The role of cold resistance and burial for winter survival and spring initiation of an *Ulva* spp. (Chlorophyta) bloom in a eutrophic lagoon (Veerse Meer lagoon, The Netherlands)

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Abstract: In the eutrophic Veerse Meer lagoon (The Netherlands) large amounts of free-floating *Ulva* thalli are present from May to October. In winter however, no algae seem to occur in the lagoon. Sexual reproduction appears to be negligible as spore formation and germling growth are observed only sporadically. Results of a field survey showed that in winter, viable *Ulva* biomass is present buried in the sediment of the shallow parts of the lagoon. Freezing experiments demonstrated that the algae are able to survive temperatures of -5 ^{0}C for two weeks when kept in darkness. In spring, the buried *Ulva* thalli are liberated out of the sediment to initiate a bloom. A field experiment indicates that bioturbation by the lugworm *Arenicola marina* does not stimulate the release of the thalli. Burial and winter survival can explain the rapid increase in *Ulva* biomass in spring and suggests that the initial spring biomass is one of the major factors determining the maximal biomass in summer.

Keywords: *Ulva*, eutrophication, freezing resistance, life history strategy, bioturbation
Abridged title: Winter survival and spring initiation of an *Ulva* bloom

Introduction

The mass development of macroalgae is one of the striking features of shallow eutrophic coastal waters. In most cases the blooms consist of green macroalgae of the genera *Ulva* and *Enteromorpha* (e.g. Ho, 1981; Lavery *et al.*, 1991; Fletcher, 1996). Several conditions have been put forward as being favourable for blooms of these macroalgae. For example, the waterbody must receive substantial nutrient supplies, and the residence time of the water must be at least a few days (Morand & Briand, 1996). In addition, the morphology of most bloom-forming species ensures rapid nutrient uptake (Littler & Littler, 1980; Hein *et al*, 1995). Furthermore, macroalgae have an advantage over microalgae because their capacity to store nutrients is much higher (Pedersen & Borum, 1996). Therefore, they can make better use of pulse supply of nutrients, which is often the case in eutrophicated areas. The life cycle of the species may also play an important role in the formation of a macroalgal bloom. For example, vegetative propagation and the ability to form free-floating or loose lying populations capable of almost unlimited vegetative growth have been related to the mass development of *Cladophora monteagneana* in Australia (Gordon *et al.*, 1985).

Ulva blooms regularly occur in the Veerse Meer lagoon in the Netherlands. This brackish lagoon is a former part of the river Rhine delta. In the early nineteensixties, two dams were constructed that separated this estuary from North Sea and river influence. The lagoon has deep gullies (up to 25 m) and shallow areas (depth of less than 5 m). The latter comprise 60 % of the area (Nienhuis, 1992). The bottom consists of sand or silt depending on the location. Dikes and some small loose stones and shells are the only hard substrates present. To increase the storage capacity for discharge of nutrient-rich polder water an artificial low water level (Mean Standard Sea Level - 0.70 m) is maintained during winter (1 November - 1 April). In summer, the water level is raised again to Mean Standard Sea Level.

In winter, the lagoon seems to be devoid of macroalgae. Nevertheless, in summer and fall large amounts of free-floating *Ulva* thalli (up to 350 gram ash-free dry weight (AFDW) m^2) are present on the sediment surface of the shallow areas (Malta & Verschuure, in press). Sexual reproduction of *Ulva* appears to be negligible in the Veerse Meer lagoon, spore formation is rarely observed. Only sporadically did we find germlings attached to hard substrates (P. Kamermans, E. Malta & J.M. Verschuure, personal observations). If sexual reproduction is not important, then the spring bloom must be based primarily on regeneration of old vegetative fragments.

Since the biomass initiating the bloom is not present on the surface of the sediment in the shallow areas it was unclear where the initiating material came from. In early spring of 1992, *Ulva* biomass (2 g AFDW m^2) was found to be buried in the sediment of a shallow location in the Veerse Meer lagoon (Malta, 1993). If these thalli are able to resume growth in spring, overwintering in the sediment could be a survival strategy of *Ulva*. Santelices *et al.* (1984) reported survival of *Gracilaria* thalli buried in sand for up to six months. The winter that preceded the observation on buried *Ulva* biomass (1991/1992) had only 6 days with minimum temperatures below - 5 ^{0}C and can be considered mild (data from KNMI - the Royal Dutch Meteorological Institute). Cold periods of 10 to 20 days with minimum temperatures of - 5 ^{0}C or more are common during severe winters (KMNI, data from 1960-1995). In these cases the lagoon is partly, or completely, frozen. Vermaat & Sand-Jensen (1987) showed that *Ulva* was unable to survive freezing to - 18 ^{0}C in the laboratory, but it did resume growth when collected in the field from fjord ice. It is thus unknown under which circumstances vegetative *Ulva* parts may be able to survive freezing. *Ulva* is capable of heterotrophic growth for at least 41 days (Markager & Sand-Jensen, 1990), and is thus able to survive prolonged periods of darkness. However, we do not know if the anoxic and dark conditions produced by burial in the sediment may affect survival of *Ulva* under freezing conditions.

For initiation of the *Ulva* bloom in spring to occur the overwintering thalli have to get onto the sediment surface. A possible machanism can be digestion survival. Santelices & Ugarte (1987) found regeneration of new cells from *Ulva rigida* thalli fragments they collected from faecal pellets of herbivorous molluscs. The lugworm (*Arenicola marina*) is common at the shallow areas of the Veerse Meer lagoon. These animals live in an L-shaped burrow, where they ingest sediment with organic material at the lower end of the head-shaft and deposit faeces at the surface of the sediment through the tail-shaft (Cadée, 1976). It may be possible that the lugworm is feeding on the buried *Ulva* material and that algal fragments survive passage through the digestive tract. When temperature rises in spring, bioturbation by the lugworm increases. The feeding behaviour of the lugworm could bring undigested *Ulva* fragments to the surface of the sediment and thus initiate the bloom. On the other hand, the opposite effect is also possible, deposition of faeces mounts produced by the lugworms on the sediment surface may rebury already uncovered thalli.

In this paper, we present the results of experiments that were carried out to test the following hypotheses: 1. *Ulva* biomass that overwinters in the Veerse Meer lagoon buried in the sediment is viable. 2. *Ulva* is

able to survive freezing in darkness and under anoxic conditions. 3. In spring, the bloom is initiated by *Ulva* fragments emerging at the sediment surface by bioturbation of the buried material.

Material and methods

Viability of overwintering Ulva

In February 1995, 2 shallow sites (0.10 m water) in the Veerse Meer lagoon were sampled for the presence of *Ulva* (site 1: N 51^0 32' 63" E 3^0 46' 89"; site 2: N 51^0 32' 61" E 3^0 50' 79"). The locations were chosen because it was known from earlier work that an *Ulva* bloom developed at those sites (Malta & Verschuure, in press). At each site, the upper 20 cm of the sediment of five 0.05 m^2 areas was sieved (2-mm mesh size). *Ulva* tissue collected was cultured to test the viability of the material. Discs of 2.2 cm diameter were cut out of the thalli. Five discs were used per incubation and four replicates were incubated for each location. Erlenmeyer flasks were filled with 1 l filtered (0.45 mm) water from the Veerse Meer lagoon and aerated continuously. All flasks were placed under the same natural light conditions in a NE window sill. The light cycle was 13 hours dark and 11 hours light and the average irradiation was 260 mE m^2 s^{-1}. The medium was renewed with fresh Veerse Meer water every two weeks. Mean salinity was 11 (psu) and the water temperature ranged from 12 to 29 ^{0}C. Every 3-13 days the total wet weight of the 5 discs in each replicate was determined. Before weighing, the discs were blotted between two paper sheets to remove adhering water. Growth rate (m) was assumed to be exponential and was calculated as:

$$\mu = \frac{\ln(W_t) - \ln(W_0)}{t}$$

in which W_0 is the initial and W_t the final wet weight after t days of incubation.

Freeze tolerance

To study the effect of darkness and anoxia on survival under freezing conditions, discs of 2.2 cm diameter were cut out of the *Ulva* thalli collected in February 1995 at site 1in the Veerse Meer lagoon. Treatments involved placing 5 *Ulva* discs in 1 l of filtered (0.45 mm) Veerse Meer lagoon water in the dark or in a 12 h dark and 12 h light cycle (100 mE m^2 s^{-1}). In addition, 5 discs were buried 5 cm in anoxic sediment (natural sediment collected in the Veerse Meer lagoon) or in oxic sediment (bird cage sand), both covered with a 2-cm layer of filtered lagoon water. Two replicates were incubated for each of the 4 treatments. All treatments were acclimatised at 2 ^{0}C for 1 week, transfered to -5 ^{0}C for 2 weeks and after that acclimatised again for 1 week at 2 ^{0}C. Viability after this 4-week period was tested by culturing the discs as described above. The light cycle during culturing was 11.5 hours dark and 12.5 hours light and the average irradiation was 490 mE m^2 s^{-1}. To check the presence or absence of oxygen in the different sediment types, oxygen concentration was measured for each 0.5 mm depth with a Clark oxygen microelectrode mounted on a micromanipulator. Measurements were done at 2 ^{0}C with an autoranging picoammeter.

Bioturbation effect

In March 1995, a total of 10 cages were placed at site 1 in the Veerse Meer lagoon (water depth of 0.10 m in winter and 0.80 m in summer). Iron frames were covered at all four sides with wire mesh (mesh size of 1 cm) leaving the top and bottom open. The cages were 1 m high and the bottom covered 1 m^2. In 5 cages a 1-mm mesh screen was inserted horizontally into the sediment at approximately 10-cm depth to force lugworms out of experimental plots. This method has been used succesfully by Reise (1983) and by Philippart (1994). As a control treatment the other 5 cages were left without the screen. To correct for the effect of sediment disturbance when placing the screens, the sediment in the control cages was also removed and shovelled back into the holes. Twice a week, the number of *Arenicola* faeces mounts in each

cage was counted with the aid of an underwater viewer. The number of faeces mounts shows good correlation with the actual number of lugworms present in the sediment ($R = 0.783$, $n = 9$, $P < 0.05$, Kamermans, unpublished data). At the same time as counting the faeces mounts, the *Ulva* coverage within the cages was visually estimated. The duration of the experiment was 63 days, until 15 May 1995. At the end of the experiment all *Ulva* biomass present in the cages was harvested and the wet weight was determined after 1 min rotation in a laundry centrifuge.

Statistical analyses

The effects of different pre-culture conditions and different culture times on the growth rate of *Ulva* was tested with two-way analyses of variance (ANOVA). The significance of differences between treatments was analysed with a Tukey-Kramer procedure as post-hoc test (Sokal & Rohlf, 1995). In the same manner the significance of differences in *Ulva* coverage and lugworm densities with or without a mesh screen buried in the sediment and different sampling dates was tested. A student t-tests was used to test the effect of mesh screen cages on *Ulva* wet weight at the end of the experiment. Assumption of normal distribution of the dependent variables was examined using box plots. Data were tested for heteroscedacity with a Bartlett's test for homogeneity of variances. Data that scored significantly were *Ulva* coverage, *Ulva* wet weight and number of *Arenoicola* faeces mounts. These data were log-transformed ($\log x + 1$), which considerably improved the data: their distribution was normal and they did not show significant heteroscedacity anymore. Statistical tests were carried out with those log-tranformed data. A significance level of 5% was used in all tests. The statistical analyses were conducted using the STATISTICA programme.

Results

Viability of overwintering Ulva

The viability test yielded growth rates of 0.03 - 0.08 d^{-1} (Fig. 1). Growth rates achieved with *Ulva* from the two sampling sites did not differ significantly (Table 1). These results show that in early spring 1995 *Ulva*

fragments that had overwintered buried in the sediment of the Veerse Meer lagoon were able to resume growth.

Freeze tolerance

Results of the oxygen measurements show that in the natural sediment oxygen concentration is reduced to zero mM at a depth of 0.5 cm while the concentration in the bird cage sand stayed comparable to the concentration in the overlying water upto a depth of more than 5 cm (Fig. 2). The viability test showed that *Ulva* spp. can survive a period of 14 days at -5 ^{0}C (Fig. 3). The different treatments resulted in significantly different growth rates (Table 2). Growth was fastest after freezing in natural anoxic sediment. This was followed by freezing in oxic sediment and then freezing in water under dark conditions. No recovery was found after freezing in water exposed to a 12 hour light and 12 hour dark regime where the *Ulva* discs had turned completely white. The post-hoc test showed significant differences in growth rates between the light-water treatment and the other three treatments (Table 3). These results demonstrate that light conditions prevent the survival of *Ulva* during freezing. Burial of *Ulva* in the sediment produces dark conditions. Thus, in the field, buried *Ulva* may be able to survive freezing conditions during winter.

The role of oxygen in survival is not clear. Growth rates after the oxic-sediment treatment did not differ significanty from those after the anoxic-sediment treatment (Table 3). However, growth rates after the dark-water treatment were significantly lower than those after the anoxic-sediment treatment (Table 3). Growth rates after the dark-water treatment did not differ significantly from the oxic-sediment treatment (Table 3), showing that, when frozen in water and in sediment, darkness and oxic conditions yield similar survival results.

Bioturbation effect

In the mesh-screen cages, faeces-mount density was significantly lower than in the control treatment without mesh screen (Fig. 4a, Table 4). This shows that the horizontal insertion of the mesh screen effectively reduced the density of *Arenicola*. Treatment and time, as well as their interaction, had a significant effect on *Ulva* coverage (Table 5). Coverage was significantly higher in the cages with a mesh screen bottom (Fig. 4b, Table 5). This can not be caused by sediment pertubation at the beginning of the experiment, since the sediment was equally mixed in all cases. At the end of the experiment, *Ulva*

biomass was also significantly higher in the screen-bottom cages (Fig. 4c, Student t-tests, $P<0.01$). These results indicate that lugworms do not play a role in uncovering *Ulva* thalli from the sediment. In contrast, they seem to have a negative effect on *Ulva* biomass.

Discussion

Our results show that in winter, viable *Ulva* biomass is present buried in the sediment of the shallow parts of the Veerse Meer lagoon. We observed that most fragments were found near holes left by foraging herbivorous birds such as coots and swans which are abundant in the lagoon (Coosen *et al.*, 1990). This suggests a possible explanation for burial of *Ulva*. Macroalgae accumulate in these depressions after which the holes may be filled with sediment by water motion. Overwintering of *Ulva* thalli is in contrast with the general view that *Ulva* is an annual plant starting with germlings attached to hard substrates (Bliding, 1968). The occurence of large loose-lying thalli that do not seem to be related to the presence of nearby hard substrates certainly supports the idea of a perennial life style.

Our freeze experiments demonstrated that *Ulva* tissue is able to survive freezing when kept in darkness. Several other macroalgal species are able to withstand a certain amount of freezing. This freeze tolerance has been attributed to the presence of antifreeze substances in their cells (Lüning, 1990). Karsten *et al.* (1990) observed that Antarctic green algae contained high amounts of DMSP, a supposed antifreeze compound. DMSP formation has also been found in *Ulva rigida* (Karsten *et al.*, 1991). Karsten *et al.* (1991) also showed that the DMSP concentration in *U. rigida* did not decrease under conditions of darkness. When frozen in light, the *Ulva* discs of our freeze experiment did not survive and turned white. This may be caused by photodamage as low temperatures reduce the ability of algae to use light. Under these circumstances excess light energy may damage the photosynthetic apparatus of the algae (Davison, 1991).

Vermaat & Sand-Jensen (1987) presented contradicting results on freeze tolerance of *Ulva lactuca*. On the one hand, they showed that 10 days of freezing at -18 ^{0}C reduced the viability of *Ulva lactuca* to virtually nil, but, on the other hand, when they collected *Ulva lactuca* frozen in ice from the the field it was able to resume growth. The authors suggest that, in the field, gradual freezing may allow the alga to

acclimate to the lower temperatures. The freeze experiment of Vermaat & Sand-Jensen (1987) was carried out with *Ulva* thalli that were frozen in the dark under oxic conditions. In the Veerse Meer lagoon overwintering may take place buried in the sediment. In that case the *Ulva* thalli are subjected to darkness and anoxic conditions. Vermaat & Sand-Jensen (1987) demonstrated that at 4 ^{0}C, anoxia lead to an increase in respiration and a decline in growth capacity of *Ulva lactuca*. However, respiration seems to be more strongly inhibited by low temperatures than photosynthesis (Kirst & Wiencke, 1995). A small decrease in temperature may considerably lower respiration rates and reduce the negative effect of anoxia. Therefore, we suggest that anoxic conditions at temperatures lower than 4 ^{0}C (that are experienced during freezing) may not be detrimental to the survival of *Ulva* thalli. Freezing under anoxic conditions was not included as a treatment in the experiments of Vermaat & Sand-Jensen (1987).

Recovery of *Ulva* tissue was best in natural anoxic sediment. Therefore, burial in natural sediment seems to offer the a good overwintering location for *Ulva*. The winter of 1994/1995 that preceded our experiments had only 4 days below -5 ^{0}C (KNMI). The winter of 1995/1996 had 28 days with temperatures below -5 ^{0}C and was thus more severe (KNMI). A preliminary survey in March 1996 showed that *Ulva* tissue was still present buried in the sediment of the shallow areas of the Veerse Meer lagoon (Kamermans *et al.*, 1996). Apparently, *Ulva* tissue is able to withstand freezing in sediment for such a long period.

In spring, buried *Ulva* thalli are not moved out of the sediment by bioturbation. Results of the cage-experiment indicate that lugworms had a negative effect on this process. The deposition of faeces mounts produced by the lugworms on the sediment surface may have reburied already uncovered macroalgae. Likewise, Philippart (1994) demonstrated a negative effect of lugworm density on seagrass survival and concluded that this effect was caused by sediment-reworking activities of lugworms resulting in burial of the seagrasses. It is more likely that wind-induced water motion frees the *Ulva* thalli out of the sediment. Recent investigations carried out in the Veerse Meer lagoon by Kamermans *et al.* (1996) support this view. In March 1996 large amounts of *Ulva* thalli were found buried in the sediment of site 1. The amount was substantial (about 90 g AFDW m^2 compared to 5 g AFDW m^2 in 1995). However, the spring of 1996 was very calm and, unlike in the spring of 1995, no thalli were uncovered from the sediment (Kamermans *et al.*, 1996). In March, April and May only 2 days had wind velocities higher than 15 m s^{-1}, compared to 19 days during the same period in 1995 (KNMI). This observation suggests that wind may play a role in uncovering *Ulva* thalli from the sediment.

Ulva biomass (up to 660 g AFDW m^{-2}) has been observed in the gullies of the Veerse Meer lagoon in summer (Hannewijk, 1988). Sand-Jensen (1988) also reports large amounts of *Ulva lactuca* occurring at great depths in eutrophicated estuaries and relates this to the capacity of *Ulva* to maintain growth at very low light intensities. In the Veerse Meer lagoon, *Ulva* was also present on the bottom of the deeper parts in winter (Kamermans, unpublished data). The gullies may thus represent another winter-survival location. A trap experiment showed that healthy looking adult *Ulva* thalli were floating near the egde of the shallow areas before resident vegetative fragments had emerged on the sediment surface of those areas (Kamermans, unpublished data). Currents may carry the deposited *Ulva* from the gullies to the shallow areas. This is supported by the fact that the largest amounts of *Ulva* were captured in the traps during the period of water-level change (Kamermans, unpublished data). In addition, the first *Ulva* usually appears on the shallow parts directly after the period of water-level change. Rising of the water level in spring may induce current-driven transport of *Ulva* from the gullies to the shallow areas.

It can be concluded that vegetative *Ulva* spp. parts that survive the winter initiate next years bloom. This can explain the rapid increase in *Ulva* biomass in spring and suggests that the initial biomass present in the Veerse Meer lagoon in spring may determine to a large extent the maximal biomass in summer. When this is the case removal of overwintering *Ulva* could be considered as a strategy to control the development of the macroalgal bloom in the Veerse Meer lagoon.

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Table 1. Statistical evaluation of influences of location (buried in sediment of two shallow sites) and culture time on growth rates of Ulva *in the viability test after winter survival. Values are degrees of freedom (df), mean square (MS) and probability (P) of ANOVA.*

source of variation	df	MS	P
location	1	0.000	0.613
day	2	0.002	0.230
location x day	2	0.001	0.325
error	18	0.001	

Table 2. Statistical evaluation of influences of treatment (anoxic sediment, oxic sediment, frozen water without light and frozen water with a 12 hour light and 12 hour dark regime) and culture time on growth rates of Ulva *in the viability test after freezing. Values are degrees of freedom (df), mean square (MS) and probability (P) of ANOVA.*

source of variation	df	MS	P
treatment	3	0.019	0.000
day	1	0.002	0.125
treatment x day	3	0.001	0.518
error	8	0.001	

Table 3. Matrix of pairwise comparison probabilities of Tukey Kramer post hoc test.

treatment	anoxic-sediment	oxic-sediment	dark-water	light-water
anoxic-sediment	1.000			
oxic-sediment	0.208	1.000		
dark-water	0.012	0.233	1.000	
light-water	0.000	0.002	0.030	1.000

Table 4. Statistical evaluation of influences of treatment (with or without mesh screen) and date on log-transformed number of Arenicola *faeces mounts. Values are degrees of freedom (df), mean square (MS) and probability (P) of ANOVA.*

source of variation	df	MS	P
treatment	1	143.5	0.000
day	10	0.2	0.724
treatment x day	10	0.1	0.955
error	88	0.4	

Table 5. Statistical evaluation of influences of treatment (with or without mesh screen) and date on log-transformed Ulva *coverage data. Values are degrees of freedom (df), mean square (MS) and probability (P) of ANOVA.*

source of variation	df	MS	P
treatment	1	15.13	0.000
day	10	3.32	0.000
treatment x day	10	0.76	0.038
error	88	0.37	

Figure legends

Fig. 1. Growth rates (d^{-1}) of *Ulva* during the viability test determined after the first 13 days, the middle 8 days and the last 3 days. Tissue was collected at two shallow locations (site 1 and 2). Bars represent mean value ± s.d. (n=4).

Fig. 2. Oxygen concentration (mM) at different depths in a beaker with submerged natural sediment (solid line) and a beaker with submerged bird-cage sand (dashed line). Depth 0 is the concentration in the overlaying water. Measurements were carried out at 2 ^{0}C.

Fig. 3. Growth rates (d^{-1}) of *Ulva* during the viability test determined after the first 6 days and the last 6 days. Tissue was subjected to four different treatments before culturing. Treatments involved keeping *Ulva* for two weeks at - 5 ^{0}C in anoxic sediment, in oxic sediment, in frozen water without light and in frozen water with a 12 hour light and 12 hour dark regime. Bars represent mean value ± s.d. (n=2).

Fig. 4. (a) Density of *Arenicola* faeces mounts (number per m^2) and (b) *Ulva* coverage (%) in cages with a 1-mm mesh screen inserted horizontally into the sediment at approximately 10 cm depth to force lugworms out of experimental plots (dashed lines and open symbols, mean value ± s.d., n=5) and in cages without a 1-mm mesh screen in the sediment as control treatment (solid lines and closed symbols, mean value ± s.d., n=5). (c) Wet weight of *Ulva* (grams) at the end of the cage experiment (mean value ± s.d., n=5).