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# Salinity-Induced Extinction of *Zostera marina* in Lake Grevelingen? How Strong Habitat Modification May Require Introduction of a Suitable Ecotype

**Marieke M. van Katwijk [1,*], Rens J. T. Cronau [2], Leon P. M. Lamers [2], Pauline Kamermans [3], Brigitta I. van Tussenbroek [1,4] and Dick J. de Jong [5,†]**

[1] Department of Environmental Science, Radboud Institute for Biological and Environmental Sciences, Heijendaalseweg 135, 6525 AJ Nijmegen, The Netherlands
[2] Department of Aquatic Ecology and Environmental Biology, Radboud Institute for Biological and Environmental Sciences, Heijendaalseweg 135, 6525 AJ Nijmegen, The Netherlands
[3] Wageningen Marine Research, Wageningen University and Research Centre, P.O. Box 77, 4400 AB Yerseke, The Netherlands
[4] Institute of Ocean Sciences and Limnology, Universidad Nacional Autónoma de México, Puerto Morelos 01000, Mexico
[5] Rijkswaterstaat, Sea and Delta, Ministry of Infrastructure and Water Management, Poelendaelesingel 18, 4335 JA Middelburg, The Netherlands
* Correspondence: marieke.vankatwijk@ru.nl
† Retired.

**Abstract:** During the 1980s–1990s, 4600 hectares of the seagrass *Zostera marina* were permanently lost from Lake Grevelingen (the Netherlands), and restoration is planned. In 1971, the lake was fully marine (salinity 30), and seagrass covered 1300 hectares. After closure in that year, the lake gradually became brackish (salinity of 23 by 1978), and the meadows expanded to 4600 hectares. However, with the creation of a sluice connection to the sea in 1978, the lake returned to marine conditions and a fatal decline initiated. We revisit traditionally suggested causes of the disappearance of the seagrass, finding them unsatisfactory. We hypothesize that during the lower salinity conditions from 1971 to 1978, selection of low-salinity genotypes occurred, and these genotypes were not adapted to returning marine conditions. This hypothesis is no longer testable through genetic analysis in Lake Grevelingen but is supported by previously unpublished experiments that found a lack of seed germination at even moderately high salinity for the now extinct population. Such processes could be relevant for, and tested in, environmentally modified water systems worldwide, particularly when isolated. Based on our assessment, the abiotic environment of Lake Grevelingen seems suitable for *Z. marina* restoration using a donor from a high salinity environment.

**Keywords:** selection; ecotype; germination success; isolation; Lake Grevelingen; population dynamics; salinity tolerance; *Zostera marina*

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## 1. Introduction

Seagrass meadows provide important ecosystem services such as carbon sequestration, coastal protection, water purification, support of biodiversity, and of global fisheries [1]. They are rapidly declining worldwide [2]. Although restoration appears to be challenging, successful restoration and natural recovery are possible [3–5]. In Europe, seagrass decline has been significantly reduced following the implementation of national and European regulations for the improvement of water quality [6], but more intense restoration is needed to meet the targets for the European Water Framework Directive. Accordingly, the Dutch Ministry of Infrastructure and Water Management aims to restore seagrass in Lake Grevelingen, a saltwater lake that once harbored up to 4600 ha of eelgrass (*Zostera marina*) meadows, during the 1970s and 1980s [7]. This ambitious restoration plan has revived the question as to why *Z. marina* disappeared during the 1990s, and why it did not recover. A comparison of 84 potential seagrass sites in northwest Europe showed that Lake Grevelingen is a highly suitable habitat with respect to a suite of 30 relevant abiotic environmental variables [8]. So, what other factors may be at stake?

Until 1971, Grevelingen was an estuary harboring approximately 1300 ha of intertidal seagrass, *Z. noltei* and annual *Z. marina* (Figure 1; [7,9]). After closure from the sea by a seaward dam in 1971, the estuary turned into an isolated saline lake (Lake Grevelingen) covering 108 $km^2$ of which 62 $km^2$ were less than 4 meters deep [10]. This initiated a salinity drop from salinity 30 in 1971 to salinity 23 in 1978. *Z. noltei* disappeared immediately in the year after closure and subsequent loss of tides and intertidal areas, corresponding to the general absence of *Z. noltei* in permanently submersed conditions in the region. In contrast, *Z. marina* initially thrived in this newly created environment and expanded rapidly to cover a maximum of 4600 hectares in 1978. Nearly all sheltered areas with a water depth between 0.3–2.5 m were colonized within a couple of years whereby the life history shifted from mainly annual to perennial [7,11,12]. (Note that the depth range of 0.0–2.5 reported in Nienhuis [7] was modified to 0.3–2.5 m following observations last author in Lake Grevelingen). In abundant years the population even expanded to depths of 4–5 m (mostly annual plants; [7]). In 1978, however, a sluice was created in the seaward dam to allow seawater to re-enter the lake. Salinity rose from 23 to over 30 within two years. In 1978/1979 an extremely cold winter occurred. Both events coincided with a drastic loss of 61% of the seagrass area in these years (Figure 2). Though not quantitatively monitored, the dieback was particularly severe in 1979 [7]. Between 1980 and 1985, salinity fluctuated around 30 and the seagrass cover stayed at between 2600 and 4000 hectares followed by its final disappearance by 1997 (Figure 2). Fluctuations in seagrass cover were related with salinity fluctuations (Figures 2 and 3), showing coverage loss at higher salinity.

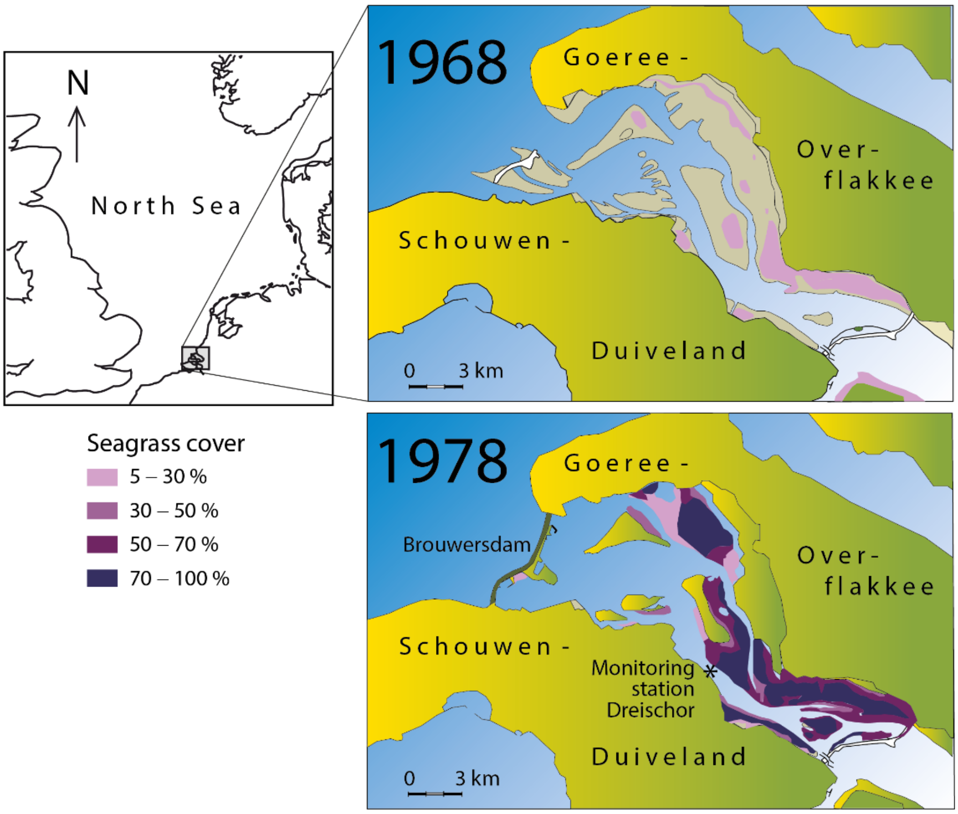

**Figure 1.** Grevelingen estuary harboring 1300 hectares of low cover *Zostera marina* and *Z. noltei* (1968, above). After closure from the sea in 1971 by the seaward dam (Brouwersdam), the *Z. marina* population expanded to 4600 hectares in the peak year 1978 (below). Data from Wielemaker-van den Dool and Herman [13]. * location of the water quality monitoring station (Dreischor).

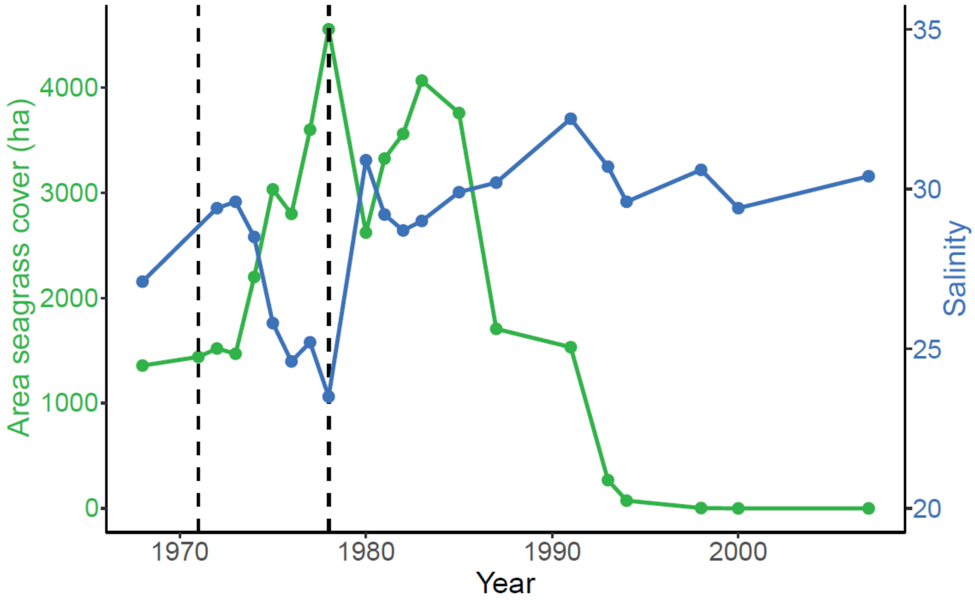

**Figure 2.** Area with seagrass cover and salinity throughout the rise and fall of *Zostera marina* in Lake Grevelingen. The two vertical dashed lines indicate the closure of the lake from the sea in 1971 and the opening of the sluice in 1978. The grey shaded area indicates an extremely cold winter [7]. Seagrass data from [13–15] and salinity data from the Dutch Ministry of Infrastructure and Water Management (waterinfo.rws.nl, accessed on 14 January 2020).

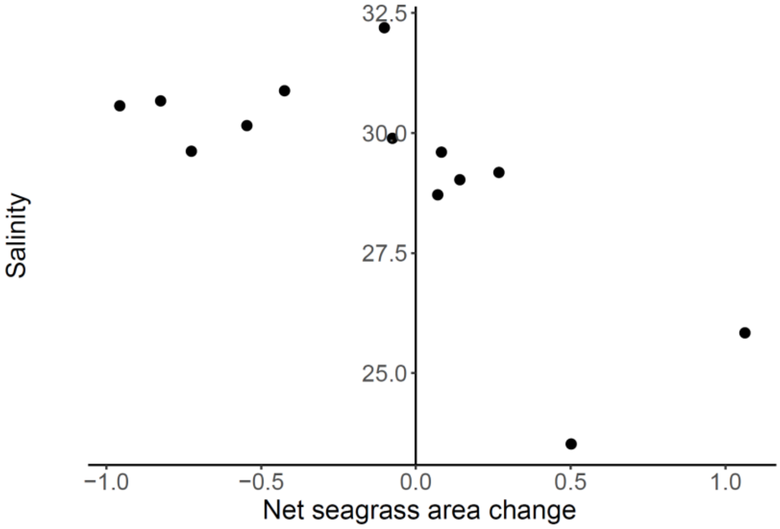

**Figure 3.** Relative change in net *Zostera marina* area in Lake Grevelingen between 1968 and 1994 in relation to average salinity in the 12 months preceding the seagrass summer monitoring showing that net seagrass area increases consistently when salinity is below 29.6 and decreases when salinity is above 29.6.

We aim to evaluate the causes of seagrass loss in Lake Grevelingen and evaluate whether Lake Grevelingen would be suitable for seagrass restoration. Specifically, we review previous explanations for the 4600 hectares loss of seagrass in Lake Grevelingen, including organic matter accumulation [16], reduced silicate levels [17], nutrient limitation [18], or a combination of cold winters, reduced silicate levels, increased salinity, and reduced ammonium levels [7]. Second, we re-analyze the correlation between salinity and seagrass cover in Lake Grevelingen over two decades and highlight a potential bottleneck event (i.e., cold winter 1978/1979). Third, we present a salinity-based hypothesis based on available data and previously unpublished results from germination experiments of the now extinct population and neighboring populations that show trait loss, i.e., an inability to germinate at salinity 30. Finally, we discuss probable mechanisms and provide recommendations for how one might proceed with restorations when causes must remain hypothetical due to total loss of the seagrass area and an inability to apply retroactive analyzes.

## 2. Materials and Methods

### 2.1. Study Area

Lake Grevelingen is a saline lake covering 10,800 ha of which 6200 ha were less than 4 m deep [10]. The lake has been closed off by dams at the landward and seaward side since the 1970s [10]. It is connected to the North Sea through a sluice, and tidal range is marginal. The water level is kept constant with a maximum fluctuation of 20 cm (not accounting for waves or temporal wind-driven increases or decreases of the water level) [19]. Season stratification and bottom hypoxia occurs in the deeper parts [20,21]. Median water temperature is 13 °C (min–max: 1–22 °C) and median visibility of the water is 2.3 m (min–max: 1.1–9.0 m) (data from the Dutch Ministry of Infrastructure and Water Management (waterinfo.rws.nl), compiled over the period 2015–2020). The salinity (2014–2017) is median 30.7 (min–max: 29.2–32.1). The lake is not only targeted for seagrass (*Zostera marina*) restoration [22], but also for recovery of fish stocks [23]. Its history and seagrass presence are described in the Introduction.

### 2.2. Correlative Study of Seagrass Cover and Salinity

Salinity of the water (at 1 m depth) was obtained from the Ministry of Infrastructure and Environment, monitoring station Dreischor and Dreischor Gemaal (waterinfo.rws.nl, accessed on 14 January 2020). Seagrass area and percentage cover were mapped during the peak of the growing season (July–August) in 5 categories. These data were obtained from the Ministry of Infrastructure and Water Management and the Netherlands Institute of Ecology [13]). The seagrass area was converted to net seagrass area by multiplying the area of each cover category with the categorical midpoint (0–5% → 2.5%; 5–30% → 17.5%; 30–50% → 40%; 50–70% → 60%; 70–90% → 80%; 90–100% → 95%) and adding them. The correlation between the average salinity in the preceding 12 months (counting from 1 August to 31 July), and the relative net area change (i.e., relative net seagrass area change [year] = (net seagrass area [year] − net seagrass area [year−1])/net seagrass area [year−1]) was analyzed.

### 2.3. Germination Experiments

Prior to the *Z. marina* extinction, in 1997, two previously unpublished germination experiments were carried out.

In September 1989, *Z. marina* seed-bearing shoots were collected at two locations, in Lake Grevelingen near Battenoord, and from a tidal area in the Wadden Sea near West-Terschelling (tidal range 1.8 m, salinity 29–31 [24]. Seeds were collected from the shoots after further ripening during 2 weeks of aeration at room temperature and stored at 4 °C in salinity 30 seawater (artificial sea salt WIMEX) in the dark for circa 2 months. On 30 November 1989, seeds were placed in petri disks at 15 °C at 4 salinity levels: 1, 10, 20, and 30, using 25 seeds in each of two replicates. Germination (breakage of the seed coat) was recorded over 109 days.

On 28 August 1997, *Z. marina* seed-bearing shoots were collected in Lake Grevelingen (salinity 32) and, on 1 September 1997, in nearby Lake Veere (salinity 18–23). The shoots were left to ripen for 3 weeks in an outdoor basin with running seawater. After that, the seeds were stored at 4 °C for 11 days. Triplicate petri dishes were used for a germination experiment testing salinity 22 and 32 with two levels of silicate (5 and 100 µM). Each petri dish contained 5 seeds, with demineralized water, artificial sea salt (creating salinity 22 and 32), and 10 µM N, 0.65 µM P, and two Si levels of 5 and 100 µM. Germination was recorded over 35 days. In a parallel experiment, seeds from the same batch were exposed to salinity 1 which was increased in weekly steps to salinity 15 over 30 days (salinity 1, 5, 10, 15).

### 2.4. Statistical Analyzes

First, we analyzed the correlation between the salinity in the preceding 12 months (from 1 August of previous year to 31 July), and the relative net area change using linear regression. The models' assumptions were met, and all tests were considered significant at an alpha level of $p \leq 0.05$. Statistical analyses were conducted in R v4.2.0 [25]. For the laboratory germination experiment, the correlation between salinity and germination was compared between both locations of origin using generalised linear modelling with a Poisson distribution containing percentage of seeds geminated as dependent variable and the interaction between location and salinity as independent variables. The Poisson model was checked for overdispersion, and the *p*-value was retrieved with the ANOVA command using Type III analysis in R.

## 3. Results

### 3.1. Correlation between Seagrass and Salinity

A strong negative relationship was found between salinity and relative net seagrass area change (t(11) = −3.24; $p$= 0.008) in Lake Grevelingen during the period from 1968 to 1995. This relationship seems to show a threshold, with a salinity higher than 29.6 consistently leading to a decrease in seagrass area cover, and when lower, leading to an increase (Figure 3).

### 3.2. Germination Experiments

The germination experiment of 1989 with seeds from the now extinct population, as well as a Wadden Sea population for comparison, showed that increasing salinity reduced germination for both locations (Chi(1) = 74.18; $p < 0.001$). Salinity, however, also showed an interaction with location (Chi(1) = 10.07; $p < 0.001$) with seeds from Lake Grevelingen showing a steeper germination decline with increasing salinity than the Wadden Sea population. Eventually, seeds of Lake Grevelingen did not germinate at salinity 30, in contrast to the Wadden Sea seeds that still showed more than 40% germination at marine salinity, and germination at salinity 20 was strongly reduced in Lake Grevelingen (Figure 4).

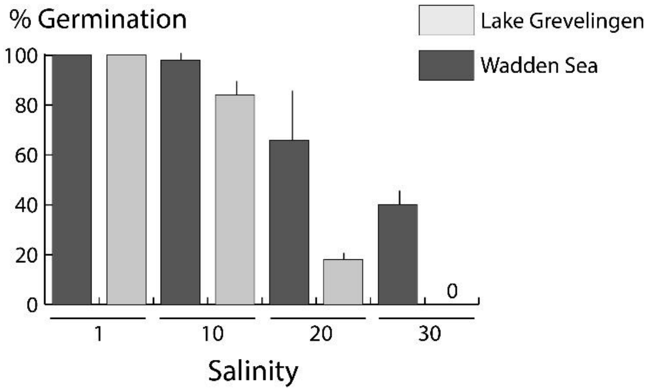

**Figure 4.** Percentage germination of *Zostera marina* at four salinities, showing a strong inhibition of the Lake Grevelingen seeds at salinity 20 and no germination at 30.

The germination experiment dating back from 1997 testing salinity 22 and 32 with two levels of silicate (5 and 100 µM) on seeds originating from two populations—Lake Grevelingen and Lake Veere—showed only 7% germination in the lowest salinity and silicate treatment for a batch from Lake Veere (i.e., 1 seed out of 15 germinated), whereas none of the seeds from Lake Grevelingen germinated. Seeds of both batches showed 35–40% germination when exposed to salinity 1–15, showing at least part of the batch consisted of viable seeds in this batch were viable (Table 1).

**Table 1.** Percentage germination of seeds of two *Z. marina* populations at different salinities and silicate (µM) concentrations.

| Origin | Salinity 1–15<br>No Si Added | Salinity 22<br>5 µM Si | Salinity 22<br>100 µM Si | Salinity 32<br>5 µM Si | Salinity 32<br>100 µM Si |
|---|---|---|---|---|---|
| Lake Grevelingen | 35–40% | 0% | 0% | 0% | 0% |
| Lake Veere | 35–40% | 7% | 0% | 0% | 0% |

## 4. Discussion

The seagrass *Zostera marina* went gradually extinct in Lake Grevelingen during the beginning of the 1990s, and three decades later, restoration plans started, although the actual causes of the decline were still not clear. Suggested but inconclusive causes were organic matter accumulation [16], nutrient limitation [18], reduced silicate levels [17], or a combination of cold winters, reduced silicate levels, increased salinity, and reduced ammonium levels [7].

Organic matter accumulations may have occurred locally, but Lake Grevelingen sediment composition is heterogeneous [26]; thus, it does not explain why the seagrasses disappeared entirely. Research in later years showed relatively low organic matter content in Lake Grevelingen populations (0.3–3.5%, [27], compared to 0.2–4.3% (5th and 95th percentile, respectively)) in NW European seagrass meadows [28]. Moreover, organic matter accumulation would likely have contributed to enhanced sediment ammonium levels, which did not occur [7,10].

Nutrient limitation may have occurred after closure from the sea [7,18,27] since water nutrient concentrations dropped following closure [10] and declined slightly between 1980 and 1990 [29]. Still, nutrient limitation is, to our knowledge, never a cause of disappearance of seagrass, although it may lead to slow growth and/or sparse cover [30]. Moreover, nitrogen was not limiting between October and April/May 1987–1989 [18,27] and seagrasses have several mechanisms to cope with low nutrient levels ([31,32]) and references therein. Moreover, porewater ammonium concentrations were still around 40 µM (0–160 µM) during this period (1987–1989 [27]), which is well within the range which can support healthy seagrass meadows [28].

Herman and coworkers [17] postulated that low silicate concentrations could be the causal factor for the observed loss; silicate levels correlated well with net seagrass area in Lake Grevelingen and are an essential element for growth [17]. However, a subsequent full factorial laboratory experiment testing silicate levels and salinity failed to demonstrate a causality between seagrass growth and silicates. Plants originating from Lake Grevelingen maintained growth under low levels of silicate similar to field conditions [33]. Instead, the experiment showed a negative effect of high salinity levels rather than low silicate levels on seagrass growth. Moreover, germination was not limited by silicate availability, as increasing silicate levels did not increase germination rates (Table 1). Apparently, the appealing postulate of Herman and coworkers on the basis of a strong correlation [17] had to be rejected on the basis of experimental evidence [33].

The extremely severe winter of 1978/1979, which lasted until the end of February, could perhaps explain the drastic loss observed in 1979 (mapped in 1980). Although *Z. marina* can locally and temporarily adapt to low temperatures [34,35], they are obviously limited by low temperatures and by the duration of a cold period [36,37]. Nienhuis [7] mentions two more cold winters that may relate to seagrass loss periods, but those were less exceptional in this region, and so it is likely that the seagrass would have been able to cope with them.

The inverse relationship between changes in net *Z. marina* area and the shifts in salinity regime is obvious (Figure 3). Herman and coworkers [17] did not find a correlation between salinity and net *Z. marina* area. However, they compared net seagrass area with median salinity (January until December) in the same year, which has two drawbacks: (1) the net seagrass area depends not only on growth conditions in a particular year, but also on the standing stock in the preceding year; (2) the median salinity in a given calendar year is not entirely relevant, considering that the mapping occurred in July/August. Therefore, we compared changes in net seagrass area at the peak of the growing season with average salinity in the 12 months preceding the peak. Note that the monitoring series has gaps for some years, so the coupling between net seagrass area change and salinity during the 12 months preceding the peak is not optimal (though probably the best possible option).

This correlation found between net seagrass area change and salinity is obviously not the same as a causality. Moreover, the species *Z. marina* is well able to grow and germinate at salinities of 30 and higher, as evidenced by its presence in saline environments all over the world including locations in the vicinity of Lake Grevelingen, such as the Wadden Sea in the north (in some seagrass beds salinity 30), and Atlantic France in the south (salinity 33) [24]. In NW European *Z. marina* meadows, the 95th percentile for salinity is 35 [28]. Therefore, salinity is not the first factor to point the finger at when trying to explain the seagrass extinction in Lake Grevelingen (but see [38]), despite its tight correlation.

In short, the previously suggested causes for the seagrass decline are not satisfactory. The correlation with high salinity is strong but cannot be the sole explanation. This leads us to the second part of this study. The germination experiments with seeds from the Lake Grevelingen population show that the seeds did not have the ability to germinate at salinity 22, 30, and 32, and had a reduced germination capacity at salinity 20, while germination still occurred at lower salinities. In contrast, seeds from other populations, i.e., the Wadden Sea (experiment 1) and Lake Veere (experiment 2) did show some capacity to germinate at higher salinities (30 and 22, respectively). The Wadden Sea population showed almost 40% germination at salinity 30, similar to germination percentages in the field [39]. Note that van Lent and Verschuure [40] still found germination in a laboratory experiment with Lake Grevelingen seeds at high salinity, and though it is unknown whether van Lent and Verschuure primed the seeds by scarring or low salinity treatment, we cannot claim that the trait loss was complete. In their detailed field measurements in Lake Grevelingen in 1988 and 1989, however, they found no (!) seedlings in 1988 and only small numbers of seedlings in 1989, in contrast to two nearby *Z. marina* populations, Lake Veere and Eastern Scheldt, where the number of seedlings was abundant. The Lake Grevelingen population was largely perennial, depending on clonal growth as well as recruitment from seed for its maintenance, recovery, and expansion [7]. Recruitment from seed must have been important for maintenance of the Grevelingen population, given the frequent strong expansions of the population (i.e., from 1500 to 3000 between 1973 and 1975, and from 2600 to 3300 between 1980 and 1981 (see Figure 2)), which can only be explained by seed dispersal and germination, given the low clonal growth rate (median 0.41 m year$^{-1}$, [41]). Therefore, population traits of seeds clearly need to be considered in addition to those of mature plants. The reduced seed germination at higher salinity implies that although the populations initially could have persisted through clonal growth, eventual decline may well have been inevitable, as recolonization from seeds was strongly impaired.

Across its biogeographic range, *Z. marina* ecotypes have been described for a range of salinities. For example, plants from several locations showed reduced germination when introduced to high salinities (e.g., [42] in Europe, [43] in China), whereas McMillan [44] found no effect of salinity on germination in the Gulf of California (testing salinity 15 and 35). Moreover, in a laboratory experiment, “marine” donor plants were better adapted to high salinity than “estuarine” donor plants [45]. Kamermans and coworkers [33] already reported that the Lake Grevelingen plants in 1996 grew better at low salinity (22) than at a salinity of 32. Therefore, Lake Grevelingen may also have been inhabited by a low salinity ecotype of *Z. marina*. The hypothetical low-salinity ecotype could have resulted from a founder effect in the remote past. However, we find that unlikely as, prior to the construction of dams, the Grevelingen estuary had only slightly reduced salinities compared to the North Sea (yearly averages between 27 and 30, and >30 in periods of low river discharge) [46]. Moreover, a population in the hydrologically connected Oosterschelde estuary had seeds that were able to germinate at salinity 30 (15%) and even salinity 40 (ca 3%) [42]. We can assume that a neighboring, hydrologically connected population is also a genetically connected population, as Olsen and co-workers [47] found genetic isolation by distance > 150 km for *Z. marina* among northern European countries. After the construction of the dams, this connection between Lake Grevelingen and neighboring populations was lost.

The low-salinity ecotype may have become dominant during the progressive lowering of the salinity during the first years after closure from the sea. This ecotype could have become the only one surviving after a mass dieback. Mass diebacks of isolated populations are known to create genetic bottlenecks [48–50], which may result in genetic impoverishment and loss of traits, such as the capacity of seed germination at high salinity for *Z. marina* in the Grevelingen. In the case of Lake Grevelingen 61% loss of the biomass occurred during or after the cold winter of 1978–1979 [7,16]. The prolonged low temperature during this winter could well have caused the die-off, as explained earlier in the Discussion. An alternative cause of the die-off could be the abrupt rise in salinity after reopening the sluice to the North Sea in 1978 (Figure 2), leading to mortality of the low salinity ecotype. The few remaining high salinity ecotypes—if still present—may not have survived the anoxia caused by the die-off of the low salinity ecotype [7,51].

We thus hypothesize that the extinction of *Z. marina* in Lake Grevelingen was due to a combination of lower salinities and environmental selection for low frequency, low salinity genotypes in the original population, leading to an impoverished population incapable of sexual recruitment under marine conditions. Unfortunately, population genetic surveys were not available in the 1980s, and samples were not preserved for a possible retrospective analysis. The hypothesis, if adopted, suggests that the present abiotic environment is suitable for marine-salinity genotypes. Experimental transcriptomic responses of *Z. marina* to experimental heat waves [52] also show that in a given population, there are pre-adapted genotypes (in this case for temperature) that can respond to environmental selection. After an extended heatwave, at least some genotypes will survive [52]. We suspect that this is what happened in the Lake Grevelingen population with the low salinity genotypes. They must have been present during the estuarine period before 1968 (for example, in the more brackish parts of the estuary), then expanded during the low salinity period and became dominant. When salinity sharply rose after reinstalling the connection to the North Sea, mass die-off occurred, coinciding with a severe winter. If few high salinity specimens were still present, the severe winter may have caused them to have gone extinct in Lake Grevelingen. Introduction of donor plants from a population that grows at a relatively high salinity would then be a feasible start for restoration in the case of Lake Grevelingen. We also note that rhizosphere microbiomes of transplanted material should also be considered [53].

## 5. Conclusions

In general, seagrass restoration is challenging. Causes of decline, as well as restoration failures, usually involve more than one factor, and are further exacerbated by large environmental variability. Nevertheless, to assess the restoration suitability of habitats that have lost seagrass, we have to collect all available information and provide a "best possible guess". For Lake Grevelingen, our review suggests that environmental selection on low salinity genotypes may likely explain the loss of *Zostera marina*. It would therefore be advised to introduce donor plants from a *Z. marina* population that grows at a relatively high salinity for restoration in the case of Lake Grevelingen.

Genetic bottlenecks creating impoverished populations with trait loss could be a serious threat to the natural recovery of seagrass populations worldwide, particularly since so many coastal systems experience rapid environmental change and—sometimes simultaneously—become hydrologically isolated from other systems due to human modifications. In addition, global seagrass losses cause increasing fragmentation and isolation of meadows [2]. At the same time, stresses like eutrophication and global warming continue to exist. The genetic component in seagrass restoration [54] will therefore become increasingly important.

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