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Contents

Summary

Seaweed and mussels are both important organisms used for aquaculture and often coexist in marine ecosystems. Amongst the many interactions between these two organisms is the potential for seaweed to benefit from additional nutrients (in particular ammonium) excreted by mussels. In addition, in mussel cultivation systems where the shellfish live in high densities, seaweed growth and production can be stimulated by the nutrient excretion from bivalves. Here we investigate co-cultivation of the green macro-algae *Ulva lactuca* and *Mytilus edulis* (blue mussels) to see whether co-cultivation with mussels increases *Ulva* production. Mesocosm experiments in which the *Ulva lactuca* and blue mussels were grown in combination as well as separately were conducted and production, C:N ratio of the *Ulva* biomass and nutrient uptake were determined. No significant differences in production rates were found between *Ulva* cultivated in monoculture or co-cultivated with blue mussels. The specific growth rate of *Ulva* was 7.7 $\pm$ 0.86 and 7.8 $\pm$ 0.89 % DW d^{-1} in mono and seaweed mussel co-cultivation respectively. The C:N ratio of the Ulva biomass (tanks containing only seaweed) measured at the start of the experiment (31/07/2018) was 17.2 (SD=2.35). This result was supported by the comparable C:N ratio of the *Ulva* biomass and no significant difference in ammonium uptake between both treatments (with and without mussels). Although an initial increase in ammonium concentration was detected by the addition of blue mussels, the nutrient uptake experiment in combination with *Ulva* did not show significant differences in the uptake of ammonium by *Ulva* cultivated in combination with mussels as opposed to *Ulva* cultivated by itself. However, a net increase in ammonium uptake by *Ulva* in combination with mussels as opposed to the uptake of ammonium in *Ulva* tanks without mussels was not ruled out. Moreover, the tanks containing only mussels did not show increased ammonium concentrations. It remains unknown why no enhanced ammonium concentrations were observed during the end of the experiment, and whether or not this has occurred throughout the experimental period, subsequently leading to little variation in growth and CN ratios between mono- and co-culture treatments. Although these results do not evidently demonstrate an advantage of seaweed cultivation in combination with mussels they do not rule out potential benefits from combined macroalgae- shellfish production.

1 Introduction

1.1 Background

Mussel farming and seaweed farming are fast-growing sectors worldwide (Wijsman *et al.*, 2019). Both mussels and various seaweed species can be grown on similar basic longline structures and several reasons exist to integrate the cultivation of these two crops. 1) better space utilization of limited permitted sites, 2) shared use of the capital costs of expensive anchors, lines and buoys, 3) better risk management via crop diversification. The additional benefits of using multiple complementary nutrient bio-extractive crops are improved ecosystem services such as (i) improved water quality, (ii) provision of structure resulting in nursery and foraging habitat for other species, and (iii) a sustainable seafood supply (Rose *et al.*, 2015). Co-cultivation of seaweed and shellfish is often mentioned as a multi-use approach to efficiently use space in offshore wind parks (Michler-Cieluch & Kodeih, 2008, van den Burg *et al.*, 2017).

Moreover, seaweed and shellfish often co-exist in marine ecosystems and display multiple interactions. Seaweeds use the supply of nutrients available in seawater for growth just like unicellular algae (phytoplankton), a major food source for shellfish, do. Nitrogen (especially ammonium) is an important component produced by shellfish that macroalgae could benefit from. In the marine environment dissolved nitrogen is also available as nitrate (NO_3). Algae are able to utilize both forms of nitrogen but show a preference for ammonium (NH_4), since the uptake of ammonium requires less energy (ATP). Marine bacteria transform ammonium into nitrate, which means ammonium is often limited in waterbodies. The ammonium produced by mussels could therefore stimulate macroalgae growth. It has been suggested that seaweed can benefit from the excess ammonium excreted in IMTA systems by shellfish (Ajjabi *et al.*, 2018, Bouwman *et al.*, 2011a, Mao *et al.*, 2009a) and fish (Buschmann *et al.*, 2009, Sanderson *et al.*, 2008). As opposed to unicellular algae, seaweed is capable to store nitrogen, which makes seaweed as a group suitable to grow under varying nutrient conditions that occur naturally as well as a result of the components produced by shellfish. In addition, shellfish improve visibility by filtering phytoplankton and other organic and inorganic material from the water column. The improved transparency can in turn positively affect seaweed growth in deeper water. Moreover, positive interaction of co-existence of seaweeds with shellfish have been claimed as mussel excretion nutrients can be exploited as a resource input (Ajjabi et al., 2018, Bouwman et al., 2011a, Mao et al., 2009a), reducing the risk of eutrophication (Bouwman *et al.*, 2011b, Mao *et al.*, 2009b). On the other hand, when seaweed production results in a strong decrease in nutrient concentration shellfish growth might be impaired. This is due to the reduced primary production that is limited by low nutrient availability, lower light availability by shading of macroalgae or high grazing pressure from filter-feeders. In an ecosystem where primary production is limited by nutrients, the nutrients utilized by seaweed are no longer available for unicellular algae, the feed supply for shellfish. This could potentially result in competition between seaweed and shellfish production in these systems. In this context it is also important to underline the differences in seasonality between seaweeds and phytoplankton (depending on the seaweed species).

1.2 *Ulva* nutrient dynamics and productivity

From May onwards, decrease of diatom biomass in the Oosterschelde is assumed to be a result of nutrient limitation (Bakker *et al.*, 1994). Low ambient total ammonium nitrogen (TAN, the amount of NH_4 and NH_3 in the water) concentrations in the Oosterschelde are assumed to be the limiting factor for summer seaweed such as *Ulva* spp. Mean TAN concentration recorded at a sampling station nearby the experimental facility (Rijkswaterstaat, Lodijkse Gat) ranges between 3 and 6 µmol L^{-1} during July, August and September. If *Ulva* spp. is nitrogen limited (de Vries, 2014), it might benefit from nutrient-N excretion. But if N is not limiting, NH_4 excretion might lead to higher growth as a result of

provision of an energy efficient N-source. Therefore, it is hypothesized that *Ulva* spp. productivity rates (Table 1) will be positively correlated with increased N-flux by mussel co-cultivation.

In addition, higher ammonium-N flux is expected to cause a shift from nutrient to light limited *Ulva* spp. growth, as was for example shown for experiments with the red algae *Gracilaria tikvahiae* (Lapointe & Duke, 1984) (Fig. 1). The C:N ratio (ratio of carbon over nitrogen) in tissue can be used to determine if macroalgae are N limited (Hurd, 2014). High C:N ratio indicates N-limitation because of a decrease in amino acids and proteins and an increase in carbohydrates (Björnsäter & Wheeler, 1990). RuBPcase, an enzyme involved in fixing CO_2 in plant tissue, is also N-dependent, with a positive correlation under N-sufficient and a negative correlation under N-limited conditions (Duke *et al.*, 1986). In sub-tropical cultivated green macroalgae such as *Ulva lactuca* (Neori *et al.*, 1991a) and *Ulva rigida* (Pinchetti *et al.*, 1998), high C:N ratio in biomass is used as an indication of N-limitation; *U. rigida* C:N ratio reduced from 35:1 to 5:1 when changing from nitrogen starvation to enrichment. In general, C:N values close to 10 have been described as optimal or normal for the nitrogen status of algae and a ratio higher than 10 indicates N-limitation (Lapointe et al., 1976). However, others indicate C:N ratio of 18 as average for all macroalgae, and describe C:N ratios >20 indicate possible N limitation (Hurd, 2014). C:N ratios below >17 have been measured for *Ulva* spp. during summer conditions, and values in August were higher compared to September (Buisman 2018), indicating that nitrogen limitation may occur in summer in the Oosterschelde.

Ulva spp. generally shows preferred uptake of ammonium-N above NO3-N, although both *U. rigida* and *U. lactuca* are able to utilize both nitrate and ammonium (Table 2). Ammonium is suggested to require less ATP compared to NO_3 (Fujita, 1985). Therefore, NH_4^+ or total ammonium nitrogen (TAN) is considered the preferred Ulva spp. N-source. This is confirmed by higher uptake rates of TAN when compared to nitrate (NO_2) (Cohen & Neori, 1991a, Cohen & Fong, 2004, Fujita, 1985). However, this pattern is not empirically found in all *Ulva* species. For example, the opposite was found for *U. rigida* that showed a preference for NO_3 (Lavery *et al.*, 1991b).

Table 1: Ulva spp. productivity as reported in literature in temperate, northern conditions

Ulva spp. species	Unit	Productivity	Reference
U. lactuca	SGR % FW d^{-1}	1.6 – 6.3	(Robertson-Andersson *et al.*, 2008b)
U. lactuca	SGR % FW d^{-1}	7.4 – 17.9[#]	(Neori *et al.*, 1991b)
U. curvata	SGR % DW d^{-1}	9.5 – 28	(Duke *et al.*, 1989)
U. lactuca	SGR % DW d^{-1}	-0.3 – 11.3[*]	(Bruhn *et al.*, 2011)
U. lactuca	kg DW ha^{-1} d^{-1}	-31 – 679[*]	(Bruhn et al., 2011)
U. lactuca	kg DW ha^{-1} d^{-1}	68 – 188[*]	(Debusk *et al.*, 1986)
U. lactuca	kg DW ha^{-1} d^{-1}	22 – 327	(Groenendijk *et al.*, 2016)

[*] Mean values as presented in the study

[#] Sub-tropical region, fertilized conditions

Table 2: Ulva spp. nitrogen uptake rates (µmol gram dry weight^{-1} hour^{-1}) of Total Ammonium Nitrogen (TAN) and nitrate (NO_3^-) as reported in literature. When cells are left empty no information is available.

Species	TAN [a]	NO_3^-	Reference
Ulva rigida	50[*] – 136[*] 250[#] – 371[#]	64	(Solidoro *et al.*, 1997)
Ulva rigida	440	820	(Lavery *et al.*, 1991a)
Ulva lactuca	138[*]– 252[#]		(Fujita, 1985)
Ulva lactuca[b]	50[*] – 390[#]		(Cohen & Neori, 1991b)
Ulva curvata	250		(Rosenberg & Ramus, 1982)
Ulva fasciata		30	(Lapointe *et al.*, 1976)

[a] TAN (Total Ammonium Nitrogen) uptake rate; [b] Sub-tropical region; * N sufficient; # N limitation

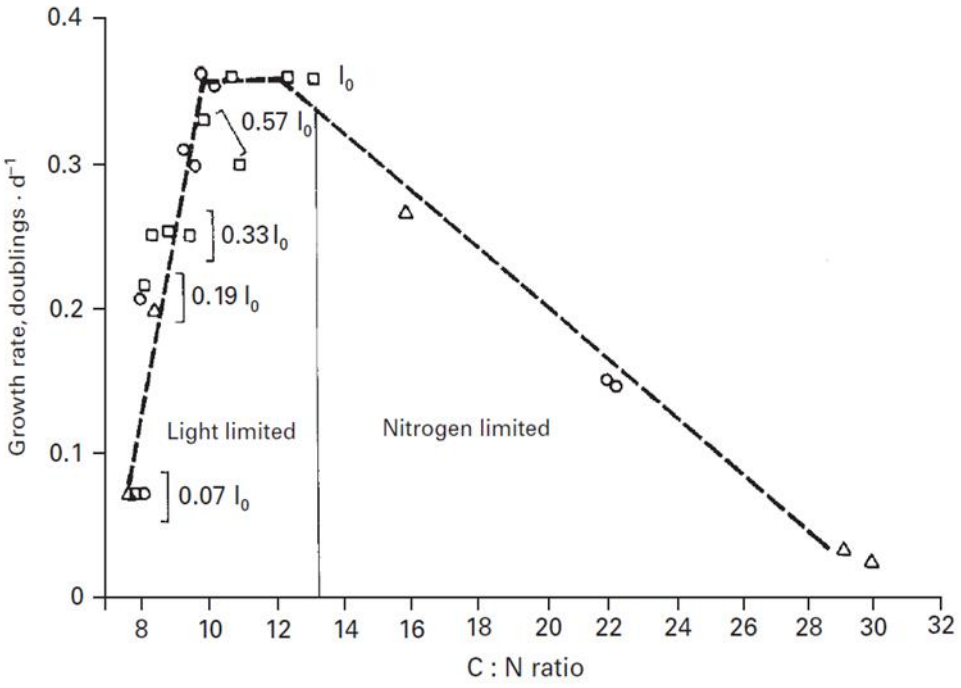

Figure 1: Correlation between growth rate of Gracilaria tikvahiae and tissue C:N ratio is dependent on light or nitrogen limitation (in Hurd et al., 2014, from Lapointe and Duke, 1985).

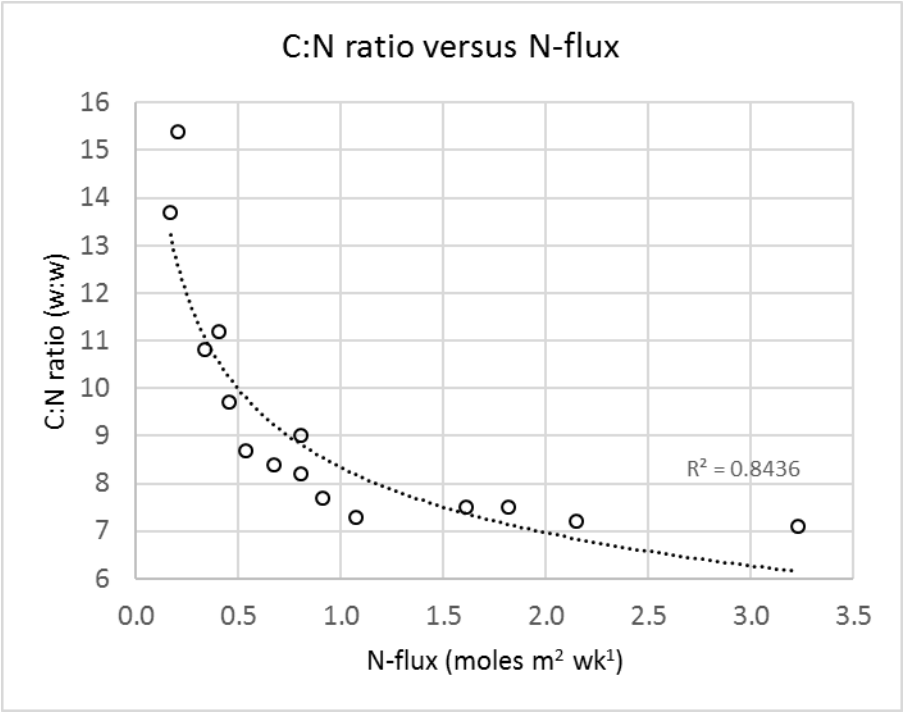

Figure 2: Correlation between C:N ratio in Ulva spp. and the N-flux in the water, data taken from (Neori et al., 1991a). The water N-flux is exponentially negatively correlated with C:N ratio in the seaweed biomass, indicating higher N level.

1.3 Research aim

In the context of a multi-use approach to increase efficient use of space in wind parks the question was posed whether co-production of seaweed and mussels is beneficial for *Ulva* growth through the uptake of metabolic byproducts (such as ammonium) excreted by mussels.

Growth enhancing effects of *Mytilus edulis* on the seaweed early nursery stages and grow out phase in the Baltic sea have been demonstrated (Rossner *et al.*, 2014). However, benefits of co-cultivation for seaweed growth and mussels have not yet been thoroughly investigated. Empirical testing of seaweed variation and mussel co-cultivation will help to understand how circular thinking in aquaculture can be implemented resulting in maximum resource output.

The current study focusses both the potential benefit of co-cultivation. In order to do this, the following research questions were formulated:

Is co-cultivation with mussels (*Mytilus edulis*) potentially beneficial for *Ulva* spp. cultivation?

a) Does *Ulva* spp. productivity increase in co-cultivation with mussels?
b) Does *Ulva* spp. biomass C:N ratio decrease in co-cultivation with mussels?
c) Do nutrient uptake rates of inorganic nutrients in the water column correlate to growth and C:N composition of seaweed

To study these research aims, mesocosm experiments in which the seaweed species *Ulva sp.* and blue mussels (*Mytilus edulis*) were grown in combination as well as separately were conducted and nutrient uptake, production and C:N ratio of the *Ulva* biomass were determined.

Figure 3: Mytilus edulis and Ulva sp.

2 Materials and Methods

2.1 Sample location & organisms

Ulva spp. were collected in April-May 2018 from 11 different locations (Fig. 4) in the Oosterschelde and at 2 locations in the Veerse Meer. A single piece of thallus (approx. size 100cm^2) was collected per location. This method ensured samples contained one single *Ulva* spp. strain. Locations were chosen at least 50 m apart in an attempt to collect different strains. The *Ulva* spp. collected from Heerenkeet (Fig. 4, sample location USHEE; strain was named "blue18") was successfully cultivated and used to compare *Ulva* spp. growth with and without mussels. Blue mussels (*Mytilus edulis*) of commercial size were obtained from commercial producer Prins & Dingemanse. Model species *Ulva lactuca* is common from tropical to polar coastal marine systems, although the strains most likely vary among regions (Bruhn et al., 2011). Season and climate effectively influence macroalgae conditions for growth, effectively determining *Ulva* spp. growth rates and biochemical composition (Lamare & Wing, 2001). The growth season of Ulva is in summer.

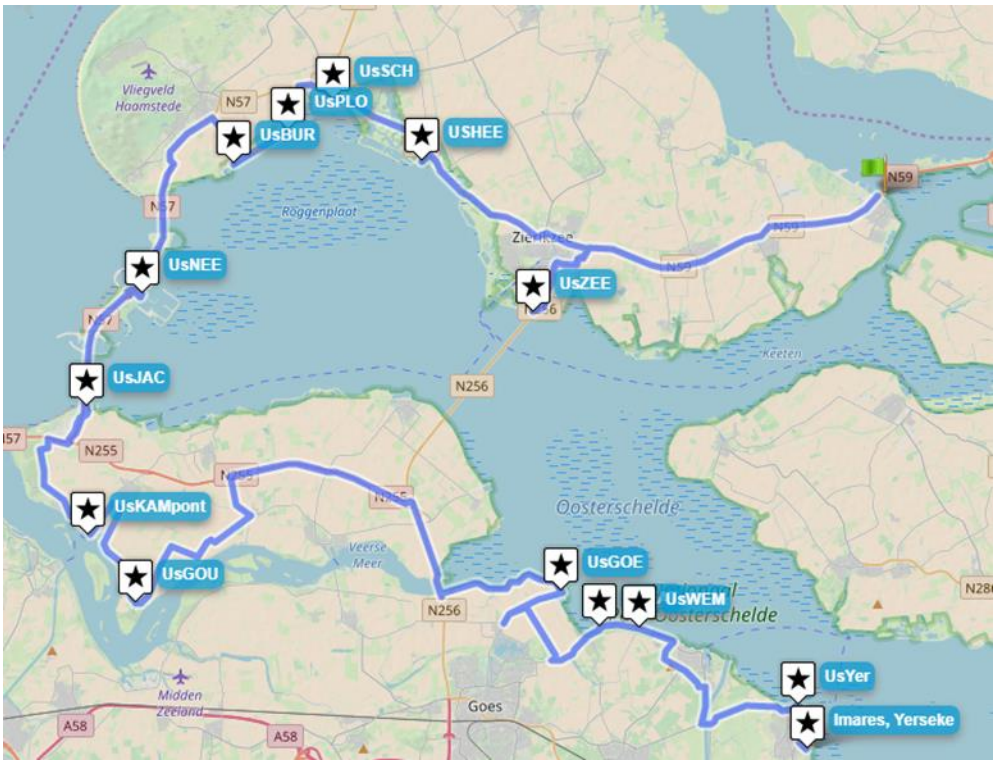

Figure 4: Sampling locations in 2018 in the Oosterschelde estuary and Veerse Meer and location of experimental set-up (Imares, Yerseke).

2.2 Experimental set-up

The experiment was conducted in July/August 2018 at the research facility of Wageningen Marine Research in Yerseke. Ulva was cultivated in outdoor, flow-through. The PCV tanks (400L, 90 x 110 x 40 cm width x length x depth) were placed outdoor and connected in a flow-through set-up (Fig. 5). Tanks were continuously supplied with water from the Oosterschelde from a 10 - 15m deep entry point (no nutrients added), and distributed via higher situated overflow tanks (headers) using tubing (Ø 15 mm). Water exchange was kept constant at approximately 7 times tank volume d^{-1} (32.5 mL sec^{-1}) by weekly measurements and fitted with an aeration system (PVC tubes) to ensure mixing inside the tanks. Tanks were cleaned every two weeks from fouling using a water vacuum cleaner and high-pressure cleaner (DiBo P50 WP, Nilfisk C 125.7-6 Home X-TRA). Netting and a bucket were fitted in front of the tank

outflow to ensure Ulva remained in the tank and an UV filter (Auga UV-c PEHD 420) was placed before the outlet into the Oosterschelde to inactivate and reduce excessive Ulva spores and material.

Figure 5: Experimental set-up of flow-through cultivation tanks (left); First cultivation of sampled single Ulva spp. thalli (right).

The experiment was designed to compare *Ulva* spp. growth and nutrient uptake rates in monoculture or in co-cultivation with blue mussels (Fig. 6). Control tanks (no seaweed, no mussels) were added to the experimental design to determine the effect of natural accumulating fouling organisms on nutrient availability. A genetically homogeneous *Ulva* spp. strain was used in this set-up in order to minimize interaction effects of growth and/or nutrient uptake variation between strains. The *Ulva* spp. used was simultaneously re-stocked with a fixed amount of fresh weight (FW) cultivated *Ulva* spp. in additional tanks (Table 3). Blue mussels were placed in a separate pvc tray at the bottom of the tank. Mussels were left to acclimatize for at least 24 hours. Additional tanks that only contained mussels (n = 4) were included to determine the effect of mussel presence.

A pre-trial was performed on 20/06/2018 to determine the amount of mussels necessary to obtain sufficient levels of ammonium in the tanks. In order to do this 2, 2.5 and 4 kg FW mussels were added to 3 tanks and DIN concentration was measured after 24 hours acclimatization to confirm the increase of ammonium-N flux by mussel presence (see nutrient analysis for more details). The pre-trial was conducted with similar flow through (32.5 mL sec^{-1}).

Each 'mussel tank' and 'seaweed plus mussel tank' contained 4 kg FW commercial mussels (length: 5.54 ± 0.54 cm). Subsequently, 280 g FW *Ulva* spp. was added to 4 of these tanks. The replicate tanks of each treatment (control, seaweed, mussels, mussels + seaweed) were organized in a randomised way to avoid potential effects of the header and shading from adjacent buildings. The experiment ran for four weeks (from 31/07/2018 until 28/08/2018). At the end of the experiment DIN concentration was measured again in order to get an indication of DIN uptake (see nutrient analysis for more details).

Figure 6: Experimental tanks: trays with blue mussels placed on the bottom of the Ulva tanks to imitate co-cultivation system conditions. Mussel trays were elevated using bricks to prevent detritus to accumulate inside the tray.

2.3 Analysis

2.3.1 Environmental parameters

Temperature and photon irradiance

Temperature (°C) and light intensity (µmol m^{-2} sec^{-1}) were logged continuously in control tanks without mussels or seaweed (HOBO logger, 15 min. interval). Water quality was checked weekly by determining temperature, pH and Dissolved Oxygen in each tank. In addition, biweekly monitoring of tank fouling was done using a qualitative scale of measurement with four levels of tank fouling (no, light, medium, heavy).

Dissolved Inorganic Nutrient concentrations

The nutrient concentration in the header tanks were determined at a standardised time (11 AM) by taking water samples for Dissolved Inorganic Nutrients: ammonium (NH_4^+), nitrite (NO_2), nitrate (NO_3), phosphate (PO_4) and silica (SiO_2). Nutrient uptake was determined: 1) prior to the experiment to confirm the increase of ammonium-N flux by mussel presence, and 2) at the end of the experiment to get an indication of DIN uptake (point measurement). DIN water samples were taken from the in- and outflow in each tank. Water samples (10ml) were filtered (0.45 µm), and stored at -20°C prior to analysis. DIN analyses (NH4+, NO3, NO2, PO_4 and SiO_2) were performed at the research facility of the Royal Netherlands Institute for Sea Research (NIOZ) in Yerseke using an autoanalyzer. These were compared to ambient Total Ammonia Nitrogen (TAN; total ammonia and ammonium combined) concentration measurements from a nearby sampling station (Lodijkse Gat) (Fig. 9).

2.3.2 Mussel related biotic parameters

Organic fouling

Fouling by organisms inside the cultivation tanks in between fortnightly cleaning was monitored by scoring the level in categories: no fouling, light, medium and heavy.

Mussel length and yield

Individual mussel length was measured before and after the experiment. The tanks containing mussels were checked regularly to ensure mussels were still alive. The total amount of mussels was weighed and individuals were counted at the beginning and at the end of the experiment.

Suspended Particulate Matter (SPM)

Sufficient food availability for the mussels was determined by weekly measuring the Suspended Particulate Matter (SPM) in the inflow of tanks from experiment 2 using pre-burned filters (3 x 2L, Whatman GF/C 47 µm). After filtration (2L), filters were rinsed with fresh water (3 x 50 ml) and dried (70 °C, >30h). Filters were weighed (W_{70C}), placed in a furnish (450 °C, 6h) and weighed again (W_{450C}).

2.3.3 Seaweed productivity and C:N analysis

To determine productivity, seaweed biomass was weighed every two weeks by collecting all biomass per tank (Fig. 7). To prevent potential damage to the Ulva by centrifuge, subsamples of 280 g FW were dabbed dry using paper, and this biomass was used to re-stock. The remaining biomass was dried using a centrifuge to get rid of excess water and to determine FW growth rate. Dry weight (DW) and ash free dry weight (AFDW) were determined by subsample using the oven (70 °C, 24h), and muffle furnace (450 °C, 6h).

To determine the C:N ratio of the Ulva biomass subsamples were dried (40ºC) for 48 hours and processed using a homogenizer. The C:N ratio was determined according to the DUMAS principle using a isotope-ratio mass spectrometry (IRMS) element analyser at the department of animal nutrition (Wageningen University & Research).

2.3.4 Calculations

Water temperature (°C) and light intensity (µmol m^{-2} sec^{-1}) measured by HOBO loggers resulted in peak values when loggers were taken out from the water. Moving averages were applied to smoothen the disrupting peak values using the rollmean(k=15) function in R, calculating average daily values. Ammonia-N flux was calculated using the NH_4^+ concentrations in the header tanks multiplied by the water exchange rate using the following formula (Fujita, 1985, Neori et al., 1991b):

$$Flux\ (\mu mol\ h^{-1}) = [DINinput] * Water\ exchange\ rate$$

Where [TAN] = ammonia-N concentration in the header tanks (µM), water exchange rate = number of times water is exchanged in the tanks (d^{-1}). Uptake was quantified as the difference between in- and outflow DIN concentrations in the tanks, and expressed as relative measure using the following formulas:

$$Uptake\ (\mu mol\ h^{-1}) = ([DIN]_{IN} * flow) - ([DIN]_{OUT} * flow)$$

$$Uptake\ (\%) = \frac{[DIN]_{IN} - [DIN]_{OUT}}{[DIN]_{IN}} * 100$$

Ulva spp. growth was expressed in fresh weight (FW) and calculated as productivity (D'Elia & DeBoer, 1978) and specific growth rate (SGR):

$$Seaweed\ productivity\ (g) = \mathrm{W}_t\ -\ \mathrm{W}_{\mathrm{re-stocked}}$$

$$SGR\ (\%d^{-1}) = 100 * [\ln\left(\frac{W_t}{W_0}\right)]/t$$

where W_0 is the initial biomass and W_t is the biomass after t days. Variations in growth periods were accounted for by comparing productivity between years by only comparing weeks when both experiments were running (week 29 – 39).

Furthermore, dry weight (DW) and ash free dry weight (AFDW) of seaweed biomass were calculated using the following formulas:

$$Dry\ Weight\ (g\ DW) = \mathrm{W}_{70C}$$

$$Ash\ Free\ Dry\ Weight\ (g\ AFDW) = \mathrm{W}_{70C} - \mathrm{W}_{450\mathrm{C}}$$

In experiment 2, suspended particulate matter (SPM) in water inflow was monitored, and water fraction Organic Material (%OM) was calculated using the following formula:

$$Fraction\ Organic\ Material\ (\%OM) = \frac{AFDW}{DW} * 100\%$$

2.3.5 Statistics

Data was statistically analysed using the R software (R Core Team, 2017). After the assumption of normality was checked for all parameters tested by one-way Analysis of Variance (ANOVA) and data was transformed when variation in the data deviated from normal distribution. If assumption of normality remained violated, alternative non-parametric methods were used (Kruskal–Wallis rank test). Differences between treatment groups were analysed with multiple comparison post-hoc tests (Tukey's tests) at $p < 0.05$.

3 Results

3.1 Environmental parameters

In Table 3, environmental conditions are listed for the following tanks: controls, with Ulva, with mussels and tanks with mussels combined with *Ulva*. Environmental water parameters (temperature, pH, dissolved oxygen concentration) were not affected by the presence of mussels in the co-cultivation set-up. However, oxygen levels were slightly higher in tanks containing *Ulva* and tanks containing *Ulva* in combination with mussels (Table 3). The mean water temperature measured in the control tanks from July until September 2018 was high compared to 2017 (2.8 and 3.3 ºC higher in August and September respectively) which is in line with the hot weather recorded in the Netherlands during the summer of 2018. Water temperature in the control tanks (400L) peaked at the end of July 2018 when day time irradiance reached a maximum light irradiation of 68550 µmol m^{-2} sec^{-1} (Fig. 7 and 8). A maximum of 35.5 °C was found in the tanks at the start of August. During two periods (end of July and beginning of August), the minimum night time water temperature in the tanks did not fall below 20 °C. In addition, the difference between maximum and minimum water temperature was larger during July when compared to August and September, indicating stronger daily fluctuations in water temperature. Figure 4 demonstrates differences in weather conditions between 2017 and 2018. The *Ulva* mussel co-cultivation was conducted in August 2018.

Mean TAN concentration in ambient Oosterschelde water was 3.6 and 5.8 µmol L^{-1} in August and September (Fig. 9). Mean NO_3/NO_2 concentration was 2.8 and 3.6 µmol L^{-1} in August and September (Fig. 9). Ammonium concentrations in the header measured during the experiments all fall within the total ammonia nitrogen (TAN) concentration range measured at the nearby sampling station (Lodijkse Gat) during the period of 2010 – 2016 (Fig. 9). Therefore, mean ambient TAN concentration could be used as a proxy for nutrient inflow concentrations in the experiment in 2018. Mean TAN concentrations increased during the experimental period, with values ranging between 3 and 6 µmol L^{-1} h^{-1} in July, August and September (Rijkswaterstaat: waterinfo.rws.nl). Nitrogen flux in the Oosterschelde generally peaks early at the start of spring (February-March) and is lowest during summer (July-August) (de Vries, 2014), reflecting the nutrient uptake by phytoplankton blooms. The average chlorophyll a concentration measured in the Oosterschelde at Lodijkse Gat (data from 2000-2016, Rijkswaterstaat: waterinfo.rws.nl) peaks in May (4.7 µg Chl *a* L^{-1}) and varies between 3.2 and 3.8 µg Chl *a* L^{-1} in July and August (Fig. 10).

Table 3: Environmental water parameters in 2018

Environmental parameters are: pH; Temperature (°C); O_2 (mg L^{-1}); light intensity (µmol m^{-2} sec^{-1}) and Total Ammonia Nitrogen (TAN; µmol-N L^{-1}). Numbers (n=4) are the mean (± SD) of the weekly measurements in the tanks (control, ulva, mussel and combination mussel + Ulva (co)) except for TAN which was measured at nearby monitoring station, Lodijkse Gat, by Rijkswaterstaat (mean ± SD, n=1-4).

	July 2018	August 2018	September 2018
$pH_{control}$	8.5 ± 0.27	7.9 ± 0.22	8.2 ± 0.05
pH_{ulva}	8.6 ± 0.23	8.2 ± 0.06	8.5 ± 0.27
pH_{mussel}	-	8.1 ± 0.06	-
pH_{co}	-	8.2 ± 0.06	-
$T_{control}$	22.4 ± 1.20	23.0 ± 0.95	18.8 ± 0.31
T_{ulva}	22.4 ± 1.45	22.8 ± 0.82	18.5 ± 0.33
T_{mussel}	-	23.0 ± 0.79	-
T_{co}	-	22.9 ± 0.81	-
$O_{2\ control}$	9.1 ± 0.83	8.3 ± 0.56	10.1 ± 0.23
$O_{2\ ulva}$	10.9 ± 0.70	9.4 ± 0.91	10.6 ± 0.50
$O_{2\ mussel}$	-	8.8 ± 0.72	-
$O_{2\ co}$	-	9.9 ± 0.94	-
Light#	283.1 ± 423.7	211.1 ± 368.6	119.7 ± 231.8
[TAN]	3.3 ± 2.5		

Measured in control tanks only

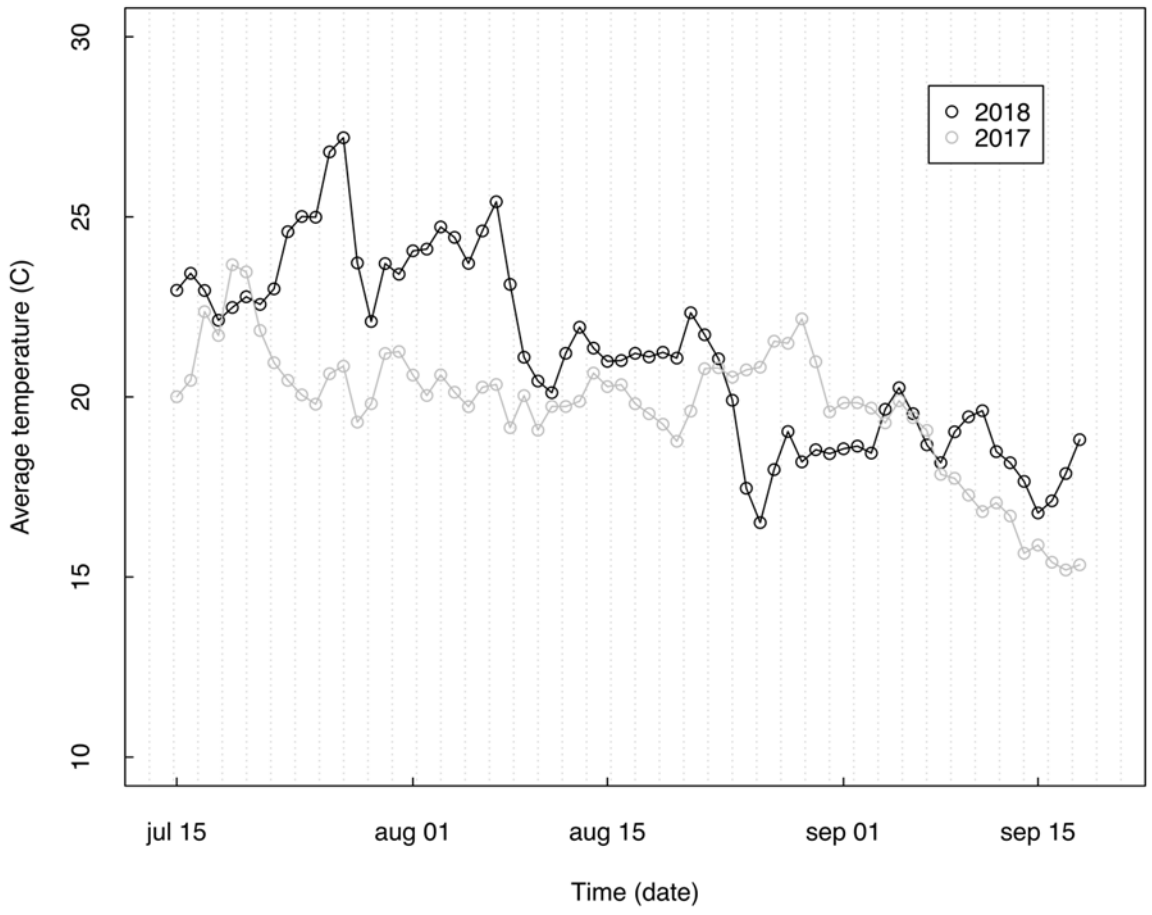

Figure 7: Average (n=4) water temperature (°C) measured in the control tanks in 2017 (light grey) and 2018 (dark grey).

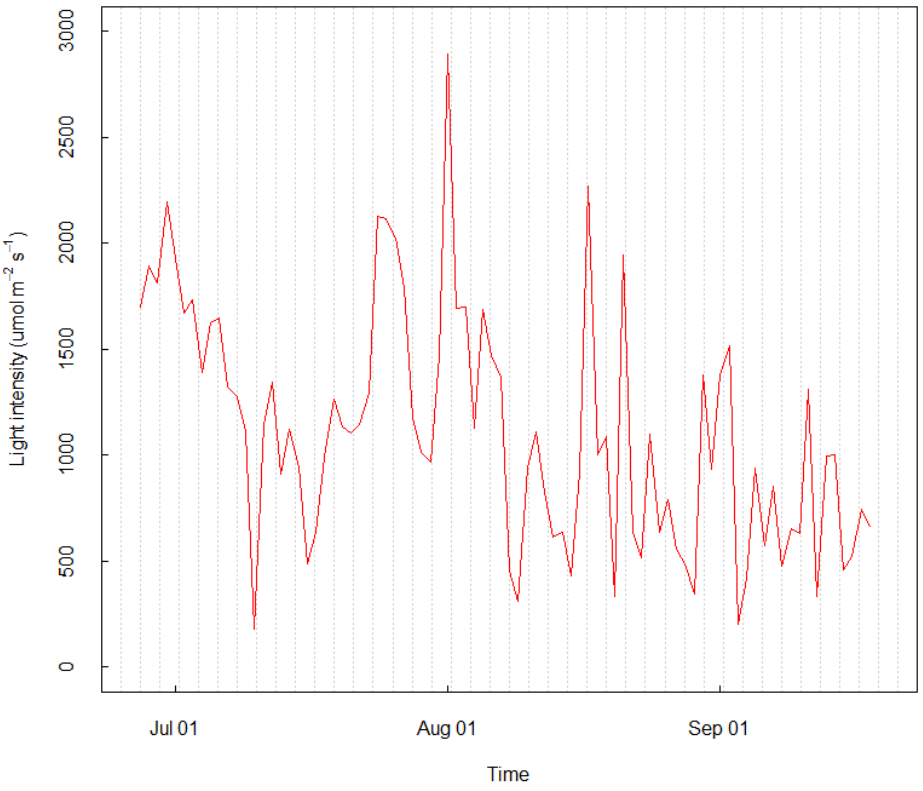

Figure 8: Daily moving average of maximum photon irradiance (measured in µmol photons m^{-2} s^{-1}) in 2018 (n=4).

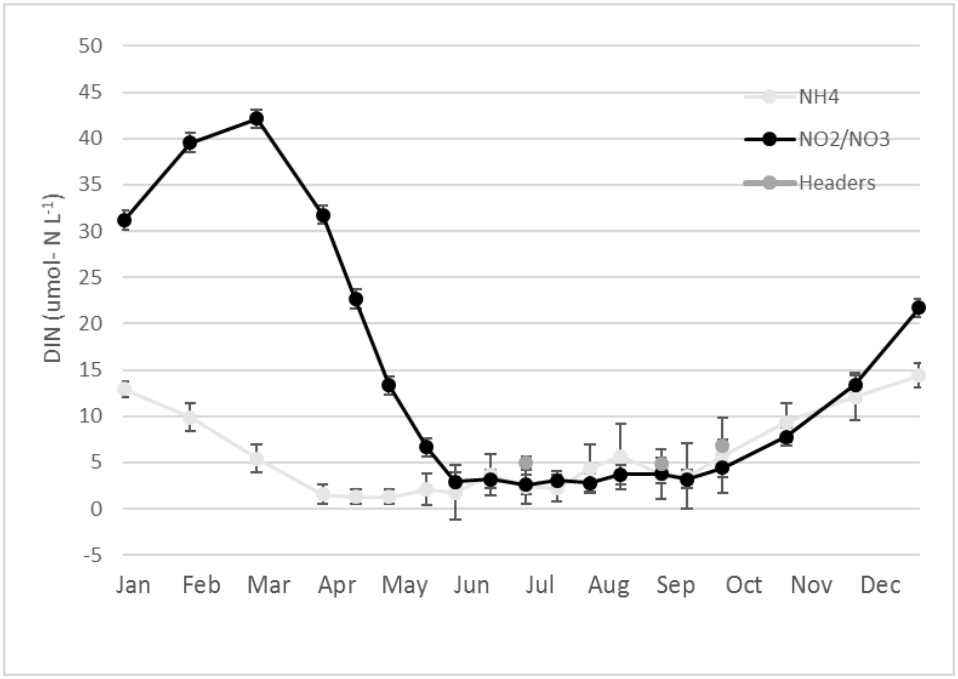

Figure 9: Mean dissolved inorganic nitrogen (DIN) concentration (NH_4 in light grey line, NH_4 headers in dark grey, NO_2/NO_3 in black µmol-N L^{-1}, mean ± SD, n=7) at Lodijkse Gat in the Oosterschelde 2010 – 2016 (waterinfo.rws.nl) and mean ammonium concentration measured in headers during the experiment (grey and black squares, µmol-N L^{-1}, mean ± SD, n=1-5) (this study).

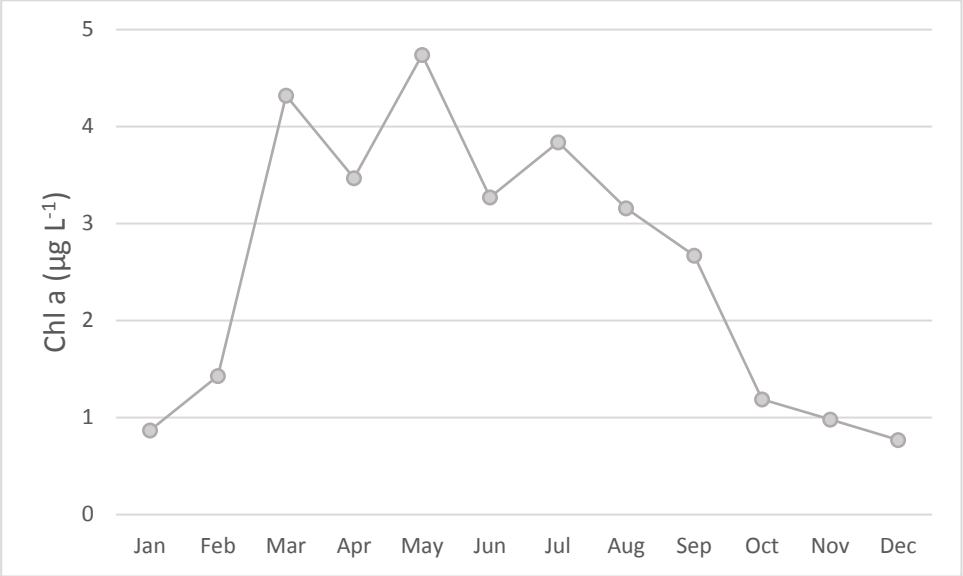

Figure 10: Chlorophyll a concentration (black line, µg Chl-a L^{-1}, mean, n=7) at Lodijkse Gat, the Oosterschelde, during 7 years: 2010 – 2016 (waterinfo.rws.nl).

3.2 Mussel related biotic parameters

The level of fouling by organisms inside the cultivation tanks in between fortnightly cleaning was monitored (Fig. 11). Most heavy organic fouling was detected in the control tanks. Tanks with mussels showed light to medium fouling, whereas tanks with both mussels and seaweed showed least fouling. Tanks containing only seaweed (*Ulva* strain blue) showed higher fouling without the addition of mussels. The content of the tank seems to be an important indicator for the appearance of micro-organisms.

Mussel survival during the experiment was >80% and equal between treatments resulting in a biomass reduction of mussels from 4 to 3.3 kg FW total weight. Individual mussel length was between 5 and 6 cm (placing them in the category of consumption size mussels) during the experimental period in both treatments (Table 4). No significant differences were found in length or weight between the start and the end of the experiment (after 5 weeks).

Suspended particulate matter (SPM) in unfiltered seawater from the Oosterschelde varied between 5.3 and 11.7 mg L^{-1}, with an average of 25.1 ± 0.05 (%) organic matter measured weekly during the experiment (Fig. 12).

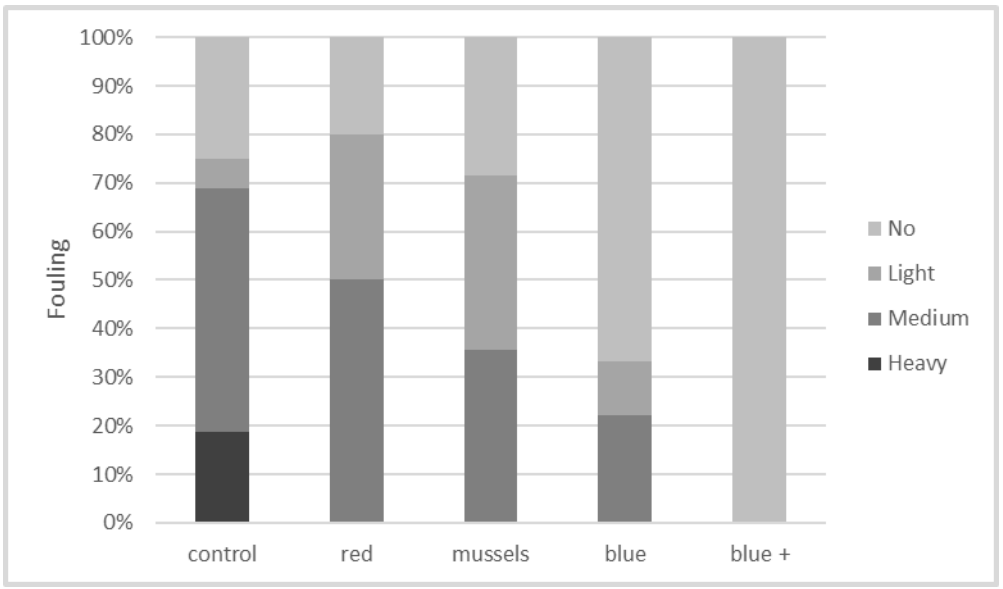

Figure 11: Tank fouling during the experiment, August 2018, week 31 - 35 (n = 4). Red, blue and green denote names of different Ulva strains (only blue was used in this experiment), blue + denotes tanks with both Ulva and mussels.

Table 4: Mussel parameters

Mussel survival (% of initial number of specimens, mean ± SD, n=8), total weight (W) (kg, mean ± SD, n=8) and individual length (L) (cm, mean ± SD, n=167 – 276) at start and end of the experiment for monoculture and co-cultivation.

	Unit	Mussels only	*Ulva* spp. and mussel co-cultivation
Survival	% of total # specimens	81 ± 10	89 ± 11
W_{start}	kg	4.0 ± 0.0	4.0 ± 0.0
W_{end}	kg	3.3 ± 0.4	3.3 ± 0.0
L_{start}	cm	55.2 ± 3.2	56.3 ± 4.6
L_{end}	cm	56.2 ± 1.9	56.4 ± 3.7

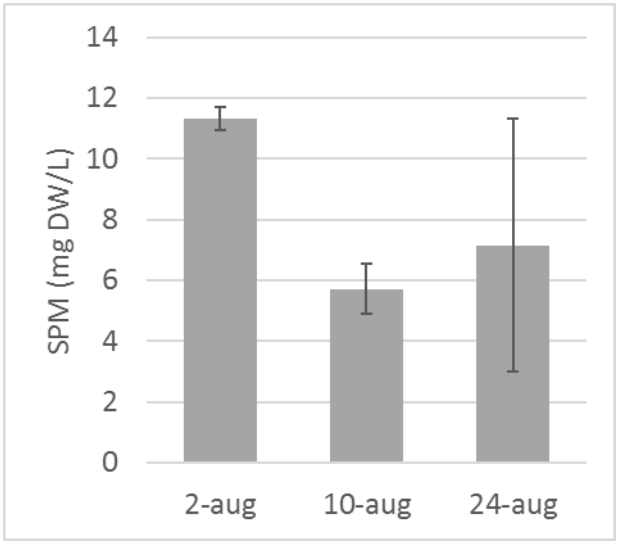

Figure 12: Grey bars indicate Suspended Particulate Matter (SPM; mg DW L^{-1}) in inflow per header tank measured during the mussel co-cultivation experiment. Error bars (n=3) indicate standard deviation.

3.3 Pre-trial: mussel Dissolved Inorganic Nutrient excretion

The pre-trial results (Fig. 13) show that the addition of 4 kg of mussels is sufficient to increase the ammonium level (from 1.5 to 7 µmol L^{-1}). A polynomial regression was chosen since an optimum biomass is expected, after this mussel feed will become limiting and re-filtration will occur. Both NH4 and PO4 concentration showed a positive correlation with mussel biomass in the tank (polynomial, R^2 = 0.99 and R^2 = 0.84 respectively) (Fig. 13).

NH4 level in the pre-trial (green bars) increased with >70% when comparing header concentration with tanks containing 4 kg FW blue mussels (Fig. 14). Mean NH4 concentration increased by 70% from 4.0 to 7.0 µmol $N\text{-}NH_4$ L^{-1} when comparing headers and mussel tanks.

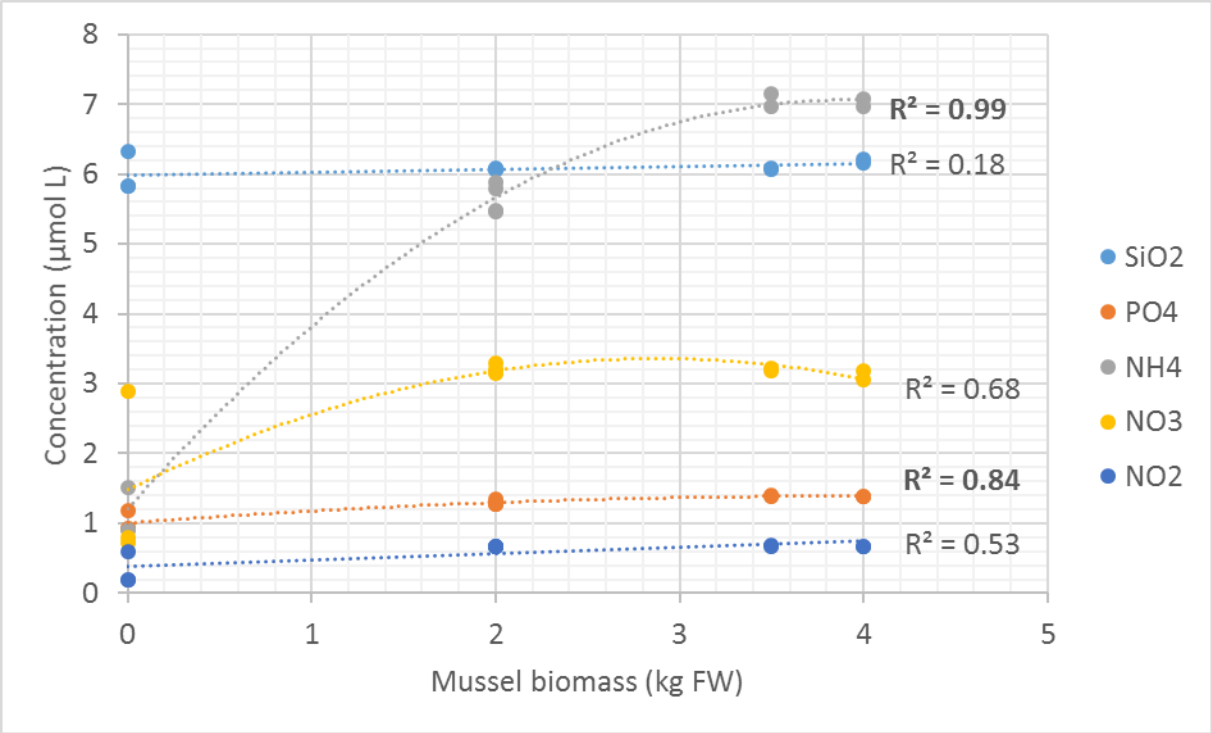

Figure 13: Concentration of dissolved silicate (blue), phosphate (orange), ammonium (grey), nitrate (yellow) and nitrite (dark blue) in the pre-trial (µmol L^{-1}) as a function of mussel biomass added to the tank (kg FW). Dotted lines are based on polynomial regression.

3.4 *Ulva* spp. productivity in monoculture and co-cultivation

Productivity rates of *Ulva* spp. reared for one month in co-cultivation with blue mussels and in monoculture were comparable. No significant differences were found between treatments (ANOVA, $p > 0.05$); *Ulva* spp. specific growth rate (SGR) was 7.7 ± 0.86 and 7.8 ± 0.89 % DW d^{-1} in mono and seaweed mussel co-cultivation respectively (Table 7).

The dry weight content of *Ulva* spp. reared in monoculture was 23.3% of fresh weight (FW) of the seaweed biomass. In co-cultivation with blue mussels, dry weight content reduced to 20.9% FW. Therefore, productivity expressed in DW was 90.4 and 80.0 kg DW ha^{-1} d^{-1} in monoculture and co-cultivation respectively (Table 7).

The C:N ratio of the Ulva biomass (tanks containing only seaweed) measured at the start of the experiment (31/07/2018) was 17.2 (SD=2.35). At the end of the experiment (on 28/08/2018) the C:N ratio measured in tanks containing just seaweed and seaweed in combination with mussels was clearly lower (13.2 and 10.4 respectively, Table 7, Fig. 14). Although the C:N ratio in seaweed cultivated in combination with mussels was slightly lower than without mussels, this result was not significant (Table 7).

Table 7: Ulva spp. productivity rate and biomass composition parameters

Ulva weight (W), specific growth rate (SGR), dry weight (DW), ash free dry weight (AFDW) (unit, mean ± SD, n=4). Letters indicate statistical significant difference between treatments, the absence of letters indicate that no statistical significant difference was observed.

	Unit	*Ulva* spp. monoculture	*Ulva* spp. Mussel co-cultivation
Productivity	kg FW ha^{-1} d^{-1}	388.1 ± 92.1	383.0 ± 102.5
Productivity	kg DW ha^{-1} d^{-1}	90.4 ± 21.5	80.0 ± 21.4
N -assimilation	mM N m^{-2} d^{-1}	38.0 ± 3.0	33.6 ± 1.7
SGR	% DW d^{-1}	7.7 ± 0.86[a]	7.8 ± 0.89[b]
DW	% of FW	23.3 ± 3.03	20.9 ± 0.66
AFDW	% of DW	68.6 ± 3.12	66.4 ± 1.12
C:N ratio	-	13.2 ± 2.80	10.4 ± 1.29

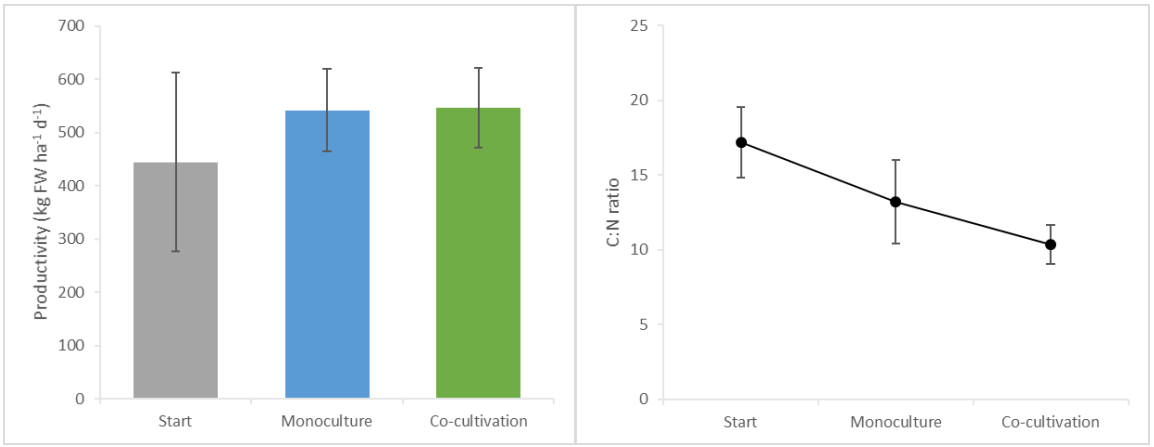

Figure 14: C:N ratio (black dots, no unit, mean SD, n=3) and productivity rate (kg FW ha^{-1} d^{-1}, mean SD, n=4) for Ulva spp. at the beginning of the experiment (grey) and after one month of monoculture (blue bar) and one month of co-cultivation with mussels (green bar).

3.5 Nutrient uptake *Ulva*-mussel experiment

At the end of the experiment (t = 30 days) nitrogen uptake was measured in all tanks containing cultured *Ulva* spp., either in monoculture or co-cultivation with mussels (Table 5). Mean *Ulva* spp. ammonium removal in the seaweed tanks was highest, 56% and 66% (33.6 or 38.0 mM N m^{-2} d^{-1} with and without mussel co-cultivation with no significant difference between the two treatments (Table 6, ANOVA, $p>0.05$). An increase in ammonium is expected in the mussel-seaweed tanks based on the presence of mussels. In order to correct for the increased ammonium concentration the ammonium uptake is measured. Surprisingly, no addition of ammonium was found in the tanks with mussels, and the mussel tanks even showed a reduction (uptake) of NH_4 (purple bars). Ammonium removal was found to some extent in the control tanks (yellow and purple bars, Fig. 15), which is not very surprising. However, it is surprising to find ammonium uptake in the mussel only tanks. PO_4 was removed in seaweed and seaweed plus mussel tanks. A high uptake of SiO_2 was found in the mussel tank (Fig. 15).

Table 5: Concentrations of Dissolved Inorganic Nutrients (DIN)

DIN concentration in pre-trial header (IN) and tanks with 4 kg of mussels (OUT) (equal units; mean, n=1-2) and in the tanks in inflow (IN) and outflow (OUT) (µmol P-PO_4 L^{-1}; µmol Si-SiO_2 L^{-1}; µmol N-NH_4^+ L^{-1}; µmol N-NO_2 L^{-1}; µmol N-NO_3 L^{-1}; mean, n=3). Dif: indicates difference IN-OUT.

DIN		Pre-trial 20/06/2018	Nutrient uptake experiment 31/08/2018				
		Mussels	Ulva mono	Ulva co-cult.	Mussels	Control	Header
NH_4^+	IN	4.04	6.07	6.31	5.64	5.86	6.85
	OUT	7.07	2.08	2.76	4.48	5.25	-
	Dif	**-3.03**	**3.99**	**3.55**	**1.16**	**0.61**	**-**
NO_2	IN	0.6	0.75	0.78	0.76	0.76	0.75
	OUT	0.67	0.32	0.42	0.76	0.76	-
	Dif	-0.07	0.43	0.36	0	0	-
NO_3	IN	2.89	3.43	3.56	3.63	3.53	3.42
	OUT	3.05	1.42	1.69	3.31	4.08	-
	Dif	**-0.16**	**2.01**	**1.87**	**0.32**	**-0.55**	**-**
PO_4	IN	1.18	1.89	1.91	1.9	1.91	1.91
	OUT	1.38	1.57	1.63	1.87	1.96	-
	Dif	**-0.20**	**0.32**	**0.28**	**0.03**	**-0.05**	**-**
SiO_2	IN	6.32	4.17	4.19	4.28	4.26	4.36
	OUT	6.16	3.58	3.72	2.96	3.78	-
	Dif	**0.16**	**0.59**	**0.47**	**1.32**	**0.48**	**-**

Table 6: DIN uptake

Dissolved inorganic nutrient (DIN) uptake (%, mean ± SD, n = 3). Significant differences are indicated with letters (for example a is sign. different from b).

DIN	Ulva monoculture	Ulva co-cultivation	Mussels	Control	ANOVA p
NH_4	65.8 ± 5.3^a	56.3 ± 4.9^a	20.5 ± 4.3^b	9.9 ± 13.8^b	< 0.001
NO_2	56.6 ± 6.9^a	46.3 ± 15.6^a	5.7 ± 3.4^b	-0.9 ± 1.5^b	< 0.001
NO_3	58.8 ± 5.5^a	52.1 ± 14.0^a	8.8 ± 3.5^b	-15.7 ± 3.5^c	< 0.001
PO_4	17.1 ± 2.5^a	14.7 ± 7.3^a	1.4 ± 1.2^b	-3.0 ± 4.3^b	< 0.01
SiO_2	14.1 ± 1.8^b	11.3 ± 2.3^b	31.0 ± 6.2^a	11.3 ± 2.9^b	< 0.001

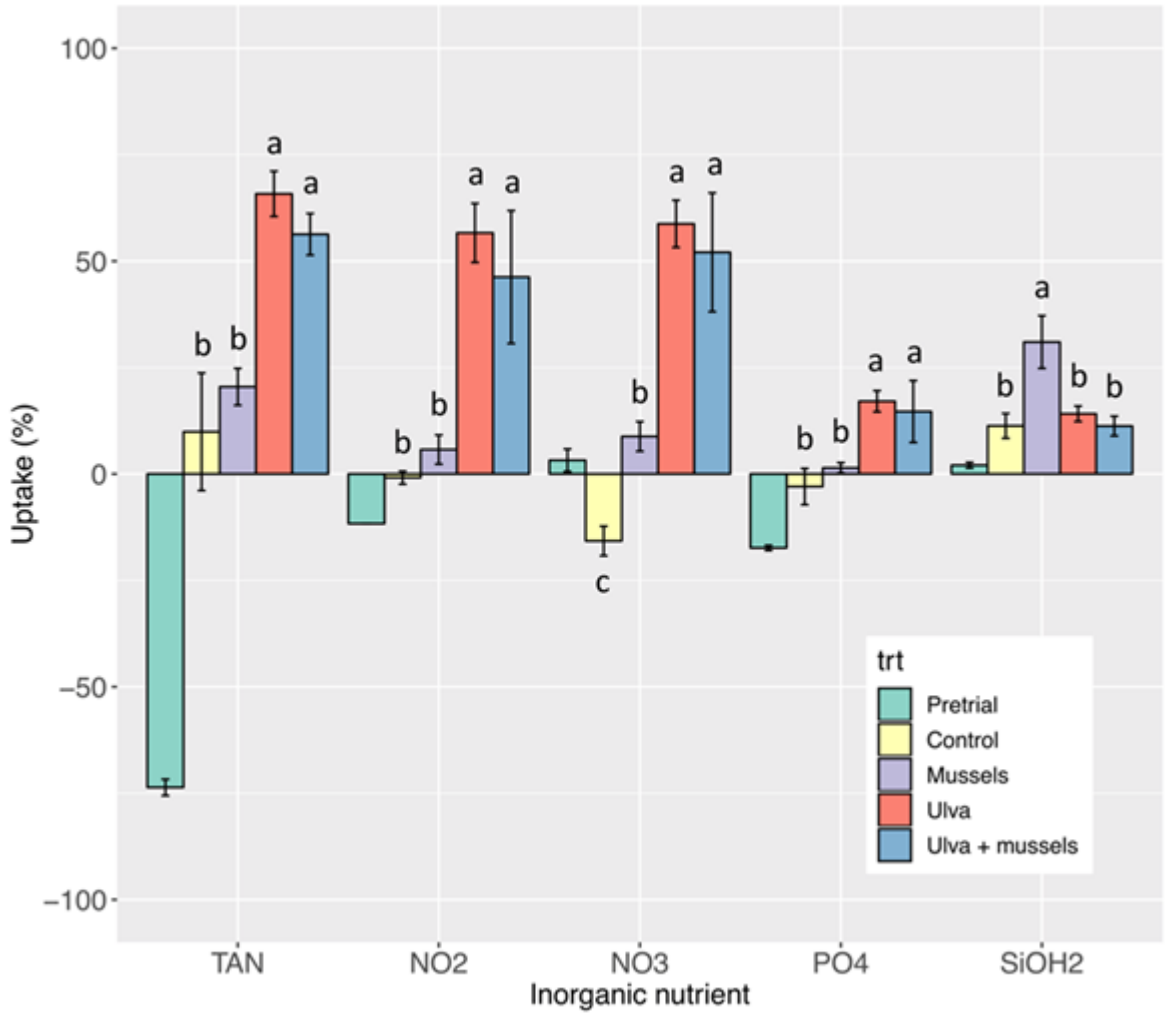

Figure 15: DIN uptake (% of inflow concentration removed, mean ± SD, N=3) for each experimental treatment; Control (no mussels + no Ulva) (yellow), Mussels (purple), Ulva (red), Ulva + mussels (blue). Pre-trial (green) is percentage nutrient addition in the tanks containing mussels when compared to header tanks (N=2). Significant differences (p<0.01) between experimental treatments (excluding pre-trial) are indicated with letters (for example: a is sign. different from b).

4 Discussion and recommendations

4.1 Main outcomes

The pre-trial experiment showed that the addition of 4kg mussels almost doubled the ammonium concentration measured in the tanks at a flow rate 0f 32.5 mL sec^{-1} , and could result in higher growth of *Ulva* under nitrogen limited conditions and/or higher availability of the low energy requesting N form (ammonium). The productivity rates of *Ulva* were, however, comparable for both treatments (with and without mussels). The C:N ratio in *Ulva* biomass did not significantly differ between treatments but was lower at the end of the experiment, indicating N was limited at the start of the experiment and limitation decreased during the experiment (concordantly with increasing N concentration over summer [Fig. 9]). The lack of significant difference between treatments might indicate that *Ulva* is not affected by higher nutrient concentration nor by availability of ammonium. However the nutrient uptake experiment showed that ammonium concentrations were not elevated by the presence of mussels. This is not in accordance to our expectation, but is in line with the absence of enhanced growth.

4.2 Benefits of *Ulva* spp. and mussel co-cultivation

Pre-trial results showed >70% increase of ammonium-N concentration in the tanks, validating our assumption that the experimental set-up enabled the mussels to be used as additional nitrogen source for seaweed growth. Ammonium concentrations in the flow-through tanks of the experimental set-up increased with 3.12 µmol-N L^{-1} when 4 kg FW mussels were added. This is equal to an ammonium-N excretion rate of 1 µmol-N g^{-1} DW h^{-1}, as is also found in literature (Jansen *et al.*, 2012, Smaal & Vonck, 1997), assuming a mussel biomass conversion ratio of 6.6 SFDW/FW (Ricciardi *et al.*, 1997). However, nutrient uptake (difference between in- and outflow NH_4 levels in the tanks) determined after the 30 days of the *Ulva* spp. and mussel co-cultivation experiment did no longer support the assumption of ammonium release by the mussels. Moreover, a de- rather than increase in NH_4 concentrations was detected when comparing in- and outflow in the mussel only tanks. This does not match increased ammonium-N concentrations as reported in proximity of mussel longlines by field observations (Jansen et al., 2012, Smaal, 2002).

The lack of nitrogen enhancement coincides with the lack in observed productivity increase for *Ulva* cultured in combination with mussels. No difference was found in productivity rates of *Ulva* cultivated with and without the addition of mussels, although 4 weeks are deemed sufficient to note an increase in *Ulva* productivity. The productivity rate of the *Ulva* (80-90 kg DW ha^{-1} d^{-1}) can be considered normal as it is in the same range of *U. lactuca* growth in temperate regions as reported in literature (Bruhn et al., 2011, Debusk et al., 1986, Robertson-Andersson *et al.*, 2008a)see also table 1) despite exceptionally warm and dry conditions during July-September 2018. Furthermore, C:N ratios were similar for *Ulva* cultivated in mono or co-culture with mussels. Though not statistically different, lowest ratio was found in combination with mussels, which is in line with an expected higher N supply and potentially indicative for the higher net ammonium uptake by *Ulva* cultivated in combination with mussels.

The question is whether the lack in growth difference and similarity in C:N ratios between monoculture and co-cultivation treatments is because N was not limited and ammonium is not preferred by *Ulva*, or if N concentrations were not enhanced by addition of mussels. The nutrient uptake experiments were only performed once (point measurement) and do not provide evidence whether N addition has been low throughout the experiment, but the lack of enhanced N concentration in mussel tanks highlight an unexpected result.

Several factors were considered that could have contributed to the unexpected result. Although no evidence to support these theories was found and these factors are merely hypothetical they will be addressed here briefly. 1) We considered the mesocosm set-up which may facilitate competition for nutrients by fouling micro-organisms such as autotrophic periphyton (microalgae, bacteria, fungi), heterotrophic bacteria or phototrophic fouling organisms that have accumulated despite regular cleaning. In this scenario, ammonium is excreted by the mussels but strong competition for nitrogen impedes assimilation by macroalgae (*Ulva* spp.). Fouling was heaviest in the control tanks where no competition for nutrients from macro-algae existed. Considering that ammonium uptake in fouled control tanks was low it is unlikely that the micro-organisms in the *Ulva* plus mussel cultivation tanks account for a large proportion of the ammonium uptake..

2) Aerobic nitrification by so-called nitrifying bacteria converts ammonium into nitrate in an aerobic environment. This process is abundant in marine systems (Wuchter *et al.*, 2006). In this transformation process ammonium is transformed into nitrite (NO_2^-, toxic and nitrite (NO_2^-) is converted into nitrate (NO_3^-). However this should result in an increase in NO_3^- which was not detected. Again, although these processes may have occurred at a small scale they are unlikely to account for the breakdown of the ammonium excreted by mussels.

A potential explanation of low ammonium-N excretion could be due to the mussels. 3) Mussels could have been affected by limited food availability. Mussels have shown to adapt to a range of 10 - 90 mg L^{-1} (SPM) (Bayne, 1998, Hawkins *et al.*, 1998). SPM levels ranged between 5.3 – 11.7 mg L^{-1} and decreased during August 2018 (Fig. 12, note that SPM levels as measured in this study are potentially underestimated due to rinsing with fresh water instead of HCO_2NH_4). However, large variation in SPM levels measured in the inflow headers at 24th of August could be an indication that header conditions (turbulence, sedimentation processes) might have been a factor contributing to changes in SPM throughout the experiment, as was accounted for by the randomized set-up of the experimental design. On the other hand, *M. edulis* is adapted to low phytoplankton concentrations and filtrates continuously when the food concentration reaches above a trigger value of about 0.5 to 0.9 µg Chl *a* L^{-1} (Pascoe *et al.*, 2009, Riisgard *et al.*, 2014). Mean Chl *a* concentrations in previous years measured at a nearby station were generally above 2.5 µg Chl *a* L^{-1} during the experimental months (July –September) (Fig. 10) making food shortage a less likely candidate.

4) Although the mussels did not show significant growth over the 5 week period of the experiment the relatively high survival rates (>80%, t = 30 days) indicate all environmental parameters in the culture tanks (temperature, pH, dissolved oxygen, SPM) were within the acceptable range for healthy mussel metabolism. During tank observations, no signs of stress were observed; the majority of shells were open when submerged and closed when mussels were taken out of the water. Mussels that remained open above water were removed from the tanks directly after observation to avoid contamination from degrading mussel biomass. Nonetheless heat stress in mussels is well documented and although temperature stress may potentially increase metabolism and ammonium excretion to a certain extent, lethal temperatures of 30-31 ºC have been indicated for *M. edulis*, while respiration rates of North Sea *Mytilus spp.* population decline above 24 ºC (Jansen *et al.*, 2007, Wallis, 1975). It is therefore possible that the extreme weather conditions (temperatures over 35 ºC) have had an effect on the mussels metabolism and thereby the excretion of ammonium. The remaining question is then whether the mussel metabolism itself may have been impaired throughout the experimental period, even if impaired they would have still excreted ammonium to some extent, considering the mussels appeared in good health with high survival rates this explanation is unlikely.

It remains unknown why no enhanced ammonium concentrations were observed during the end of the experiment, and whether or not this has occurred throughout the experimental period, subsequently leading to little variation in growth and C:N ratios between mono- and co-culture treatments.

4.3 Relevance for commercial cultivation

Although this study does not show a clear benefit from co-cultivation of *Ulva* with mussels to increase productivity, a potential benefit can also not be ruled out as a result of uncertainties in the outcomes and absence of ammonia excretion during the co-cultivation experiment in comparison to the pre-trial.

Lower C:N ratios (though not significant) in co-cultivated *Ulva* may hint towards higher uptake of nitrogen in treatments with mussels.

4.4 Recommendations

More frequent DIN measurements may have given a clearer pattern and potentially resolve uncertainties following the nutrient uptake experiments. Budget calculations using simple models to understand the system can provide insight in whether the mesocosms used in this study could have acted as a complex ecotype which is not fully understood. Unforeseen extreme conditions such as the high temperatures may have had consequences for the mussel metabolism. Repeat measurements throughout the summer season could resolve this. A more direct or theoretical approach can be chosen by which the mussels itself are not included in the growth experiment but instead ammonium concentrations are increased directly. Greater effects may be obtained by using young sporophytes that appear to be more susceptible to the uptake of added nutrients (Mortensen, 2017).

5 Conclusion

Co-cultivation with mussels did not increase *Ulva* production. Despite the addition of mussels was deemed sufficient to increase initial ammonium concentrations in a pre-trial experiment. This result was supported by the C:N ratios of the *Ulva* biomass and the results from the nutrient uptake experiment. It remains unknown why no enhanced ammonium concentrations were observed during the end of the experiment, and whether or not this has occurred throughout the experimental period, subsequently leading to little variation in growth and C:N ratios between mono- and co-culture treatments.

6 Quality Assurance

Wageningen Marine Research utilises an ISO 9001:2015 certified quality management system. This certificate is valid until 15 December 2021. The organisation has been certified since 27 February 2001. The certification was issued by DNV GL.

Furthermore, the chemical laboratory at IJmuiden has NEN-EN-ISO/IEC 17025:2005 accreditation for test laboratories with number L097. This accreditation is valid until 1th of April 2021 and was first issued on 27 March 1997. Accreditation was granted by the Council for Accreditation. The chemical laboratory at IJmuiden has thus demonstrated its ability to provide valid results according a technically competent manner and to work according to the ISO 17025 standard. The scope (L097) of de accredited analytical methods can be found at the website of the Council for Accreditation (www.rva.nl).

On the basis of this accreditation, the quality characteristic Q is awarded to the results of those components which are incorporated in the scope, provided they comply with all quality requirements. The quality characteristic Q is stated in the tables with the results. If, the quality characteristic Q is not mentioned, the reason why is explained.

The quality of the test methods is ensured in various ways. The accuracy of the analysis is regularly assessed by participation in inter-laboratory performance studies including those organized by QUASIMEME. If no inter-laboratory study is available, a second-level control is performed. In addition, a first-level control is performed for each series of measurements.
In addition to the line controls the following general quality controls are carried out:

- Blank research.
- Recovery.
- Internal standard
- Injection standard.
- Sensitivity.

The above controls are described in Wageningen Marine Research working instruction ISW 2.10.2.105. If desired, information regarding the performance characteristics of the analytical methods is available at the chemical laboratory at IJmuiden.

If the quality cannot be guaranteed, appropriate measures are taken.

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Justification

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The scientific quality of this report has been peer reviewed by a colleague scientist and a member of the Management Team of Wageningen Marine Research

Approved: Dr. ir. J. Wijsman
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