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ORIGINAL ARTICLE

In situ and experimental evidence for effects of elevated pH on protistan and metazoan grazers

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Plankton succession was studied in a hyper-eutrophic stratified estuary, Mariager Fjord, Denmark. Above the pycnocline (15 m) pH increased from 8.5 to 9.2 and the oxygen increased to super saturation after 5 d of sunny weather due to high primary production. The protistan grazers were dominated by heterotrophic dinoflagellates and mixotrophic and heterotrophic ciliates. Metazooplankton was dominated by meroplankton, rotifers and the copepod, *Acartia tonsa*, all with a relatively low biomass. Cirriped nauplii occupied the upper strata while polychaete larvae populated the whole water column. Bivalve larvae occurred occasionally above the pycnocline even at very high pH. In pH challenge experiments, the mixotrophic ciliate *Mesodinium rubrum* was the least pH tolerant species, followed by *Strombidium* spp., which did not cope well with seawater pH > 8.5. Some heterotrophic dinoflagellates were more tolerant with net growth at pH > 9. The predominant rotifer *Synchaeta* sp. tolerated up to pH 9.5 and the copepod survived pH 10 but stopped producing eggs at pH 9.5 with unaffected egg hatching success. The polychaete and cirriped larvae tolerated pH 9.5, but bivalve larvae showed decreased survival already at pH 8.5. *In situ* distribution patterns and pH challenge experiments suggest that pH indeed contribute to structuring zooplankton distribution.

KEYWORDS: Mariager Fjord; eutrophication; structuring factor; zooplankton; incubation experiments

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INTRODUCTION

Mariager Fjord is 35 km long, with a maximum depth of 30 m and a sill at its mouth. Located at 56° 39.08' N, 9° 58.50′ E, it is a hypereutrophic estuary. Its inner basin has a permanent halocline at 10–15 m overlying hypoxic water. The estuary is characterized by elevated pH of up to 9.75 in the photic zone during the high productive summer period (Hansen *et al.*, 2002; Tiselius *et al.*, 2008). This potentially may affect the productivity of the system as well as cause a reduction in species diversity.

It has been suggested that at high pH levels the availability of inorganic carbon in a form that phytoplankton can utilize (CO_2 and $HCO3^-$) may become limiting to both freshwater and marine phytoplankton growth and photosynthesis (Talling, 1976; Chen and Durbin, 1994; Hinga, 2002; Hansen *et al.*, 2007). In addition, the elevated pH itself may affect the growth of the algae probably due to inability to maintain a constant pH cell environment, which may be crucial for enzymatic processes inside the cells (e.g. Hansen *et al.*, 2007).

The current information of how heterotrophic organisms respond to high pH is very sparse. Droop (1959) found that the heterotrophic dinoflagellate *Oxyrrhis marina* is highly pH-tolerant and could grow well at pH 10. Pedersen and Hansen (2003b) studied the effect of high pH on four ciliates and two heterotrophic dinoflagellates in laboratory cultures, and found that some species are highly pH-tolerant and grew well at pH 9.5–10, while others were quite sensitive to elevated pH. Their growth was affected above pH 8.5 and they could not survive at pH exceeding 8.9. The tolerance among copepods also differ. Species with an oceanic-neritic distribution like *Oithona similis* had a LC_{50} at pH 8.4, while some estuarine species like *Eurytemora affinis* were much more tolerant and did not exhibit LC_{50} before pH reached 9.5 (Hansen *et al.*, 2017). Ringwood and Keppler (2002) even report a stimulated growth response in juvenile clams, *Mercenaria mercenaria* at slightly elevated pH. The exact reasons for the differences in tolerances to elevated pH are however still unknown. Ringwood and Keppler (2002) stated '*It is time to reconsider the potential role of pH in estuaries—it may be more important than previously appreciated*'.

The pelagic environment in Mariager Fjord seems quite hostile at times. During summer, the estuary is characterized by high primary production mainly by chain-forming diatoms, and the heterotrophic protistan and metazoan plankton benefit from the surplus food always above saturation levels. However, taxa that cannot feed on large chain-forming diatoms (e.g. ciliary feeders like bivalve larvae and ciliates) suffer from food limitation (e.g. Petersen *et al.*, 2002). In late summer, the zooplankton community additionally experiences deteriorating environmental conditions including anoxia and sulfide (Fenchel *et al.*, 1995). Due to frequent severe hypoxic and even anoxic events, the inner section of Mariager Fjord is susceptible to recurring defaunation (Hansen *et al.*, 2002) followed by recolonization by propagules brought in by water intrusions from source populations in the outer estuary and open waters in Kattegat. The metazoan plankton is characterized by a low diversity, with essentially only one opportunistic copepod species (*Acartia tonsa*) showing low biomass despite high reproduction (Tiselius *et al.*, 2008). The protistan grazers, with short generation times, are dominated by a few completely heterotrophic species. The meroplankton are composed of several taxa (Hansen *et al.*, 2002; Petersen *et al.*, 2002), supplied by local populations and by the import from open water habitats. Predation pressure from pelagic zooplanktivores (fish and jellyfish) is modest and so is diurnal vertical migration due to anoxia below the pycnocline. More specifically, we expected that oxygen availability and in particular pH, determined the structure and distribution of the plankton community. To test this hypothesis we monitored environmental parameters and plankton in time and space over 9 days during late August 2005. Moreover, we conducted short-term challenge experiments on zooplankton pH tolerance. Our experimental variables were survival, and in addition for the protistan grazers and rotifers, their population growth, and for the copepods, egg production and egg hatching success. We aimed to resolve the spatial and temporal zooplankton distribution in the estuary, with special focus upon pH. This was achieved by linking *in situ* observations with obtained pH tolerance results.

METHOD

In situ sampling program

Samples were taken around true noon on 16, 18, 20, 22 and 24 August 2005 at a station in the deepest, central part of Mariager Fjord (Fig. 1). Water column structure was identified by Conductivity, Temperature, Density (CTD) casts (ME-profiler, Meerestechnik) measuring temperature, salinity and fluorescence as a proxy for chlorophyll *a*. Aliquots of 3 × 0.5 L were sampled every second meter through the water column and filtered onto 25 mm GFC-filters. The filters were extracted in 5 mL 96% ethanol overnight (Jespersen and Christoffersen, 1987), and pigments were measured on a Chl *a* TD-700 fluorometer (Turner) calibrated against a chl *a* standard before and after acidification (Yentsch and Menzel, 1963). Oxygen concentration profiles were from the surface down to

10 m of water depth measured using an oxygen meter (WTW oxi 196) equipped with a probe (WTW 196-10) inserted into a stirrer unit (WTW BR190). At water depths of 11–20 m, samples were taken every second meter from 10 L Niskin sampling bottles and oxygen concentrations were measured with spectrophotometric Winkler determination as described by Labasque *et al.* (2004). Based on the water column structure, zooplankton sampling depths were identified and collected using a 30-L Niskin sampling bottle at discrete depths (0, 2, 4, 6, 8, 10, 12, 15 and 20 m). The water samples were decanted to 100 mL brown medicine bottles and fixed with acid Lugol solution (1% final concentration) for enumeration of ciliates and heterotrophic dinoflagellates, and the rest of the whole 30 L sample was buffered formalin concentrated on a 45 μm mesh size sieve and fixed in formaldehyde (5% final concentration) for enumeration of meroplankton and copepods. For protistan grazers and rotifers, up to 10 aliquots of 10 mL were analysed under inverted microscope (×400 magnification, Üthermöhl 1958). The heterotrophic protists were so characteristic that they were easily recognizable in Lugol fixated samples. For meroplankton, all samples were analyzed under a dissection microscope (×50 magnification) and all organisms were counted and determined to major taxa at the level of Class. All zooplankton abundance data no matter numerical levels are presented. In the case of relatively low counting numbers, we are aware that these are imprecisely determined; however, data indicate low abundance at the location. Copepods were determined to species (see Tiselius *et al.*, 2008). Depth-integrated zooplankton biomasses (g C m^{-2}) were calculated by trapezoidal integrations, based on abundance and length measurements of the organisms. To obtain biomass the protistan cell volumes were calculated separately for each species/-size group on the basis of linear dimensions, assuming simple geometrical shapes. Cell carbon was estimated by multiplying volumes by 0.11 pgC μm^{-3} for athecate dinoflagellate and ciliate species and 0.13 pgC μm^{-3} for thecate dinoflagellate species (Edler, 1979; Lessard, 1991). The carbon conversion of multicellular zooplankton was made using literature regressions; *A. tonsa* (Berggreen *et al.*, 1988), planula larvae (as naked ciliates), gastropod larvae (Hansen and Ockelmann, 1991), bivalve larvae (Fotel *et al.*, 1999), polychaeta larvae (Hansen, 1999), cirriped nauplii (as *A. tonsa* nauplii *sensu* Berggreen *et al.*, 1988) and rotifers (Hansen *et al.*, 1997). To verify the diurnal pH shift in the upper 20 m, we conducted two diurnal studies (20–21 and 24–25 August). The oxygen (same oxygen meter as above) and the pH was measured directly in the Niskin sampling bottle on each sampling occasion using a Sentron® Argus pH meter equipped with a HOT-Fet line pH probe calibrated using standard buffers of pH 7 and 10 at all sampling depths every fourth hour from 10–10 a.m.

pH challenge experiments conducted in microosms and bottles

pH in seawater can in principle be changed in different ways. The most common methods are (i) by bubbling with gasses, i.e CO_2 for lowering the pH and free N to wash out the inorganic carbon pool to create elevated pH or (ii) the addition of an acid (HCl) or a base (often NaOH). Each method has its strengths and weaknesses. The bubbling with gasses mimics the removal of inorganic carbon in a similar way as the algae does. However, bubbling natural communities will strongly affect especially the protist community, since they are very sensitive to turbulence and air bubbles (e.g. Rost *et al.*, 2008). Addition of a base allows for changes in seawater pH without, in principle any effect on the composition of the plankton community. This method has been applied with success in cases where large differences in seawater pH are required for experimental purposes (e.g. Pedersen and Hansen, 2003a; Berge *et al.*, 2010; Nielsen *et al.*, 2010). The downside of this method is that the pool of inorganic carbon stays more or less constant because it is not removed from the water, thus potentially underestimating the effects of elevated pH on photosynthetic protists. The focus in the present contribution is mainly on the heterotrophic organisms that are not expected to be affected by the inorganic carbon pool in the water, but rather pH itself. Therefore, we chose the method with base adjustments. We only observed minor diurnal changes in pH in the challenge experiments (<0.1 pH unit each time).

The pH challenge experiments were conducted for monitoring the effect on survival and growth of various zooplankton taxa. *In situ* microcosms (30 L polycarbonate bottles) for protistan grazers and rotifers, and bottles (50 mL NUNC plastic bottles) for meroplankton and 500 ml polycarbonate bottles for copepods were used, respectively. For the 30 L bottles, water from 2 m was collected on 18th August. In brief, the pH in each microcosm was measured and adjusted by adding 0.1 N NaOH or HCl to pH of 8.0, 8.5, 9.0, 9.5 and likewise in the bottle experiments that also contained pH 10 and 10.5. The collected water had an initial pH of 8.65. The adjustments for the 8.0 and 8.5 experiments were done instantaneously. For the 9.0 treatment, they were first subjected to 8.5 for 6 h where after pH was raised and for the 9.5 treatments they were adjusted from 9.0 to 9.5 after another 6 hours and so forth for the 10 and 10.5 treatments. The pH was measured directly in the microcosms using a Sentron® Argus pH meter equipped with a HOT-Fet line pH probe calibrated using standard buffers of pH 7 and 10.

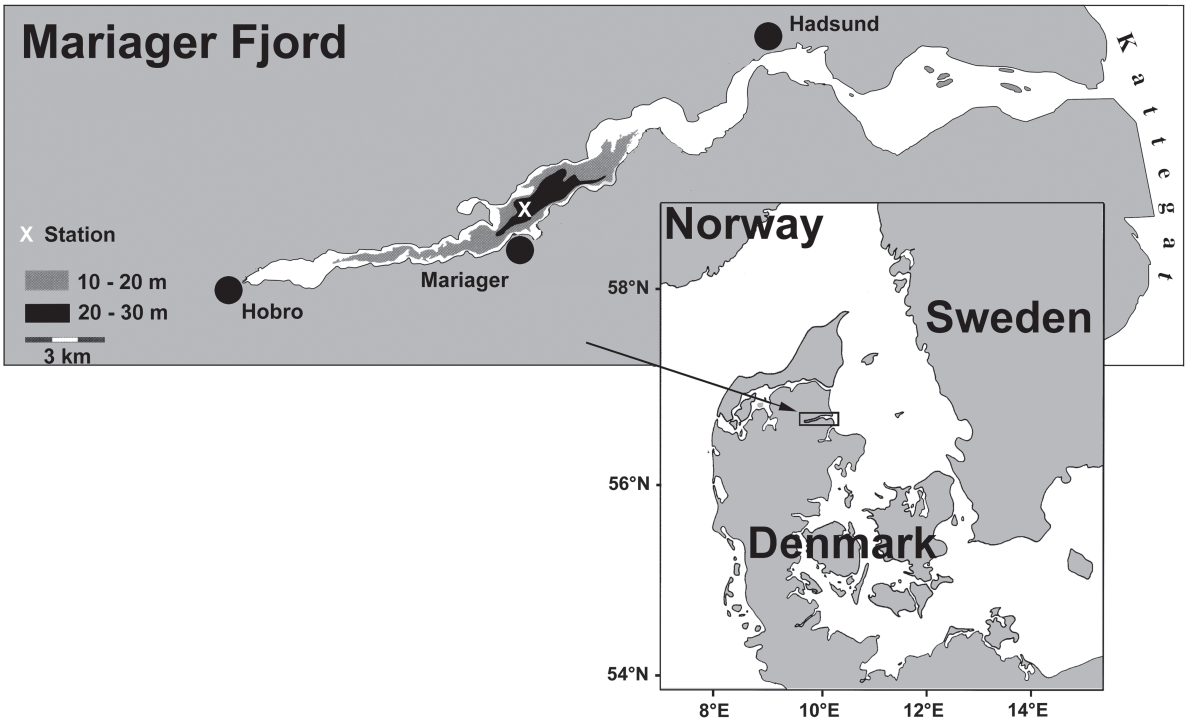

Fig. 1. Map of Mariager Fjord on the east coast of Jutland, Denmark. Sampling station is indicated by X (position: 56 ° 39.08' N, 9 ° 58.50′ E). The area considered in the present paper is the inner part of the estuary sampled through a 9 d campaign in August 2005.

Microcosmos incubations in the estuary

Twelve microcosms were filled with 200-μm pre-screened estuary water from 2 m depth. Three replicates of each pH treatment were adjusted to pH 8, 8.5, 9 and 9.5 initially as stated above as well as once every day the following days. The microcosms were incubated for 72 h (18–21 August) floating at the surface of the estuary. Initial and final samples (50 mL) were taken and zooplankton fixated with acid Lugol solution (1% final concentration) and analyzed by inverted microscopy.

Bottle incubations in the laboratory

The experiments were conducted in a temperature controlled walk-in container set to *in situ* estuary surface water temperature (17–20 °C) and dim light. The pH range was from 8.0 to 10.5 in steps of 0.5 units. For all incubations, the pH was adjusted stepwise as described above for the microcosm experiments. Meroplankton experiments were done in 70-μm pre-screened estuary water in triplicate and three control bottles were not manipulated. For the bivalve larvae only one start bottle and one final bottle per treatment were used as too few larvae were available. Larvae were individually picked with 25 bivalve larvae (*Mytilus edulis*, D-stage velichoncha larvae originating from Limfjord situated north of Mariager Fjord), 50 polychaete larvae (dominated by spionid larvae of variable sizes) and 50 cirriped nauplii (*Balanus* sp.) both collected from Mariager Fjord was added to each incubation bottle. We inspected the animals directly in the incubation bottles under a dissecting microscope after acclimation to pH's and observed that all were alive when the 24-h challenge experiments were initiated.

In the challenge experiments testing effects of pH on the copepod *A. tonsa* on survival, egg production and egg hatching, specimens (females) were collected by 200 μm mesh size closed cod-end WP-2 net hauls. The catch from the hauls were gently brought to the temperature controlled laboratory container in an insulated box. Thereafter individuals were individually pipetted under a dissecting microscope, and transferred to 2-L polycarbonate bottles (20 per bottle) containing 45 μm filtered estuary water. The pH was adjusted gradually, every 6 h in triplicate as above, 8.0, 8.5, 9.0, 9.5, 10.0 and 10.5. After acclimation, the copepods ($n = 20$) were picked individually and transferred to three replicate 500 mL polycarbonate bottles and incubated for 24 h at their respective pH treatment. The protocol for death determination was to poke the copepods with a dissecting

needle after leaving them in a Petri dish for 3 minutes. If no movement at all, the copepod was considered dead.

From the same incubation bottles, eggs were separated from copepods, by sequential filtration (200 μm followed by 45 μm), transferred to Petri dishes with 10 mL water from the respective bottles, and counted (egg production). Afterwards the eggs were incubated for 24 h in Petri dishes using the same media as for the survival experiment. After incubation, the hatching success was determined by counting the eggs and live and dead nauplii under a dissecting microscope.

Statistical methods

The effect of elevated pH on survival and growth, egg production, hatching rates in the challenge experiments was evaluated using one-factor ANOVA with pH levels as fixed factors. The following three comparisons were made: (i) survival of animals, (ii) egg production and hatching success for *A. tonsa* and (iii) microcosm experiments over 3 days in which abundances in exposed communities of plankton were recorded. For survival of meroplankton, copepods and organisms in the bottle incubations, the number of living animals at the end of the incubations at pH = 8 was used as a common control for increased pH levels. Differences between the initial abundances and those after 72 h at pH 8 were tested with *t*-tests. Egg production and hatching success at elevated pH levels were compared to rates at pH = 8 that served as the common control. For both microcosm and bottle experiments, the effect of elevated pH levels were compared to the respective controls (pH 8) using ANOVA and Dunnett's *post-hoc* test (two-sided). Significant differences were assigned when $P \leq 0.05$ indicated in figures by an asterisk (∗). Number of replicates were too few ($n = 3$) to conduct rigorous tests for normality, and the requirement of equal variances for all treatments were judged from box-plots rather than from formal tests. Indeed, variances differed significantly in some survival experiments (*Lohmaniella oviformis*, polychaete larvae and *A. tonsa*), but in all cases the means were also very different. All statistical tests were performed using IBM SPSS Statistics (v. 25).

RESULTS

In situ observations

Mariager Fjord zooplankton assemblage was mainly characterized by protozoan grazers and meroplankton. The meroplankton accounted four major groups, Gastropoda-, Bivalvia-, Polychaeta- and Cirripedia larvae (Fig. 2). The inner estuary was strongly stratified during the campaign, with a warm (17 to 20 °C); mixed surface layer down to 12–15 m and cold (<5 °C), deep waters below 20 m (Fig. 3). Salinity profiles were stable throughout the campaign and ranged from 15 to16 in the upper mixed layer, to 20 in the deep water. The strong thermocline was located at 15 m and coincided with the weaker halocline together forming a pycnocline at ∼15 m of depth. Fig. 4 depict that oxygen was present above 15 m, but declined rapidly below the pycnocline. The condition reached anoxia at 20 m and below (data not shown). Based on the smell from the water samples we observed that sulfide was present at 20 m and episodically occurred at 15 m. pH was ∼8 at the pycnocline dropping to pH 7.5 close to the bottom. pH increased from 8.5 on 17th August to pH 9.2 on 22 August in the upper water column, where after it decreased slightly toward the end of the investigation (Fig. 4). Oxygen concentration below the pycnocline demonstrated anoxia. Just above the pycnocline it was touching severe hypoxia (2 mg L^{-1}) during afternoon and increased with time to reach hypoxia (4 mg L^{-1}) during evening (Fig. 5). Further up in the water column oxygen fluctuated diurnally always above adequate concentration for zooplankton and it build up to supersaturated level > 10 reaching 16 mg L^{-1} during afternoon. However, pH were more constant diurnally with only minor drops in pH during nighttime (which is only 6 h at this time of the year). The pH in 0 m depth ranged from 8.92 to 9.13, in 10 m depth from 8.32 to 8.50 and in 20 m depth, it was constant 7.8 throughout 24 h (Fig. 5). There were no major exchanges of water with the Kattegat judged from the CTD profiles that showed a stable stratified water column.

During the campaign, a bloom of the chain-forming diatom *Skeletonema marinoi* resulted in increasing chlorophyll *a* concentrations. From already high values (15–18 μg chl *a* L^{-1}) on the first date, the bloom more than doubled in biomass in 2 d, reaching peak values of 46 μg chl *a* L^{-1} on 18 August (Fig. 4). Within 6 days, the bloom disappeared, chl *a* concentrations went back to the original values, but the phytoplankton biomass was now mainly dominated by the phototrophic dinoflagellate *Heterocapsa triquetra* instead of *S. marinoi*.

Protistan grazers were dominated by heterotrophic and mixotrophic ciliates (*L. oviformis*, and especially *Strombidium* spp. and *Mesodinium rubrum*) consistently found above the pycnocline and by heterotrophic dinoflagellates, *Gymnodinium dominans* and *Oblea rotunda*, suspended in the entire water column. We depict the distribution patterns of the most dominant groups of protistan grazers in Fig. 6. In the upper 10 m metazoan holoplanktonic grazers

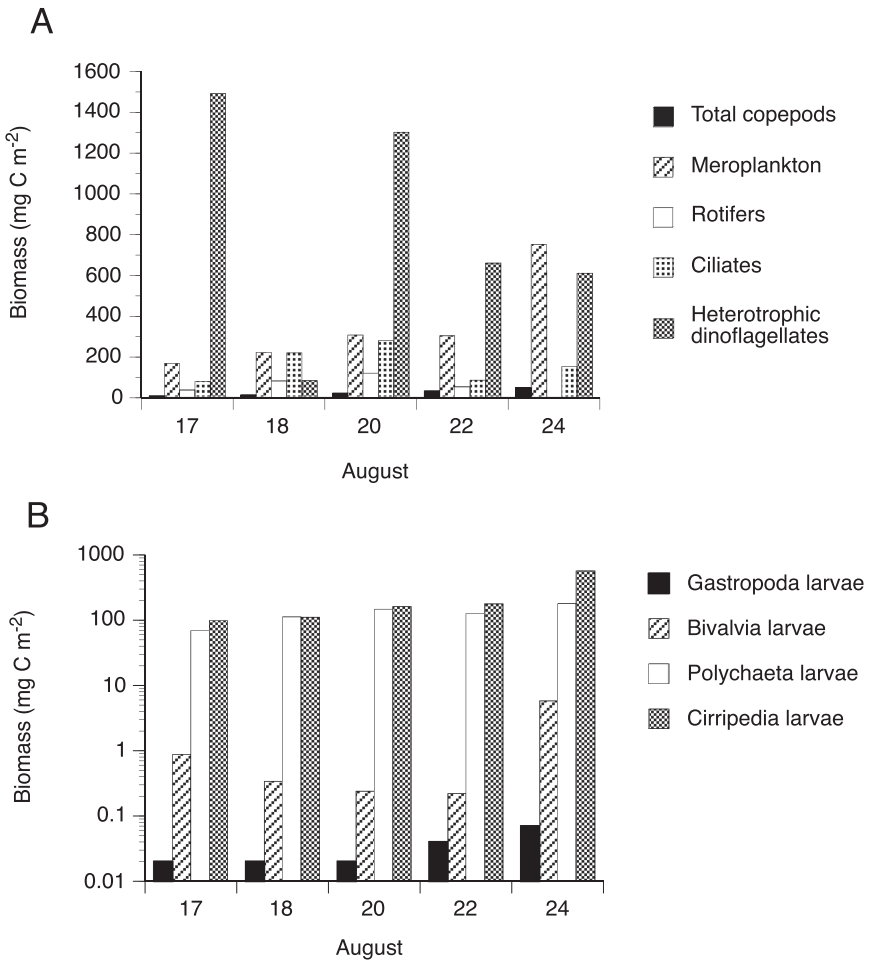

Fig. 2. Depth integrated biomass estimated from size of **A**: main holozooplankton taxa and **B**: main meroplankton groups present in the inner part of Mariager Fjord. Note log scale in B.

were dominated by rotifers *Synchaeta* and Trichocercidae species and others (Fig. 7). The meroplankton exhibited somewhat different distribution among taxa in both time and space (Fig. 7). The ciliated planula larvae were present first half of the campaign and situated from 15 to 5 m depth. Other ciliated larvae were represented by (i) gastropoda larvae, present all through the campaign in 10–5 m depth; (ii) bivalvia larvae, present in the upper 10 m just at the beginning and the very end of the campaign; and (iii) polychaeta larvae (mainly spionid metatrochophorans of variable sizes), with the most abundant presence all through the water column, but mostly in the upper 7 m. The predominant cirriped nauplii larvae, *Balanus* sp., were most frequent the first half of the campaign in the upper 7–8 m of the water column.

Among larger mesozooplankton, the copepod *A. tonsa* was the dominant species (see Tiselius *et al.*, 2008 for details). Only a few *Centropages* spp., *E. affinis*, *Oithona* spp. and harpacticoids were recorded, and together they constituted <5% of the copepod abundance.

pH challenge experiments

Microcosm experiment

The microcosm experiment investigated potential effects of Mariager Fjord relevant elevated pH on protistan grazers and the rotifers (Fig. 8). After 3 days of incubation the pH 8 treatment was significantly different from the start concentration. The mixotrophic ciliate *M. rubrum* had declined significantly in number from the start (*t*-test, $P = 0.043$). Nevertheless, this species was found in the pH 8 and 8.5 treatments after 3 days of incubation, while it had disappeared completely from the pH treatments 9 and above. The other protistan grazers and

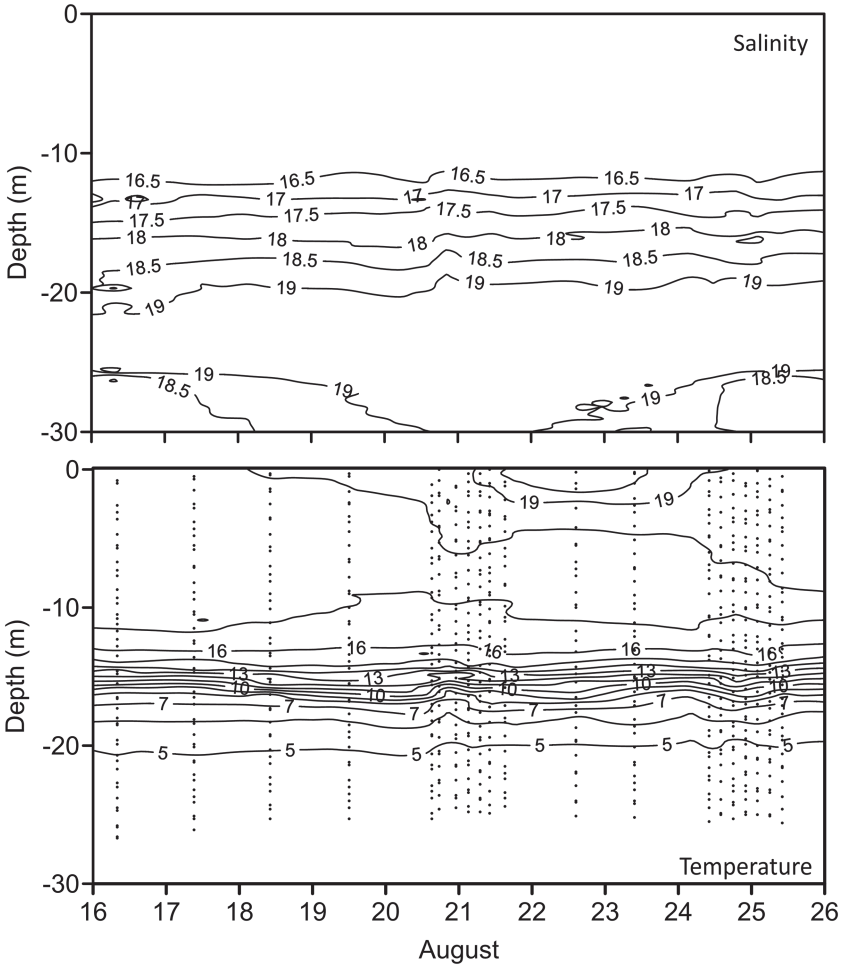

Fig. 3. Isopleths of salinity and temperature (°C), in the inner part of Mariager Fjord.

the rotifer *Synchaeta* sp. were more pH resistant. All of these species increased in numbers during the incubation at pH 8, significantly for *Strombidium* spp. ($P = 0.022$), *L. oviformis* ($P = 0.0002$) and *Synchaeta* sp. ($P = 0.002$), while there were significant declines in abundance at higher pH. Declines in population numbers were observed at pH 8.5 for *Strombidium* spp., 9.0 for *L. oviformis* and 9.5 for *O. rotunda* and *Synchaeta* sp. Hence, apart from *M. rubrum* and *Strombidium* spp. a pH tolerance to pH 9 was observed; evidently higher pH levels led to declines in populations numbers.

Bottle experiments

The 24-h bottle experiments were conducted to monitor possible effects of Mariager Fjord relevant elevated pH on three groups of meroplankton. These organisms were collected individually and incubated for 24 h in the pH range. Bivalvia larvae showed a gradual response, starting to decline already at pH 8.5 reaching almost zero survival at pH ≥ 9.5, but lack of replicates prevent statistical analysis. The polychaeta larvae and cirripedia nauplii survived pH levels up to 10, above which they significantly declined or were all dead (Fig. 9).

Copepod survival was unaffected up to pH 10, followed by a drastic mortality at pH 10.5. They exhibited equal egg production at all pH treatments up to pH 9.5, and no significant effect on egg hatching success was observed (Fig. 9).

DISCUSSION

The ocean acidification phenomenon caused by increasing anthropogenic CO_2 loading is expected to have its greatest impact in the open ocean (Hofmann *et al.*, 2011;

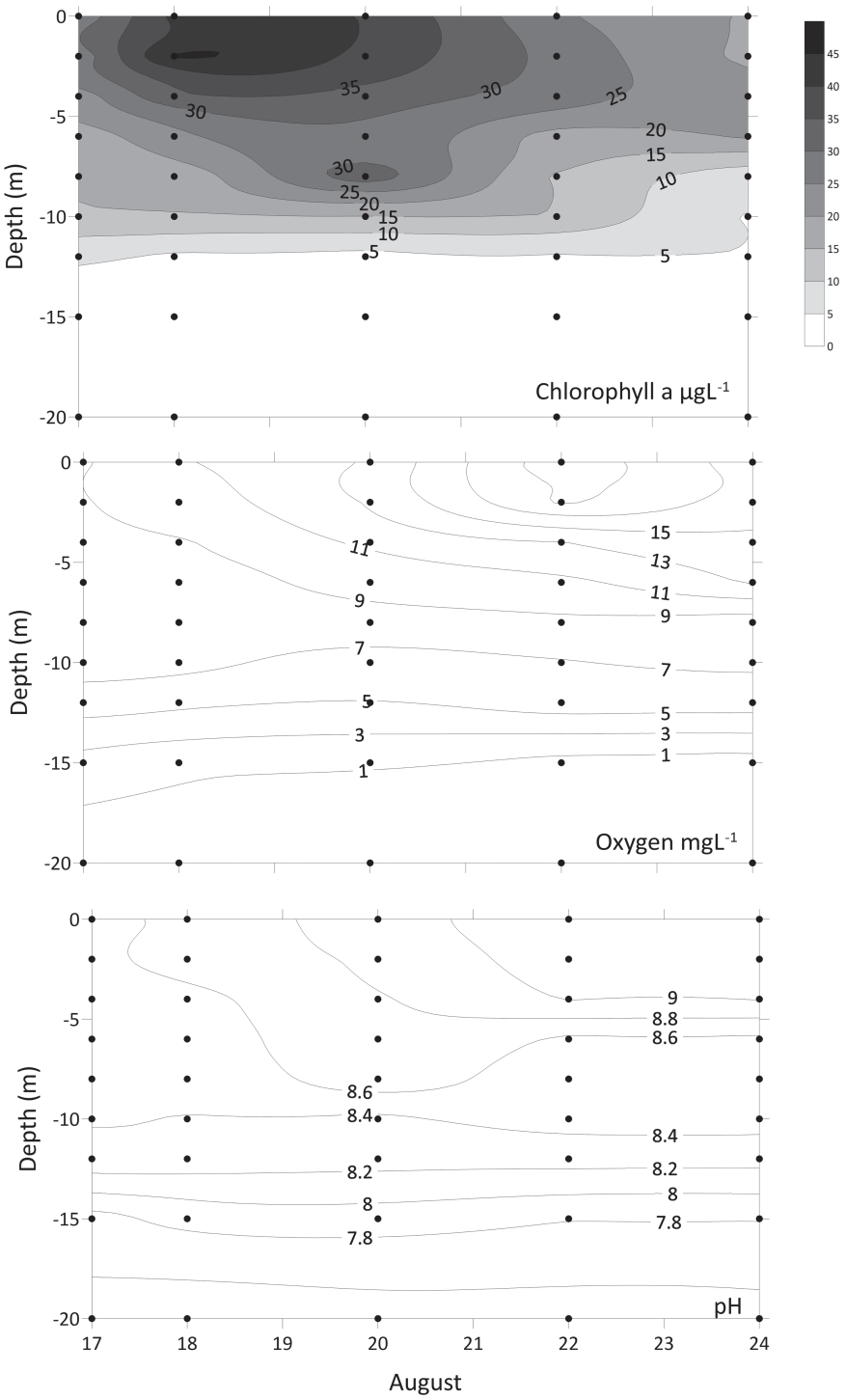

Fig. 4. (Isopleths of chlorophyll µg chl *a* L^{-1}) as proxy for phytoplankton biomass, oxygen concentration (mg L^{-1}) and pH in the inner part of Mariager Fjord.

Duarte *et al.*, 2013). However, eutrophic estuaries in e.g. Denmark and the UK are described to be prone to significant pH changes during summer (Howland *et al.*, 2000; Carstensen *et al.*, 2018) and coastal phytoplankton may be negatively affected by elevated pH (e.g. Hinga, 2002; Hansen, 2002; Hansen *et al.*, 2007).

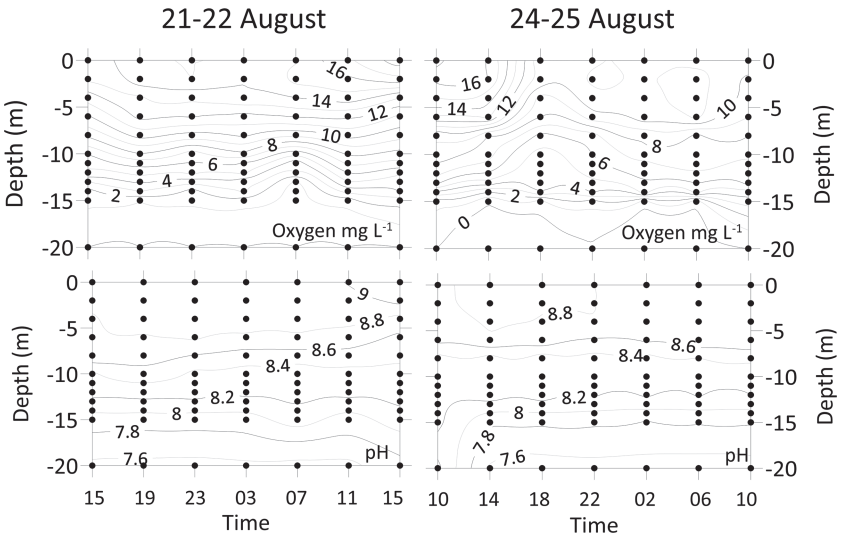

Fig. 5. Diurnal campaigns showing fluctuations of oxygen (mg L^{-1}) in the upper panels and pH in the lower panels in the inner part of Mariager Fjord.

The critical environmental parameter for zooplankton in the model system here, Mariager Fjord, is the unusually high pH in surface waters during the productive season. Since hardly any zooplankton resides below the pycnocline, the zone above the pycnocline with surplus of food particles, elevated pH and negligible predation was our primary focus. To our knowledge, this study is the first to compare *in situ* observations of elevated pH with spatial and temporal distribution to pH challenge experiments of zooplankton, spanning from protistan to metazoan plankton. The ambition of the present study was to test the hypothesis that the zooplankton distribution pattern can be explained by the alkalinity of the water and the corresponding sensitivity of the organisms to elevated pH levels. An abundant literature exists on organismal responses to anthropogenic low pH (e.g. Duarte *et al.*, 2013), but there is a clear lack of data for the common and natural conditions of high pH.

The hydrography during the sampling campaign revealed a very stable scenario with hardly any changes in salinity in the water column. According to Hansen *et al.* (2002) the water change in the inner estuary is limited. This narrow fjord receives land runoff and regular intrusions of water from the open Kattegat. The latter can be identified by salt balance calculations and by changes in phytoplankton composition. Major water intrusion and freshwater runoff to Mariager inner fjord in 1998 took place in May and minor intrusions in June–July. Hence, there are reasons to believe that negligible water intrusions took place during our campaign in late August 2005. Moreover, it was a dry summer with hardly any wind and where limited freshwater runoff took place, why we strongly believe we sampled in the same water mass during the present study.

Elevated pH in estuarine systems is often associated with high photosynthetic activity, which is indeed characteristic for Mariager Fjord. It is considered one of the most productive estuaries in the world and the primary production reaches on an annual basis ~1000 g C m^{-2} in the inner part of the estuary (Conley *et al.* 2000; Olesen 2001). For comparison, >30 Danish estuaries and the Gullmar Fjord on the nearby Swedish west coast has an annual production ranging from 200–350 g C m^{-2} (Conley *et al.*, 2000; Tiselius *et al.*, 2016). The massive primary production cause oxygen depletion below the pycnocline (Olesen 2001) as verified here. Mariager Fjord hosts a high biomass of benthic suspension feeding animals but metazoan plankton populations are in general small (Tiselius *et al.*, 2008).

The substantial primary production generates a high pH due to uptake of inorganic carbon by the phytoplankton. The inorganic carbon-drain exceeds the delivery rate from the bicarbonate system, which challenges the estuarine buffer system driving the pH upwards (Chen and Durbin, 1994). The elevated pH has been suggested to structure the phytoplankton community during the productive season (April–October) in Mariager Fjord leaving only a few species able to tolerate the pH often reaching well above 9 and even 9.75 during daytime (Hansen, 2002). The phytoplankton community is often totally dominated by the chain-forming diatom *S. marinoi* (Hansen *et al.*, 2002) as described for the present campaign by Tiselius *et al.* (2008) or by the phototrophic dinoflagellates *H. triquetra* and

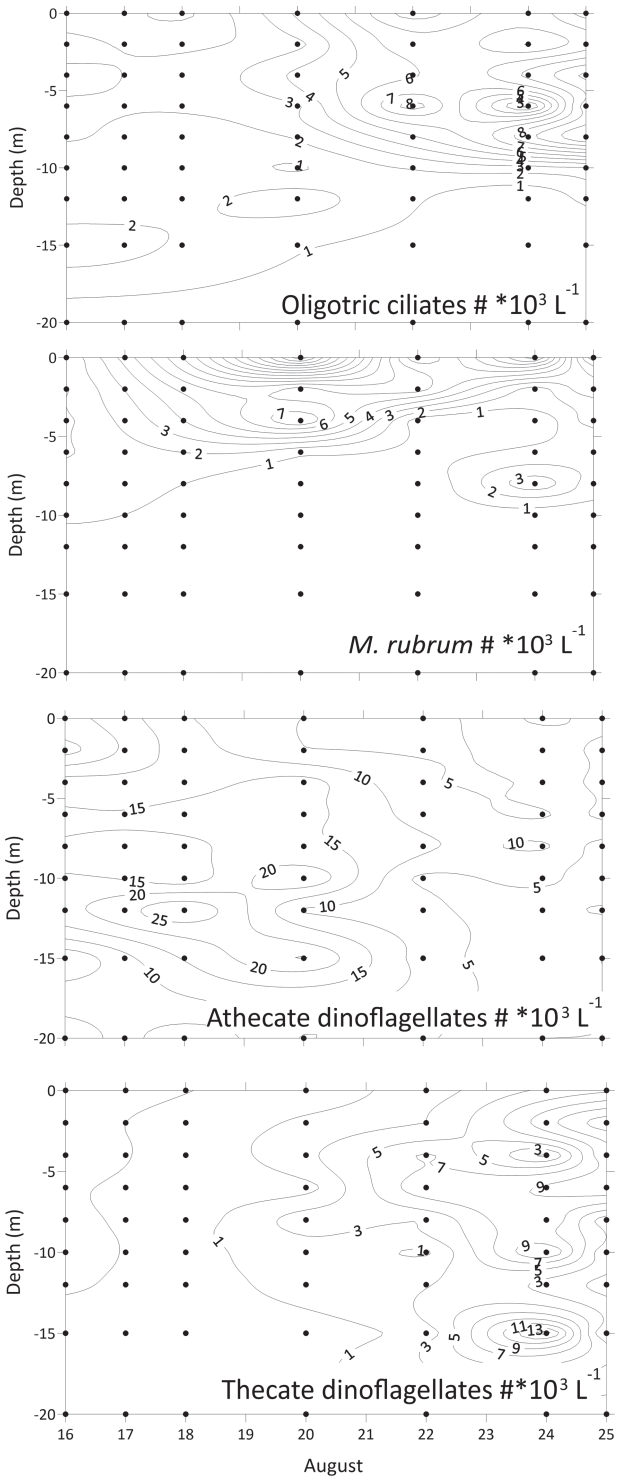

Fig. 6. Isopleths of the distribution of the major taxa of protistan grazers in the inner part of Mariager Fjord (abundance in individuals L^{-1}).

Prorocentrum minimum (Hansen *et al.*, 2002). During the period, late autumn to early spring, the phytoplankton community is more diverse (Olesen, 2001). Some of the dinoflagellates and especially the colonial diatoms are too large prey items for many of the pelagic suspension feeders. Polychaete (spionid) metatrochophora larvae and

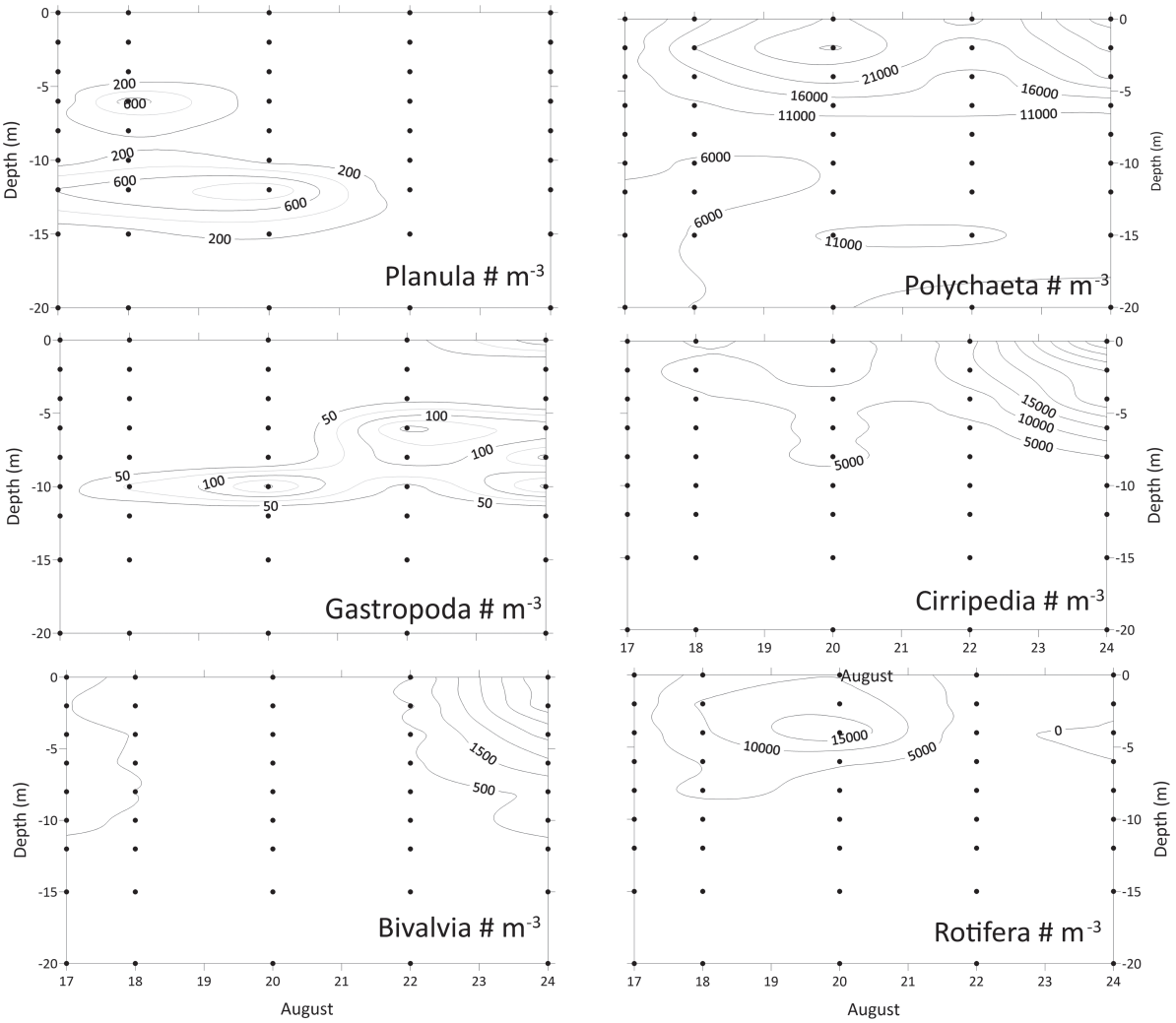

Fig. 7. Isopleths of the predominant meroplankton and rotifers distribution in the inner part of Mariager Fjord (abundance in individuals m^{-3}).

the advanced copepodite stages of the copepod *A. tonsa* can feed on a large spectrum of food particles including the ones from the present system (Berggreen *et al.*, 1988; Hansen, 1999). However, gastropod and bivalve veligers cannot feed on the larger particles (Hansen, 1991; Petersen *et al.*, 2002) that even obstruct their feeding and swimming (Hansen *et al.*, 1991). Cirriped nauplii as well as the rotifer *Brachionus plicatilis*, which can be used as a proxy for pelagic rotifers in general, prefer small-sized algae (2–8 μm) (e.g. Tackx *et al.*, 1990; Hansen *et al.*, 1997). Hence, even though the pelagic grazers physiologically might tolerate elevated pH they could be indirectly challenged by facing functional lack of food and even starvation due to a mismatch between their retention spectrum and the sizes of the ambient prey available (e.g. Petersen *et al.*, 2002).

Since the extent and persistence of elevated pH, as observed in the inner Mariager Fjord during the productive season, has been suggested to structure the phytoplankton community (Hansen, 2002), it is likewise (directly or indirectly) possibly structuring various components of the zooplankton community. However, the literature on the effects of elevated pH on the different protistan and metazoan grazers is still quite limited (Pedersen and Hansen, 2003a, 2003b; Hansen *et al.*, 2017).

The abundance and biomass of protistan grazers were quite low during this field campaign, taking the high biomass of primary producers into consideration (see for comparison Zervoudaki *et al.*, 2009 for open water Kattegat). This indicates that some of the protistan grazers are limited in their production rates, which is also what we

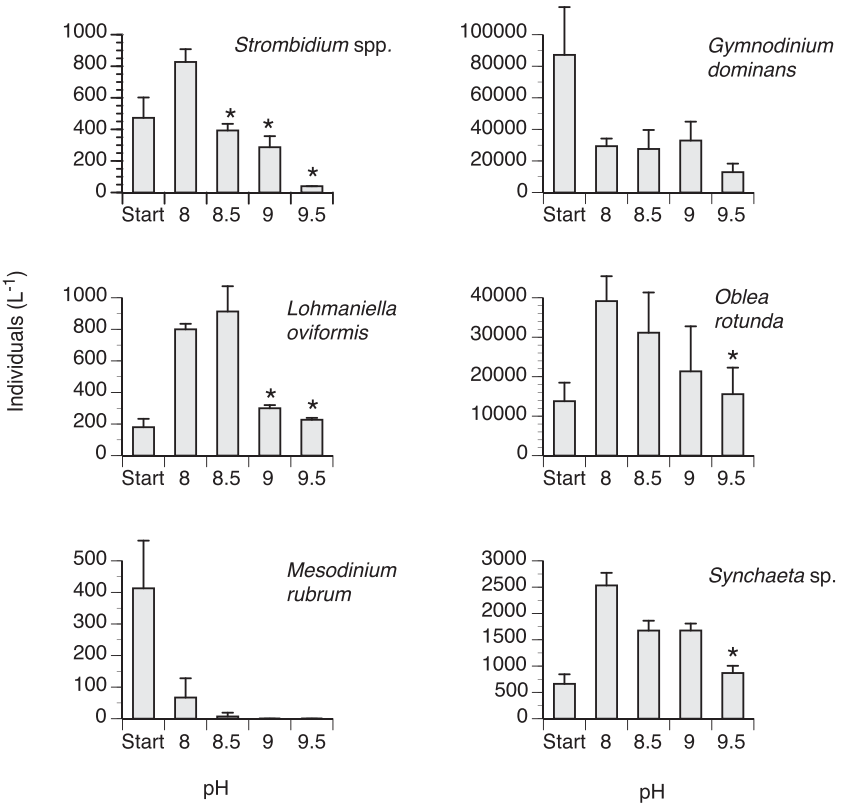

Fig. 8. Results of 30 L microcosm pH challenge experiment on the growth of heterotrophic dinoflagellates (*Gyrodinium dominans* and *O. rotunda*), heterotrophic ciliates (*L. oviformis* and *Strombidium* spp,), mixotrophic ciliates (*M. rubrum*) and the rotifer *Synchaeta* sp. ($n = 3$, mean ± SD).

found during our incubation experiments. It is, however, notice worthy that initial abundance in our challenge experiments was lower than *in situ* presumably due to initial screening and some container effects in a few cases (i.e. the protistan grazers *M. rubrum* and *G. dominans*). This presumably did not influence the recorded growth rates. Elsewhere it has been observed in laboratory studies on *M. rubrum*, which have positive growth rates above pH 8.5, that this species cannot grow at pH exceeding 8.7–8.8 (Hansen and Fenchel, 2006; Smith and Hansen, 2007). In addition, our experimental data indicate that some succession of species of protistan grazers will occur during such high pH events. Similar observations have been made in incubations of eutrophic coastal brackish water field populations (Øresund; part of the Baltic Sea) as well as observed in natural environments in hypertrophic enclosed brackish waters experiencing pH of up to 9.3 (Chomérat *et al.*, 2004). This pH tolerance supports our *in situ* observations of athecate and thecate dinoflagellates presence in the entire water column. Some initially abundant species were indeed very sensitive to pH above 9; this we observed both in the natural environment and in the pH incubation experiments. The tolerance to high pH among protistan grazers is, consequently, species-specific, and the upper pH growth limits for heterotrophic dinoflagellates and ciliates have been found to vary between 8.4 and 10.2 (see Pedersen *et al.*, 2003b). New populations of protistan grazers will of course be supplied by water intrusions from the Kattegat area during westerly storms. This means that when pH in the inner estuary start to decline during autumn, organisms will be supplied to the inner estuary from the Kattegat, resetting the community structure every year.

Meroplankton was present with few groups. The predominant taxa was gastropods, polychaetes and cirriped nauplii (Figs 2 and 7). In addition, a few bivalve larvae were present but with such low abundance, not enough to conduct pH challenge experiments, which is why we recruited organisms from a neighboring estuary. We did no exposure experiments with gastropod larvae, but they reside in the depth strata 5–10 m where the pH never reached higher than 8.4–8.8. However, we challenged polychaete larvae and cirriped larvae, both residing above10 m depth and some polychaete larvae were distributed in the entire water column. They could

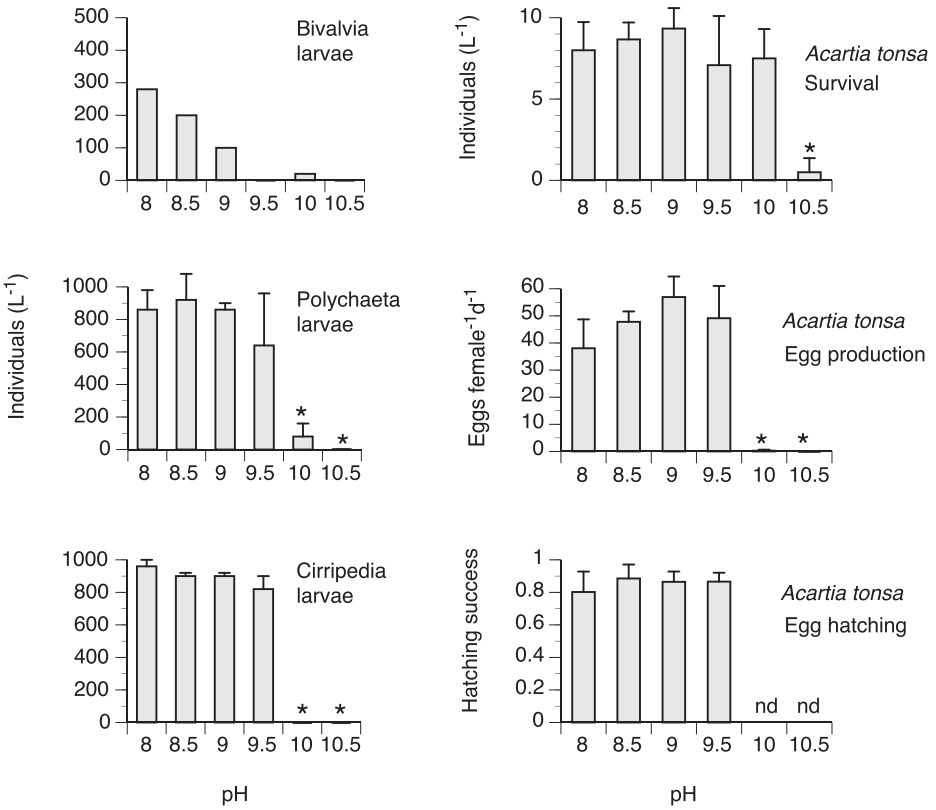

Fig. 9. Results of 50-ml bottle pH challenge experiments with meroplankton and in 500-mL bottle experiments for the only copepod species, which are predominant in the inner part of Mariager Fjord. ($n = 3$, mean ± SD). Treatments marked with asterisks are statistically different from pH 8.

all tolerate very high pH and apparently low oxygen and as such seem well adapted to the situation in the inner estuary. Bivalve larvae are possibly quite vulnerable to elevated pH based on our tentative experiment, and additionally not well adapted to the prevalent food particle regime. They were only present in the beginning and the end of our campaign; however, they nevertheless resided in the surface water and were exposed to pH > 9. This position in the water column is non-viable for the larvae over an entire pelagic phase of 3–4 weeks, when coinciding with elevated pH during summer. Hence, either local mussels provide the necessary quantum of propagules at other times of the year where pH is lower or the larvae stems from populations inhabiting the outer part of the estuary. This input of propagules is likely supplying the inner part with blue mussel recruits and other larvae from benthic invertebrates (see Hansen *et al.*, 2002).

Moreover, it is well documented that such an extreme pH elevation (in combination with anoxia) as documented in the present study, kills a number of copepod species (Pedersen and Hansen, 2003a; Hansen *et al.*, 2017). Hence, practically only one species is present in the inner section of Mariager Fjord (Tiselius *et al.*, 2008) which species assemblage is significantly reduced compared to open water Kattegat assemblages (Zervoudaki *et al.*, 2009). This is most likely an indirect effect, possibly due to lack of ability to cope with the available phytoplankton food by the juvenile stages of *A. tonsa* (Berggreen *et al.*, 1988). In other words, it is not necessarily a direct effect since it recently has been documented that when compared to *A. tonsa* some of the other copepod species present in the estuary at low numbers are just as—or even more tolerant to elevated pH (Hansen *et al.*, 2017). One could speculate that those copepod species cannot cope with chain-forming diatoms, but that remains to be verified. The *A. tonsa* spatial distribution is life-stage dependent, but at a species level, they inhabit the entire water column (see Tiselius *et al.*, 2008 for stage specific distribution). Their response to hypoxia was well studied by Marcus *et al.* (2004) showing although egg production and population growth is reduced at oxygen concentrations of 0.7 and 1.5 mg L^{-1} just the lowest oxygen concentration caused significant death. The presence of *A. tonsa* in the entire

water column is supported by the present laboratory observation of tolerance to pH ≥ 9.5 with good survival, reproduction and egg hatching. The same tolerance to elevated pH is also demonstrated by wild as well as long-term culture specimens of the same species originating from Øresund, Denmark where they are not adapted to elevated pH (Hansen *et al.*, 2017). Why are they not abundant then, but only present by 5–10% as compared to open water habitats just outside the estuary? Tiselius *et al.* (2008) proposed a significant recruitment loss due to the bulk part of their eggs sinking to the anoxic bottom layer preventing hatching combined with eggs and juvenile stages being grazed by the band of filtrating blue mussels situated at 5–10 m depth in the inner section of the estuary (Lomstein, 1999; Tiselius *et al.*, 2008). Moreover, Hansen *et al.* (2017) reported that even though *A. tonsa* eggs should hatch at pH 9.5 the nauplii die at elevated pH when leaving their protective embryonic membrane in the eggs.

CONCLUSION

We demonstrated that there are a few but persistent protistan and metazooplankton organisms robust enough to combat the hostile environmental conditions of the inner part of Mariager Fjord, with anoxic bottom water and elevated pH above the pycnocline. Mariager Fjord has a long and slender shallow-water outer section in open connection toward Kattegat. Both these habitats are typically well oxygenated, with moderate pH fluctuations and a species-rich benthic community hosting a healthy and diverse invertebrate fauna, which presumably supply the inner estuary with propagules. We suggest that these propagules are advected to the inner estuary, but to a large extend are eliminated by environmental challenges, leaving only the 'real opportunistic survivors' to inhabit the high-pH inner part of Mariager Fjord. Moreover, we suggest that our observations are not only restricted to the present ecosystem but of more general character for hypereutrophic estuaries.

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