Combined effects of salinity and temperature on grazing activity of invasive *Gammarus tigrinus* on charophytes

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Abstract

Charophytes are a refuge for zooplankton and stabilize sediments, but they are also a food source for various animal species (water birds, fishes, invertebrates). This grazing pressure may be an important factor prohibiting macrophyte re-colonization in eutrophic coastal waters. Especially the introduction of new species, like *Gammarus tigrinus*, into the Baltic Sea led to yet not understood changes in the food web. Furthermore, the occurrence of native and invasive species is determined by their capacity to acclimatize to changing abiotic factors. In this study we investigated the influence of temperature and salinity on the grazing pressure of *Gammarus tigrinus* on two charophyte species: *Chara tomentosa* and *Chara aspera*. The grazing experiments were conducted in a full factorial design with the factors salinity (3 – 13 PSU), temperature (5 – 30 °C), and charophyte species. Grazing rates were determined as mass deviation within 48 hours considering simultaneous growth of the charophytes. Experimental results were further used to determine gross macrophyte biomass within the ecosystem, using field data. Gammarids preferred *C. aspera* before *C. tomentosa*, but both were grazed within their respective growth optima. The potential grazing peak of 24 °C is not yet reached in these coastal waters. Furthermore, calculation of gross production revealed that macrophytes could show up to 60% and 15% more biomass without grazing in deeper light-

limiting, and shallow, not light limited water depths, respectively. The impact of food web on coastal water restoration should therefore be equally considered, as the nutrient control.

Keywords: charophytes, grazing, *Gammarus tigrinus*, temperature, salinity

Introduction

Anthropogenic-induced eutrophication affected coastal water bodies worldwide, through increased growth of phytoplankton mediated by elevated nutrient inputs from river, diffuse run-off and precipitation. This increased blooming of phytoplankton subsequently increased turbidity within the water column and led to a loss of macrophyte cover (González Sagrario et al. 2005, Barker et al. 2008). Such primary production shifts from benthic to pelagic zone were described as alternative stable states, and can be very stable especially in shallow aquatic systems. This loss of submerged vegetation had several implications on ecosystem functioning and services, e.g. , altered species composition (Suikkanen et al. 2013) and possibility of potentially harmful algal blooms (Paerl and Paul 2012). To counteract these developments, nutrient inputs as one eutrophication-causing factor were reduced (Gulati and Van Donk 2002, Nausch et al. 2011). However, these reductions worked only partly and, in some cases, macrophytes need a long time frame to re-colonize (Sand-Jensen et al. 2017). One theory emphasizes, that the current phytoplankton dominated state has strong own feedback mechanisms, like increasing attenuation, that inhibit a return to pre-eutrophication conditions (Scheffer et al. 1997). Furthermore, Global Change will affect water temperatures worldwide, as well as altering run-off from land, leading to possibly different salinity conditions within the next decades (Schindler 2006). The Baltic Sea is such a system prone to eutrophication and Global Change, due to already increased sear surface temperatures, compared to other oceans worldwide (Reusch et al. 2018). The Baltic is subject to large phytoplankton blooms since decades, with the riparian states working to reduce the impact of

nutrient-run-off to the sea (Janssen et al. 2004, HELCOM 2018). Similar, the coastal water bodies of the Baltic Sea were impacted by eutrophication and shifts on primary producer dominance, i.e. from submerged macrophytes to phytoplankton (Schiewer 2007). Even though nutrient inputs declined in the Baltic and its adjacent coastal water bodies through the last decades, most coastal water bodies (CWB) remain eutrophied (Berthold et al. 2018a).

However, there were also hypotheses that not only nutrients, but also top-down control (i.e. grazing) may affect re-mesotrophication and macrophyte recolonization (Körner and Dugdale 2003, Östman et al. 2016).

Besides (accidental) grazing from native species (Körner and Dugdale 2003), there is also an increasing grazing pressure of invasive species introduced through, e.g. ballast water of ships. One of these species is the gammarid *Gammarus tigrinus* (Sexton 1939), a successful new species introduced from North America to European waters (Berezina 2007). This gammarid was described to colonize shallow waters, especially within reed, and soft bottom systems of the Baltic Sea (Daunys and Zettler 2006, Kotta et al. 2011). Amid Global Change, invasive species can alter known food web interactions and may counteract re-colonization of former native primary producers (Puntila 2016).

Charophytes receive attention as indicator species for oligotrophic water bodies (e.g. Melzer 1999), and were listed as ‘endangered species’ in Germany. However, this view has been relativized by the fact that during a Germany-wide survey, an increased number of charophyte populations were reported at strongly eutrophic sites (Doege et al. 2016). Blümel et al. (2002) found charophyte species in the Darss-Zingst lagoon system (DZLS) even with high nutrient levels and turbidity (Selig et al. 2009). An explanation was offered by Schubert et al. (2018), who concluded that many charophyte species are "pioneer plants" reacting particularly sensitive to the competitive pressure of higher plants. Shading in particular reduces the competition with other species. This distribution pattern means that charophytes can be found

in shallow water areas even under highly eutrophic conditions, if the level of light exposure and the water level stability admits it. Nonetheless, charophytes are also under pressure in other parts of the Baltic Sea. Comparisons of historical and recent distributions in the northern Baltic found that *C. aspera* and *C. tomentosa* were found 8 and 86% times less in the most recent transect, respectively (Pitkänen et al. 2013).

Salinity constitutes another factor in brackish water, where the increased salinity prevents the growth of macrophyte species (Lindner 1972). This means that regardless of the degree of trophism, a niche is created in which charophytes can be established. It was assumed that charophytes have several turbidity-reducing traits, like e.g. dissolved nutrient competition with phytoplankton, refuge function for zooplankton and young fish, or reducing of resuspension of particulate matter (Kufel and Kufel 2002). Therefore, re-colonization with those species could create feedback mechanism, favoring a macrophyte-dominated stable state. Charophytes at the southern Baltic Sea coast diminished in some CWB already in the 1930ies (Schlungbaum et al. 2000), and later in adjacent CWB as well (Blümel et al. 2002). Even though nutrient concentrations were reduced to a comparable level between today and the 1930ies, no dense macrophyte cover was able to form in e.g. the Darss-Zingst lagoon system (DZLS) (Gessner 1957, Berthold et al. 2018a, Paar et al. 2021). Indeed, the maximum depth distribution of charophytes has not increased significantly in the DZLS during the last years, and charophytes are still found only in very shallow waters, where light is not limiting (Piepho 2017). The DZLS can be considered as model ecosystem for the southern Baltic Sea, and is monitored for decades (see Schiewer 2007). The process of sudden alternative stable state shifts were observed in the 1930s in the innermost part, and in the 1970s in the outer parts (Gessner 1957, Schiewer 2007, Berthold 2016). This state-shift caused a major decline in submerged vegetation cover (Blümel et al. 2002). Restoration measures starting thirty years ago, lowered the nutrient inputs by up to 90% for Phosphorus and 60% for Nitrogen (Nausch

et al. 2011, LUNG 2013). However, the macrophyte covers were not able to recover until today.

The question evolves, if re-colonization of macrophytes is additionally hampered by grazing pressure of (newly introduced) mesograzers, and how this pressure changes throughout the year. *Gammarus tigrinus* and charophytes occur within the same depth distribution, and grazing can potentially happen. It was questioned what for effects the variable temperature, and salinities in lagoons have on grazing behavior on charophytes. Therefore, a full-factorial design was chosen with the variables water temperature and salinity at four levels. This design would also allow to discuss future developments within CWB at e.g. rising water temperatures, or reduced salinities at increasing precipitation events. Shallow CWB are likely to be more affected by such events due to their relatively lower water column and close coupling with the adjacent land. Therefore, we combined the laboratory determined grazing potentials with field-determined elemental budgets of submerged macrophytes of the DZLS. The data sets were further used to calculate potential nutrient flows from the macrophytes to the water column, therefore putting macrophytes as nutrient mediators between the sediment and higher trophic levels. It was hypothesized that there is either a combination of factors (abiotic and biotic) that amplify mass loss on charophytes, or that some abiotic factors quench each other, e.g. lower salinity amplifies/buffers effects of higher temperatures. Those mass losses may depend on the acclimation potential of the local population.

Material and Methods

Experimental design

We chose a full factorial design with three factors, for which one factor relates to species and includes two levels (*Chara aspera* and *C. tomentosa*), and the other two factors each with four levels are water temperature (9, 15, 24, and 30 °C), and salinity (3, 5, 7, 13). These salinity levels represent the salinity gradient from the Western belt Sea to the Gulf of Finland, covering most of the Baltic Sea area (HELCOM 2010). The water temperature levels represent the blooming period of shallow coastal waters (winter to summer +1 – +22 °C, Schiewer 2007), with 30 ° C as prospective endpoint of possible charophyte growth. Charophytes were cultured from intact plants extracted from the DZLS. The DZLS is formed by four consecutive shallow lagoons, and shows a salinity and trophic gradient from the main river inflows Recknitz and Barthe (inner and middle part, salinity of 1 – 6, eutrophic) to the open Baltic Sea (salinity of 10 – 12, mesotrophic, Schumann et al. 2006). Salinity varies around 6 to 8 at the respective sampling locations in the Bodstedter Bodden (54°22'30,5" N, 12°34'14,9" E) and the Grabow (54°22'2,3" N, 12°48'27" E) (Schumann et al. 2006). During the experiment, two charophyte species were used, *Chara tomentosa* and *Chara aspera*. Both species are common in the Baltic Sea, with *C. aspera* covering the widest salinity range among charophytes (Blindow 2000). Along the west coast of Sweden, *C. aspera* is regularly found at sites with salinities up to 15 (temporarily up to 20), but it is also common in freshwater (Hasslow 1931, Blindow 2000). *Chara tomentosa* is among the largest charophyte species and can be found in freshwaters all over the world. Only in the Baltic Sea, *C. tomentosa* also occurs in brackish water, with a salinity range between 0-7.5 (Björkman 1947, Torn et al. 2003). Gammarids were derived from the Aquaculture section in Born of the State Research Centre for Agriculture and Fishery Mecklenburg-Vorpommern. This research facility is located at the DZLS and uses brackish water from the lagoon with a salinity of 4 –

6. Gammarids were identified by morphology and genetic analyses (see below).

Charophytes were cultured in beakers (vol. 500 ml), containing sediment containers (vol. 64 ml) with acid-rinsed, phosphorus-enriched sediment (6 g P kg^{-1} sediment) according to Wüstenberg et al. (2011). Mineral growth media was prepared to culture charophytes (Pohl et al. 1987, Wüstenberg et al. 2011), but was adjusted to the respective salinities by adding marine salt (Instant Ocean, Aquarium Systems, Sarrebourg). Salinity was checked with a salinometer (Multiline P4, WTW). Each of the 16 combinations were replicated eight times with gammarids and without gammarids present (control) (Fig. 1). Gammarids were pre-acclimatized to experimental conditions by culturing them at the respective temperature and salinity combinations. Ten to 20 individuals were simultaneously cultured per temperature-salinity combination, aerating all cultures. Charophytes were added as food supplement, as well as resting surface for the gammarids.

Field sampling and analyses

In addition, we used elemental and biomass composition of submerged macrophytes from the DZLS, to compare the experimental derived-grazing rates to the actual ecosystem. Field sampling was conducted in the Bodstedter Bodden and the Grabow in 2014, as part of the BACOSA project. Submerged macrophytes were sampled throughout the year, first in 54x54 cm plots, and later in the year in 25×25 cm plots at nine different depths. Here, three sampling locations were pooled into three depth zones, according to water depth and proximity to the reed belt. Water depths for both stations varied with water levels in the respective lagoons and were shallow (30 – 60 cm), intermediate (60 – 90 cm), and deep (90 – 120 cm). The pooling became necessary, as macrophytes grew patchy at each sampling station. Sampled macrophytes were separated on the genus level. Whole plants, including roots, were wet-weighed and then dried at 60 °C for 24 h. Afterwards, plants dry mass (DM) were re-weighed again to calculate water content. Dried plant material was ground with a ball mill. The powder

was treated with 1M HCl to remove inorganic carbon residues and then weighed in tin-container (1×0.5 cm). These samples were analyzed for their carbon and nitrogen content using an elemental analyzer (varioEL III). The analyzer was calibrated using acetanilide (~5 mg per sample).

Aliquots of dried plant material were weighed in crucibles and combusted at 550 °C for 4 h. The ash was re-weighed to calculate ash-free dry mass. Ashes were used to determine total phosphorus content (mg P g^{-1} dry mass), using an acid persulfate extraction (see Berthold et al. 2015).

Charophyte growth experiment

Growth responses of *C. tomentosa* and *C. aspera* to the different culture conditions were followed. Fresh mass was determined before and at the end of the experiment by blotting them dry with a paper towel. Experiments lasted 48 to 72 hours and were simultaneously the gammarid-free controls of the grazing experiment. The differences in mass were used to calculate short-term developments and compensate for possible growth in grazing experiments (see below).

Gammarid grazing experiments

For grazing experiments, charophytes were wet weighed and set into beakers filled with sediment and growth media of the respective salinity. Gammarids were photographed before and after the experiment to determine possible increases in length. One charophyte and five gammarids were set together in one beaker. Each replicate run lasted for 48 hours and in four cases over the weekend, i.e. for 72 hours. Replicate runs for each *Chara* species were performed over a period of four weeks, with two replicates of each respective temperature-salinity combination at a time. Charophytes were wet weighed after each replicate run to calculate mass differences during the replicate run. Dead gammarids were counted at the end

of each replicate run and replaced for the next replicate run. If possible, the same gammarids were used throughout all replicate runs. Equations 1 – 3 were used to extract grazing rates of gammarids from growth rates of charophytes during the experiments. Grazing rates were treated with an error-propagation to account for experimental variability.

Equation 1 subtract the mean effect of pure growth of the control (C) from the treatment which also includes grazing (T):

$$G_i = T_i - \bar{C} \; \{i = 1..n$$

Equation 2 standardizes the grazing effect from prior formula (**z**-score):

$$Gz_i = \frac{(G_i - \bar{G})}{\sigma_G} \; \{i: 1..n$$

Equation 3 calculates the mean of pure grazing and include its error caused by the experiment:

$$Gf_i = \bar{G} + Gz_i \cdot \sqrt{|(\sigma_T^2 - \sigma_C^2)|} \; \{i: 1..n$$

T = treatment, C = control, G= detrended grazing, G_z = standardized grazing, G_f = real grazing with included experimental error, n = replicates

<u>Data analyses</u>

We further used the experimental grazing data to calculate grazing rates based on salinity and water temperature at two sites of the DZLS. The data set failed to show homogeneity of the residuals, and was therefore Box-Cox power transformed (*bcnPower* function, R package "*car*", Fox and Weisberg 2019). Finally, general linear models (GLM, *glm* function, R package "stats", R Core Team 2019) and generalized additive models (GAM, *gam* function, R package *"mcgv"*, Wood 2011), both with Gaussian distributional assumptions, were compared by the Akaike Information Criterion (AIC, R package "stats", R Core Team 2019) and the Bayesian Information Criterion (BIC, R package *"stats"*, R Core Team 2019),

choosing the model with low AIC and BIC values. We chose the GAM (with knots, k = 16) as the best model to reflect the experimental data (see Results). Recent biomass and individual numbers of gammarids were modelled according to described numbers within the DZLS and nearby lagoons (Zettler 1995, M. Paar personal communication). We assumed that *Gammarus tigrinus* populations followed the same seasonal pattern as described in Chambers (1977). We used again a GAM model to calculate the seasonal population development of *Gammarus tigrinus*, normalized the predicted seasonal data to have a maximum of 1. Then, we used this normalized prediction to calculate population densities with a maximum of 400 Ind m^{-2} (Zettler 1995, M. Paar personal communication, supplement figure S3). To calculate seasonal grazing rates within the DZLS, these population numbers were combined with the individual grazing rates of the temperature-salinity model with field data from the two sites of the DZLS (Equation 4). We used a fixed grazing preference of 20% macrophyte share in gammarid diets (Pellan et al. 2016). Furthermore, we used the GAM to predict the charophyte development as function of sampling date (k = 8) at two sites of the DZLS. We calculated daily biomass increase and subsequently added gammarid grazing rates to determine the potential gross biomass production per site (see Results and Discussion below).

Equation 4 calculates the seasonal grazing rates per square meter and day for specific sites:

$$Graz_t = G_{GAM_t}\{T_t; S_t\} \cdot \delta_t \cdot 0.2 \quad \{t: 1..n$$

t = day of the year, $Graz_t$ = grazing at day t ($mg \cdot m^{-2} \cdot d^{-1}$), G_{GAMt} = modelled grazing rate of one individual at time t with given temperature (T) and salinity (S) from specific site at time t, δ_t = population density at time t (individuals$\cdot m^{-2}$), 0.2 = reflects the 20 % fixed grazing preference, n = length of the year in days

Genetic analyses

At the end of the grazing experiment, 69 gammarids were determined as *Gammarus tigrinus* following Zettler and Zettler (2017) and preserved in 80 % ethanol. To verify the morphological analyses, a genetic approach was conducted on 15 individuals. Total genomic DNA was extracted using a silica spin column procedure with the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) following the protocol provided by the manufacturer. Partial sequences of the COI gene were amplified with the universal primers LCO 1490 and HCO 2198 (Folmer et al. 1994).

PCR amplifications were performed with a denaturation step for 60 s at 94°C, followed by 5 cycles of: 60 s at 94°C, 90 s at 45°C and 60 s at 72°C, 35 cycles of: 60 s at 94°C, 90 s at 51°C and 60 s at 72°C, and completed with 5 min at 72°C as a final extension step. PCR was performed in a 30 µl reaction volume with a Taq PCR Master Mix (Qiagen, Hilden, Germany) consisting 2.5 mM MgCl2, and 0.5 pmol of each primer (final concentration). The PCR products were extracted from agarose gels according to the protocol of the Biometra-innuPrep Gel Extraction Kit (Analytik Jena, Jena, Germany), and were sequenced directly using a 3130×L Genetic Analyzer (Applied Biosystems, NY, USA) with sequencing primers identical to the primers that were used for the PCR reaction. Obtained sequences were quality controlled and aligned via the BIOEDIT software (Hall 1999). The COI gene is widely used for inter- and intraspecific diversification questions concerning amphipod taxa. For the taxonomic determination addition GenBank sequences of *Gammarus tigrinus* (see Supplement Table S1), *G. duebeni* Liljeborg, 1852 (EU421779), *G. locusta* Linnaeus, 1758 (KT209211), *G. oceanicus* Segerstråle, 1947 (GQ341809), *G. pulex* Linnaeus, 1758 (MN400977), *G. roeseli* Gervais, 1835 (EF570337) *G. salinus* Spooner, 1947 (KT208533), and *G. zaddachi* Sexton, 1912 (KU845083) were used for the phylogenetic analyses.

In order to estimate the evolutionary divergence between haplotypes, pair-wise uncorrected p-

distances and the number of substitutions were conducted using MEGA version X (Kumar et al. 2018). To uncover phylogenetic relationships, a Maximum likelihood tree was constructed using MEGA version X (Kumar et al. 2018) on the basis of the Kimura 2-parameter model. Branch support for the nodes was calculated from 1000 bootstrap replicates.

To study the distribution of mtDNA sequence diversity, a haplotype network was constructed using the Median-joining algorithm (Bandelt et al. 1999) implemented in PopART (Leigh and Bryant 2015). The distribution basemap was created with QGIS.org software (http://www.qgis.org) and modified in CorelDRAW (Corel Corporation, Ottawa, Ontario, Canada).

Results

Genetic data

The present study analysed 15 specimens of *Gammarus tigrinus* collected at the DZLS with sequence data from previous studies (see Table S1) to validate morphological species determination and identify their genetic haplotypes. Based on the 407 bp region of the COI, the 15 analysed individuals could be assigned to the northern species of *G. tigrinus*. The phylogenetic analyses revealed that the individuals of this study belonged to two different, very closely related haplotypes: N4a & N4e (Fig 2&3). Both haplotypes differed by a single base pair substitution and were identical with *G. tigrinus* specimens from Europe (N4a&N4e), the British Isles (N4e-1) and North Amerika (N4a-4). Both haplotypes were sympatrically present in the DZLS and were observed in both fresh and brackish water habitats. Haplotype N4e-1 comprised 4 individuals of the DZLS and was already identified in previous studies in the Baltic Sea and in freshwater habitats in Northern Ireland. 11 DZLS individuals corresponded to haplotype N4a-4, which has been described at the Baltic Sea coast, but also in North America and moreover it was found in the northern Rhine (freshwater habitat).

Growth and survival rate

Starting biomasses of *C. aspera* in control and experimental beaker ranged from 50 to up to 300 mg, with the most macrophytes being within the range of 100 – 175 mg (70%). After two days, biomass distribution of *C. aspera* in control beacons stayed relatively the same, but smaller and larger biomass proportions increased. Biomass of *C. aspera* decreased with increasing temperature at salinities of 3 and 13. Contrary, the biomass stayed almost constant at all temperatures at salinities of 5. A small possible biomass increase was detected at temperatures 15 and 24 degrees at salinities of 7, and a strong decrease at 30 degrees for almost all salinities (Supplement Figure S1).

Starting biomass of *C. tomentosa* in control beacons ranged from 75 to up to 600 mg, and 40% of all macrophytes were within the range of 100 – 125 mg at the beginning. Contrary to *C. aspera*, biomass of *C. tomentosa* in experimental beacons was close to a normal distribution with 50% of all macrophytes being within the range 175 – 225 mg at the beginning. The biomass distribution of *C. tomentosa* in control beacons stayed almost the same, but with a trend to smaller biomasses, indicating biomass loss even without grazing. Biomass of *C. tomentosa* stayed very constant at almost every temperature-salinity combination. There was a possible growth at temperatures of 15 to 24 degrees at salinities of 7 (Supplement Figure 1). In total, these results indicate an overall stable experimental approach.

Two thirds of the time (65%) all gammarids survived the incubation period at all temperature-salinity-macrophyte combinations. Additionally, in 30% of the time, only one gammarid died during incubation. These results indicate overall stable culture conditions and the capability of these gammarids to survive a wide range of temperature-salinity gradients.

Grazing rate

Growth-corrected grazing ranged between 1 to 10 mg DM per individual and day on *C. aspera*. Grazing rates peaked at 24 °C, except for salinity concentrations of 5, where it probably peaked at 30 °C. This grazing rate was contrary to all other salinities, were grazing rates at 30 °C were lower compared to 24 °C. Furthermore, growth of *C. aspera* was less affected of high temperatures at salinity of 5, compared to other salinities (see Figure 4 and Supplement Figure S2). Growth-corrected grazing ranged between 1 to 5 mg DM per individual and day on *C. tomentosa*. Grazing rates peaked at 24 °C for all salinity levels and at 15 °C for salinities of 7. Interestingly, grazing rates of gammarids coincided with growth optima of the charophytes in this study. These results may indicate a grazing preference on actively growing charophytes. In general, grazing rates on *C. aspera* were up to two times higher compared to *C. tomentosa*. The grazing rate considering both charophytes to be present, would have been up to 5.5 mg Ind^{-1} d^{-1}, with a general larger temperature-salinity range of highest observed grazing.

Field data results and model

Individual daily grazing rates within the system would range from 0.1 up to 5 mg Ind^{-1} d^{-1}, when applying the predicted GAM output on field observations of temperature and salinity (Figure 5). Grazing rates would peak during summer and be lowest at winter at the two sampling stations of the DZLS (see Figure 5). The here used individuals of *G. tigrinus* grazed therefore twice as much at the experimental 24 °C, as would be expected from their initial harvesting season in April and May, indicating acclimation processes. Interestingly, grazing rates differ between both stations, even though the difference in salinity is only 1 – 2 PSU. The yearly potential grazing rate for *G. tigrinus* on charophytes ranges from 37.2 to 42.5 g m^{-2}, considering regional salinity and temperature patterns within the DZLS. These values are

based on the assumptions of a maximum of 400 Ind m^{-2} and a food preference of 20% for macrophytes compared to other available food sources (see discussion below).

Charophyte biomass was highest in the shallow areas, close to the reed belt at both sampling (Figure 6). Biomass in the shallow zone peaked in July in the less eutrophic Grabow, and in October in the highly eutrophic Bodstedter Bodden. Contrary, charophyte biomass was higher in the Bodstedter Bodden at intermediate distances/water depths compared to the Grabow. However, Grabow biomass peaked during summer, whereas biomass in the Bodstedter Bodden became depressed during that time. There were no regular biomasses of charophytes in the deeper water areas at both sampling locations, indicating a possible light limitation from shallow to deep.

There were no significant differences for the C:N and C:P ratios along the depth distribution, or between stations (see pooled values in Fig 7). Interestingly, charophytes showed either a weak seasonal trend in elemental composition (C:N), or the ratio decreased (C:P). Lowest C:P ratios were found from July to October. Contrary, co-occurring tracheophytes increased their C:N ratio during summer, as well as their C:P ratios. These findings indicate that charophytes had probably a higher protein content per biomass compared to tracheophytes.

Discussion

Grazing rates in our experiment and in the field depend on several factors, like the population structure of gammarids, available food and food quality, occurrence/absence of (competing) predators, and ultimately habitat choice and niche occupation.

Populations of *G. tigrinus* were described to turn-over at least three times per year, with a peak of large animals (6 – 9 mm) in spring, and a concomitant shift to smaller animals in late summer (2 – 4 mm) (Chambers 1977). We used for all our experiments animals in the size range of 6 – 12 mm, i.e. large adults. This size class is only dominant by up to 60% until May,

and is almost completely replaced by smaller animals from June to September (Chambers 1977). This pre-selection had likely an effect on the described grazing rates, as metabolic rates of *G. tigrinus* increase with lower weight in form of a power function (Normant et al. 2007). However, the gammarids in our study weight from 10 – 90 mg wet mass Ind^{-1} (data not shown), whereas the gammarids of Normant et al. were caught in August, and weighed only 5 – 25 mg wet weight Ind^{-1}. We therefore calculated with the provided power function, that our animals showed even lower specific metabolic rates of 0.25 – 1.5 mW g^{-1} wet mass, compared to 1 – 3.5 mW g^{-1} wet mass in smaller gammarids (Normant et al. 2007). This reduced metabolic rate had likely an impact on the grazing rates we found in this study, as grazing for metabolic upkeep is lower in large adult animals. Our rates are therefore only representative for springtime, if similar population dynamics are assumed for our system like described in Chambers (1977).

To our knowledge, published studies on seasonal population dynamics of *G. tigrinus* are currently missing in the system studied here, as elsewhere in the Baltic Sea. However, recent field studies found 5 to 2200 Ind m^{-2} (gammarids on genus level) in the DZLS and the adjacent Westrügensche lagoon system. Largest, but fewest animals were found early in the year (22 – 28 mg Ind^{-1}), with decreasing mass, but increasing abundance in summer (3.5 mg Ind^{-1}) (M. Paar, personal communication). These patterns point to the same seasonal development as described in Chambers (1977). If we assume that *G. tigrinus* constitutes 20% of the local gammarid population in the DZLS (Zettler 2001), we get a population size of up to 450 Ind m^{-2}. These numbers are in agreement with first described abundances of *G. tigrinus* of 300 – 500 Ind m^{-2} in the phytal and reed belt of the DZLS (Zettler 1995), or 30 – 110 Ind m^{-2} in the Neva estuary (Baltic Sea, Berezina 2007).

Nonetheless, springtime is a crucial period for charophyte sprouting, as the active growth of for example *C. tomentosa* takes place during a relatively short period at the beginning of

summer (Torn et al. 2006). Furthermore, young sprouted charophytes show a higher elemental content per biomass, than for example tracheophytes (Volkmann 2016, this study), making them a preferred grazing opportunity (Kraufvelin et al. 2006). *G. locusta* follows a preference for high value plant material and selectively feeds on more nutrient-rich macrophytes, especially periphyton, brown and green algae (Kraufvelin et al. 2006). If *G. tigrinus* shows similar grazing preferences, periphyton from tracheophytes would be the first choice followed by charophytes in our system. Periphyton (Sanudo-Wilhelmy et al. 2004) and possibly charophytes (Kufel and Kufel 2002) can take up fast amounts of nutrient pulses that are likely to happen close to the wetland in this area (Berthold et al. 2018b). The charophytes in our study area showed higher C:N and N:P ratios, then tracheophytes, making them more likely to gammarid grazing as high-value food. Furthermore, this pre-selection of high-value food may explain the differences in grazing rates on *C. tomentosa* vs. *C. aspera* found in this study. *C. tomentosa* can show higher C:N ratios (44:1) than *C. aspera* (27:1), making it maybe a less favorable food. Furthermore, *C. aspera* showed in our study signs of early decay, contrary to *C. tomentosa* (see trends in growth control beacons, supplement figure S1). Such beginning decay can weaken *C. aspera* and make it more susceptible to grazing (Buchsbaum et al. 1991), explaining the deviation of grazing rates between both charophytes found in this study. Tracheophytes in our and adjacent lagoons can show high colonization rates by epiphytes (Paar et al. 2021), offering an additional plant food source to charophytes. Furthermore, food preferences can change throughout the year depending on gammarid life stage and temperature (Felten et al. 2008, Pellan et al. 2016). Plant biomass becomes increasingly important, when temperatures increase and can reach up to 20 – 30% of total food consumed (Pellan et al. 2016). Detritus, plant litter, or animal matter, like chironomides, are usually important food sources during winter time (Felten et al. 2008, Pellan et al. 2016). Sediment organic content can be as high as 20% in sediments of the DZLS (Berthold et al.

2018c). Sediment detritus was not an alternative source in our experiments, as we used only inorganic sand. Plant litter occurs abundantly within the reed belt during winter time in the DZLS (Karstens et al. 2016). Chironodmides biomass can be as high as 40 g m^{-3} and is positively impacted by the occurrence of another invasive species, *Marenzellaeria viridis* (Zettler 1995, Schiewer 2007). Functional feeding in gammarids relies furthermore on population composition and food availability (Kelly et al. 2002). Our experimental results show therefore only a potential grazing rate, as we did not represent the variety and abundance of alternative food sources from within the ecosystem. However, our suggested weighed grazing rates of only 20% macrophyte biomass may still be a realistic assumption (see Figure 6).

Furthermore, grazing rates depend on niche occupation and competition with native and other invasive species. *G. tigrinus* can probably outcompete the native *G. salinus*, if both depend on *P. littoralis* in the northern Baltic Sea (Orav-Kotta et al. 2009). Both gammarids had the potential to exceed daily macrophyte production during summer (Orav-Kotta et al. 2009). Comparably, we found that macrophyte biomass could have been at least 25% higher during summer, without the impact of *G. tigrinus* grazing. However, these results are only valid, if *G. tigrinus* outcompeted other gammarids in our study area. Otherwise, grazing rates on charopytes could be even higher.

Additionally, microhabitats, like macrophyte patches, are important for native species to co-exist with invasive species (Korpinen and Westerbom 2009). Nonetheless, recent studies showed that *G. tigrinus* would compete with native species, either directly through food choices (Orav-Kotta et al. 2009), or grazing on native juveniles (Jänes et al. 2015). *G. tigrinus* may be more tolerant against heat waves and sub-oxic conditions, than native species (Lenz et al. 2011). These broader temperature acclimation may help to permanently introduce *G. tigrinus* within the Baltic Sea, as sea surface temperature there are already higher, then in

other ocean parts (Reusch et al. 2018). Furthermore, endurance of sub-oxic conditions is an advantage in eutrophic coastal waters of the Baltic, as redox conditions within the reed belt can change fast (Karstens et al. 2015), and an increase of nutrients is challenging submerged macrophytes in the coastal water bodies of the southern Baltic Sea (Paar et al. 2021), stressing habitats of native gammarids. Contrary, an *in silico* study assumed that *G. tigrinus* has actually a narrower niche than native gammarids in the norther Baltic (Herkül et al. 2016). These results are somewhat contradictory to other studies in this area (Korpinen and Westerbom 2009, Kotta et al. 2011, Lenz et al. 2011), and may have not considered the impact of haplotype-diversity, that means locally adapted populations of *G. tigrinus*. Different studies revealed high genetic variability of *G. tigrinus* in the Baltic Sea (this study, Kelly et al. 2006) and Paiva et al. (2018) indicated that the invasive species may have different tolerance and different limits to salinities due spatially varying selection among populations. The patchy macrophyte stands found in the DZLS, and in other eutrophic water bodies, may affect the occurrence of *G. tigrinus*, and therefore the grazing rates, especially in intermediate and deeper waters. Palaemonid prawns put a higher grazing pressure on *G. tigrinus* in unvegetated mesocosms, then in vegetated ones (Kuprijanov et al. 2015). It is likely that *G. tigrinus* stays close to the reed belt, as described for this lagoon and other coastal waters (Zettler 1995, 2001). This spatial distribution would further hamper charophyte recolonization, as the shallow areas in the DZLS are the only spots, where light availability is high enough to support charophyte biomass (see Figure 6, Piepho (2017). Furthermore, elemental concentrations of reed, macrophytes and sediment is highest close to the land (Berthold et al. 2018b). Grazing on macrophytes can impact nutrient sequestration rates by increasing grazing-mediated release rates, therefore slowing down restoration measures to reduce eutrophication.

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Figures

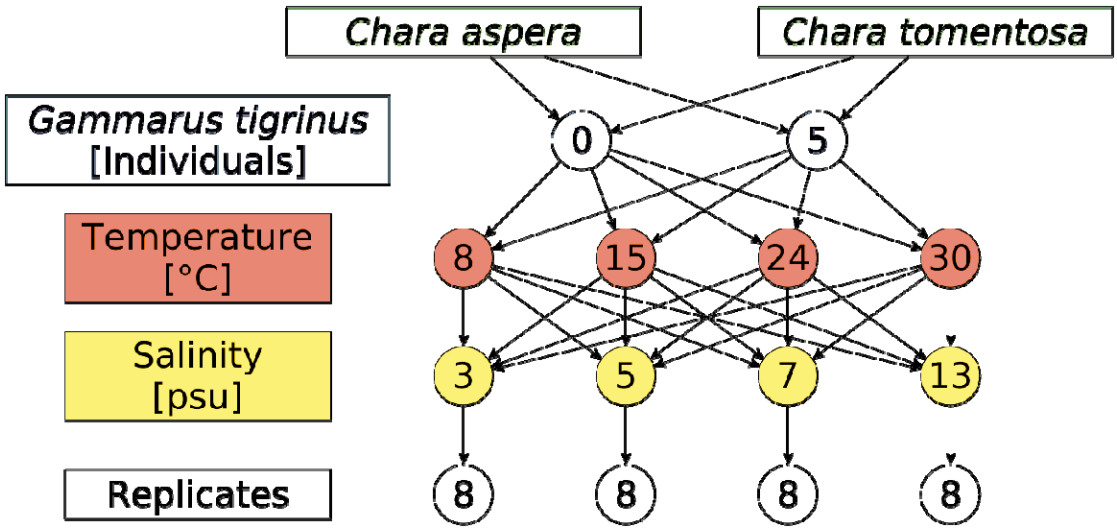


Figure 1 Flow scheme of the full factorial experimental design for temperature, and salinity with *Chara aspera* and *C. tomentosa*, with and without *Gammarus tigrinus*.

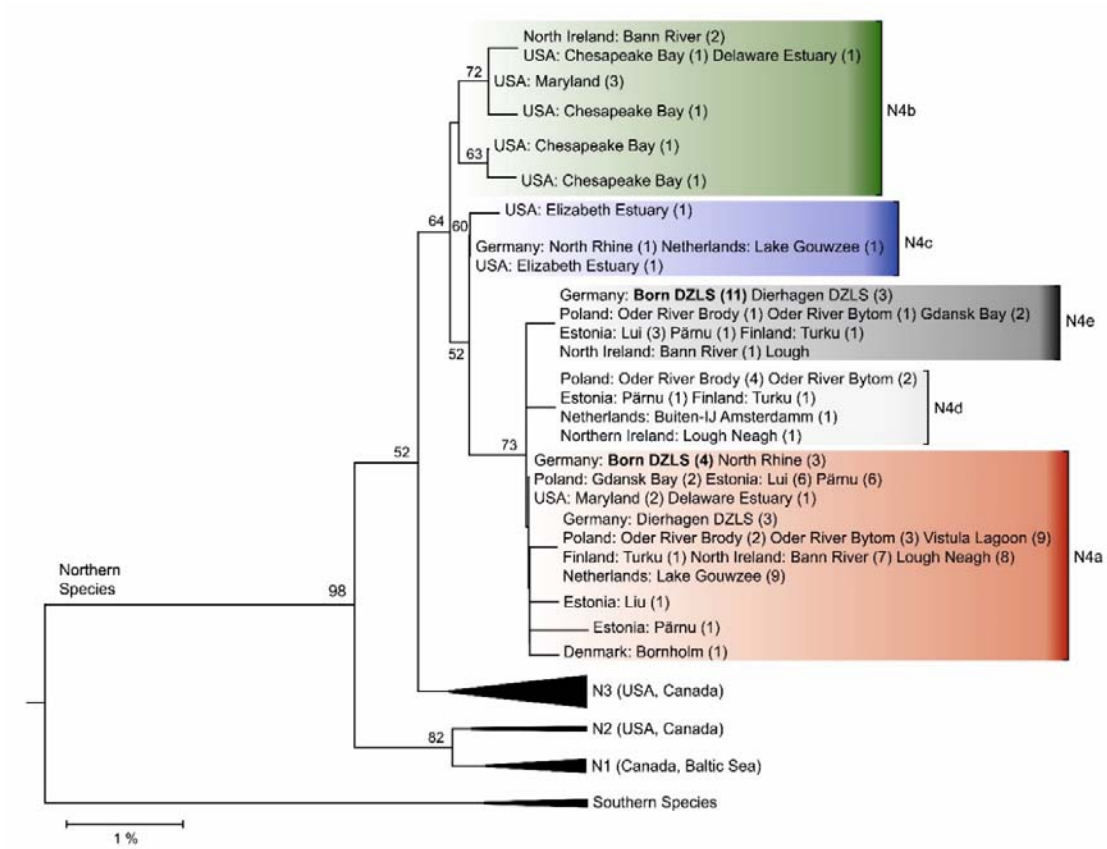


Figure 2 Phylogeny of *Gammarus tigrinus* COI haplotypes inferred by using the Maximum Likelihood method (Tamura-Nei model +I+Γ) with bootstrap values above the branches (1000 replicates). The clade numbers are identical to Kelly et al. (2006) and the coloured boxes indicate haplotypes of clade N4. Haplotypes N4a and N4e comprised the individuals from the DZLS.

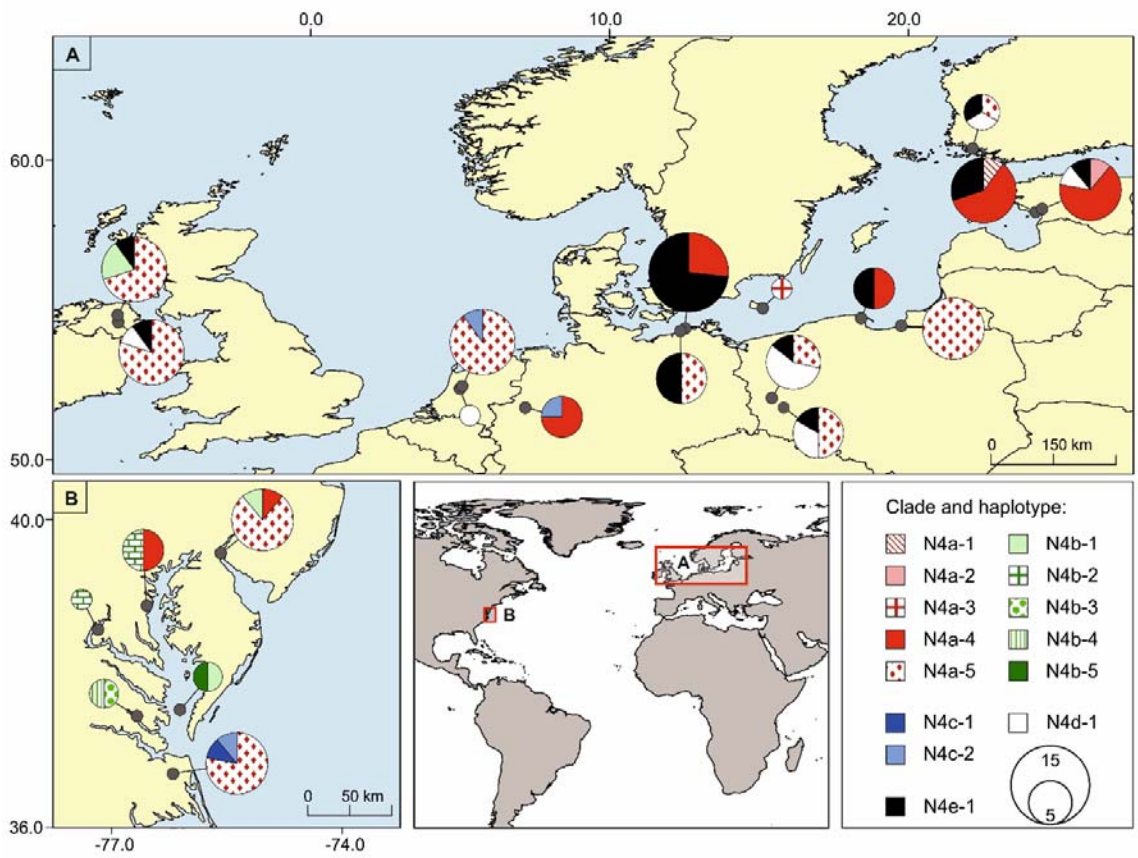


Figure 3 Distribution of *Gammarus tigrinus* populations of clade N4 identified by COI network analysis. Pie charts indicated haplotype frequencies in European (A) and North American (B) populations.

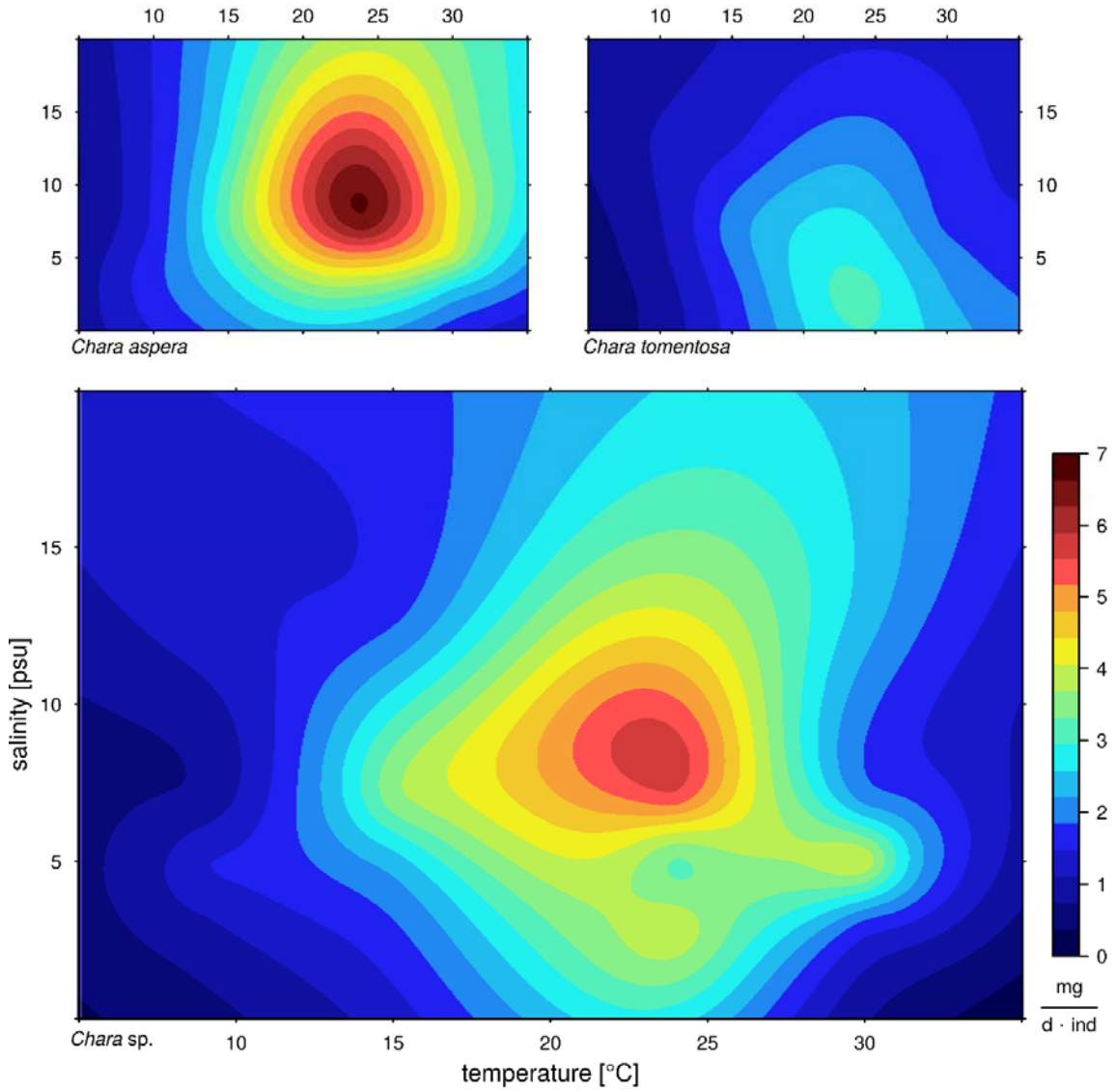


Figure 4 Generalized additive model for grazing rates of *Gammarus tigrinus* on *Chara aspera* (top left), *Chara tomentosa* (top right) and on both Chara species (bottom) in respect to different salinity and temperature levels.

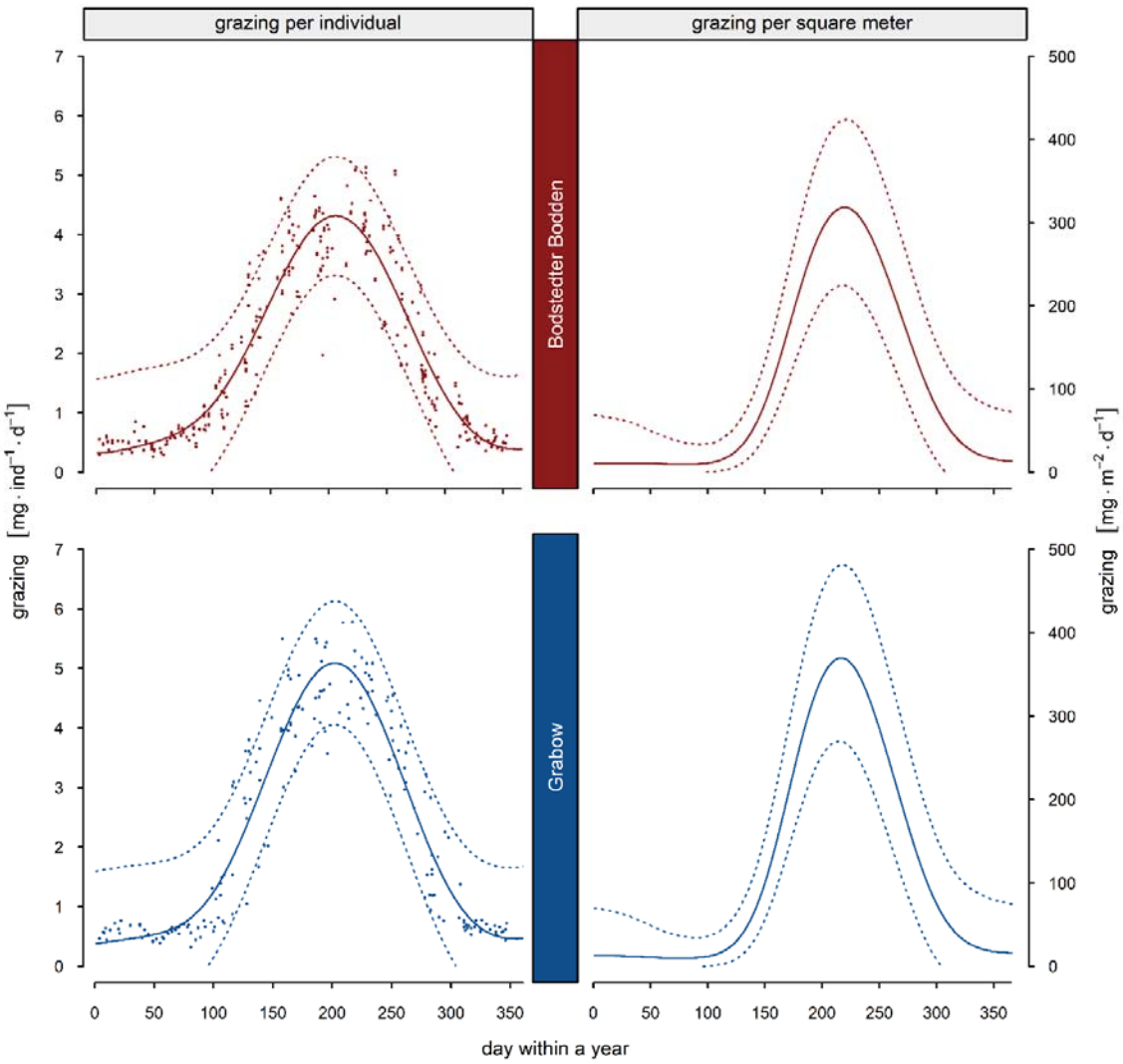


Figure 5: Seasonal grazing rates of *Gammarus tigrinus* in the Grabow and Bodstedter Bodden over the years of 2000 to 2018, whereas individual grazing rates (points) were modelled depending on temperature and salinity conditions at the respective time point in the water body (left). The loss by grazing per square meter were calculated as the product of the left side model and the seasonal pattern of *Gammarus tigrinus* (Supplement Figure S3) with a maximum abundance of 400 individuals per square meter (right).

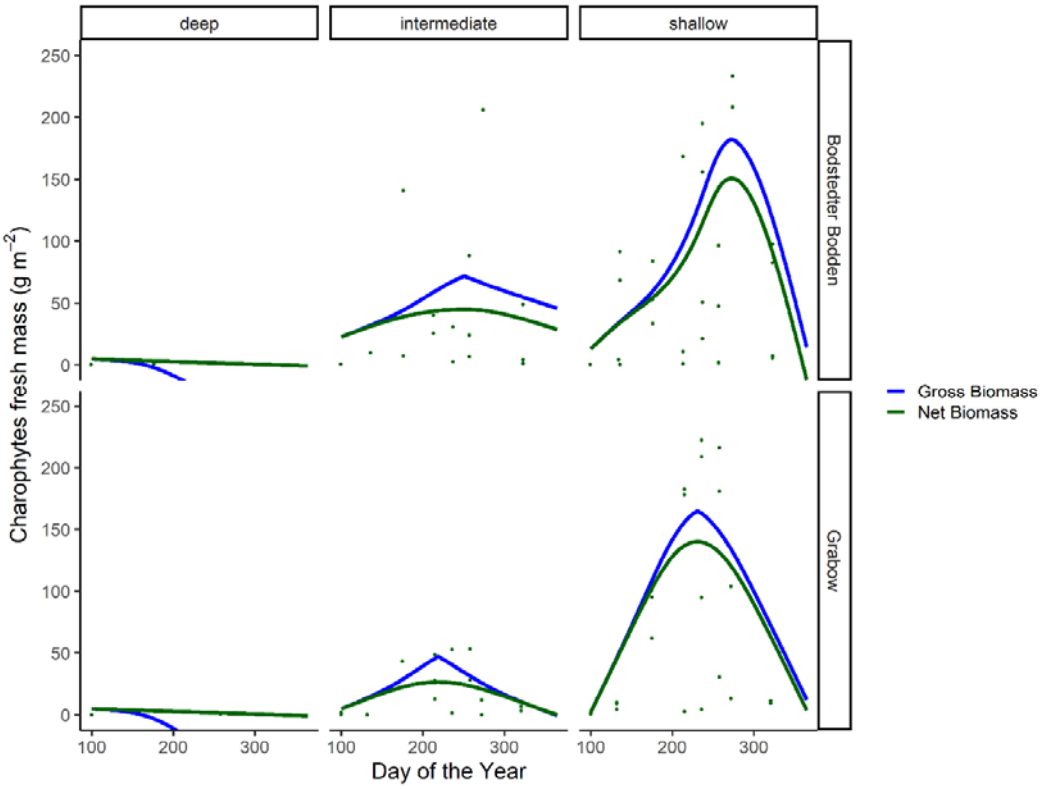


Figure 6 Charophyte fresh mass in gram per square meter at two sampling locations of the Darss-Zingst lagoon system along a water depth/distance to reed belt transect. Shallow depth (30 – 60 cm) and close to reed belt, intermediate depth (60 – 90 cm) and distance to reed belt, deep depth (90 – 120 cm) and farthest distance to reed belt. Net biomass represents model-fitted values (generalized additive model) of observed charophyte biomass. Green points represent observed charophyte biomass at the respective day. Gross biomass represents the sum of net biomass plus biomass possibly lost to *Gammarus tigrinus* grazing.

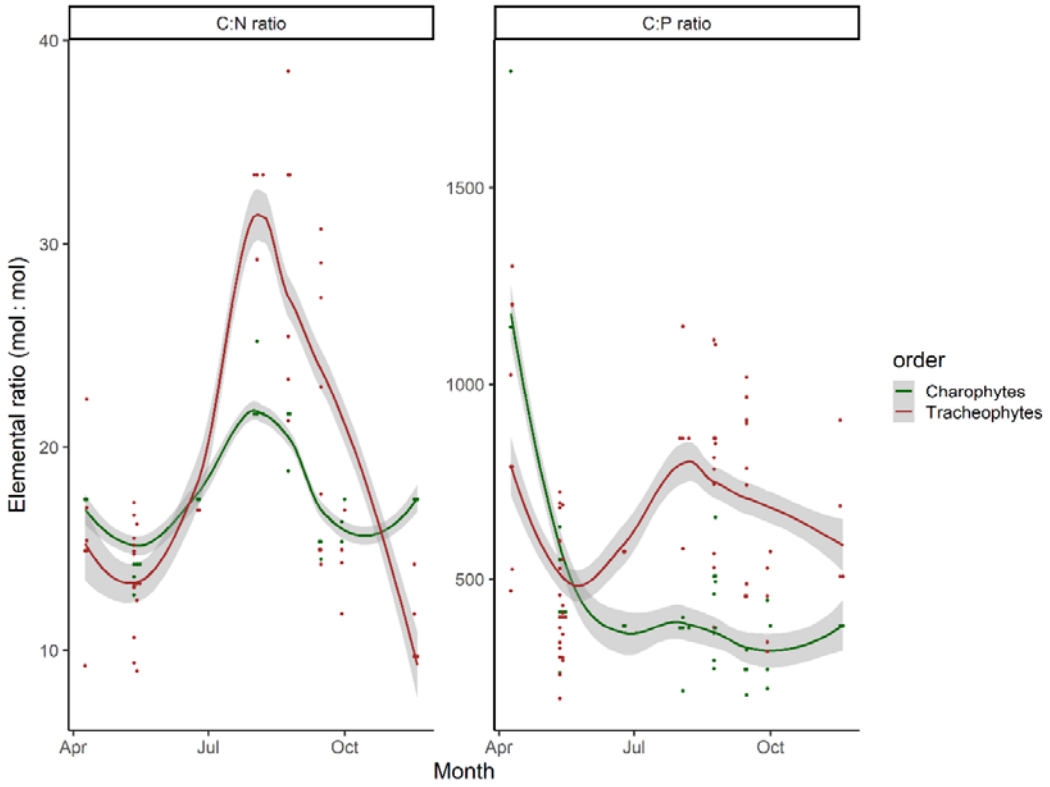


Figure 7 Elemental ratio of Carbon to Nitrogen (C:N) and Carbon to Phosphorus (C:P) of macrophyte biomass (separated by order) at two stations of the Darss-Zingst lagoon system across all sampling stations (Bodstedter Bodden and Grabow) and sampling depths in 2014. Please note the different scaling. Points represent measured elemental ratios, lines a local regression (LOESS – locally estimated scatter smoothing), and the ribbons the 95% confidence intervals (Wickham 2016).

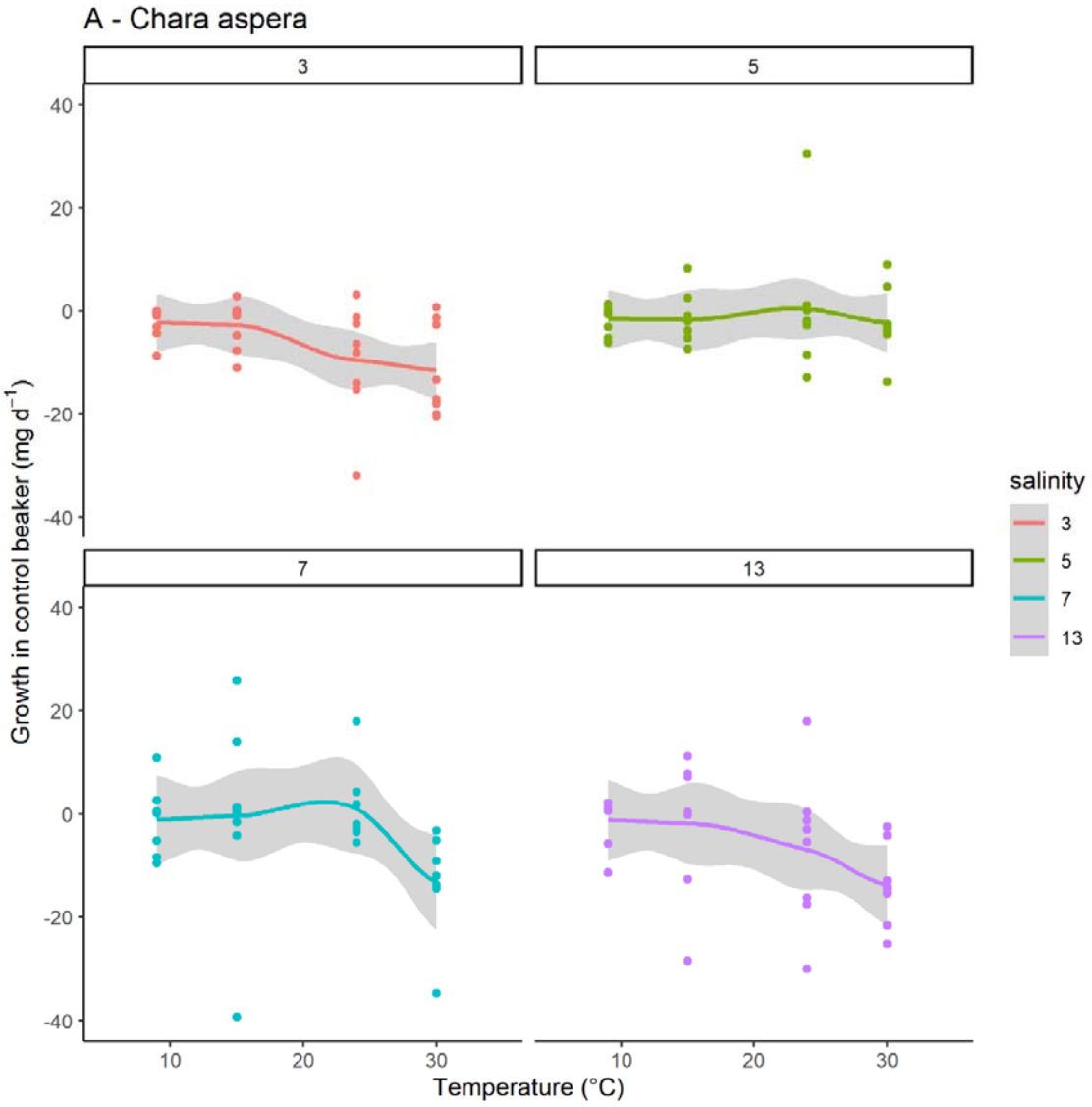
Supplement

Supplement Table 1: Sample list of specimens used for the phylogenetic tree and the determination of haplotypes. Indicated are the localities, if available the salinity of the sampling site, the identified haplotypes and the accession numbers (F=freshwater sites).

Country	**Site**	**Lat., Lon.**	**Sal.**	**haplotype**	**AccessionNo**	**Ref.**
Denmark	Bornholm, Hovedstaden, Baltic Sea, Svaneke	55.1329, 15.152		N1	MK403734	Baltazar-Soares et al. (2017)
	Bornholm, Hovedstaden, Ostersoen stream, Nexo	55.0569, 15.127		N1, N4a	MK403733, MK403736	Baltazar-Soares et al. (2017)
Estonia	Liu	58.2744, 24.2528	4.7	N1, N4a, N4e	KU845009-KU845015, KU845017-KU845021, KU845023-KU845027	Paiva et al. 2018
	Pärnu	58.3748, 24.4734	4.28	N1, N4a, N4d, N4e	KU845029, KU845030, KU845033, KU845035-KU845038, KU845040-KU845043, KU845046, KU845049, KU845050	Paiva et al. 2018
Finland	Turku	60.4, 22.2	4.4	N4a, N4d, N4e	DQ523177, DQ523178, DQ523183	Kelly et al. 2006
Germany	**Born, DZLS**	**54.379434, 12.524819**	**5.5**	**N4a, N4e**	**MW509071-MW509085**	**this study**
	Dierhagen lagoon	54.2, 12.3	1.5	N4a, N4e	DQ523178, DQ523183	Kelly et al. 2006
	Travemümde	53.9655, 10.8851	10	N1	KU844994-KU845006	Paiva et al. 2018
	North Rhine	51.7328950, 7.1774890	F	N1, N4a, N4c	KT075215, KT075216, KT075218-KT075220	Grabner et al. 2015
Netherlands	Buiten-IJ, Amsterdamm	52.369028, 4.990722	F	N4d	EF570348	Hou et al. 2007
	Lake Gouwzee,	52.4, 5.0	F	N4a, N4c	DQ300227, DQ523183	Kelly et al. 2006
North Ireland	Bann river	54.8, -6.4	F	N4a, N4b, N4e	DQ523178, DQ523181, DQ523183	Kelly et al. 2006

	Lough Neagh,	54.7, -6.5	F	N4a, N4d, N4e	DQ523177, DQ523178, DQ523183	Kelly et al. 2006
Poland	Brody, Oder river	52.0, 15.4	F	N4a, N4d, N4e	DQ523177, DQ523178, DQ523183	Kelly et al. 2006
	Bytom, Oder river	51.7, 15.8	F	N4a, N4d, N4e	DQ523177, DQ523178, DQ523183	Kelly et al. 2006
	Gdansk Bay, Puck	54.723394, 18.416633		N4a, N4e	GQ341858-GQ341861	Costa et al. 2009
	Vistula lagoon	54.3, 19.7	4.5	N1, N4a	DQ300212, DQ523183	Kelly et al. 2006
Canada	New Brunswick	47.037389, -65.163250		N1, N2	FJ581678, FJ581679	Radulovici et al. 2009
	Nova Scotia	45.631983, -61.958414		N2	FJ581680-FJ581683	Radulovici et al. 2009
	Quebec	47.149100, -70.521900		N1, N3	FJ581684-FJ581690	Radulovici et al. 2009
	St. John estuary	45.3, -66.2	4.1	N1	DQ300250, DQ300251	Kelly et al. 2006
	St. Lawrence, d/s Quebec, Cap Brule	46.9, -70.5	6.5	N3	DQ300211	Kelly et al. 2006
USA	Berrys creek, New Hampshire	43.0, -70.7	16	N1	DQ300208-DQ300210	Kelly et al. 2006
	Chesapeake Bay, Virginia	37.45, -76.67		N4b	DQ300219, DQ300220, DQ523180, DQ523181	Kelly et al. 2006
	Delaware estuary, Deemers Beach, Delaware	39.6, -75.5	5	N3,N4a, N4b	DQ523181-DQ523184, DQ523189	Kelly et al. 2006
	Delaware estuary, Delaware	39.57, -75.58		N3, N4a	DQ300221, DQ300222, DQ300225	Kelly et al. 2006
	Elizabeth estuary, Virginia	36.7, -76.2	10.2	N3, N4a, N4c	DQ300224, DQ300226, DQ300227-DQ300231, DQ523183, DQ523186-DQ523188, DQ523190-DQ523195	Kelly et al. 2006

	Hudson estuary, New York	40.9, -73.8	7.8	N3	DQ523179	Kelly et al. 2006
	Maryland, Rhode River, SERC Education Dock	38.885, -76.542		N4a, N4b	KU905729, KU905737, KU905742, KU905952	unpublished, direct submission
	Maryland, Thoroughfare Island, Potomac River, Chesapeake Bay	38.5731, -77.175		N4b	MH235818	Baltazar-Soares et al. (2017)
	Neuse River: d/s New Bern, N. Carolina	35.1, -77.0	14.2	Southern Species	DQ300240-DQ300244	Kelly et al. 2006
	Pawcatuck estuary, Rhode Island	41.3, -71.8	11.2	N2	DQ300245-DQ300249	Kelly et al. 2006


A - Chara aspera
3
5
7
13
40
20
0
-20
-40
10
20
30
Growth in control beaker (mg d^{-1})
Temperature (°C)
salinity
3
5
7
13

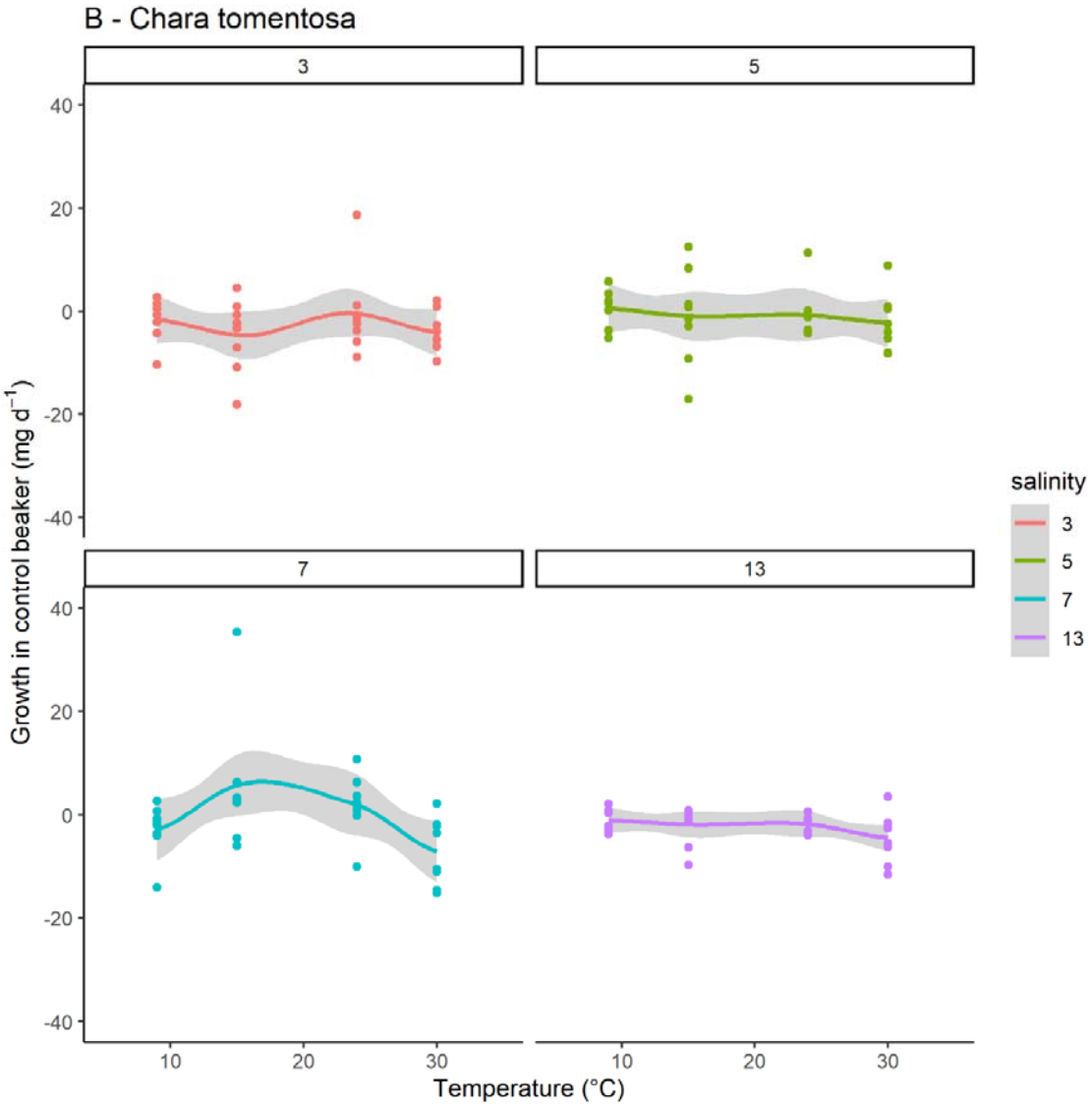
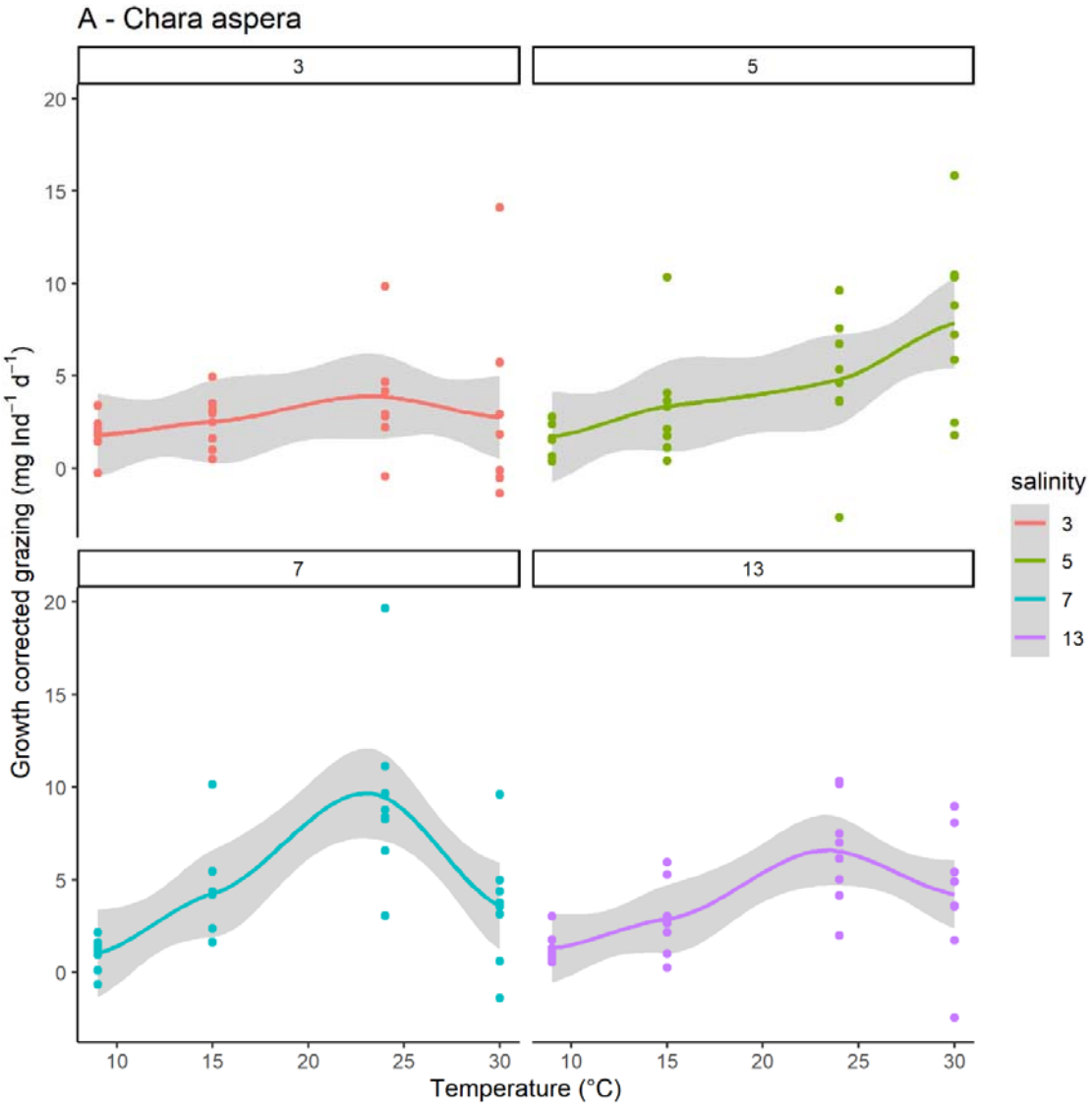


Figure S1 Growth of *Chara aspera* (A) and *Chara tomentosa* (B) in beakers without gammarids after 48 hours (n = 8).


A - Chara aspera
3
5
7
13
Growth corrected grazing (mg Ind^{-1} d^{-1})
Temperature (°C)
salinity
3
5
7
13

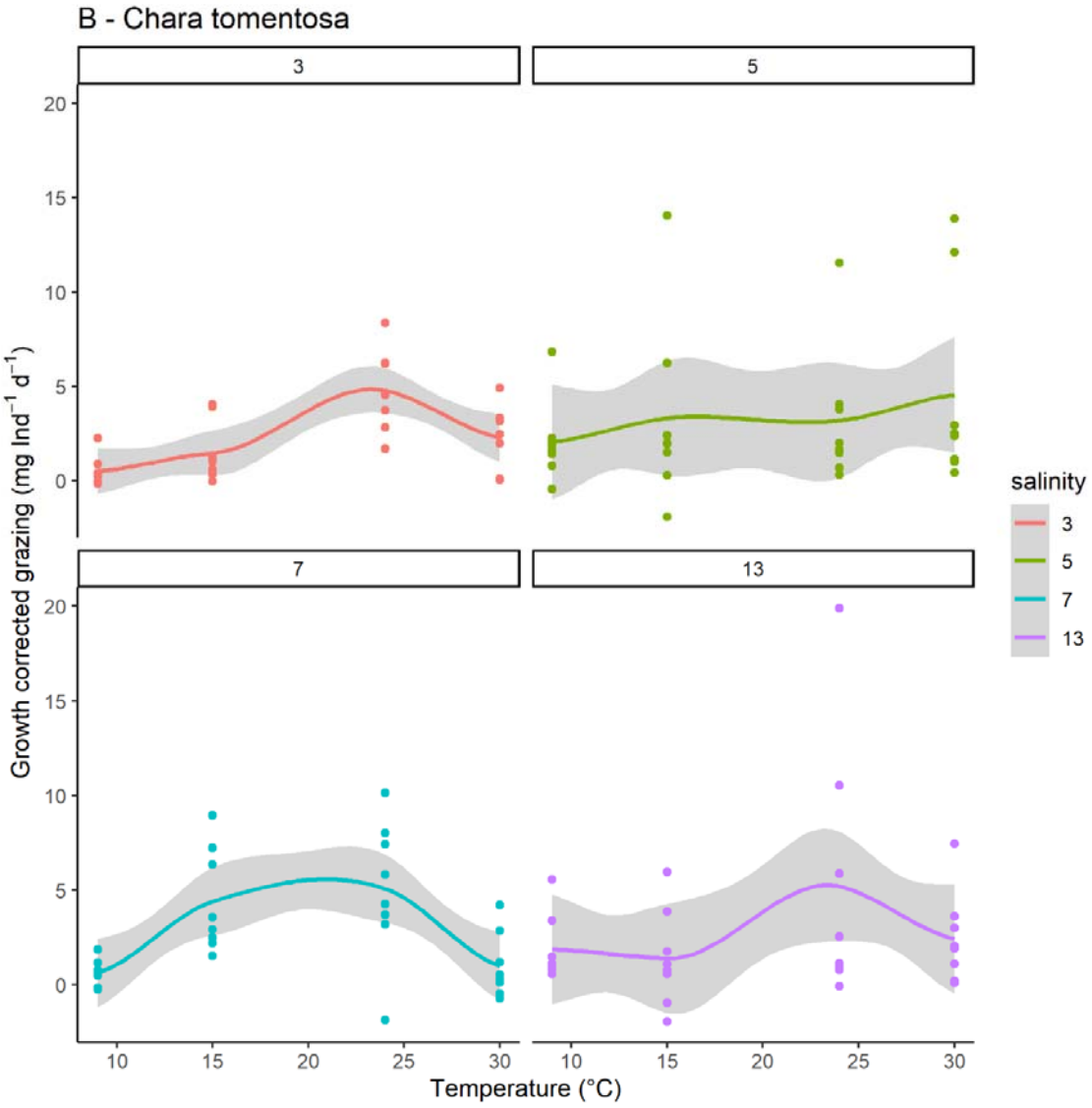


Figure S2 Growth-corrected grazing rates of *Gammarus tigrinus* on *Chara aspera* (A) and *Chara tomentosa* (B) in beakers after 48 hours (n = 8).

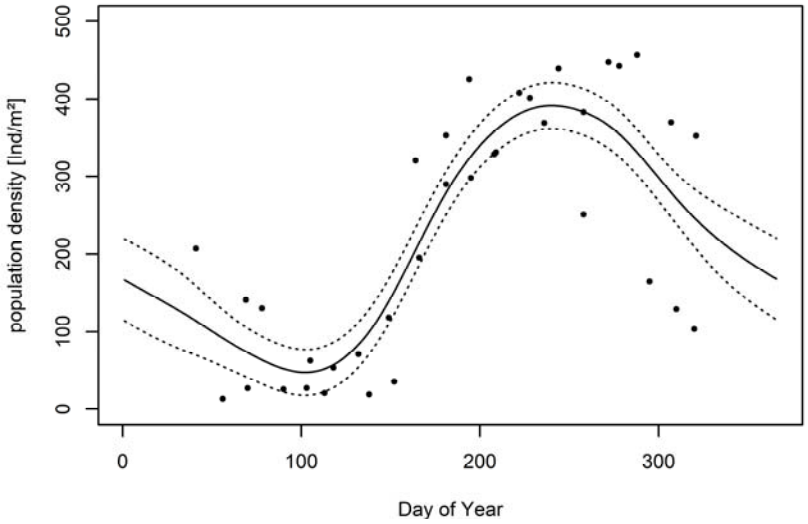


Figure S3 Model of seasonal pattern of *Gammarus tigrinus* calculated with population densities from Chambers (1977) and modified to have a maximum of 400 individuals per square meter based on values of Zettler (1995) and of M. Paar (personal communication).