2 m	3 m	4 m	5 m
2004	<i>C. crispus</i> and <i>F. lumbricalis</i>	Musholm Exp 1	218	146	98	66	44
		3.Aug-2.Sept					
		Musholm Exp 2	141	98	67	47	32
		2.Sept-30.Sept					
2005	<i>Chondrus. crispus</i>	Snaptun	165	108	71	47	31
		25.Aug-7.Oct					
		Musholm Exp 3	256	187	136	99	72
		7.July-28.July					
		Musholm Exp 4	191	151	119	94	74
		28.July-22.Aug					
		Musholm Exp 5	166	129	100	78	60
		22.Aug-19.Sept					
		Musholm Exp 6	167	130	101	78	61
		15.Aug-7.Sept					
	<i>Furcellaria lumbricalis</i>	Musholm Exp 7	126	99	78	62	48
		7.Sept-5.Oct					
		Musholm Exp 3	222	169	129	98	75
		15.July-15.Aug					
		Musholm Exp 4	167	130	101	78	61
2006	<i>Gracilaria vermiculophylla</i>	15.Aug-7.Sept					
		Musholm Exp 5	126	99	78	62	48
		7.Sept-5.Oct					
		Snaptun Exp 1	351	262	196	146	109
		1.June-5.July					
		Snaptun Exp 2	354	280	221	175	138
		5.July-16.Aug					
		Snaptun Exp 4	354	280	221	175	138
		5.July-16.Aug					
		Snaptun Exp 5	137	110	87	70	56
		16.Aug-10.Oct					

