BIO-C3
Biodiversity changes: causes, consequences and management implications

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BIO-C3 overview

The importance of biodiversity for ecosystems on land has long been acknowledged. In contrast, its role for marine ecosystems has gained less research attention. The overarching aim of BIO-C3 is to address biodiversity changes, their causes, consequences and possible management implications for the Baltic Sea. Scientists from 7 European countries and 13 partner institutes are involved. Project coordinator is the GEOMAR Helmholtz Centre for Ocean Research Kiel, Germany, assisted by DTU Aqua, National Institute of Aquatic Resources, Technical University of Denmark.

Why is Biodiversity important?

An estimated 130 animal and plant species go extinct every day. In 1992 the United Nations tried countering this process with the "Biodiversity Convention". It labeled biodiversity as worthy of preservation – at land as well as at sea. Biological variety should not only be preserved for ethical reasons: It also fulfils key ecosystem functions and provides ecosystem services. In the sea this includes healthy fish stocks, clear water without algal blooms but also the absorption of nutrients from agriculture.

Biodiversity and BIO-C3

To assess the role of biodiversity in marine ecosystems, BIO-C3 uses a natural laboratory: the Baltic Sea. The Baltic is perfectly suited since its species composition is very young, with current salt level persisting for only a few thousand years. It is also relatively species poor, and extinctions of residents or invasions of new species is therefore expected to have a more dramatic effect compared to species rich and presumably more stable ecosystems.

Moreover, human impacts on the Baltic ecosystem are larger than in most other sea regions, as this marginal sea is surrounded by densely populated areas. A further BIO-C3 focus is to predict and assess future anthropogenic impacts such as fishing and eutrophication, as well as changes related to global (climate) change using a suite of models.

If talking about biological variety, it is important to consider genetic diversity as well, a largely neglected issue. A central question is whether important organisms such as zooplankton and fish can cope or even adapt on contemporary time scales to changed environmental conditions anticipated under different global change scenarios.

BIO-C3 aims to increase understanding of both temporal changes in biodiversity - on all levels from genetic diversity to ecosystem composition - and of the environmental and anthropogenic pressures driving this change. For this purpose, we are able to exploit numerous long term data sets available from the project partners, including on fish stocks, plankton and benthos organisms as well as abiotic environmental conditions. Data series are extended and expanded through a network of Baltic cruises with the research vessels linked to the consortium, and complemented by extensive experimental, laboratory, and modeling work.

From science to management

The ultimate BIO-C3 goal is to use understanding of what happened in the past to predict what will happen in the future, under different climate projections and management scenarios: essential information for resource managers and politicians to decide on the course of actions to maintain and improve the biodiversity status of the Baltic Sea for future generations.

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1. Executive Summary BIOC3 Task 1.2

This deliverable is part of WP 1, “Genetic adaptation and eco-physiology”, which investigates physiological tolerances and adaptive variation of key Baltic Sea species. The goal is to provide a general understanding of principal determinants of the distribution of species and populations, which ultimately determine the functional diversity and resilience of Baltic Sea ecosystems in response to environmental drivers. WP1 output will feed into WP2, 3 and 4, and is therefore crucial for the incorporation of evolutionary processes into future projections of the Baltic Sea.

The Baltic Sea experienced large changes in community composition, yet it is largely unknown how these changes affect ecosystem functioning and how organisms are able to cope regarding physiological tolerance, preference and phenotypic plasticity in relation to environmental factors. To help answer these questions, a series of experiments and field studies have been performed for a range of different species including invasive/non-indigenous ones at different positions in the food web.

1.1 Zooplankton

Zooplankton as a critical link for energy and nutritional transfer from phytoplankton to upper trophic levels as well as microzooplankton as another important part in the food web.
Understanding physiological tolerance and zooplankton responses to predicted climate change in the Baltic Sea is a prerequisite for prediction of change in biodiversity. It was decided in this task to specifically look at the responses of offshore deep basin copepods (*Acartia* and *Temora*), as well as nearshore coastal copopods (*Eurytemora)*. The results show that females of two different populations of *Acartia longiremis* originating from the Arkona and the Bornholm Basins displayed a broad salinity tolerance. Survival, feeding and egg production were strongly related to salinity and decreased with decreasing salinity. A salinity of 5 is critical to the population of *A. longiremis* with regard to the species vital rates. Although females were still able to produce some eggs at this salinity and survival was generally high, the rate of reproduction is likely too low to sustain populations in the Baltic and account for additional mortality by predation. At a salinity of 4 decreased feeding rates were not sufficient anymore to sustain reproduction and high survival of the species. This suggests that the species might not be robust enough for the anticipated worst-case changes in salinity values in the central Baltic Sea (e.g., Meier et al. (2012)).

The comparison of survival to instantaneous changes in salinity suggests that population differences of *A. longiremis* exist in the physiological ability to respond to salinity changes. Female copepods originating from a salinity of 14-16 PSU in the Arkona Basin displayed a higher mortality to the reduction of salinity to 6 and 5 compared to females isolated at a salinity of 7.8 in the Bornholm Basin. However, female copepods from Bornholm that were acclimated to salinities of 6-4 before their response to instantaneous reduction in salinity was tested, displayed no differences in sensitivity to low salinity and had a broad salinity tolerance. Such difference between populations may be caused by the use of those females surviving the acclimation to low salinity. Nevertheless, more than 70% of females from Arkona Basin survived the instantaneous reduction of salinity from 16 to 5 after 5d of incubation. This indicates physiological and potentially genetic diversity exists in the Arkona population that allows the species to prosper in areas with lower salinity such as the Bornholm Basin. Investigations of reaction norms and common garden experiments are required to evaluate this and will be continued in the project and reported in Deliverable 1.3.

Experiments with individuals of the coastal copepod (*Eurytemora affinis*) from three different areas in the Baltic sea (Askö, Pärnu Bay (part of Gulf of Riga) and Bothnian Bay) revealed how survival, development time and hatching success are affected by temperature, salinity and the interaction between these two environmental factors. Our results revealed the most beneficial and detrimental conditions, and the manner in which stress manifested on our endpoints. Depending on the degree of stress to which they were subjected, the optimum condition proved to be at an experimental temperature of 15°C and a salinity of 6, while the combination of high temperature with low salinity was the most detrimental (20°C and salinity 2). Osmotic and heat shock has been shown for *E. affinis* and has been associated with changes in protein expression. Even though copepods can adapt to osmotic stress by osmoregulation, it is costly in terms of energy and dependent on the degree of stress. Considering this, our findings for the survival analysis suggest that at 15°C and a salinity of 2 and at a temperature of 20°C and a salinity of 6 the degree of stress was intermediate between the lowest (15°C and salinity of 6) and highest (20°C and salinity of 2). The information gained from this study helps to get a better insight into functioning of the pelagic system and relevant responses in the higher trophic levels.

Within the project a comprehensive micro- and mesozooplankton survey in the Lithuanian water has been conducted along at salinity gradient where largest change in pelagic patterns are expected, By providing the data on the zooplankton distribution within the Lithuanian coastal zone as an example, new knowledge on spatial and temporal zooplankton variability was achieved.

1.2 Invasive/ non-indigenous species:
Besides the comb jelly, *Mnemiopsis leidyi*, which is going to be dealt with in Task 2.3, the benthic fish species, the round goby (*Neogobius melanostomus*) was in the focus in our studies. This is because the introduction of an efficient predator, that has become a dominant element in many shallow water ecosystems, is likely to change the abundance of benthic organisms across many trophic levels; they could also modify the physical environment and thereby modulate ecosystem processes such as nutrient fluxes, primary production and sedimentation rates. As the species is not monitored data on its current spread and environmental tolerance is needed. The current range of the round goby observations is extensive, covering all major sub-basins of the Baltic Sea. The most recent observations appeared in the northern regions (Northern Baltic Proper, the Gulf of Bothnia and the Gulf of Finland) and on the eastern and western coasts of southern Sweden. Modelling results show that the distribution of the round goby is primarily related to local abiotic hydrological conditions (wave exposure). Furthermore, the probability of round goby occurrence was very high in areas in close proximity to large cargo ports. This links patterns of the round goby distribution in the Baltic Sea to shipping traffic and suggests that human factors together with natural environmental conditions are responsible for the spread of NIS at a regional sea scale. The hand line survey demonstrated the occurrence of round goby at the Baltic coastline of Schleswig Holstein. This revealed that the actual position of the invasion front is in the area of Kiel Bight. Results based on physiological tolerance and aerobic scope (AS, the difference between maximum and standard metabolic rate) measurements suggest that round goby has the capacity to occupy full oceanic

environments. Therefore, we predict that the species will continue to spread into the Baltic Sea-North sea transition zone, although the depressed physiological performance at high salinities might reduce its competitive potential as it approaches the North Sea. However, a pronounced high inter-individual variation in physiological performance at the highest salinities suggests that some individuals are able to offset the negative effects of high salinity, thus increasing the dispersal potential at the invasion front. According to our results, round and black goby do not compete for food.

1.3 Fish and climate change

What are potential drivers of change, which may affect survival, development, duration or (spatial) distribution of early life stages of cod, sprat and other commercially important key species? To address some of these complex processes we have selected, (partly preliminary) results from three case-studies. The objective of the first study was to compare the potential dimensions of egg buoyancy differences over several spawning seasons to assess general patterns. The objective of the second study was to investigate the importance of different oxygen and salinity conditions in relation to the available habitat determined by the eggs´ specific density. This is particularly interesting by comparing different hydrographic conditions which have recently been changed due to the Major Baltic Inflow (MBI) end of 2014. The third case study is an example how to use field-derived egg diameter data in combination with buoyancy data and to link this with stock characteristics to improve the current estimation method of a spawning stock biomass.

In sprat, egg survival is reduced due to critical temperature conditions, which act as the main driver. Being a “large” egg early (April) in the spawning season might not be optimal for sprat; being “small” at that time however, increases thermal survival conditions in a still tolerable environment concerning oxygen. This size-pattern turns into the other direction in the continuation of the spawning season in May/June. During late spring and early summer, the temperature of the water column above the halocline increases and the larger eggs now experience the warmer conditions (Nissling et al., 2003; Petereit et al., 2009) and thus are outside of critical tolerance limits. In cod, oxygen related egg survival is one of the key drivers determining egg abundances in different years. The new approach presented combines directly cod age structure with mean egg diameter and resulting mean density characteristics. This relationship is used as a feedback loop to model the number of effectively contributing females under known water density characteristics including the application of the well-accepted physiological tolerance limits for especially oxygen, temperature and salinity.

Despite the great economic and ecological significance of sprat, relationships between feeding rates and temperature have not been investigated so far, although growth and recruitment success of sprat depend strongly on water temperature and food availability (MacKenzie & Köster 2004 Peck et al. 2012). Therefore, a basis for estimations of food densities required for optimal growth and good recruitment success is needed. Strong relationships between functional response and temperature in sprat dependent on the sizes of the fish could be shown. Since small sprat exhibit other environmental conditions than larger fish, which undertake daily vertical migrations associated with rapid changes in temperature, different functional response curves had to be fitted. Juvenile fish, however, are mainly abundant in the coastal zones or upper water layers with higher and more constant temperatures. Thus, they are able to feed faster at higher temperatures, whereas larger individuals (adults) may reach their maximum possible biting rates at even lower temperatures. The proportion of feeding sprat in laboratory experiments was high at a temperature range from 20°C to 8°C. This corresponds to field observations made in spring and summer (Bernreuther 2007). However, at 5°C only 26.3% of sprat were feeding in our study. Stomach data from March 2013 (western Baltic Sea) revealed a similar rate of 25.24% at 1.07 ± 0.28 °C (unpublished data). This indicates that sprat is able to feed over the entire year even at relatively low temperatures, but benefits from higher water temperatures above 8°C.

To investigate physiological boundaries that might emerge in juvenile sprat nursery habitats and can have consequences for growth performance, survival and recruitment of Baltic sprat the fate of different seasonal cohorts originating from the extended spawning season was studied. Two different simulation approaches modeling the growth of seasonal cohorts as a function of temperature were followed. In a backwards approach (1) the energy demand of seasonal cohorts was constructed. In a forward approach (2), the maintenance and optimum ration young sprat need during the seasons were calculated. To validate the model results four different years (2002, 2003, 2006, and 2007) were analyzed, where previous studies uncovered the temporal origin of autumn survivors by the microstructure analysis of otoliths (Baumann et al 2008, Günther et al 2012). The high total energy demand of earlier cohorts calculated in the backwards simulation implies that earlier cohorts need a higher amount of food. However, the prey concentration for maintenance is similar for early and late spawned cohorts. The reason for that is that early cohorts experience higher temperatures when they become larger. This increases snatching rates enabling these cohorts to eat more prey items at a similar prey concentration. Thus, maintenance concentrations of seasonal cohorts do not reflect the higher total demand of energy for earlier spawned cohorts. The strongest determinants for the concentration necessary to achieve maintenance ration are fish size and season (interaction between temperature and day length). In summer, when early cohorts enter the juvenile stage, necessary concentrations for larger individuals are smallest. In contrast, later spawned cohorts need larger concentrations at the same length as season progresses. At a first glance, the lower concentration needed for early cohorts in contrast to later cohorts are contradicting to the hypothesis that later cohorts have an advantage. However, individuals that are large early in the season rely on high prey concentrations for fulfill their maintenance for the rest of the growing season. In other words, the risk to starve before wintertime increases. Individuals that are born later in the season need a slightly higher prey concentration for maintenance. However the period they need to bypass before winter is reduced and thus, the risk to starve before winter. Being too large too early in the season is unprofitable, which might explain why the share of early cohorts in autumn caught survivors is of minor importance. The results of both simulation approaches underline the advantages of later born individuals in the juvenile life-stage of Baltic sprat. On the one hand the total energy demand is lower for late seasonal cohorts. On the other hand, early cohorts might suffer from the fact that they achieve large body size early in the season and rely on high prey concentrations for the rest of the growing season. Thus, early cohorts suffer during the juvenile stage in summer and their probability to survive until autumn is reduced. The interaction between temperature in nurseries and food demand of juvenile sprat determines the temporal origin of cohorts dominating autumn recruits.

Cod is crucial for the Baltic Sea, with large ecosystem and socio-economic effects. Thus, an improved knowledge about factors affecting cod dynamics is critical. Concerning early life stages, biochemically based estimates of larval nutritional condition and growth showed relationships with prey abundance and stock recruitment and are thus a promising tool to validate bottlenecks for recruitment success and determine "highest survival potential" from time series of zoo- and ichthyoplankton as was shown in Task 2.1 (see deliverable 2.1).

Through the increased usage of fossil fuels and changed land use, the concentration of atmospheric carbon dioxide has been steadily rising since the onset of the industrial revolution. A portion of this CO_2 is dissolving into the oceans causing a decrease in pH, e.g. acidifying a habitat that covers 2/3 of this planet. This process has been termed "the other CO2 problem" or ocean acidification. The experiments performed within in this Task 1.2 clearly show that larvae of Western Baltic and coastal Barents Sea cod in 2014 are impacted by near future levels of ocean acidification. Our findings additionally suggest that even at ad libitum feeding, e.g. a richer energy budget that would allow for more efficient acid-base regulation, cod larvae cannot mediate the adverse effects of ocean acidification. However this apparently does not hold true for growth patterns. At high fed availability, larvae in ambient and end-of-century treatment show no apparent differences in standard length and dry weight by the end of the experiment (36 dph). Further these experimental results with cod larvae highlight the importance of investigating several responses to changes in the physiological environment in the same experiment, as in our case mortality and growth. Looked at separately the growth patterns of the high CO_2 treatment could be interpreted as ocean acidification having a positive effect on cod larvae through increased growth; yet if one includes the detrimental increase in mortality it will most likely have several effects on the population dynamics of both cod populations.

2. Introduction to BIOC3 Task 1.2

Changes in community composition of nearly all trophic levels ranging from plankton to fish and benthos have been described in the Baltic Sea (Ojaveer et al. 2010). The underlying physiological tolerance (including capacity for acclimatization), preference and phenotypic plasticity in relation to environmental factors needs to be evaluated in selected planktonic, benthic and bentho-pelagic species considering different life stages in order to predict ecosystem-wide consequences of changing biodiversity under spatio-temporally varying drivers. In this task, physiological tolerance and preference have been experimentally studied in populations sampled across geographical (local to regional) scales and ecological gradients (e.g. salinity) in the field. The number of investigated populations and influencing drivers are varying among taxa. Focal species include copepods (*Temora longicornis, Acartia spp*. *Eurytemora affinis*), comb jellies (non-indigenous *Mnemiopsis leidyi*) and fish (the non-indigenous round goby, *Neogobius melanostomus*; the native clupeid sprat (*Sprattus sprattus*); flounder (*Platichthys flesus*) and the key piscivorous fish cod, *Gadus morhua*. Focus was on critical life-stages for example fish egg and larval gobies, cod & herring (*Clupea harengus*) in relation to population origin and dispersal potential.

3. Core Activities

Major activities of task 1.2 "**Documentation on key drivers and physiological tolerance limits for resident and invasive/non-indigenous species**" per partner have been as follows:

P8 (KU-CORPI): contributes with field studies to analyse non-indigenous species (NIS) thresholds. Distribution ranges of meso- and microzooplankton species have been examined by conducting field sampling along an estuarine-marine salinity gradient and within vertically stratified water column, focusing on community level responses and non-indogenous zooplankton species occurrence along these gradients.

P2 (DTU-Aqua): is responsible for physiological tolerance experiments on Baltic Sea offshore basin copepod species -*Temora longicornis* and *Acartia longiremis*.

P4 (SU): is responsible for experiments to evaluate physiological tolerance to temperature and salinity change of a key copepod coastal, estuarine species in the Baltic Sea - *Eurytemora affinis*

P1 (GEOMAR): is responsible for laboratory and field experiment on the comb jelly *Mnemiopsis* for studying tolerance levels.

P6 (UT-EMI): is responsible for studying non-indigenous round goby using a field approach. The aim of the study was to quantify the effect of different anthropogenic and environmental drivers on the abundance and distribution of round goby.

P7 (SYKE): is responsible for studying non-indigenous round goby (*Neogobius melanostomus*) populations in the Baltic Sea based on samples of round goby populations in five distinct locations representing most of the species range in the Baltic Sea: Guldborgsund (Denmark), Hel (Poland), Palanga (Lithuania) Muuga Bay (Estonia) and Mariehamn (Åland Islands).

P2 (DTU-Aqua): is responsible for studies on non-indigenous round goby with an experimental approach aiming at determining the salinity tolerance to be able to predict invasion patterns in the Baltic Sea-North Sea transition zone.

P3 (UHH- IHF): is responsible for performing experiments on temperature and salinity dependency of round goby egg development and functional response curves related to feeding for sprat. Energetically derived growth models were developed to analyze critical periods during juvenile sprat development and to study the importance of timing of the early juvenile stage for the potential growth performance in Baltic sprat.

P1 (GEOMAR): is responsible for performing experiments on fish eggs buoyancy and studying responses of cod larvae from populations across geographical scales in experimental setups to evaluate potential drivers of change, which may affect survival, development duration or (spatial) distribution of early life stages of cod and other commercially important key species.

4. Methods and results

Major results are highlighted in section (5), detailed methods and results for each respective study are described in the Appendices. A list of appendices is given in section (8). All appendices are attached in section (9).

5. Scientific highlights

5.1 Community level responses and invasive zooplankton species performance along gradients (for details see Appendix I)

Two field surveys were conducted in 2014, representing 17 locations along a salinity gradient from the Nemunas river mouth to the Curonian lagoon to the western edge of the Lithuanian EEZ. Zooplankton and phytoplankton samples were collected within vertically stratified water columns above and below the halocline. The results have shown that zooplankton biomass increased from Nemunas River avandelta towards the Baltic Sea. Community shift from cladocerans in the Curonian Lagoon to rotifers in the Baltic Sea was observed. The most significant factors determining shifts in zooplankton community structure were salinity and chlorophyll-a concentration (Griniene *et al.* in prep.). Zooplankton biodiversity within the Lithuanian coastal zone was assessed from the samples collected in 2013, applying novel molecular techniques High-Throughput Sequencing (HTS) metabarcoding for the surveillance of plankton communities within the SE Baltic Sea coastal zone was applied. Results were compared to those from routine monitoring survey and morphological analyses. Four of five non-indigenous species (NIS) found in the samples were identified exclusively by metabarcoding. All of them are considered as invasive in the Baltic Sea with reported impacts on ecosystem and biodiversity. The proportion of identified NIS was significantly higher in metabarcoding results. Most of them were detected in the transitional zone between the Curonian Lagoon and the Baltic Sea, with the most variable salinities. In all three sampling locations there were sequences attributed with high confidence to the invasive polychaete *Marenzelleria viridis*. Based on the results of the earlier molecular identification and areal distribution assessment of three sibling *Marenzelleria* species within the Baltic Sea, only *M. neglecta* was unambiguously reported from the eastern and south-eastern regions (where Lithuanian cost belongs to). These findings contribute to the update of the current distributional maps of the species as well as national inventories of the non-indigenous organisms. However, further ground-truthing studies are required to verify the particular distribution of these two species in the benthic habitats. (Zaiko *et al*. in prep.)

5.2 Physiological tolerance: *Temora longicornis – Acartia longiremis* (for details see Appendix II)

In a series of laboratory experiments metabolic and reproductive responses of two populations of the calanoid copepod *Temora longicornis* from the Bornholm- and the Gotland Basin were determined. Ingestion, respiration, egestion, egg production and egg hatching success were compared at salinities ranging from 10 to 5. Both populations showed a decreasing ingestion and egg production with decreasing salinity, down to a critical salinity of 6 below which mortality increased to 100%. However, hatching success of eggs was high and respiration was generally constant at all salinities. Our results suggest that energy partitioning of *T. longicornis* is significantly affected by decreased salinity.

In contrast to *Temora longicornis*, cultures of the calanoid copepod *Acartia longiremis* could not be established in the laboratory. The salinity tolerance was, therefore, investigated during a cruise to the Arkona and Bornholm Basin in September 2015 on R/V Dana. Feeding, fecundity and instantaneous survival to lowered salinity was compared in two populations originating from a salinity of 16 and 7.8. Females of both populations displayed a broad salinity tolerance to lowered salinity. The lower lethal salinity, however, depend strongly on the population origin indicating local acclimation/adaption to salinity. Metabolic and reproductive rates were strongly related to salinity and decreased with lowered salinity. Similar to *Temora longicornis*, a salinity of 5 is critical to the population with regard to the species vital rates. Below it, decreased feeding rates were not sufficient anymore to sustain reproduction and high survival of the species. (Dutz *et al*.)

5.3 Physiological tolerance to temperature and salinity change of a key copepod species in the Baltic Sea - *Eurytemora affinis* (for more details see **Appendix III**)

Populations of the calanoid copepod *Eurytemora affinis* from locations across the Baltic Sea (Bothnian Bay, Pärnu Bay (part of Gulf of Riga), Askö) have been collected and exposed to different salinities and temperatures in common garden experiments to investigated their fitness response. Lowering the salinity (from 6 to 2 PSU) increased mortality, delayed development time and reduced egg hatching success in several populations. The combined effect of increased temperature and low salinity even further increased mortality. Increased temperature is especially stressful in the most northern populations as they are at their outmost temperature range. This suggests that some copepod populations likely have low tolerance levels to future climate change. The project has been performed within a master and PhD thesis and is currently being prepared for publication (Winder *et al*.).

5.4 *Mnemiopsis leidyi* - range expansion salinity and temperature limits

Mnemiopsis leidyi was present in the Baltic Sea from 2006 to the winter of 2010/2011. However, thereafter reports are sporadic and indicate that it could not establish a permanent population in the low saline Baltic Sea region. On the other hand, areas with higher salinity and higher winter temperatures, like the Dutch Wadden Sea and German Bight, support year-round populations and animals have been present there since its first sightings. Significant temperature effect on the distribution throughout Europe - especially for the Baltic Sea could be shown (Jaspers *et al*. in preparation)

Investigations regarding *M. leidyi* are also a component of Task 2.3 where results will be presented in more detail.

5.5. Effects of natural environmental conditions and shipping on the distribution of the invasive round goby (for more details see **Appendix IV** and paper within)

Introductions of non-indigenous species (NIS) are considered a major threat to aquatic ecosystems worldwide. While it is valuable to know the distributions and ranges of NIS, predictive spatial models along different environmental gradients are more useful for management of these species. In this study we modelled how external drivers and local environmental conditions contribute to the spatial distribution of an invasive species using the distribution of the round goby *Neogobius melanostomus* in the Baltic Sea as an example. Using the collected distribution data, an updated map on the species distribution and its invasion progress in the Baltic Sea was produced. The current range of the round goby observations is extensive, covering all major sub-basins of the Baltic Sea. The most recent observations appeared in the northern regions (Northern Baltic Proper, the Gulf of Bothnia and the Gulf of Finland) and on the eastern and western coasts of southern Sweden. Modelling results show that the distribution of the round goby is primarily related to local abiotic hydrological conditions (wave exposure). Furthermore, the probability of round goby occurrence was very high in areas in close proximity to large cargo ports. This links patterns of the round goby distribution in the Baltic Sea to shipping traffic and suggests that human factors together with natural environmental conditions are responsible for the spread of NIS at a regional sea scale.

5.6 Differences in the invasive round goby (*Neogobius melanostomus*) populations in the Baltic Sea

In the past 25 years, the non-indigenous round goby has invaded almost all Baltic Sea sub-basins. The species was first observed in Hel, Gulf of Gdansk in Poland in 1990. The most recent observations were made in the Åland islands and Bothnian Bay (2011). The round goby populations in different parts of the Baltic Sea are experiencing very different abiotic conditions and have very different invasion histories: some populations have existed 25 year whereas some only few years.

In our study we sampled round goby populations in five distinct locations representing most of the species range in the Baltic Sea: Guldborgsund (Denmark), Hel (Poland), Palanga (Lithuania) Muuga Bay (Estonia) and Mariehamn (Åland Islands). The populations were sampled using the same combination of fishing equipment in all sites. Samples of the populations differed significantly from each other: CPUE (catch/day) varied from 24.5 fish (in Hel) to 123 fish (in Guldborgsund). Some of the variation may be due to abiotic variation such as in weather conditions (especially wind) but some is definitely due to variation in population sizes. Condition of the fish (Fulton's K) varied among the populations. The fish in Hel had the highest condition factor, Fulton's K values (average in fall 2014 1.70 and in early summer 2015 1.67). The fish in Palanga and Mariehamn were in the poorest condition (average in fall 2014 1.43 and in early summer 2015 1.37 in Palanga and fall 2014 1.32 and in early summer 2015 1.45 in Mariehamn).

Moreover, in 2015 a subset of 25 round gobies at each location (except from Poland) were inspected for presence of parasites and their conditions were measured using the hepatosomatic index. Out of a total of 100 fish that were examined 32 were females and 68 males (table 2). Both sexes were smallest in Guldborgsund (TL 64-133 mm) whereas males were largest in Mariehamn (TL 93-205 mm) and females in Palanga (TL 77-198mm). Mariehamn sample was biased towards males and no females were included in the random subsample. Overall the infection rate was higher in females than males (pairwise t-test, $p<0.05$). In Muuga 73 % of all females were infected and the total infection prevalence was 60 % in both Muuga and Mariehamn. These sites had the highest frequency of infection compared with 56 % in GBS and 28 % in Palanga. Conversely, the round goby males in Mariehamn were on average larger (TL 152.5 ±23.5 mm), had a higher HSI and were visually in better condition (e.g., less skin damage) than round gobies sampled from other sites. In contrast, the GSI of males was lowest in Mariehamn and highest in Palanga and GBS, whereas the trend was opposite regarding the HSI, which was lowest at these two sites. Age analyses are still pending, but they should provide more insights to the differences in population characteristics among these populations (P7: Maiju Lehtiniemi and Riikka Puntila)

5.7 Salinity tolerance of round goby (for more details see **Appendix V**)

Non-indigenous species (NIS) can have strong impacts on marine biodiversity and ecosystem structure and function, including their services. Once introduced into a new region, secondary dispersal of NIS depends on a suite of ecological factors such as presence of predators, competitors, and parasites, yet with the most fundamental constraints on the distribution arising from the organism's physiological limitations in relation to the ambient environment. Predicting dispersal however remains a challenge. It was possible to show that physiological traits, namely aerobic scope and osmoregulation, can be used to predict performance and dispersal potential of an aquatic invasive/non indigenous species in novel environments. It was shown that round goby *Neogobius melanostomus*, one of the most wide-ranging invasive fish species in Europe and North America, has the capacity to occupy full oceanic environments. Currently round goby thrives in brackish and freshwaters, while it hitherto has remained unclear if the species will endure high salinity waters. Our results demonstrate that key physiological traits provides a tool to predict dispersal and hence 'area of impact' at an earlier state. Early predictions are a great asset in relation to taking appropriate management actions. While eradication of round goby is unrealistic, population control that leads to minimizing the risk of further secondary dispersal is feasible (Behrens et al. submitted).

5.8 Salinity and temperature effects on egg development in round goby & Hand line survey in the Western Baltic (for detail see **Appendix VI**)

Round goby *Negobius melanostomus* was first found in Puck Bay, Poland at the beginning of the 1990s (Skora & Stolarski, 1993). Since these first findings round goby managed to be one of the most successful invasive species within the Baltic Sea. The current distribution includes coast segments of all Baltic Sea bordering countries. However, the physiological limits, especially in relation to reproduction are not well understood. As a first step to estimate the potential spreading of round goby in saltier waters of the Western Baltic and the Kattegat, experiments were performed to test egg development and hatching success in different combinations of salinity and temperature. The experiment showed that salinity has a strong effect on the hatching success. The hatching success decreases with increasing salinity. Hatching success is also influenced by temperature. The highest hatching rates were observed at 15 °C. Furthermore, the status of invasion by round goby along the German coastline of the Western Baltic was investigated by a hand line survey. This revealed that the current position of the invasion front is in the area of Kiel Bight (Niemax, Temming *et al*.).

5.9 Temperature and size-dependent functional response of sprat, *Sprattus sprattus* L (for more details see **Appendix VIIa**)

Laboratory experiments were conducted to study the effects of temperature and body size on functional response of sprat using *Artemia salina nauplii* as prey to determine the maximum feeding rates. Functional response curves help to understand and quantify the impact of sprat on zooplankton communities in the Baltic Sea and serve as a basis for the estimation on food densities required for optimal growth of early juveniles. The present results indicate a strong influence of temperature on feeding success with lower numbers of sprat feeding at low temperatures. Feeding rates increased with both, temperature and fish body size. The relation between snatching rate and prey concentration, temperature and body weight has been summarized in a mathematical model. Smaller fish had higher Q10 values than larger conspecifics, suggesting that larger fish reach maximum feeding rates at even lower temperatures. This may be reflected in the utilization of habitats with small juveniles living near the coasts in summer with high temperatures compared to larger ones, which exhibit lower temperatures in the deeper regions (Kulke, Temming *et al.)*

5.10 Another critical period: the importance of timing of the early juvenile stage for the potential growth performance in Baltic sprat (for more details see **Appendix VIIb**)

Most recruitment determining mechanisms in marine fish species act in the earliest life-stages, when mortality is highest. In Baltic sprat, a clupeid schooling fish with an extended spawning season, extensive research effort was invested to uncover processes in the larval stage that regulate year-class strength. However, the large amount of recruitment variability is still unexplained. Previous studies showed that the amount of larvae is unrelated to the number of YoY-recruits, highlighting the importance of the post-larval life-stage as a critical period modulating year-class-strength. In order to detect recruitment regulating mechanisms in the post-larval life-stages of Baltic sprat we performed a simulation study on growth and food demand of YoY-sprat wrapping up a comprehensive database on otolith-derived growth rates and experimental investigations on feeding habits. We assumed a spawning time from February to August and modeled growth of various seasonal cohorts in relation to temperature. In a first simulation approach, we converted the daily length increases into the equivalent required amount of energy, applying a bio-energetic budget approach. We found that seasonal cohorts originating from the first half of the spawning season have a high total energy demand in the juvenile stage in contrast to later spawned cohorts despite a similar growth performance of early and late born sprat. Later spawned cohorts experience comparatively lower temperatures as early juveniles which reduces their metabolic costs in an already food demanding life stage. In a second simulation approach, we calculated maintenance concentrations for seasonal cohorts applying a length and temperature dependent relationship between prey concentrations and snatching rates. We found that cohorts from the first half of the spawning season, which have the potential to grow fast, soon rely on high maintenance rations to fulfill the metabolic demands of a large body size. In contrast, later cohorts with a similar growth potential reach large body sizes accompanied by high maintenance rations later in the season. Thus, the risk of starvation and depletion of energy reserves before the onset of winter is higher for earlier than for later born cohorts. A high cumulative energy demand and the disadvantage of being too large too early in the season, have the potential to decrease survival rates of early born cohorts and thus underline the importance of the right timing of the post-larval, early juvenile stage. Our results are supported by previous studies showing that successful YoY-recruits of Baltic sprat stem from the second half of the spawning season only.

5.11 Fish egg - buoyancy– experimental and field approach (for more details see **Appendix VIII)**

Data from the 6-year time series (data analysis from up to four different stations) confirm a positive relation between egg size and egg buoyancy for sprat during April spawning seasons. Large eggs float higher in the water column compared to small eggs. The inter-annual variation in the specific density layer was lowest for the largest diameter sprat eggs, thus reflecting rather stable density conditions. Egg density increased in 2014 and 2015 compared to previous years, although with large variation in 2014. In 2015, the highest significant mean value in the egg-density time series for sprat could be measured. The years 2012 and 2013 were characterized by low April temperature in general, 2014 and 2015 by significantly higher temperatures. The oxygen conditions also changed during years on the analyzed density layers – however, if considering the threshold level of 2mg/l oxygen (Nissling *et al.,* 2003), all egg diameter classes in all years and stations experienced values above that condition.

The general characteristics of the pelagic **flounder eggs** in relation to environmental and depth related variables) showed statistical differences between the Bornholm Basin and the Gdansk/Gotland areas. Accordingly, the cumulative survival probabilities of egg batches might have changed in those areas. The results showed greatly enhanced survival probabilities in Bornholm basin 2015 as egg survival increased from 47% in 2014 to 100% the following year. In Gdansk Deep the situation was similar, and survival probability increased from 13% to 100%. In Gotland Basin no difference in survival probability was identified, although the dominant cause of mortality shifted from sedimentation, i.e. due to low salinity conditions in 2014, to oxygen deficiency in 2015.

Hinrichsen et al. 2016 (paper in Appendix VIII) used field-derived **cod egg** diameter data in combination with buoyancy data to link these with stock characteristics to improve the current estimation method of a spawning stock biomass. The newly established relationship between egg diameter and buoyancy (floating depth) allowed quantifying the number of effective spawners able to successfully reproduce under certain hydrographic conditions. This study used eastern Baltic cod (*Gadus morhua*) eggs sampled during 8 years in the central Bornholm Basin. For the time period 1993-2010, the results revealed large variations in the horizontal extent of spawning habitat (1000-20000 km²) and oxygen-dependent egg-survival (10-80%).

5.12 Cod larvae – populations across geographical scales – experiments (for more details see **Appendix IX)**

Through the increased usage of fossil fuels and changed land use, the concentration of atmospheric carbon dioxide has been steadily rising since the onset of the industrial revolution. A portion of this CO2 is dissolving into the oceans causing a decrease in pH, e.g. acidifying a habitat that covers 2/3 of this planet. This process has been coined “the other CO2 problem” or ocean acidification. Three experiments were performed, one at the Sven Lovén Centre in Kristineberg, Sweden in 2013 and two at the Centre for Marine Aquaculture, Tromsø, Norway in 2014 and 2015. In collaboration with researchers from the Heinrich Heine University, Düsseldorf, Germany and NOFIMA AS, Tromsö/Norway the effect of an increase in pCO_2 concentrations, representing a climate change stressor, on cod (adults, eggs and larvae) was studied. At the first location, the larvae were fed natural plankton from the adjacent fjord, while in Tromsø in 2014 two variants of aquaculture feeding protocols were applied. In 2015 adult cod where kept under control (400 ppm CO_2) and treatment (1000 ppm CO_2) for 20 weeks prior to spawning to investigate potential effects of parental acclimatization, e.g. transgenerational effects.

The experimentally estimated daily mortality rates were consistent among all stocks and feeding conditions, the end-of-century CO2 treatment resulted in an approximately doubling of mortality. Our findings additionally suggest that even at ad libitum feeding, e.g. a richer energy budget that would allow for more efficient acid-base regulation, cod larvae cannot mediate the adverse effects of ocean acidification. This strengthens the hypothesis that larvae will be negatively affected by ocean acidification independent from food availability in a patchy or match-mismatch environment. However, this apparently does not hold true for growth patterns. At high fed availability, larvae in ambient and end-of-century treatment show no apparent differences in standard length and dry weight by the end of the experiment (36 dph). The experiments performed clearly show that larvae of Western Baltic and coastal Barents Sea cod in 2014 are impacted by near future levels of ocean acidification. While some results are easy and directly to interpret, namely the massively increased daily mortalities, other variables such as the growth patterns need to be analyzed in more detail to understand the underlying physiological and genomic mechanisms as well as their implications, may they be ecological or socio-economic.

6. Progress and next steps

Studies and work-tasks were performed according to the workplan and original objectives were obtained. A wealth of new information now available and is presented in detail in the appendices. In the next steps this knowledge gain will be used for management, modeling and links to WP(2), 3, 4 and 5. There it can be further analysed and exploited. All future outcomes of Task 1.2 will be included in upcoming annual reports

7. References

Baumann H, Voss R, Hinrichsen HH, Mohrholz V, Schmidt JO, Temming A (2008) Investigating the selective survival of summer-over spring-born sprat, *Sprattus sprattus*, in the Baltic Sea. Fish Res 91:1-14

Bernreuther M. 2007. Investigations on the feeding ecology of Baltic Sea herring (*Clupea harengus* L.) and sprat (*Sprattus sprattus* L.). Dissertation, Universität Hamburg

Günther C, Temming A, Baumann H, Huwer B, Möllmann C, Clemmesen C, Herrmann J-P (2012) A novel length back-calculation approach accounting for ontogenetic changes in the fish length – otolith size relationship during the early life of sprat (*Sprattus sprattus*). Can J Fish Aquat Sci 69:1214-1229

Hinrichsen, H.-H, von Dewitz, B, Dierking, J, Haslob, H, Makarchouk, A, Petereit, C, Voss R. (2016): Oxygen depletion in coastal seas and the effective spawning stock biomass of an exploited fish species. R. Soc. open sci. 3: 150338. http://dx.doi.org/10.1098/rsos.150338

Mackenzie, B.R., Köster, F.W. (2004) Fish production and climate: Sprat in the Baltic Sea. Ecology 85(3), 784-794.

Meier HEM, Andersson HC, Arheimer B et al. (2012) Comparing reconstructed past variations and future projections of the Baltic Sea ecosystem—first results from multi-model ensemble simulations. Environ. Res. Lett. 7 034005 (http://iopscience.iop.org/1748-9326/7/3/034005)

Nissling, A., Müller, A., Hinrichsen, H.-H. (2003): Specific gravity and vertical distribution of sprat eggs in the Baltic Sea. J Fish Biol 63: 80-299.

Ojaveer H., Jaanus A., MacKenzie B.R., Martin G., Olenin S., Radziejewska T., Telesh I., Zettler M.L., Zaiko A. (2010) Status of Biodiversity in the Baltic Sea (Y Ropert-Coudert, Ed.). PLoS One 5:e12467

Peck, M.A., Baumann H., Bernreuther , M., Clemmesen , C., Herrmann , J.-P., Haslob, H., Huwer, B., Kanstinger, P., Köster, F.W., Petereit, C., Temming , A., Voss, R. (2012) The Ecophysiology of *Sprattus sprattus* in the Baltic and North Seas. Progress in Oceanography 103, 42-57.

Petereit, C., Hinrichsen, H.-H., Voss, R., Clemmesen, C., Haslob, H., Freese, M., Kraus, G., (2009): The influence of different salinity conditions on egg buoyancy and development and yolk sac larval survival and morphometric traits of Baltic Sea sprat (*Sprattus sprattus balticus* Schneider). In: Clemmesen, C., Malzahn, A.M., Peck, M.A., Schnack, D. (Eds.), Advances in Early Life History Study of Fish. Sci Mar 73(S1), 59–72.

Skora, K. E., & Stolarski, J. (1993). New fish species in the Gulf of Gdansk, *Neogobius sp.* (cf. *Neogobius melanostomus* (Pallas 1811)). Bulletin of the Sea Fisheries Institute, 1(128), 83–84.

8. List of Appendices

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APPENDIX IX: Cod larvae – populations across geographical scales – experiments (P1, GEOMAR)

APPENDIX I

Community level responses and invasive zooplankton species performance along gradients

Evelina Griniene, Aurelija Samuiloviene, Anastasija Zaiko
Klaipeda University, Marine Research and Technology Centre (KU-CORPI, P8)

There were two field studies conducted, addressing the task's objective.

1. A study on contribution of NIS species to zooplankton communities at 3 coastal habitats was performed employing high-throughput sequencing (HTS) metabarcoding approach and comparing those data to the conventional surveillance results. A paper ”Metabarcoding approach for nonindigenous species surveillance in marine coastal waters” is accepted for publication in Marine Pollution Bulletin (Zaiko *et al*., 2015) and attached as part of this appendix.

2. Another study involved two surveys along a salinity gradient from Nemunas river mouth, the Curonian lagoon to the western edge of the Lithuanian EEZ. Micro- and mesozooplankton samples were collected within vertically stratified water columns above and below halocline. The results of the study were presented at the Baltic Sea Science Congress and ICES Annual Science Conference, in 2015. Data analysis is finalized and the corresponding paper is in preparation. Preliminary title of the manuscript is "Abundance and composition of zooplankton communities in the river-lagoon-Baltic Sea continuum” (Griniene *et al*., in prep).

Methods and results

1. Along with the routine zooplankton monitoring (LNM), six samples for HTS metabarcoding were collected at 3 locations within the Lithuanian coastal zone, influenced by the Curonian Lagoon plume: Klaipeda Strait area (KS), out of the strait from the sea side (SG) and at the northern edge of the plume area (PA). Sampling effort and environmental conditions at the locations are summarized in Table 1.

The conventional (morphotaxonomic) analysis of the monitoring samples was conducted following the HELCOM guidelines (HELCOM 2005). For HTS metabarcoding, the modified universal COI primers (Geller et al. 2013) were used for PCR amplification of a fragment of approx. 658 base pairs (bp) within the mitochondrial gene coding for the cytochrome oxidase subunit I (COI). PCR reactions were undertaken by Macrogen based on the original protocol described by Geller et al. (2013). They were sequenced using a Genome Sequencer FLX (Roche) by Macrogen (Korea). Raw data were then processed using MOTHUR v.1.34.4 software (Schloss et al. 2009), and taxonomy was assigned using BOLD Systems reference sequence database

Table 1. Sampling effort as number of samples analyzed by each method; environmental conditions over the observation period; number of species/taxa detected (proportion of NIS in parentheses), in three sampling sites.

		KS	SG	PA
Sampling effort	LNM	4	2	2
	HTS	2	3	1
Environmental conditions	Salinity, PSU	0.2-1.9	6.2-6.6	4.8-6.2
	Temperature, C°	17-23	11-21	11-22
	pH	7.2-7.5	7.1-8.1	6.0-8.8
Species (NIS proportion)	LNM	23 (0)	13 (0.08)	12 (0.08)
	HTS	24 (0.13)	24 (0.17)	16 (0.13)

2. Ciliates and zooplankton samples were collected during two cruises in April and July 2014. The stations were chosen to form the gradient along Nemunas river-Curonian lagoon-Baltic Sea (Fig. 1). Zooplankton samples were taken and analyzed according HELCOM (2005) recommendations. Ciliate counts were performed in Lugol fixed samples by Utermöhl's (1958) method.

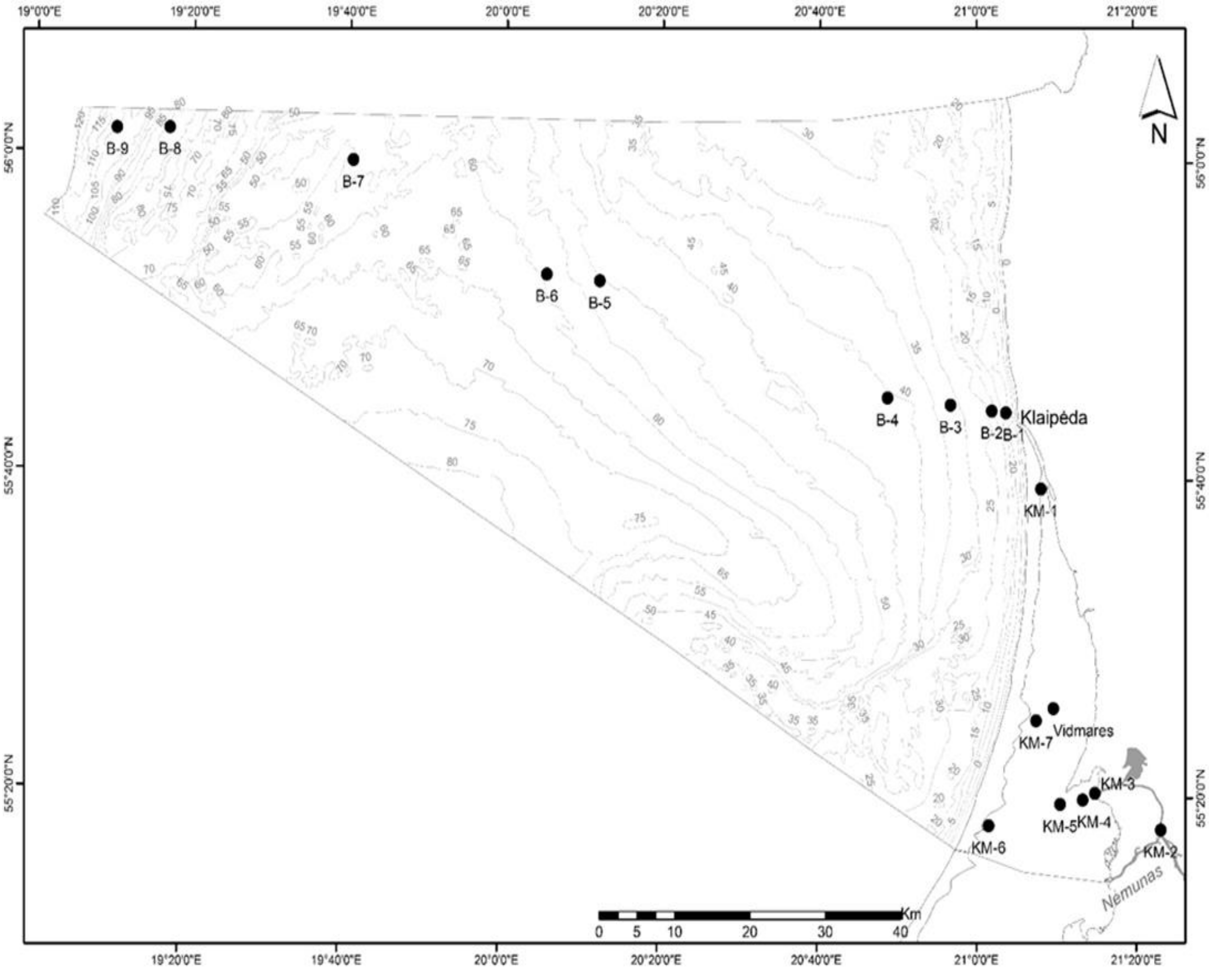

Figure 1: Locations of the sampling stations along the river-lagoon-Baltic Sea continuum (Griniene et al., in prep).

Results

1. HTS results were compared to those from routine monitoring survey and morphological analyses. Comparison of the zooplankton surveillance results obtained by two approaches indicated some discrepancies in retrieved species data, although the taxonomical composition was rather consistent at higher levels (Fig. 2). All the genera and species identified in our survey had been reported also from other studies in the area.

Noticeably, among the species identified exclusively by metabarcoding, five were the benthic organisms with planktonic larval stage (*Dreissena polymorpha, Hydrobia ulvae, Marenzelleria neglecta, M. viridis and Mytilus sp*.). Usually, larvae of benthos are not identified in monitoring samples due to their cryptic morphology and lack of specific taxonomical expertise. This implies potential biosecurity risks, since many invasive sessile organisms have dispersive planktonic stage (like three of the aforementioned species – *D. polymorpha, M. neglecta* and *M. viridis*), and might be overlooked in morphologically analyzed monitoring samples.

Four of five non-indigenous species found in the samples were identified exclusively by metabarcoding. All of them are considered as invasive in the Baltic Sea with reported impacts on ecosystem and biodiversity. The proportion of identified NIS was significantly higher in metabarcoding results. Most of them were detected in the transitional zone between the Curonian Lagoon and the Baltic Sea, characterized by the most variable salinities.
In all three sampling locations there were sequences attributed with high confidence to the invasive polychaete *M. viridis.* Based on the results of the earlier molecular identification and areal distribution assessment of three sibling *Marenzelleria* species within the Baltic Sea, only *M. neglecta* was unambiguously reported from the eastern and south-eastern regions (where Lithuanian cost belongs to).

2. From the comprehensive zooplankton survey along the salinity gradient, three groups of samples: Nemunas River avandelta, Curonian Lagoon and Baltic Sea were distinguished by ordination (MDS) on the basis of similarity of zooplankton and ciliate community structure (Fig. 3).

Zooplankton biomass increased from Nemunas River avandelta towards the Baltic Sea. Community shift from cladocerans in the Curonian Lagoon to rotifers in the Baltic Sea was observed. Ciliate biomass was 1-3 order of magnitude higher compare to zooplankton biomass and tended to decrease from Nemunas river avandelta stations towards the Baltic Sea, but this pattern was less pronounced compare to zooplankton biomass. The most significant factors determining shifts in zooplankton community structure are salinity and chlorophyl-a concentration. Zooplankton community was homogeneous in the sea, without notable effects of plume zone.

Conclusions and future perspectives

1. Our findings contributed to the update of the current distributional maps of the species as well as national inventories of the non-indigenous organisms. Further groundtruthing studies are anticipated to verify the particular distribution of these two species in the benthic habitats.
As a complementary monitoring measure, HTS is advantageous for determining the identities of marine NIS, uncovering new or earlier overlooked invasions, monitoring invasion dynamics, assessing and predicting the secondary spread and thus NIS effect on recipient communities. HTS data obtained from the non-targeted metabarcoding survey can provide information on the number of NIS in a given area and their temporal and spatial occurrence necessary for the environmental status assessment within the MSFD.

2. This was the first comprehensive micro- and mesozooplankton survey conducted in the Lithuanian water along the entire salinity gradient. The information gained from this study will help to get better insight into functioning of the pelagic system and relevant responses in the higher trophic levels. By providing the data on the zooplankton distribution within the Lithuanian coastal zone, we have contributed to the study on spatial and temporal zooplankton variability led by EMI team. A publication I was submitted to the Journal of Plankton Research (Klais *et al*., 2016).

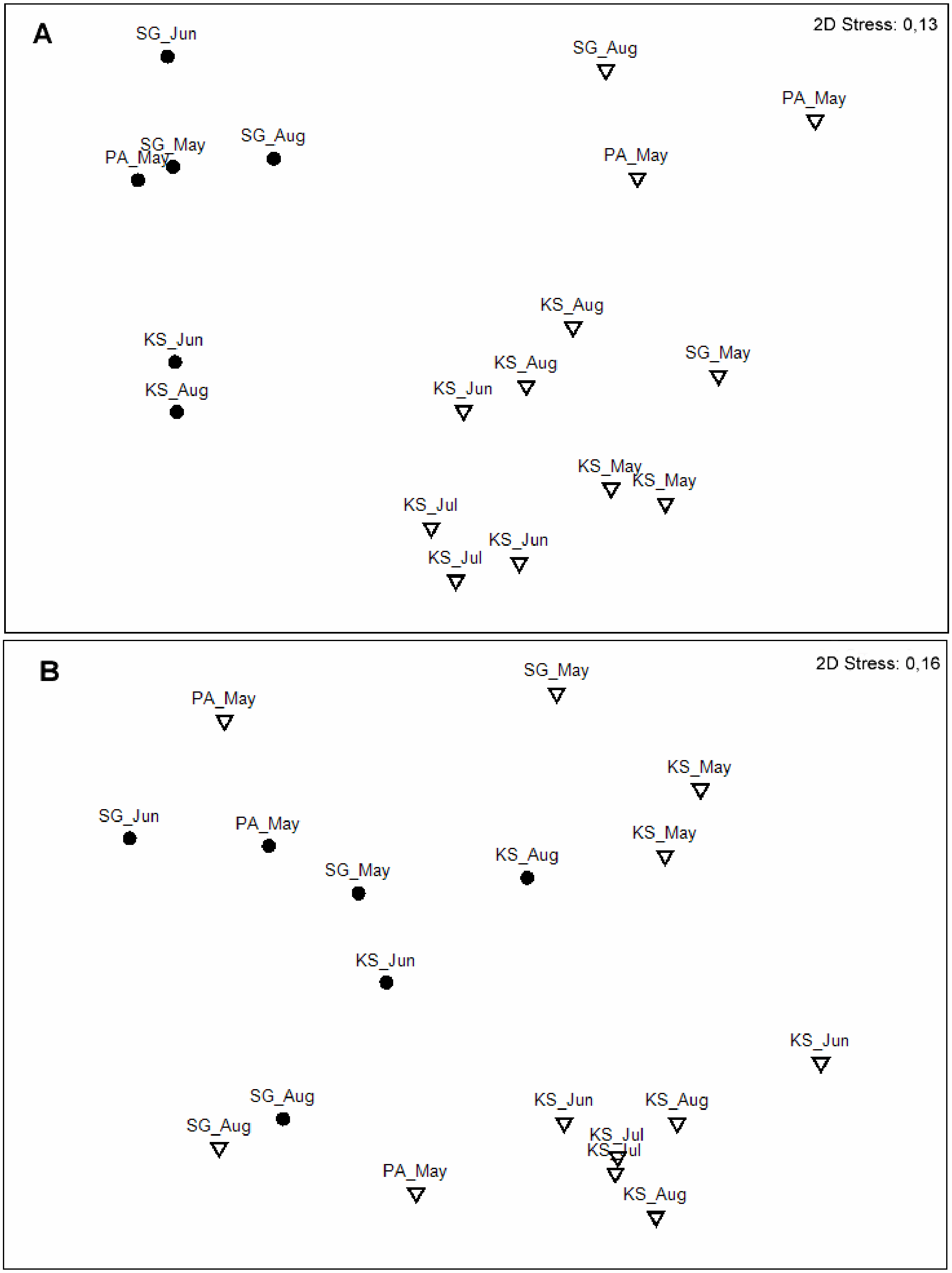

Figure 2: MDS plots showing similarities in the composition of taxa (A- at a species (lowest assigned taxonomy) level, B- at a family level) identified by two approaches at three sampling locations. Black dots correspond to HTS samples, open triangles – LNM samples, labels indicate sampling site and month (Zaiko *et al*., 2015).

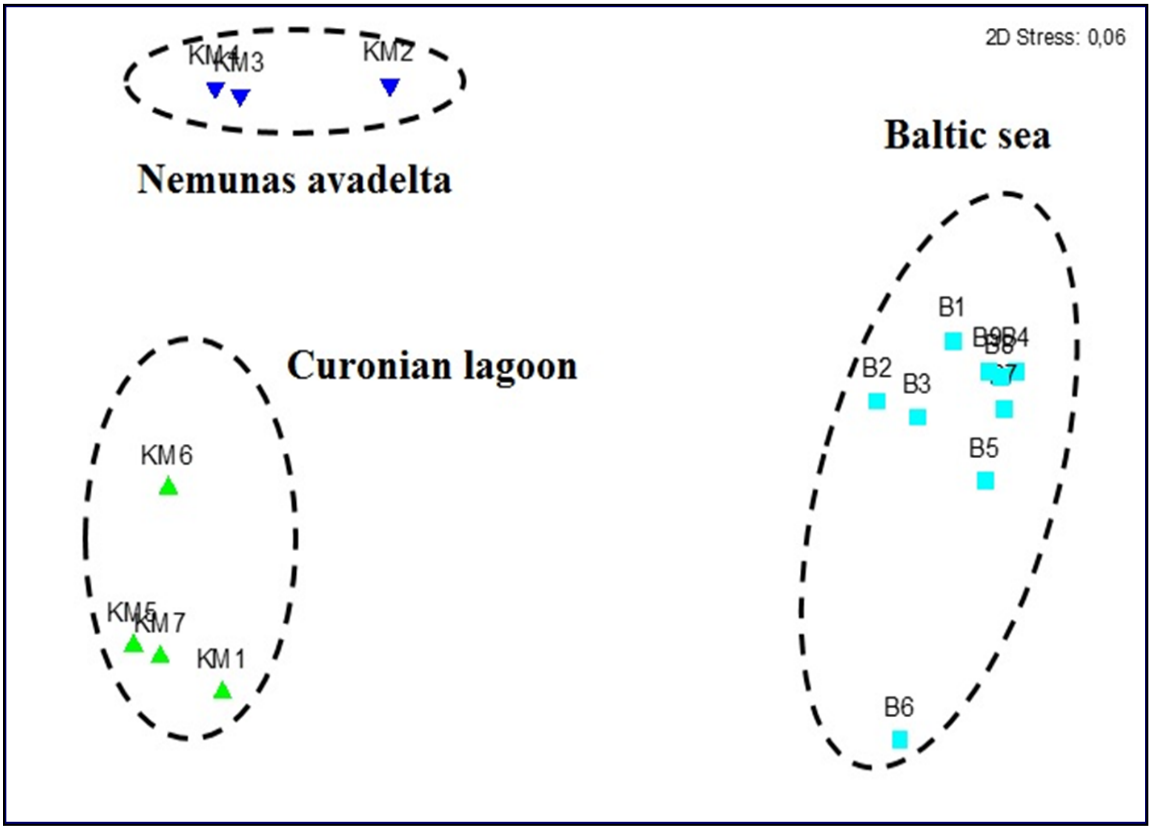

Figure 3: MDS plot based on zooplankton and ciliates biomass data.

References

Geller, J., C. Meyer, M. Parker, and H. Hawk. 2013. Redesign of PCR primers for mitochondrial cytochrome c oxidase subunit I for marine invertebrates and application in all-taxa biotic surveys. Molecular Ecology 13:851-861.

HELCOM. 2005. Manual for marine monitoring in the COMBINE programme of HELCOM, Part C. http://sea.helcom.fi/Monas/CombineManual2/PartC/CFrame.htm.

Klais R., Lehtiniemi M., Rubene G., Semenova A., Margonski P., Ikauniece A., Simm M., Põllumäe A., Grinienė E., Makinen K., Ojaveer H. 2016. Comparison of spatial and temporal variability of zooplankton in a temperate semi-enclosed sea: implications for monitoring design and long-term studies. Journal of Plankton Research (*in press*)

Schloss, P.D., S. Westcott, T. Ryabin, J.R. Hall, M. Hartmann, E.B. Hollister, R.A. Lesniewski, B.B. Oakley, D.H. Parks, C.J. Robinson, J.W. Sahl, B. Stres, G.G. Thallinger, D.J. Van Horn, and C.F. Weber. 2009. Introducing mother: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Applied and Environmental Microbiology 75(23): 7537-7541.

Zaiko A., Samulioviene A., Ardura A., Garcia-Vazquez E. 2016. Metabarcoding approach for non-indigenous species surveillance in marine coastal waters. Marine Pollution Bulletin, 100: 53-59

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Metabarcoding approach for the ballast water surveillance – An advantageous solution or an awkward challenge?

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ABSTRACT

Transfer of organisms with ships' ballast water is recognized as a major pathway of non-indigenous species introduction and addressed in a few recent legislative initiatives. Among other they imply scientific and technical research and monitoring to be conducted in a efficient and reliable way. The recent development of DNA barcoding and metabarcoding technologies opens new opportunities for biodiversity and biosecurity surveillance. In the current study, the performance of metabarcoding approach was assessed in comparison to the conventional (visual) observations, during the *en route* experimental ballast water survey. Opportunities and limitations of the molecular method were identified from taxonomical datasets rendered by two molecular markers of different degree of universality – the universal cytochrome oxydase sub-unit I gene and a fragment of RuBisCO gene. The cost-efficacy and possible improvements of these methods are discussed for the further successful development and implementation of the approach in ballast water control and NIS surveillance.

1. Introduction

Non-Indigenous Species (NIS) are recognized as one of the greatest threats to biodiversity worldwide (IUCN, 2000). These are non-native species introduced from outside of their natural, past or present distributional range, deliberately or unintentionally by humans or other agents (Martin and Hines, 2008). Part of them may spread in the recipient region and become invasive, acting as biological pollutants with adverse effects on biological diversity, ecosystem functioning and socio-economic values (Elliott, 2003; Olenin et al., 2007). Records of new observations and established NIS have been increasing steadily in different marine ecosystems during the two last centuries and are still rising. In European marine ecosystems, on average two new NIS records occurred annually during the past decade (Olenin et al., 2014). Taking into account that global shipping activities have increased dramatically over the last decades, with >20% annual growth rate (Endersen et al., 2008), shipping is believed to be one of the most important pathways for species introductions (i.e., transport of organisms in ballast waters and/or in sediments of ballast tanks and biofouling) (Wonham et al., 2001; Leppakoski et al., 2002; Hewitt et al., 2009). It has been estimated that the major cargo vessels annually transport nearly 10 billion tons of ballast water (Gollasch et al., 2002), with thousands of stowaway organisms being transported every day (Carlton and Geller, 1993; Gollasch et al., 2000b). This results in worldwide NIS exchange and growing risks of marine biological pollution (Elliott, 2003).

The significance of NIS transfer is presently acknowledged by international organizations and is addressed in a number of recent legislative initiatives (e.g. EU Strategy on Invasive Alien Species, Marine Strategy Framework Directive). In particular, the importance of ballast water as a vector of species translocation is recognized through the Ballast Water Management (BWM) Convention, adopted in 2004 by the International Maritime Organization (IMO). The BWM Convention is aimed to prevent, minimize and ultimately eliminate the transfer of organisms via shipping, through the control and management of ships' ballast water and sediments (IMO, 2004). IMO has formulated a number of special regulations

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in order to reduce the risk of organism transport. It also implies scientific and technical research and monitoring to be conducted by Parties of the BWM Convention. This includes observation, measurement, sampling, evaluation and analysis of the effectiveness of any management measure applied as well as analysis of any adverse impacts caused by such organisms and pathogens that have been transferred through ships' ballast water. On the other hand, the robust detection of NIS is crucial for implementing timely and cost-effective management measures including preborder or early incursion control (Simberloff, 2001; Darling and Blum, 2007; Darling and Mahon, 2011). Therefore there is a demand for rapid, standardized, reliable and cost-effective diagnostic tools that are able to identify and quantify the full range of NIS assemblages (King and Tamburri, 2010; Olenin et al., 2011). It is expected that this demand will increase tremendously in the nearest future after updated marine monitoring programs are launched and BWM Convention enters into force.

The accuracy and resolution of NIS data needed for different policy measures varies. For instance, Regulation D-2 Ballast Water Performance Standard limits the acceptable concentration of all organisms in the discharge of ballast water, disregarding the species composition. Therefore, the species identification is not required for compliance control tests (IMO, 2004). Uneven distribution and damage of organisms during the sampling procedure may however bias the test results. Particularly challenging is BWM Convention compliance verification for organisms of minimum dimension $\geqslant 50$ μm, since less than 10 viable organisms of that size per cubic meter are acceptable by BWM Convention (Gollasch, 2006; Gollasch et al., 2007). Therefore, intensive and extensive sampling is needed to ensure that violations of BWM Convention for discharges are detected by direct ballast water measurements (King and Tamburri, 2010). Application of alternative techniques such as remote sensors, flow cytometry and molecular methods are being increasingly discussed and addressed in experimental ballast water surveys (Gollasch et al., 2007; Harvey et al., 2009; Briski et al., 2012; Ojaveer et al., 2014).

On the other hand, simple counts of observed organisms do not provide any additional information on biosecurity risks and are of minor scientific value for bioinvasion researchers. More detailed information (including taxonomic structure) is highly recommended for development of pressure indicators (e.g. rate of anthropogenic transport of NIS propagules), environmental status assessment, port baseline surveys, and species-specific risk assessments (Gollasch et al., 2007; Olenin et al., 2010, 2011; Ojaveer et al., 2014). This requires specific taxonomic expertise which is costly and extremely laborious, both in terms of representative sample collection and identification of dispersive life forms of NIS – eggs or larvae (Darling and Blum, 2007; King and Tamburri, 2010). It is believed that the application of rapidly developing molecular methods can substantially improve species identification capacities and aid NIS surveillance in the nearest future (Mountfort et al., 2012; Kelly et al., 2014; Wood et al., 2014).

Among molecular techniques, traditional DNA-based taxon identification approaches (e.g., PCR-based fingerprinting, quantitative PCR, Sanger DNA sequencing) can be efficient for detecting and identifying targeted NIS (Bott et al., 2010; Darling and Mahon, 2011; Mountfort et al., 2012; Collins et al., 2013). However, these methods are often limited to a single species detection, and therefore not effective enough for biodiversity assessment. The recent development of the high-throughput DNA sequencing technology, also called Next Generation Sequencing (NGS), opened new opportunities for life sciences in general (Ansorge, 2009) and demonstrated a great potential in marine biological and environmental studies in particular (Chariton et al., 2010). The major advance offered by this approach is the ability to operatively produce large numbers of comparatively low-cost sequences. This opens many different application opportunities, including metabarcoding studies: species detection and identification from bulk samples, using species-specific gene markers – the DNA barcodes (Hajibabaei et al., 2011; Andersen et al., 2012). DNA barcoding and NGS have already been recommended as a prospective tool for identifying NIS from environmental samples (Mountfort et al., 2012; Kelly et al., 2014; Ojaveer et al., 2014; Wood et al., 2014).

Here we address the applicability of metabarcoding methodology for the biosecurity surveillance, and particularly detection of organisms in ships' ballast waters. For this purpose the performance of metabarcoding approach was assessed in comparison to the conventional (visual) methodology, during the *en route* ballast waters survey onboard R/V "Polarstern". Opportunities and limitations of the molecular approach were identified from taxonomical datasets rendered by two molecular markers of different degree of universality – the universal cytochrome oxydase subunit I gene (COI) (Hebert et al., 2003) and a fragment of RuBisCO (RBC) gene, designed for diatom identification (Stoof-Leichsenring et al., 2012). The cost-efficacy and possible improvements are discussed for the further successful development and implementation of the approach in ballast water control and NIS surveillance.

2. Methods

The study was conducted during the ANT XXIX-1 EUROPA cruise onboard R/V "Polarstern", hosted by the Alfred Wegener Institute for Polar and Marine Research (Germany). The vessel left Bremerhaven port on October 28 2012, called at Las Palmas, Gran Canaria, on November 4 and left the next day, crossed the equator on November 14 and ended the cruise on November 27 in Cape Town, South Africa (Fig. 1). For the purpose of the ballast water experimental study, the aft ballast tank (70 m^3) was filled with the North Sea water on October 28, out of Bremerhaven port. At the time of the ballast water upload, water temperature and salinity were 13.1 °C and 34 ppt, correspondingly.

Twenty samples of the ballast water were collected daily via the sounding pipe (20 mm diameter) starting from the 2nd day of the cruise, October 29 till November 17 (Fig. 1). Ballast water was extracted from approximately 1.5 m depth, through the build-in ballast pump (operational pressure up to 6 bar, loading capacity *ca.* 20 L/min), taking care to thoroughly flush the pipes before sampling. For each sample, 100 L of ballast water (measured with clean 10 L buckets) were concentrated by filtering through a plankton net (30 cm diameter, 55 μm mesh size) and instantly analyzed using a stereo microscope (60× magnification). The observed organisms were counted and identified to lowest possible taxonomic level. Only undamaged individuals without decay signatures were assessed, assuming them being viable or recently alive. Additional samples on days 2, 11 and 21 of the cruise were collected and vacuum-filtered through sterile 0.12 μm Nuclepore™ membrane, which was thereafter preserved with 96% ethanol and stored at 4 °C until the further land-based metabarcoding analysis of the settled material.

Simultaneously with the sample collection, environmental conditions in the ballast water were recorded, measuring salinity, temperature, pH, dissolved oxygen concentration with Ysi Professional Plus Multimeter. The sea surface temperatures were recorded automatically by the build-in onboard sensors.

2.1. Genetic and bioinformatics analyses

The precipitates from membrane filters were collected with sterile blades, then DNA was extracted from the filters using QIAamp DNA Mini Kit (Qiagen) and following the manufacturer

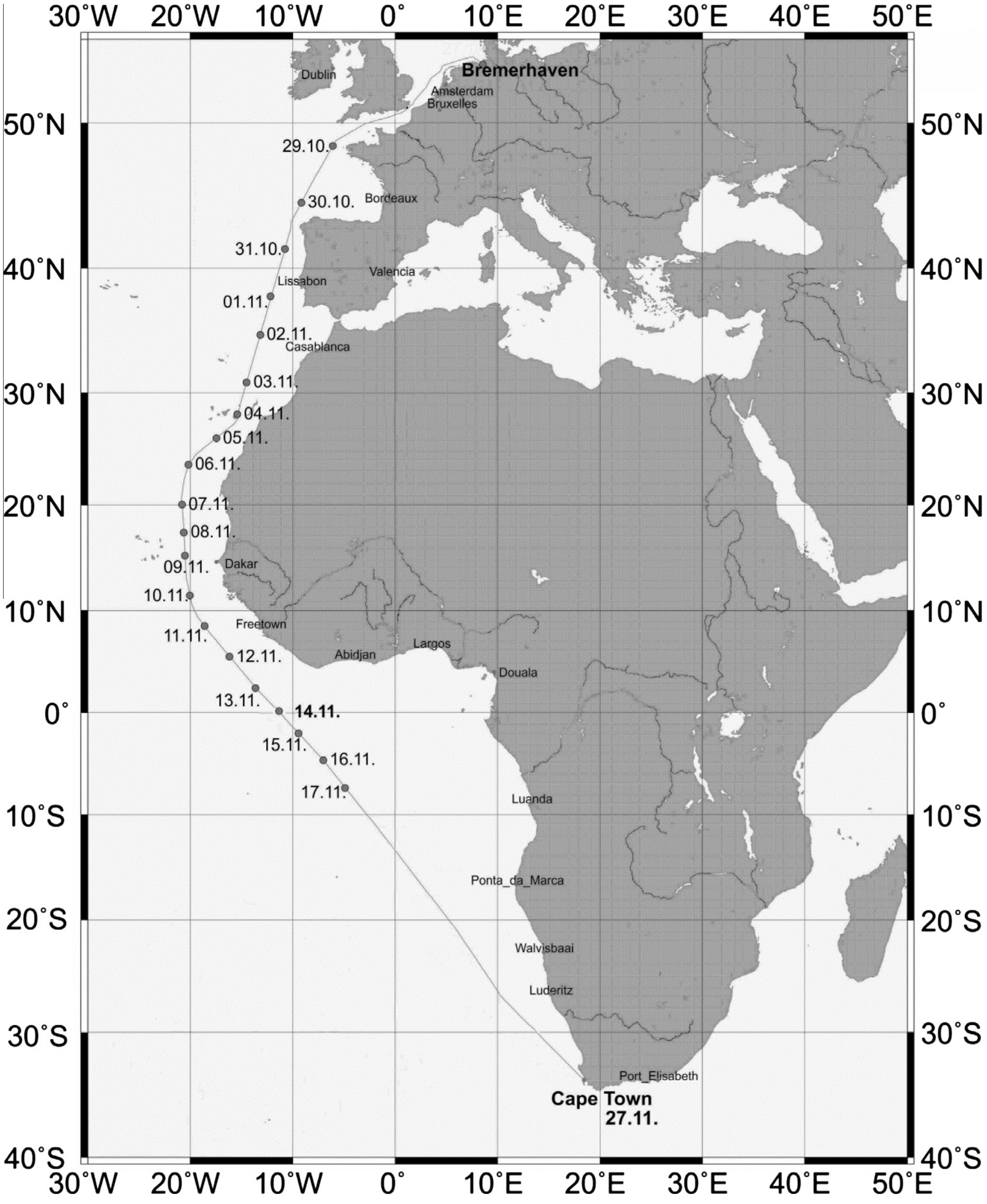

Fig. 1. ANT XXIX-1 EUROPA cruise route with indicated days and locations of ballast water sampling.

extraction protocol. COI was amplified using the universal primers miniCOI (Meusnier et al., 2008) for PCR amplification. The fragment RBC gene was PCR amplified using a pair of primers designed for diatom identification (Stoof-Leichsenring et al., 2012). The comparison between the NGS results obtained from the two markers served to further explore the biases in ballast water biodiversity assessment due to primer specificity.

High-throughput sequencing was performed using the next generation sequencing platform Ion Personal Genome Machine System (PGM. Lifetechnologies) at Sequencing unit of the Oviedo University. For multiplexing purposes, the PCR products were labeled separately for each sample using short DNA sequences. Libraries were constructed using the Ion Plus Fragment Library Kit (Lifetechnologies) and templates were obtained using the Ion PGM™ Template OT2 200 Kit (Lifetechnologies). The templates were loaded in a 314 chip and sequenced using the Ion PGM Sequencing 200 Kit v2 (Lifetechnologies).

The yielded sequences where filtered by length (between 130 and 200 bp for COI and 80–130 for RBC gene) and quality (+20) and taxonomic classification (best hit, max e value = 0.001, min percent identity = 90.0) was assigned BLAST-aligning sequences against NCBI database using QIIME platform (Caporaso et al., 2010).

After initial inspection, the sequences of the organisms unlikely to be present alive in ballast water (e.g. vertebrates and non-aquatic species) were eliminated from the dataset. Most probably, sequences of those organisms were derived from body remains such as scales or feathers that can occur in marine water uploaded in the tank, but they will have no biological significance as NIS.

2.2. Statistical analysis

For quantification summary and statistical analyses of biodiversity the species data were pooled to superior taxonomic ranks (Class or higher), as in Smith et al. (1999), for a conservative

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estimate of biodiversity in the ballast water assemblages. This way, possible uncertainties in the visual taxonomic assessment (due to for example the ambiguous phenotypes in species with phenotypic plasticity and uncertain identification of some larvae and algae propagules) were minimized. The composition of species (presence–absence data) identified from visual analysis was compared between sampling days using nonmetric multidimensional scaling (NMDS) based on Jaccard similarity matrix. NMDS cannot process identical samples, so a dummy variable (value 1) was added to all samples when constructing the similarity matrix. NMDS was undertaken with 100 random restarts and visualized in two-dimensional plot. Linear regression was applied to ascertain the trends in densities of metazoans, protozoans and algae visually identified from ballast water samples.

To verify the differences in taxonomic diversity reported from visually analyzed samples and metabarcoding results (with COI and RBC barcodes), the canonical discriminant analysis of principal coordinates (CAP) was applied, followed by a permutation test and two-dimensional visualization. The Gower's distance measure was used for dissimilarity matrix construction, based on densities (number of observed individuals and number of yielded sequences for visual and metabarcoding data correspondingly) and standardized by total number per sample. The Gower's distance is considered robust enough for standardized data analysis, flexible and non-sensitive to missing observations and double zeros (Quinn and Keough, 2002; Anderson and Willis, 2003). Samples from days 16 to 19 with no visually detected specimens were excluded from the analysis.

The analyses were implemented in PRIMER 6 software package (PRIMER-E, Ltd., UK) and the R v3 statistical computing environment (R-project, 2014).

3. Results

3.1. Environmental conditions within the ballast tank

The temperature within the ballast tank showed a steady increase over the first 14 days of observation, in consistence with the overboard temperature and reached the maximum of 29.9 °C on day 15. During the following six days the temperature dropped gradually by 6 °C, exceeding the sea surface temperature by 2 °C on average (Fig. 2). Dissolved oxygen concentration decreased from the normoxic conditions (7.3 mg/L; 91.5% saturation) at the beginning of the voyage, to anoxia (0.3 mg/L; 5% saturation) on the 20th day of observations. The pH values also demonstrated a gradual decrease in the range from 7.9 to 7.1.

3.2. Visual analysis

From the visually analyzed samples 14 taxa were identified during the observation period (Table 1). The noticeable change in community composition was reported starting from the second week of the observations (Fig. 3).

In the beginning, samples were dominated by numerous microalgae (diatoms and dinoflagellates), with a significant negative trend ($R^2 = 42\%$, $p = 0.002$) – more than tenfold decrease in a week and further drop down to zero values after few more days (Fig. 4). The last record of algae (dinoflagellate) cells was reported on the 13th day of the cruise (9 November).

The metazoan taxa have also demonstrated significant negative trend ($R^2 = 26\%$, $p = 0.02$) over the observation period, yet individual organisms (arrow worms) were reported from the samples on day 20. It is worth noting however that increasing number of damaged, partly decayed individuals was registered starting from the second week of observations (day 8 and thereafter).

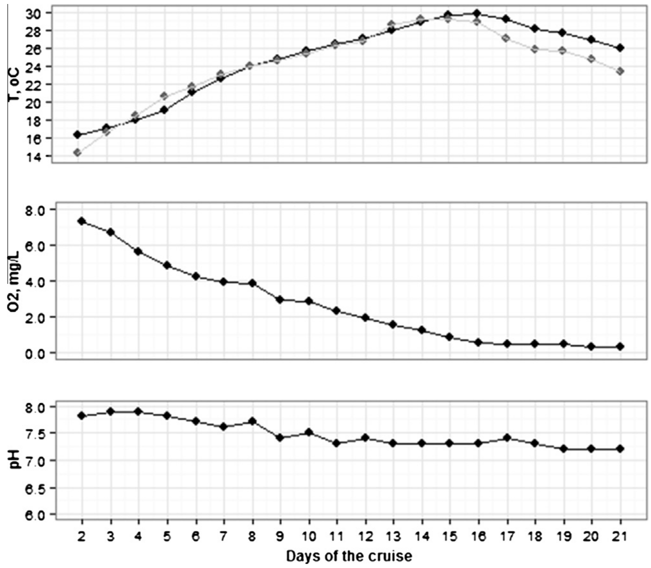

Fig. 2. Registered environmental conditions in the ballast tank: water temperature (black dots – ballast water, grey dots – sea surface), dissolved oxygen concentration and pH.

Single protozoan specimens were steadily reported from the samples with no apparent trend in densities ($R^2 = 14\%$, $p = 0.09$), except for an abrupt outbreak on the last sampling day when 122 actively moving protozoans with cilia-like protrusions were observed in the sample.

3.3. NGS results

PCR amplifications of both COI and RuBisCO (RBC) genes resulted in amplicons (145 and 97 nucleotide long correspondingly) from day 2 (COI1, RBC1), day 11 (COI2, RBC2) and day 21 (COI3, RBC3) samples. The raw high-throughput sequencing of the amplicons produced 159,039 (COI) and 137,518 (RBC) reads. The stringent quality check and filtering parameters resulted in the removal of 28.7% (COI) and 32.3% (RBC) sequences. The number of high-quality sequences used for the further downstream analysis was 113,267 and 93,060 for COI and RBC samples correspondingly. The bioinformatics analysis resulted in the clustering of sequences into 29 and 136 OTUs, for COI and RBC respectively, yielding positive assignment hits against NCBI database with >90% sequence similarity and identified at genus level for conservative approach (Suppl. 1 and 2, summarized in Table 1).

Absolute majority of assigned sequences (96%) from COI1 sample matched with metazoan species, while most (55%) of COI3 sample sequences were assigned to oomycetes (fungus-like eukaryotic microorganisms), followed by unidentified marine invertebrates (22%) and protozoans (10%). The RBC samples were highly dominated (nearly 90%) by diatom sequences as expected from primers' specificity, followed by yellow–green algae.

Generally, there was apparent decrease in a number of yielded sequences from day 21 sample for both applied markers (comparing to the day 2 sample). However, when partitioned by large taxonomic groups based on COI results, a remarkable drop in number of sequences was apparent for algae (−60%) and metazoans (−77%), while protozoans demonstrated more than tenfold increase (Fig. 4). The oomycetes (not detected by visual analysis) have also noticeably increased in number of yielded sequences by the end of observation period.

At a lower taxonomic level, there were a few taxa that have demonstrated increase in sequence number by more than 100%

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Table 1
Reported biodiversity from the ballast tank during the cruise (represented by the number of observed specimens and number of yielded sequences for the visual and metabarcoding data correspondingly, counts per 100 L of ballast water). COI1, COI2, COI3 and RBC1, RBC2, RBC3 samples correspond to days 2, 11 and 21 samples. Unident. Invert: Unidentified invertebrates. Summarized values for the highest taxonomic ranks are emphasized in bold.

Taxa	Visual observations (days of voyage)																				Metabarcoding samples					
	2	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**COI1**	**COI2**	**COI3**	**RBC1**	**RBC2**	**RBC3**
Algae (total)	**457**	**309**	**107**	**82**	**17**	**37**	**15**	**10**	**5**	**6**	**0**	**2**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**113**	**15**	**45**	**325,013**	**26,476**	**33,209**
Bacillariaphyceae	404	308	105	79	14	34	12	10	3	5	0	0	0	0	0	0	0	0	0	0	113	15	0	285,687	22,993	29,056
Dinophyceae	53	1	1	3	3	3	3	0	2	1	0	2	0	0	0	0	0	0	0	0	0	0	0	12,829	976	1044
Phaeophyta	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2306	110	118
Rhodophyta	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	45	86	8	63
Xantophyta	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	24135	2389	2928
Metazoa (total)	**17**	**4**	**2**	**4**	**5**	**7**	**6**	**1**	**2**	**1**	**2**	**5**	**0**	**9**	**0**	**0**	**0**	**0**	**5**	**0**	**12217**	**16068**	**2795**	**0**	**0**	**0**
Annelida	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	10	0	0	0
Chaetognatha	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	5	0	0	0	0	0	0	0
Copepoda	13	0	0	3	3	1	1	0	1	0	2	5	0	7	0	0	0	0	0	0	0	0	17	0	0	0
Cnidaria	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Mollusca	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1154	179	0	0	0
Nematoda	1	2	2	0	2	3	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Ostracoda	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Porifera	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1871	2564	474	0	0	0
Rotifera	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	412	0	0	0
Unident.invert.	2	1	0	1	0	3	1	0	1	0	0	0	0	1	0	0	0	0	0	0	10344	12344	1856	0	0	0
Protozoa	**1**	**0**	**1**	**0**	**0**	**0**	**0**	**1**	**0**	**1**	**1**	**0**	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**122**	**49**	**11**	**862**	**0**	**0**	**0**
Oomycota	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**310**	**17**	**4611**	**0**	**0**	**0**

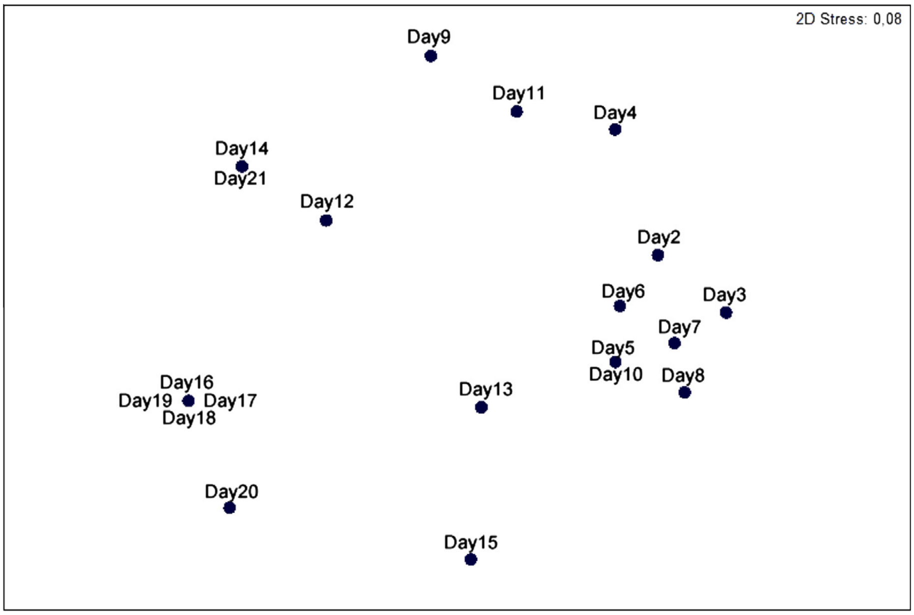

Fig. 3. NMDS plot of the visually observed ballast water community, based on presence/absence of observed taxa (Jaccard index similarity matrix).

over the observation period (Suppl. 1 and 2). The bigger increases of DNA sequences detected with COI primers were those assigned to the water mold *Achlya,* Arcellinidae protozoan *Hyalosphenia* and the rotifer *Brachionus* (Suppl. 1). The copepod *Cyclopodia,* red algae, protozoans, gastropods and most of the oomycetes have demonstrated somewhat increase in later samples. On the other hand, the algae diversity derived by NGS with RBC primers (Suppl. 2) was more consistent among samples. Several diatom genera (namely, *Eunotogramma*, *Minidiscus*, *Skeletonema* and *Thalassiosira*) did show higher than ten-fold increase in the number of sequences during the observation period. Red algae and yellow–green *Botrydiopsis* have also demonstrated moderate increase by day 21.

The diatoms detected with COI primers (*Nitzchia* spp.) were reported from the RBC samples as well. However red algae assignments showed discrepancy between two applied markers. Two genera of Ceramiales (*Polysiphonia* and *Dasya*) and *Plocamium* (Plocamiales) were obtained with COI, while RBC has resulted in *Ceramium* (Ceramiales) and *Delisea* (Bonnemaisoniales).

The permutation test carried out by CAP produced a *p*-value of 0.001 (based on 999 permutations). It means that no randomly permuted data set had more extreme assemblage than that of the original data set. It is noticeable that visually analyzed samples from days 2 to 11 with a major component of algae were located on the positive-valued side of the Axis 1 (Fig. 5). Expectedly, the RBC samples with applied diatom-specific barcode grouped on the positive-valued side as well. The taxonomic diversity reported from the ballast water samples on 12th day of the cruise onwards grouped on the negative-valued side of the Axis 1, correlating with protozoan abundance and moderately – with

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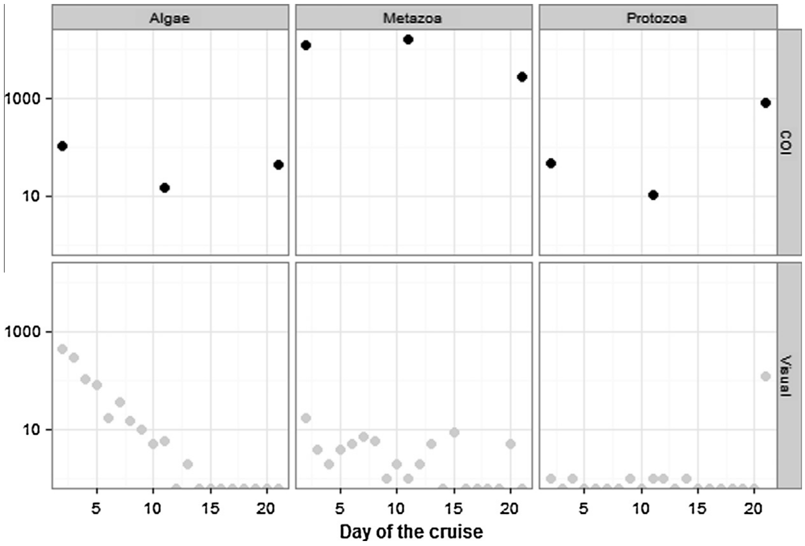

Fig. 4. Densities of algae, metazoan and protozoan organisms reported from the visual analysis of the ballast water samples (number of individuals counted from the 100 L sample) and metabarcoding analysis with COI barcode gene applied (number of sequences yielded from NGS).

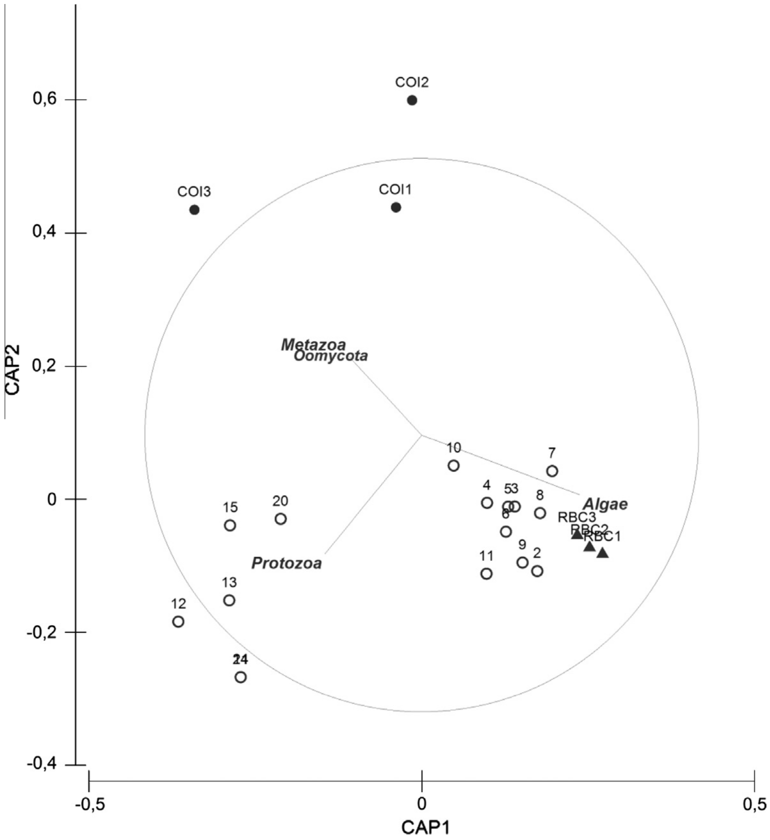

Fig. 5. The first two canonical axes of the CAP analysis, based on taxonomic diversity identified from the ballast water samples applying visual analysis, COI and RBC barcodes (only major taxonomic groups are displayed for clear reading). The labels indicate the sampling day for visually analyzed samples (open circles) and sample code for RBC and COI samples (black triangles and filled circles correspondingly).

metazoans and oomycetes. Yet, the COI samples were apparently distinct from others, demonstrating the highest positive correlation (ranging between 0.4 and 0.6) with the Axis 2 and correlating mostly with metazoans and oomycetes.

4. Discussion

This study evidences again that despite rather harsh environmental conditions (darkness, low oxygen, temperature fluctuations) during the long cross-latitudinal voyage there is a possibility for some eukaryote species to survive and even flourish in ballast waters (Gollasch et al., 2000a,b; Olenin et al., 2000; Duggan et al., 2005). Such organisms are likely to remain viable upon discharge, thus posing a high risk of incursion to a recipient ecosystem. The combination of metabarcoding and conventional (visual) taxonomic analysis let us assess the dynamics in ballast water plankton community and identify taxa that have the highest potential of survival.

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In general, the biodiversity revealed by both approaches at a higher taxonomic level (Table 1) coincided with that reported from other studies (Gollasch et al., 2000a,b, 2002; Olenin et al., 2000; Duggan et al., 2005; Flagella et al., 2007; Briski et al., 2012). As reported also by other researchers, a rapid decline in plankton abundances was noticed during the first several days of the voyage, with a more pronounced decrease for the phytoplankton taxa (Gollasch et al., 2000a,b; Olenin et al., 2000). The metazoan organisms have demonstrated higher persistence with evident drop at the most adverse environmental conditions within the tank (anoxic, high temperature) during the second week of the cruise (Figs. 2–4). Protozoans however were able to withstand the harsh ballast water environment and even increased in abundance by the end of the observation period. Such apparent shift in the ballast water community from algae-dominated to metazoan- and further microplankton-dominated (Fig. 5) can be explained by phytoplankton and zooplankton mortality increasing with time due to exposure to stressful conditions, and proliferation of saprophagous water molds and protists resistant to high temperatures, lack of light and oxygen (Jobard et al., 2010). On the other hand, these organisms were largely underestimated in previous ballast water surveys (Gollasch et al., 2000b, 2002; Duggan et al., 2005; Flagella et al., 2007) and in most NIS inventories in general (Wyatt and Carlton, 2002). For instance, the absolute majority of species currently reported as associated with vessel vectors in the AquaNIS database are metazoans (Olenin et al., 2014). Small multicellular and unicellular organisms (as well as dispersible living stages of many taxa) are easy to overlook and confound in the conventional taxonomic assessment (Foissner, 2006) or due to unrepresentative sampling strategy (Gollasch et al., 2007). In this case metabarcoding and NGS technologies are advantageous being able to detect and identify species from a single cell present in the sample (Jerde et al., 2011; Kelly et al., 2014).

At the lower taxonomic level, as resulted from the NGS data, there were a few genera that have not been reported from the ballast water surveys previously (Suppl. 1 and 2). Some of those are known to contain NIS or even invasive alien species. For instance, the red algae *Dasya baillouviana* recorded in the Baltic Sea since 1960s (Maggs and Stegenga, 1999), several species of *Polysiphonia* are recognized as NIS from North Atlantic, Mediterranean, Australia, New Zealand and Japan (Hurd et al., 2004; Minchin, 2007; Geoffroy et al., 2012). However, the exact pathway or vector of spread is still largely undetermined for those species (Thomsen et al., 2007). The invasive oomycetes of *Phytophthora* genus are known to be associated with plant twig blight disease in Europe (Werres et al., 2001) and Sudden Oak Death disease in USA (Rizzo et al., 2005). Although it is believed that *Phytophthora* species are distributed predominantly via the terrestrial pathways (wind, land-based transport, planting material from infected nurseries), they are known to remain viable in water for years (Ko, 2003) thus potentially could be transported with ballast water loaded from estuarine or coastal areas.

In the current study we did not assess specifically the viability of the organisms observed, as it is required for BWM Convention compliance control (e.g. Regulation D-2). In visual analysis we assumed that entire, undamaged individuals are likely to be alive at the sampling time or shortly before. However DNA molecules can resist for some days inside dead cells and even naked until degradation, as demonstrated in some previous studies of environmental DNA (e.g. Dejean et al., 2011). Therefore finding evidence of DNA from a species in environmental samples does not mean that it belong to a living organism. This, and inability of providing the measure of minimum dimension of observed organisms are probably the main weaknesses of metabarcoding application for the BWM Convention compliance control. However, DNA can be a signal of living organisms when its density increases with time. This could be the case of *Achlya*, *Brachionus* and *Hyalosphenia* in the present study (Suppl. 1). On the other hand, an increase of these taxa would be not surprising, since *Achlya* belongs to hardy water molds (Willoughby, 1965), *Brachionus* adults have been previously found surviving the long voyages within the ballast water (Gollasch et al., 2002; Duggan et al., 2005) and *Hyalosphenia,* a widespread and rather resistant representative of testate amoebas (Heger et al., 2013), known to be transported in ballast water as far as to the Great Lakes (e.g. Nicholls and MacIsaac, 2004). Among other organisms that have not decreased (or even slightly increased) in density (Suppl. 1 and 2) as evidenced by both by COI and RBC markers, the red algae (e.g. *Polysiphonia*) have free-living life-history phases (Kaczmarska and Dowe, 1997) and can tolerate high temperatures, low salinities and lack of light for extended periods (Fralick and Mathieson, 1975); diatoms are capable of survival in darkness 12 days and more (laboratory experiment at 18 °C temperature, Jochem, 1999), maintaining cell abundance up to 90 days (laboratory experiment, at 15 °C temperature, Smayda and Mitchell-Innes, 1974) and reported repeatedly from the ballast water samples (Gollasch et al., 2002).

However, there is another pitfall in metabarcoding application, related to the method's quantification capacity. Although eDNA concentration and number of sequences yielded from NGS are positively correlated with biomass or population density, estimates of absolute abundance remain elusive (Kelly et al., 2014). So far this approach cannot be applied independently for robust quantification and assessment of surviving taxa, but rather used as additional technique for biodiversity screening (e.g. if there are some doubts of non-compliance with BWM Convention, or a need for species-based risk assessment). For the putative samples or taxa more detailed further molecular analysis would be advised.

The use of multiple markers is often recommended for metabarcoding purposes, since it allows reducing amplification bias (Kelly et al., 2014). For instance, in the current dataset some inconsistences between visual analysis, RBC and COI could be explained by the specificity of the applied primers (e.g. Wilcox et al., 2013). In NGS results, copepods, arrow worms, nematodes were highly underrepresented. On the other hand, water molds, amoebas and rotifers were largely overlooked in the conventional analysis, but detected with COI marker instead. Genetic detection of most algae was possible only with the specific RBC primers. Indeed, the difference between the results obtained here from specific (RBC specific for diatoms; Stoof-Leichsenring et al., 2012) and more generalist (miniCOI; Meusnier et al., 2008) primers is enormous. Without the specialist primers diatoms, but also green and yellow algae, would remain inadvertent or highly underestimated in this study.

Since true universal primers annealing with same preference to all living taxa do not exist yet, we would suggest using primers cocktails for targeting a wider taxonomic spectrum as recommended by other authors as well (e.g. Valentini et al., 2009; Ivanova et al., 2007). Particularly, targeting diatoms more specifically is highly desirable on the short voyage legs, while community remains phytoplankton-dominated (Fig. 5). Diatoms are generally overlooked in conventional ballast water surveys due to light silicification of some taxa or resting stages present (Antia and Cheng, 1970; McCarthy and Crowder, 2000). We would also suggest replicating NGS from the same environmental sample employing different primer sets, specifically designed for the more problematic taxonomic groups (Jerde et al., 2011; Wilcox et al., 2013) and more particularly those expected to be found and survive in ballast water. This might improve the utility of this novel methodology for ballast water management issues.

This study was an experimental survey and not a real experience of ballast water monitoring. For the robust risk assessment, a bigger sampling effort, targeting different areas of the tanks and including sediments is recommended (Gollasch, 2006). The results of a survey

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Table 2
Preliminary comparison of strengths and weaknesses of metabarcoding and visual analysis approaches in ballast water surveys (based on the current study experience and literature review).

Metabarcoding	Visual analysis
Cost	
Moderate: $10,000–$50,000[a]/vessel/sampling voyage, with tendency to further decrease due to increasing NGS capacities	Very high: $75,000–$125,000/vessel/sampling voyage[b]
Effort (time consumption)	
Moderate: Tendency to further decrease with rapid technology development[c]	Low-moderate: Depending on volumes and aims of the survey[b,d]
Quantification	
Moderate: Increases with use of multiple markers, replication and internal control[e]	High: The accuracy however is highly dependent on sampling effort[d,f]
Taxonomic resolution	
High-very high: Increases with use of multiple markers, development of reference databases for barcode genes[e]	Low-moderate: Highly dependent on taxonomical expertize of the researcher, low for early life stages (larvae, eggs, resting stages)[g,h]
Assessment of viability	
Low-moderate: Could be improved with sequential sampling (analysis of OTU dynamics) or RNA-based analyses	Moderate: Conservative assessment by visual inspection, intact individuals or dye-based methods (high uncertainty for some groups and life stages)
Sensitivity/detectability	
High-very high: Able to detect species at low abundances (individual cells, trace material)[i,j]	Low-moderate: Rare or particularly small organisms are likely to be overlooked or underestimated[h]
Added value	
Very high: Massive amount of “non-target” data on biodiversity might be obtained	Low: Generally, only targeted range of organisms (size- or taxonomically-based) is detected

[a] Costs are given as a proxy since may vary enormously depending on the particular molecular methodologies employed (price reduction is expected as long as new technical improvements), salary conditions, aim of the survey, vessel type, etc.
[b] King and Tamburri (2010).
[c] Wood et al. (2014).
[d] Gollasch (2006).
[e] Kelly et al. (2014).
[f] Gollasch et al. (2007).
[g] Ardura et al. (2010).
[h] Pochon et al. (2013).
[i] Bott et al. (2010).
[j] Darling and Mahon (2011).

(counts of living biota) might also be affected by the uneven distribution of organisms within ballast tanks, sampling induced damage and mortality (particularly when pumping the water through the sounding pipe) or organism loss during the sample concentration (Gollasch et al., 2007). Hence, the application of more sensitive and specific molecular techniques would be particularly advantageous as a complementary measure for species detection and identification. NGS application has resulted in interesting findings and provided significant added value to the study outcome, even within the comparatively small-scale experiment. As a conclusion we would like to summarize the strengths and weaknesses of metabarcoding application for ballast water surveys in comparison to the conventional (visual) approach (Table 2).

5. Conclusions

Although based on a single experimental study, the results allow us to recommend some actions for improving the efficacy of ballast water surveillance and management employing next generation molecular technologies. First, using metabarcoding or any other molecular methodology for ascertaining the taxonomic status of organisms contained in ballast water samples is desirable, especially for taxa where microscopic identification is doubtful or very laborious. Second, molecular ballast water monitoring is recommended to occasionally verify the positive results of control surveys (zero counts of ballast water organisms), because outbreaks of resistant species can be produced even in adverse conditions and overlooked by conventional analysis. Third, combining different methods (e.g. *de visu* surveys and metabarcoding) considerably improves the power of monitoring protocols.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.marpolbul.2015.01.008.

References

Andersen, K., Bird, K.L., Rasmussen, M., Haile, J., Breuning-Mdsen, H., Kjaer, K.H., Orlando, L., Gilbert, M.T.P., Willerslev, E., 2012. Meta-barcoding of ‘dirt’ DNA from soil reflects vertebrate biodiversity. Mol. Ecol. 21, 1966–1979.

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Anderson, M.J., Willis, T.J., 2003. Canonical analysis of principal coordinates: a useful method of constrained ordination for ecology. Ecology 84, 511–525.

Ansorge, W.J., 2009. Next-generation DNA sequencing techniques. New Biotechnol. 25, 195–203.

Antia, N.J., Cheng, J.Y., 1970. The survival of axenic cultures of marine planktonic algae from prolonged exposure to darkness at 20 C. Phycologia 9, 179–183.

Ardura, A., Linde, A.R., Moreira, J.C., Garcia-Vazquez, E., 2010. DNA barcoding for conservation and management of Amazonian commercial fish. Biol. Conservation 143, 1438–1443.

AquaNIS.Information system on aquatic non-indigenous and cryptogenic species.<www.corpi.ku.lt/databases/aquanis> Version 2.36+ (accessed 28.10.14).

Bott, N.J., Ophel-Keller, K.M., Sierp, M.T., Herdina, Rowling, K.P., McKay, A.C., Loo, M.G.K., Tanner, J.E., Deveney, M.R., 2010. Toward routine, DNA-based detection methods for marine pests. Biotechnol. Adv. 28, 706–714.

Briski, E., Ghabooli, S., Bailey, S.A., MacIsaac, H.J., 2012. Invasion risk posed by macroinvertebrates transported in ships' ballast tanks. Biol. Invas. 14, 1843–1850.

Carlton, J.T., Geller, J.B., 1993. Ecological roulette: the global transport of non-indigenous marine organisms. Science 261, 78–82.

Chariton, A.A., Court, L.N., Hartley, D.M., Colloff, M.J., Hardy, C.M., 2010. Ecological assessment of estuarine sediments by pyrosequencing eukaryotic ribosomal DNA. Front. Ecol. Environ. 8, 233–238.

Collins, R., Armstrong, K., Holyoake, A., Keeling, S., 2013. Something in the water: biosecurity monitoring of ornamental fish imports using environmental DNA. Biol. Invas. 15, 1209–1215.

Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., Fierer, N., Gonzalez Pena, A., Goodrich, J.K., Gordon, J.I., Huttley, G.A., Kelley, S.T., Knights, D., Koenig, J.E., Ley, R.E., Lozupone, C.A., McDonald, D., Muegge, B.D., Pirrung, M., Reeder, J., Sevinsky, J.R., Turnbaugh, P.J., Walters, W.A., Widmann, J., Yatsunenko, T., Zaneveld, J., Knight, R., 2010. QIIME allows analysis of high-throughput community sequencing data. Nat. Methods 7, 335–336.

Darling, J.A., Blum, M.J., 2007. DNA-based methods for monitoring invasive species: a review and prospectus. Biol. Invas. 9, 751–765.

Darling, J.A., Mahon, A.R., 2011. From molecules to management: adopting DNA-based methods for monitoring biological invasions in aquatic environments. Environ. Res. 111, 978–988.

Dejean, T., Valentini, A., Duparc, A., Pellier-Cuit, S., Pompanon, F., et al., 2011. Persistence of environmental DNA in freshwater ecosystems. PLoS One 6 (8), e23398. http://dx.doi.org/10.1371/journal.pone.0023398.

Duggan, I.C., van Overdijk, C.D.A., Bailey, S.A., Jenkins, P.T., Limen, H., MacIsaac, H.J., 2005. Invertebrates associated with residual ballast water and sediments of cargo-carrying ships entering the Great Lakes. Can. J. Fisher. Aquat. Sci. 62, 2463–2474.

Elliott, M., 2003. Biological pollutants and biological pollution – an increasing cause for concern. Mar. Pollut. Bull. 46, 275–280.

Endersen, O., Eide, M., Dalsoren, S., saksen, I.S., Sorgard, E., 2008. The environmental impacts of increased international maritime shipping. Pages 43 in Global Forum on Transport and Environment in a Globalising World, Guadalajara, Mexico.

Flagella, M.M., Verlaque, M., Soria, A., Buia, M.C., 2007. Macroalgal survival in ballast water tanks. Mar. Pollut. Bull. 54, 1395–1401.

Foissner, W., 2006. Biogeography and dispersal of micro-organisms: a review emphasizing protists. Acta Zool. 45, 111–136.

Fralick, R.A., Mathieson, A.C., 1975. Physiological ecology of four *Polysiphonia* species (Rhodophyta, Ceramiales). Mar. Biol. 29, 29–36.

Geoffroy, A., Le Gall, L., Destombe, C., 2012. Cryptic introduction of the red alga *Polysiphoniamorrowii* Harvey (Rhodomelaceae, Rhodophyta) in the North Atlantic Ocean highlighted by a DNA barcoding approach. Aquat. Bot. 100, 67–71.

Gollasch, S., 2006. A new ballast water sampling device for sampling organisms above 50 μm. Aquat. Invas. 1, 46–50.

Gollasch, S., David, M., Voigt, M., Dragsund, E., Hewitt, C.L., Fukuyo, Y., 2007. Critical review of the IMO international convention on the management of ships' ballast water and sediments. Harmful Algae 6, 585–600.

Gollasch, S., Macdonald, E., Belson, S., Botnen, H., Christensen, J.T., Hamer, J.P., Houvenaghel, G., Jelmert, A., Lucas, I., Masson, D., McCollin, T., Olenin, S., Persson, A., Wallentinus, I., Wetsteyn, L.P.M.J., Wittling, T., 2002. Life in Ballast Tanks. In: Leppakoski, E., Gollasch, S., Olenin, S. (Eds.), Invasive Aquatic Species of Europe. Distribution, Impacts and Management. Springer, Netherlands, pp. 217–231.

Gollasch, S., Lenz, J., Dammer, M., Andres, H.-G., 2000a. Survival of tropical ballast water organisms during a cruise from the Indian Ocean to the North Sea. J. Plankton Res. 22, 923–937.

Gollasch, S., Rosenthal, H., Botnen, H., Hamer, J., Laing, I., Leppakoski, E., Macdonald, E., Minchin, D., Nauke, M., Olenin, S., Utting, S., Voigt, M., Wallentinus, I., 2000b. Fluctuations of zooplankton taxa in ballast water during short-term and long-term ocean-going voyages. Int. Rev. Hydrobiol. 85, 597–608.

Hajibabaei, M., Shokralla, S., Zhou, X., Singer, G.A., Baird, D.J., 2011. Environmental barcoding: a next-generation sequencing approach for biomonitoring applications using river benthos. PLoS One 6, e17497.

Harvey, J.B.J., Hoy, M.S., Rodriguez, R.J., 2009. Molecular detection of native and invasive marine invertebrate larvae present in ballast and open water environmental samples collected in Puget Sound. J. Exp. Mar. Biol. Ecol. 369, 93–99.

Hebert, P.D.N., Cywinska, A., Ball, S., deWaard, J., 2003. Biological identifications through DNA barcodes. Proc. Biol. Sci. 1512, 313–321.

Heger, T.J., Mitchell, E.A., Leander, B.S., 2013. Holarctic phylogeography of the testate amoeba *Hyalospheniapapilio* (Amoebozoa: Arcellinida) reveals extensive genetic diversity explained more by environment than dispersal limitation. Mol. Ecol. 22, 5172–5184.

Hewitt, C.L., Gollasch, S., Minchin, D., 2009. The vessel as a vector – biofouling, ballast water and sediments. In: Rilov, G., Crooks, J.A. (Eds.), Biological Invasions in Marine Ecosystems. Springer-Verlag, Berlin Heidelberg, pp. 117–129.

Hurd, C.L., Nelson, W.A., Falshaw, R., Neill, K.F., 2004. History, current status and future of marine macroalgal research in New Zealand: taxonomy, ecology, physiology and human uses. Phycol. Res. 52 (2), 80–106.

IMO, 2004. International Convention for the Control and Management of Ships' ballast water and sediments. IMO.

IUCN guidelines for the prevention of biodiversity loss caused by alien invasive species. Approved by the 51st Meeting of the IUCN Council, February 2000, 2000. Approved by the 51st Meeting of the IUCN Council. IUCN, Gland, Switzerland.

Ivanova, N.V., Zemlak, T.S., Hanner, R.H., Hebert, P.D.N., 2007. Universal primer cocktails for fish DNA barcoding. Mol. Ecol. Notes 7, 544–548.

Jerde, C.L., Mahon, A.R., Chadderton, W.L., Lodge, D.M., 2011. "Sight-unseen" detection of rare aquatic species using environmental DNA. Conserv. Lett. 4, 150–157.

Jobard, M., Rasconi, S., Sime-Ngando, T., 2010. Diversity and functions of microscopic fungi: a missing component in pelagic food webs. Aquat. Sci. 72, 255–268.

Jochem, F.J., 1999. Dark survival strategies in marine phytoplankton assessed by cytometric measurement of metabolic activity with fluorescein diacetate. Mar. Biol. 135, 721–728.

Kaczmarska, I., Dowe, L.L., 1997. Reproductive biology of the red alga *Polysiphonialanosa* (Ceramiales) in the Bay of Fundy, Canada. Mar. Biol. 128, 695–703.

Kelly, R.P., Port, J.A., Yamahara, K.M., Martone, R.G., Lowell, N., Thomsen, P.F., Mach, M.E., Bennet, M., Prahler, E., Caldwell, M.R., Crowder, L.B., 2014. Harnessing DNA to improve environmental management. Science 344, 1455–1456.

King, D.M., Tamburri, M.N., 2010. Verifying compliance with ballast water discharge regulations. Ocean Dev. Int. Law 41, 152–165.

Ko, W.-H., 2003. Long-term storage and survival structure of three species of *Phytophthora* in water. J. Gen. Plant Pathol. 69 (3), 186–188.

Leppakoski, E., Gollasch, S., Olenin, S., 2002. Invasive aquatic species of Europe – distribution, impact and management. Kluwer Academic Publishers, Dordrecht, Boston, London.

Martin, E., Hines, R., 2008. Oxford Dictionary of Biology, sixth ed. Oxford University Press.

Maggs, C., Stegenga, H., 1999. Red algal exotics on North Sea coasts. Helgoland Mar. Res. 52, 243–258.

McCarthy, H.P., Crowder, L.B., 2000. An overlooked scale of global transport: phytoplankton species richness in ships' ballast water. Biol. Invas. 2, 321–322.

Meusnier, I., Singer, G.A.C., Landry, J.-F., Hickey, D.A., Hebert, P.D.N., Hajibabaei, M., 2008. A universal DNA mini-barcode for biodiversity analysis. BMC Genomics 9, 214.

Minchin, D., 2007. A checklist of alien and cryptogenic aquatic species in Ireland. Aquat. Invas. 2 (4), 341–366.

Mountfort, D., Smith, K.F., Kirs, M., Kuhajek, J., Adamson, J.E., Wood, S.A., 2012. Development of single and multispecies detection methods for the surveillance and monitoring of marine pests in New Zealand. Aquat. Invas. 7, 125–128.

Nicholls, K.H., MacIsaac, H.J., 2004. Euryhaline, sand-dwelling, testate Rhizopods in the Great Lakes. J. Great Lakes Res. 30, 123–132.

NOBANIS.European Network on Invasive Alien Species. <http://www.nobanis.org> (accessed 28.10.14).

Ojaveer, H., Galil, B.S., Minchin, D., Olenin, S., Amorim, A., Canning-Clode, J., Chainho, P., Copp, G.H., Gollasch, S., Jelmert, A., Lehtiniemi, M., McKenzie, C., Mikuš, J., Miossec, L., Occhipinti-Ambrogi, A., Pećarević, M., Pederson, J., Quilez-Badia, G., Mijsman, J.W., Zenetos, A., 2014. Ten recommendations for advancing the assessment and management of non-indigenous species in marine ecosystems. Mar. Pol. 44, 160–165.

Olenin, S., Alemany, F., Cardoso, A.C., Gollasch, S., Goulletquer, P., Lehtiniemi, M., McCollin, T., Minchin, D., Miossec, L., Occhipinti-Ambrogi, A., Ojaveer, H., Jensen, K.R., Stankiewicz, M., Wallentinus, I., Aleksandrov, B., 2010. Marine Strategy Framework Directive Task Group 2 Report. JRC Scientific and Technical Reports JRC, DG ENV, ICES, 52pp.

Olenin, S., Elliott, M., Bysveen, I., Culverhouse, P.F., Daunys, D., Dubelaar, G.B.J., Gollasch, S., Goulletquer, P., Jelmert, A., Kantor, Y., BringsvorMezeth, K., Minchin, D., Occhipinti-Ambrogi, A., Olenina, I., Vandekerkhove, J., 2011. Recommendations on methods for the detection and control of biological pollution in marine coastal waters. Mar. Pollut. Bull. 62, 2598–2604.

Olenin, S., Gollasch, S., Jonusas, S., Rimkute, I., 2000. En-route investigations of plankton in ballast water on a ship's voyage from the Baltic Sea to the open Atlantic coast of Europe. Int. Rev. Hydrobiol. 5–6, 577–596.

Olenin, S., Minchin, D., Daunys, D., 2007. Assessment of biopollution in aquatic ecosystems. Mar. Pollut. Bull. 55, 379–394.

Olenin, S., Narscius, A., Minchin, D., David, M., Galil, B., Gollasch, S., Marchini, A., Occhipinti-Ambrogi, A., Ojaveer, H., Zaiko, A., 2014. Making non-indigenous species information systems practical for management and useful for research: an aquatic perspective. Biol. Conserv. 173, 98–107.

Quinn, G.P., Keough, M.J., 2002. Experimental Design and Data Analysis for Biologists. Cambridge University Press, p. 537.

Please cite this article in press as: Zaiko, A., et al. Metabarcoding approach for the ballast water surveillance – An advantageous solution or an awkward challenge?. Mar. Pollut. Bull. (2015), http://dx.doi.org/10.1016/j.marpolbul.2015.01.008

Pochon, X., Bott, N.J., Smith, K.F., Wood, S.A., 2013. Evaluating detection limits of next-generation sequencing for the surveillance and monitoring of international marine pests. PLoS One 8, e73935, 73910.71371/journal.pone.0073935.

Rizzo, D.M., Garbelotto, M., Hansen, E.A., 2005. *Phytophthoraramorum*: integrative research and management of an emerging pathogen in California and Oregon forests. Annu. Rev. Phytopathol. 43, 309–335.

R-project, 2014. R: A language and environment for statistical computing. In: R Foundation for Statistical Computing, Vienna, Austria. <http://www.R-project.org> [ISBN 3-900051-07-0].

Simberloff, D., 2001. Eradication of island invasives: practical actions and results achieved. Trends Ecol. Evol. 16, 273–274.

Smayda, T.J., Mitchell-Innes, B., 1974. Dark survival of autotrophic, planktonic marine diatoms. Mar. Biol. 25, 195–202.

Smith, L.D., Wonham, M.J., McCann, L.D., Ruiz, G.M., Hines, A.H., Carlton, J.T., 1999. Invasion pressure to a ballast-flooded estuary and an assessment of inoculant survival. Biol. Invas. 1, 67–87.

Stoof-Leichsenring, K.R., Epp, L.S., Trauth, M.H., Tiedemann, R., 2012. Hidden diversity in diatoms of Kenyan Lake Naivasha: a genetic approach detects temporal variation. Mol. Ecol. 21, 1918–1930.

Thomsen, M.S., Wernberg, T., Staehr, P., Krause-Jensen, D., Risgaard-Petersen, N., Silliman, B.R., 2007. Alien macroalgae in Denmark – a broad-scale national perspective. Mar. Biol. Res. 3, 61–72.

Valentini, A., Pompanon, F., Taberlet, P., 2009. DNA barcoding for ecologists. Trends Ecol. Evol. 24, 110–117.

Werres, S., Marwitz, R., Man In't Veld, W.A., De Cock, W.A.M., Bonants, P.J.M., De Weert, M., Themann, K., Ilieva, E., Baayen, R.P., 2001. *Phytophthoraramorum*sp. nov., a new pathogen on *Rhododendron* and *Viburnum*. Mycol. Res. 105, 115–1165.

Wilcox, T.M., McKelvey, K.S., Young, M.K., Jane, S.F., Lowe, W.H., et al., 2013. Robust detection of rare species using environmental DNA: the importance of primer specificity. PLoS One 8 (3), e59520. http://dx.doi.org/10.1371/journal.pone.0059520.

Willoughby, L.G., 1965. Some observations on the location of sites of fungal activity at Blelham Tarn. Hydrobiologia 25, 352–356.

Wood, S.A., Smith, K.F., Banks, J.C., Tremblay, L.A., Rhodes, L., Mountfort, D., Cary, S.C., Pochon, X., 2014. Molecular genetic tools for environmental monitoring of New Zealand's aquatic habitats, past, present and the future. N.Z. J. Mar. Freshwater Res. 47, 90–119.

Wonham, M.J., Walton, W.C., Ruiz, G.M., Frese, A.M., Galil, B.S., 2001. Going to the source: role of the invasion pathway in determining potential invaders. Mar. Ecol. Prog. Ser. 215, 1–12.

Wyatt, T., Carlton, J.T., 2002. Phytoplankton introductions in European coastal waters: why are so few invasions reported? In: Briand, F. (Ed.), Alien Marine Organisms Introduced by Ships in the Mediterranean and Black Seas. CIESM Workshop Monographs 20, Monaco, pp. 41–46.

Please cite this article in press as: Zaiko, A., et al. Metabarcoding approach for the ballast water surveillance – An advantageous solution or an awkward challenge?. Mar. Pollut. Bull. (2015), http://dx.doi.org/10.1016/j.marpolbul.2015.01.008

Appendix II

Physiological tolerance: *Temora longicornis – Acartia longiremis*

Anette Maria Christensen (DTU-Aqua, P2), Jörg Dutz (IOW, associated with P2)

Methods and results

Methods

Temora longicornis

Rates of ingestion, respiration, egestion, egg production and egg hatching success of two populations of *Temora longicornis* were compared at 4 different salinities of 10 (control), 8, 7 and 6. Experimental animals were obtained from laboratory cultures originating from the Bornholm- (55°15,0'N – 15°59,0'E) and the Gotland Basin (57°19.2'N – 20°03.0'E), and are, hereafter termed *Temora longicornis* "Bornholm" and "Gotland", respectively. Both cultures were established >3 months prior to experiments and kept for several generations at a salinity of 10 (control). The temperature during culturing and experiments was 10°C which is within the natural range experienced by *T. longicornis* in the Baltic Sea (Dutz *et al*. 2010, Dutz *et al*. 2012). Seawater dilutions were prepared by mixing 0.2 µm-filtered sea water of 34 and MilliQ water. Final salinity was checked using a WTW 3210 conductivity meter. In order to minimize stress to experimental animals, they were gradually acclimated to experimental conditions in dilution steps of 1 every 24 h. The final acclimation to experimental salinity and food conditions lasted for 24 h. During the experiments, copepods were fed the cryptophyte *Rhodomonas salina* taken from batch cultures during exponential growth, grown at the various experimental salinities and supplied at a concentration of 150 µg C l^{-1}. This food level is below saturation for *T. longicornis* (Gonçalves *et al*. 2014), which should allow for a better detection of salinity effects on copepod physiology, as excess food may mask the effect of salinity on physiological adaptations and energy budget (Jonasdottir *et al*. 1998). Nonetheless, this food level is commonly found in the Bornholm Basin (Dutz *et al* 2010).

Feeding, egg production and egestion experiments: For each salinity treatment, 7 recently molted adult females and 2 adult males were transferred to each of 4 replicate 610 ml bottles filled with the experimental suspension. Three control bottles contained the same algal suspension but without copepods to determine algal growth. All bottles were sealed to prevent air bubbles, and placed on a rotating wheel (1 rpm) with a natural light:dark cycle. Feeding was estimated as ingestion (I; µg C ind.$^{-1}$ d^{-1}) according to Frost (1972) by counting initial and final cell numbers after 24 h with a CoulterCounter Multisizer3 Coulter counter in both experimental and control bottles; ingestion was then calculated as the product of filtration rate times food concentration averaged during the incubation period, and the number of surviving copepods recorded. Surviving females were transferred to 4 new replicate 610 ml bottles for egg production experiments. After 24 h, the contents of the bottles were sieved onto a 30 µm mesh and rinsed into culture plates, the number of surviving females recorded and removed, and all the eggs and fecal pellets counted (dissection microscope at 40×) to estimate egg production rates (eggs ind.$^{-1}$ d^{-1}) and pellet production rates (pellets ind.$^{-1}$ d^{-1}). Also, 30 eggs (diameter) and 30 pellets (length and width) were measured for each experimental culture and salinity (inverted microscope at 40×).

Respiration experiments: The surviving females were placed into 250 ml beakers with experimental food suspension for 2 h prior to respiration measurements. For each of the cultures and salinity treatments respiration was measured in groups of 3-4 females. Respiration rates were measured with a UniSense "MicroRespiration System" with a micro-sensor (Clark-type) that measures oxygen partial pressure every 20 seconds and the software delivers all measured oxygen values and an average rate of consumption. Females were placed in respirometer glass chambers (volume ~2000 µl) filled with new pasteurized filtered seawater matching the various salinity treatments but without any food added. The glass chambers were completely sealed when immersed in the water bath with a constant temperature of 10 ^{0}C (±0.1). Respiration was then measured through a thin capillary in the chamber lids with the micro-sensor. The first 5 minutes after entering the micro-sensor were used for the animals to adapt to the new environment, and the next 10 minutes were for measuring the respiration. When a sample was finished the copepods were stored in Petri dishes for later measurements of prosome length for carbon content calculations. Background respiration from the pasteurized filtered seawater was measured at least 4 times for all experiments in order to subtract respiration from bacteria or other material in the seawater and glass chambers.

Results

Ingestion rates of both *Temora longicornis* 'Bornholm' and 'Gotland' were related to environmental salinity and decreased by more than 50% over the experimental salinity range (Fig.1). A disproportional large reduction was observed from 10 to 8. Rates decreased from 2.14 and 2.02 µg C ind.$^{-1}$ d^{-1} at a salinity of 10 to 0.87 and 1.06 µg C ind.$^{-1}$ d^{-1} at a salinity of 8 for *T. longicornis* "Bornholm" and "Gotland", respectively. For the "Bornholm" culture, ingestion in the 7 PSU treatment was 0.96 µg C ind.$^{-1}$ d^{-1}, thus in the same range as the 8 PSU treatment, but decreased to 0.54 µg C ind.$^{-1}$ d^{-1} in the 6 PSU treatment. For the "Gotland" culture, ingestion rate in the 6 PSU treatment was 1.09 µg C ind.$^{-1}$ d^{-1} thus approximately equal to that of the 8 PSU treatment.

In contrast to ingestion rates, egg production at a salinity of 8 did not differ substantially from that at a salinity of 10 (Fig.2). Differences between *Temora longicornis* "Bornholm" and "Gotland" were neglectible. At a salinity of 8 egg production was 5.88 eggs and 5.46 eggs female^{-1} d^{-} for "Bornholm" and "Gotland", respectively; at a salinity of 10, rates were only slightly higher with 6.82 and 6.02 eggs female^{-1} d^{-1} for "Bornholm" and "Gotland", respectively. A substantial decrease in egg production by more than 55% occurred in *T. longicornis* "Bornholm" at both a salinity of 7 and 6. Values decreased to 3.02 and 2.92 eggs female^{-1} d^{-1}, respectively. At a salinity of 6 egg production was reduced by 66% (2.03 eggs female^{-1} d^{-1}) in females originating from the "Gotland" culture.

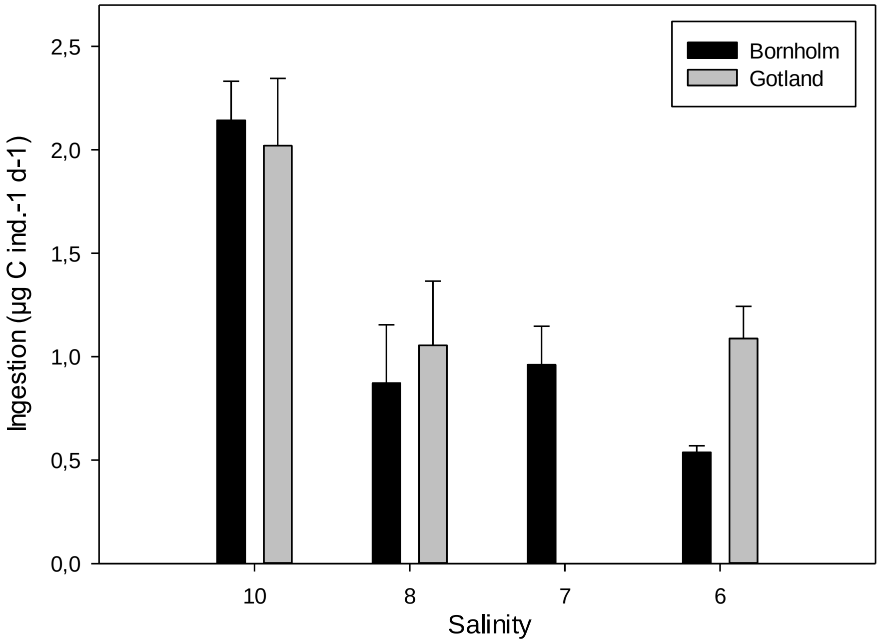

Figure 1: Ingestion rates for the two different *Temora longicornis* cultures; x-axis represents the salinity treatments and y-axis denotes the amount of carbon ingested per individual per day.

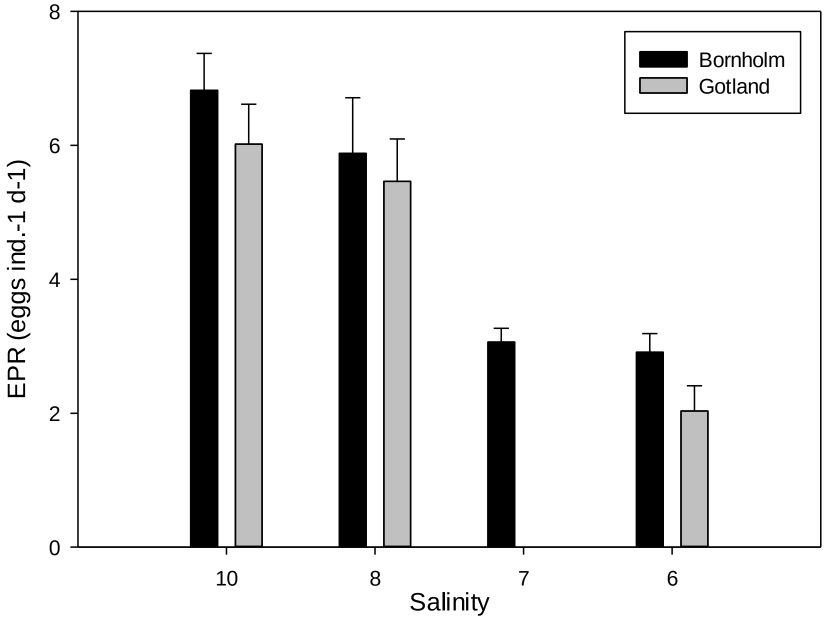

Figure 2: Egg production for the two different *Temora longicornis* cultures; x-axis represents the salinity treatments and y-axis denotes the amount of eggs produced per female per day.

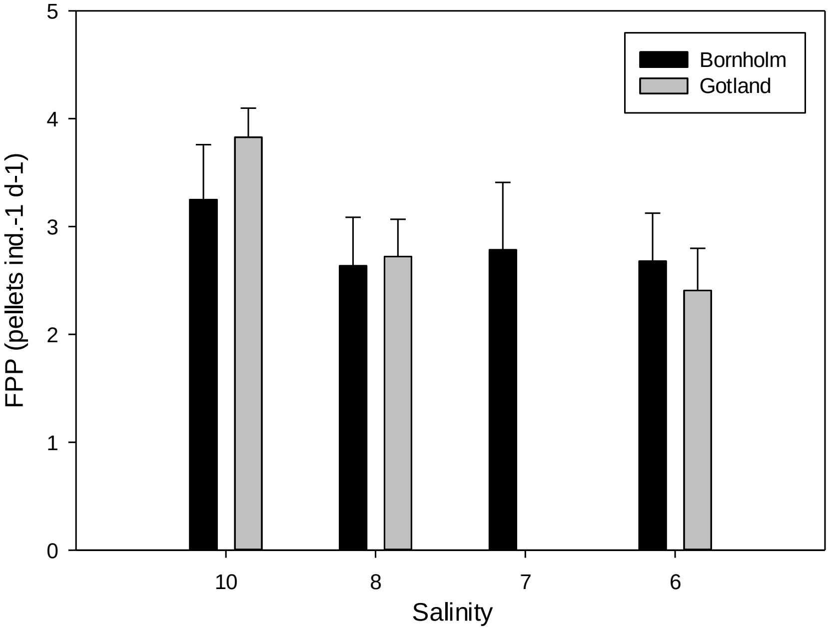

Figure 3: Fecal pellet production for the two *Temora longicornis* cultures; x-axis represents the salinity treatments and y-axis denotes the amount of pellets produced per individual per day.

The tendencies for metabolic demand between the two experimental cultures were different (Fig.4); the “Bornholm” culture showed somewhat stable metabolic demand in 10, 8 and 7 PSU, with respiration rates of 0.089, 0.094 and 0.1 µL O_2 ind.$^{-1}$ h^{-1} but with a decreased respiration rate in the 6 PSU treatment, with 0.079 µL O_2 ind.$^{-1}$ h^{-1}. For the “Gotland” culture the metabolic demand increased in both the 8 and 6 PSU treatment with 0.106 and 0.11 µL O_2 ind.$^{-1}$ h^{-1} compared to the 10 PSU treatment (0.096 µL O_2 ind.$^{-1}$ h^{-1}).

Conclusions and outlook

The two different populations of *Temora longicornis* originating from the Bornholm and the Gotland Basins generally displayed high acclimation tolerance to salinities as low as 6, which seems to be a critical limit in terms of survival as none of the animals from the Bornholm population survived the acclimation to a salinity of 5. However, feeding rates were strongly related to salinity and decreased with decreasing salinity, while egg production was strongly affected when salinity was below 7. A salinity of approximately 7 appears critical to both populations with regard to the species vital rates as the decreased feeding rates cannot sustain reproduction. Due to climate change with future projected increased precipitation and river run-off, the salinity is expected to decrease. Our results suggest that decreasing salinity is likely to have a strong influence on distribution and abundance of *T. longicornis* in the Baltic Sea.

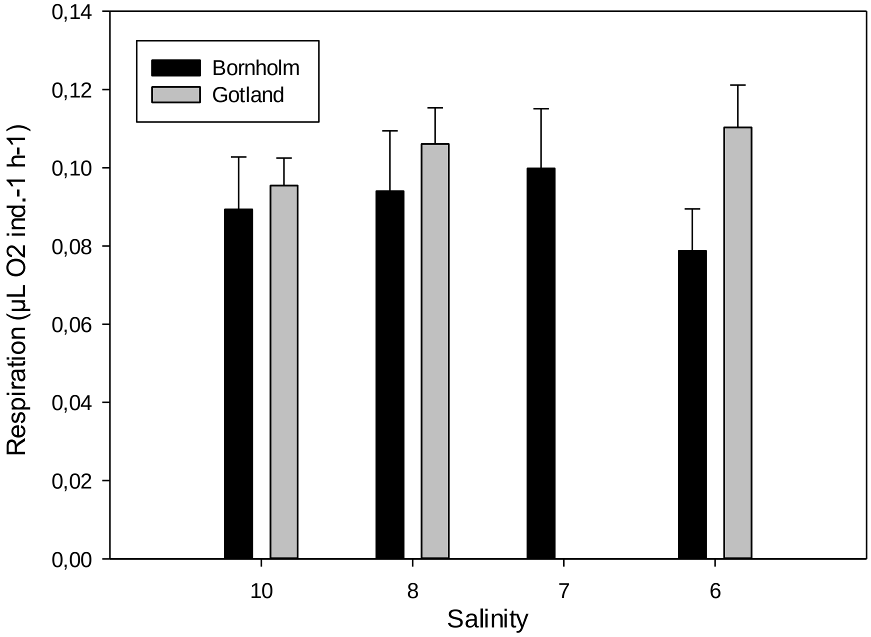

Figure 4: Respiration rates for the two cultures of *Temora longicornis*; x-axis shows the various salinity treatments, and y-axis shows respiration rate in µL oxygen used per individual per hour.

Methods

Acartia longiremis

Due to general difficulties in culturing *Acartia longiremis* in the laboratory, experiments to evaluate the species salinity tolerance and lower threshold were conducted in the field. During a cruise of R/V Dana to the Baltic Sea in September 2015, rates of ingestion, egg production/hatching, respiration and survival of females in relation to environmental salinity were determined. *A. longiremis* was sampled with a 100 µm WP-2 net equipped with a non-filtering cod end and a closing device. Because females inhabit generally the cooler water layer below the seasonal thermocline during summer, net hauls were generally done in the deeper bottom or intermediate water layer and closed before entering the warm surface layer. Net catches were immediately transferred into water from those depth layers at which they were sampled and brought into a walk-in cooling chamber set to a common temperature of 10 ˚C. Experiments were done at two locations. In the Arkona Basin, females were caught in the bottom layer (54° 58' N, 14° 03' E) at a salinity of 14-16 and temperature of 10-14 ˚C. These animals were used in experiments to determine vital rates of A. *longiremis* occurring at higher salinity. Feeding, egg production and respiration of females were determined at a salinity of 16 and the survival thresholds of females subjected to immediate salinity change were tested at a salinity of 16, 12, 10, 8, 7, 6 and 5. In the Bornholm Basin (55° 17' N, 15° 43' E), females caught at a salinity of 7.5-7.9 and a temperature of 7.3-10.5 ˚C were used to determine vital rates as well as survival thresholds of females either subjected to immediate salinity change or after acclimation at salinities of 7.7 (in-situ), 6, 5, 4 and 3. For acclimation, females were kept in several groups of 200-300 in 10 L buckets. Salinity was lowered by additions of MilliQ water at a rate of 1 d^{-1}.

Feeding and egg production: Specimens used in experiments were picked from net catches within 2 hours following the catch or from the acclimated cultures. For each salinity treatment, between 10-20 females were placed in each of 4 replicate 610 ml bottles filled with the experimental food suspension. The variable number of females was used in order to ensure a significant difference between grazing and control bottles at decreasing salinity when feeding rates decreased as well. Food suspensions consisted of mixtures of the cryptophyte *Rhodomonas salina* of 50 µg C L^{-1} and the heterothrophic dinoflagellate *Oxyrrhis marina* of 100 µg C L^{-1}, each grown at the respective salinity used in experiments. Females were acclimated to experimental conditions for 24 h and subsequently transferred to new bottles in order to determine feeding rates and, after an additional transfer, egg production for another 24h. In addition to grazing bottles, two types of controls were conducted. One consisted of pure suspensions of *R. salina* in order to control its growth rate; the other consisted of the same mixture of *R. salina* and *O. marina* as used in grazing experiments but without grazers in order to determine *O. marina* growth and its consumption of *R. salina*. Controls were run in triplicate. Samples for initial and final cell concentrations were counted using a Coulter Multisizer Model 3 (*R. salina*) or the microscope (in the case of *O. marina*) using the Utermöhl technique after settling 3.5 mL of the sample. Ingestion rates of *O. marina* and A*. longiremis* were estimated from changes in prey cell numbers in treatments compared to those in controls using an iterative approach (Tang et al. 2001). In order to estimate egg production rates (eggs $ind.^{-1}$ d^{-1}), the contents of the bottles were sieved onto a 30 µm mesh and rinsed into plastic petri dishes. Eggs were counted and incubated until eggs started to hatch. After additional 48 hours, eggs and nauplii were fixed with Lugol and counted to estimate egg hatching success.

Respiration experiments: Surviving females were placed into 250 ml beakers with experimental food suspension for 2 h prior to respiration measurements. For each of the cultures and salinity treatments respiration was measured in groups of 3-4 females. Respiration rates were measured using a UniSense “MicroRespiration System” with a micro-sensor (Clark-type) that measures oxygen partial pressure every 20 seconds and the software delivers all measured oxygen values and an average rate of consumption. Females were placed in respirometer glass chambers (volume ~2000 µl) filled with new pasteurized filtered seawater matching the various salinity treatments but without any food added. The glass chambers were completely sealed when immersed in the water bath with a constant temperature of 10 ^{0}C (±0.1). Respiration was then measured through a thin capillary in the chamber lids with the micro-sensor. The first 5 minutes after entering the micro-sensor were used for the animals to adapt to the new environment, and the next 10 minutes were for measuring the respiration. When a sample was finished the copepods were stored in Petri dishes for later measurements of prosome length for carbon content calculations. Background respiration from the pasteurized filtered seawater was measured at least 4 times for all experiments in order to subtract respiration from bacteria or other material in the seawater and glass chambers.

Survival: For the survival experiments, groups of 200-300 females were sorted into 10 L buckets filled with seawater of a salinity of 16 (Arkona Basin) or of 7.7 (Bornholm Basin) and fed with the similar food suspension as in feeding experiments. Two types of experiments were conducted. In the first type, 30-40 females taken from the 10 L cultures were subjected to an immediate change in salinity to 12, 10, 8, 7, 6 and 5 in case of females from the Arkona Basin and 7, 6, 5, 4, and 3 in case of females from the Bornholm Basin. Females kept in original salinity served as control. In the second type conducted only with females from Bornholm Basin, the groups of 200-300 females were slowly acclimated to a reduced salinity of 7, 6, 5 and 4 at a rate of 1 per day. After acclimation, females were again subject to an immediate reduction in salinity as in the former experiments. All females were incubated in groups of 5-7 in dishes of 20 ml and checked for survival every 2 hours during the first 12-16 h of the incubation and thereafter every 6-8 hours over a period of 5 days. Females were assigned to 3 categories: active, incapacitated and dead. Healthy and active females showed regular hop and sink behavior and responded immediately to fluid disturbance induced by pipette suction. Incapacitated females showed reduced pipette avoidance and activity.

Results

Ingestion rates of female *Acartia longiremis* were related to salinity (Figure 5). At a salinity of 16 females from the Arkona Basin ingested on average 0.56 µg C Ind.$^{-1}$ d.$^{-1}$. This is about 30% above those rates of 0.41 µg C Ind.$^{-1}$ d.$^{-1}$ of females from the Bornholm Basin determined at an *in-situ* salinity of 7. When the salinity was decreased to 6, 5 and 4, ingestion rates substantially decreased to 0.28, 0.18 and 0.15, respectively, which is 63-31% of those rates at a salinity of 7. The diet *A. longiremis* consisted of both *Rhodomonas salina* and *Oxyrrhis marina,* except at a salinity of 4 at which only *Rhodomonas* was ingested.

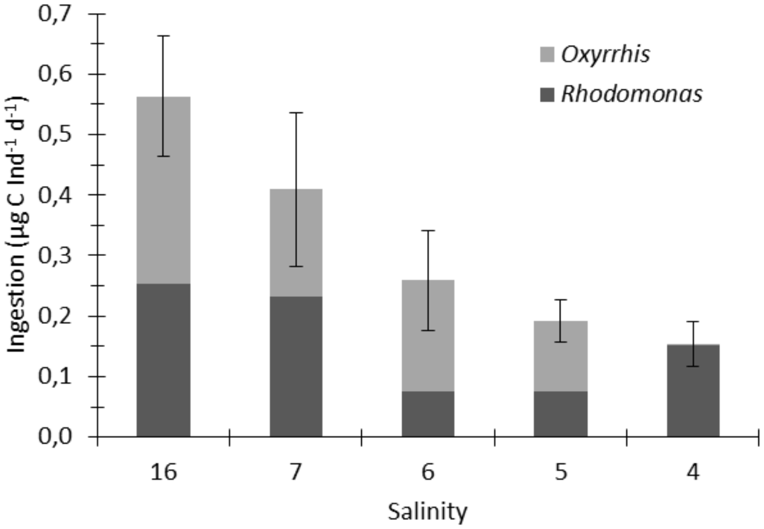

Figure 5: Ingestion rates (µg C fem-1 d-1) of *Acartia longiremis* females at different salinity.

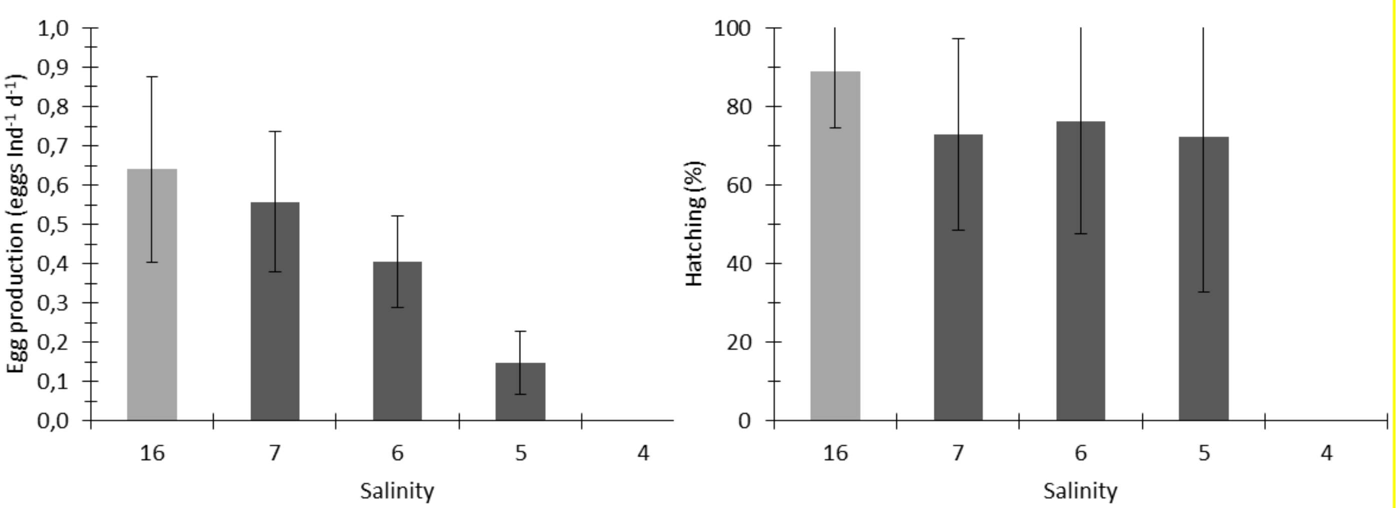

Figure 6: Egg production and egg hatching rates (eggs fem-1 d-1) of *Acartia longiremis* females at different salinity.Note that no eggs were produced at a salinity of 4.

Egg production of females was generally low. Females produced eggs at a salinity ranging from 16 to 5, at a salinity of 4 reproduction ceased. At a salinity of 16 to 5, egg production was related to salinity and decreased from 0.64 to 0.15 eggs Ind.$^{-1}$ d.$^{-1}$. Hatching of eggs was based on a low number of eggs produced by females and, therefore, has to be interpreted with care. Nevertheless, no relationship of hatching success to salinity was detected. Hatching rates of egg at a salinity of 16 were on average 89 ± 14 % and only slightly higher than that of females incubated at an *in-situ* salinity of 7 or reduced salinities of 6-5 ranging from 72± 39 % to 76± 24 %.

Respiration was highest at a salinity of 16 (0.10 µl O_2 Ind.$^{-1}$ h^{-1}) and lowest at a salinity of 4 (0.55 µl O_2 Ind.$^{-1}$ h^{-1}). Nevertheless, there was no clear relationship of respiration to salinity because rates at a salinity of 5-7 were more or less similar (Figure 7).

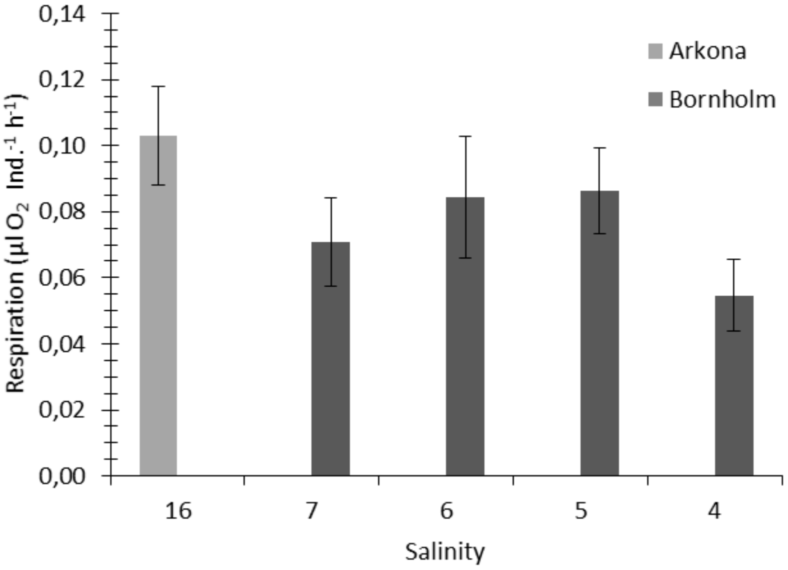

Figure 7: Respiration rates (µl O2 Ind.-1 h-1) of *Acartia longiremis* females at different salinity.

Acartia longiremis collected from the Arkona Basin showed a broad tolerance to an immediate reduction in salinity. When females were exposed to salinity of 16-10, no visible incapacitation was detected and survival remained >90 % at the end of the 5 day incubation period. Females exposed to a lower salinity of 8-5 were incapacitated only during the first 24 hours of the exposure. The visible effects vanished with time and mortality remained generally low. At a salinity of 7-8, survival rates of >88% were still high and similar to those at higher salinity, while survival decreased to 83% and 70% after 5 days at a salinity of 6 and 5, respectively (Figure 8).

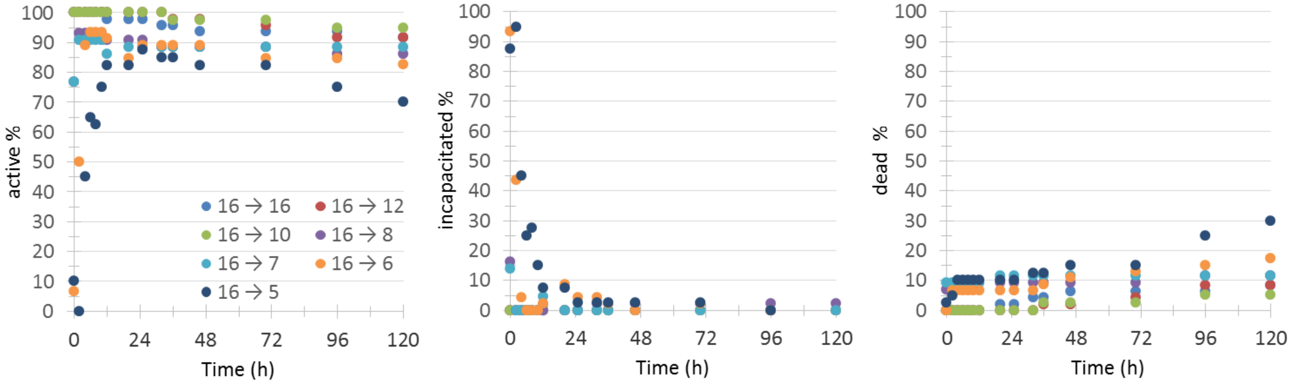

Figure 8: Effects of instantaneous decrease in salinity on activity and survival of females originating from a salinity of 16 (Arkona Basin).

Acartia longiremis collected in the Bornholm Basin at a salinity of 7.5-7.9 were less sensitive to low salinity than those from the Arkona Basin. Survival was generally high at salinities from 7.7 to 5; after 5 days of incubation more than 90% remained alive and healthy. A salinity below 5 was critical for the species survival. At a salinity of 4, more than 36 % of the females were incapacitated or dead after 5 days. At a salinity of 3, less than 25% of the females were active during the first 6h of the incubation, none of the females survived. Acclimation of the population to lower salinity prior to the experiment had only small effects on survival. When females were acclimated to a salinity of 5, survival remained at 100% during the incubation. Mortality at a salinity of 4 and 3 was also less severe during the first days when females were acclimated to salinities less than 7.7. Nevertheless, the proportion of females that were dead after 5 days was similar in all experiments (Figure 9).

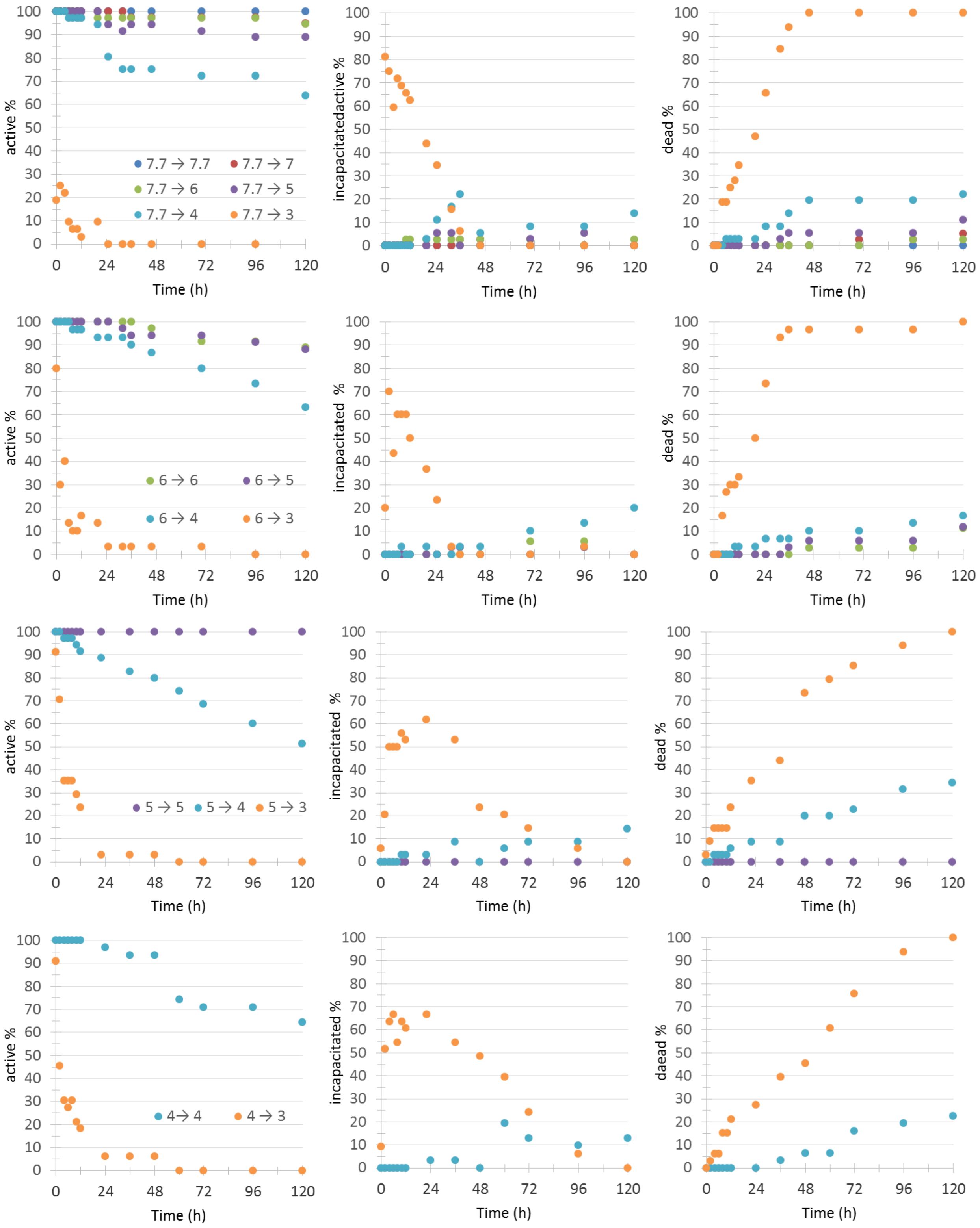

Figure 9: Effects of instantaneous decrease in salinity on activity and survival of females originating from a salinity of 7.7 (Bornholm Basin) and acclimated to a lower salinity of 7 to 3 at 1 d^{-1}.

Conclusions and future perspectives

Females of two different populations of *Acartia longiremis* originating from the Arkona and the Bornholm Basins displayed a broad salinity tolerance. Survival, feeding and egg production were strongly related to salinity and decreased with decreasing salinity. The results suggest a similar response to decreasing salinity as already described for other brackish *Acartia*, with higher rates at intermediate salinity (>10) and gradual decreases at lower salinity (Calliari *et al*. 2006). Nevertheless, *A. longiremis* appears to be more tolerant to decreasing salinity compared to another marine *Acartia* congener, *A. clausi* (Calliari *et al*. 2006). This explains the restriction of the distribution of the *A. clausi* to the Kattegat, while *A. longiremis* has a broad distribution in the Baltic Sea. A salinity of 5 appears critical to the population of *A. longiremis* with regard to the species vital rates. Although females were still able to produce some eggs at this salinity and survival was generally high, the rate of reproduction is likely too low to sustain populations in the Baltic and account for additional mortality by predation. At a salinity of 4 decreased feeding rates were not sufficient anymore to sustain reproduction and high survival of the species. This suggests that the species might not be robust enough against the anticipated worst case changes in of salinity in the central Baltic Sea (e.g., Meier *et al*. (2012)). The comparison of survival to instantaneous changes in salinity suggests that population differences exist in the physiological ability to respond to salinity changes. Females originating from a salinity of 14-16 PSU in the Arkona Basin displayed a higher mortality to the reduction of salinity to 6 and 5 compared to females isolated at a salinity of 7.8 in the Bornholm Basin. However, females from Bornholm that were acclimated to salinities of 6-4 before their response to instantaneous reduction in salinity was tested, displayed no differences in sensitivity to low salinity and had a broad salinity tolerance. Such difference between populations may be caused by the use of those females surviving the acclimation to low salinity. Nevertheless, more than 70% of females from Arkona Basin survived the instantaneous reduction of salinity from 16 to 5 after 5d of incubation. This indicates physiological and potentially genetic diversity exists in the Arkona population that allows the species to prosper in areas with lower salinity such as the Bornholm Basin. Investigations of reaction norms and common garden experiments are required to evaluate this.

References

Calliari D, Andersen CM, Thor P, Gorokhova E, Tiselius P (2006) Salinity modulates the energy balance and reproductive success of co-occurring copepods *Acartia tonsa* and *A. clausi* in different ways. Mar Ecol Prog Ser 312:177-188

Dutz J, Mohrholz V, van Beusekom JEE (2010) Life cycle and spring phenology of *Temora longicornis* in the Baltic Sea. Mar Ecol Prog Ser 406:223-238

Dutz J, van Beusekom JEE, Hinrichs R (2012) Seasonal dynamics of fecundity and recruitment of *Temora longicornis* in the Baltic Sea. Mar Ecol Prog Ser 462:51-66

Frost BW (1972) Effects of size and concentration of food particles on the feeding behavior of the marine planktonic copepod *Calanus pacificus.* Limnol Oceanogr 17: 805−815

Gonçalves RJ, van Someren Gréve H, Couespel D, Kiørboe T (2014) Mechanisms of prey size selection in a suspension-feeding copepod,*Temora longicornis*. Mar Ecol Prog Ser 517:61-74

Jónasdóttir SH, Kiørboe T, Tang KW, St. John M, Visser AW, Saiz E, Dam HG (1998) Role of diatoms in copepod production: good, harmless or toxic? Mar Ecol Prog Ser 172:305–308

Meier HEM, Andersson HC, Arheimer B et al. (2012) Comparing reconstructed past variations and future projections of the Baltic Sea ecosystem—first results from multi-model ensemble simulations. Environ. Res. Lett. 7 034005 (http://iopscience.iop.org/1748-9326/7/3/034005)

Tang KW, Jakobsen HH, Visser AW (2001) *Phaeocystis globosa* (Prymnesiophyceae) and the planktonic food web: Feeding,growth, and trophic interactions among grazers. Limnol Oceanogr 36:1860-1870

APPENDIX III

Effects of salinity and temperature on the development of the Baltic Sea copepod *Eurytemora affinis*

Simona Puiac, Konrad Karlsson, Monika Winder (Stockholm University, P4)

Introduction

In aquatic systems, species physiological tolerance to environmental variables affects their spatial and seasonal distribution, which will be highly influenced by future climate change. For the Baltic Sea, predictions are that the system is getting warmer and fresher. A key question is how species will be affected by these changes and what the consequences at the communities and ecosystem level would be. Copepods are a major trophic link between primary producers and fish. Calanoid copepods (along with cladocerans) are primary prey and highly important for the growth and survival of herring (*Clupea harengus*) and sprat (*Sprattus sprattus*) in the Baltic Sea. *Eurytemora affinis* is one of the key species with major influence on herring recruitment. Due to their role in maintaining the fish stocks it is important to understand copepods ability to cope with future climate change.

Eurytemora affinis (Poppe) is a euryhaline calanoid copepod with a widely distribution in the Northern hemisphere. It commonly inhabits brackish systems, often being a dominant species in zooplankton communities of European estuaries and North America. Despite its preference for brackish conditions, this species is also known to invade freshwater, demonstrating high adaptability, capable of using osmoregulation as a response to fluctuating salinities. *E. affinis* is an egg-bearing species and after hatching undergoes a nauplius phase (larval phase) and a copepodite phase (juvenile phase) before reaching adulthood. After the first phase consisting of 6 naupliar stages, larva metamorphose into juvenile with 5 distinct stages (copepodite stages), which ends with the adult state. Feeding, mortality, swimming behavior or predation pressure differs between development stages. For these reasons assessing the effects of environmental factors should be made considering each stage or group of stages.

Considering their key role in ecosystem functioning understanding the effects of climate change on copepods populations is highly important. Here we investigated the tolerance of different *E. affinis* populations from the Baltic Sea to changing temperature and salinity. For this copepod species our study is the first to assess temperature and salinity effects on development by individual follow up.

Materials and methods

Zooplankton samples were collected at the Askö area (sampling station B1), the Pärnu Bay (part of the Gulf of Riga) and Bothnian Bay. Individual *E. affinis* (different copepodite stages and adults) were used to establish a base culture in a 10L plastic bucket at 15°C and salinity of 6 ppt under constant gentle aeration and a 12L: 12D photoperiod. Artificial seawater was obtained by mixing Instant Ocean® sea salt with tap water. Water was changed twice a week. Copepods were fed *ad libitum* with the cryptophyte algae *Rhodomonas sp*, cultured on a F2 media (20 ppt). For the Askö population we conducted four experiments in a factorial design with two different temperatures (15 and 20°C) and two salinities (6 ± 0.5 and 2± 0.5). The treatments were named as follows: T15/S6, T15/S2, T20/S6 and T20/S2. For each treatment 15-20 egg bearing females were pooled from the base culture and placed individually in 25 ml glass flasks at the experimental conditions (Fig. 1). After the eggs hatched females were removed and nauplii were checked (water change, feeding) every day. When the majority of the individuals reached juvenile phase, 10-15 pairs (1 C5 female and 1C5 male) were formed and each was placed individually in 25 ml glass flask. Clutch size, hatching success and number of spermatophores were recorded every day. Four nauplii from each female from the 1st clutches and three from the following 2nd to 5th clutches were placed individually in 10 ml plastic beakers (Fig. 1). Their developmental stage was checked every day or every second day until adulthood or death. Different nauplii (N1, N2, N3, N4, N5 and N6), copepodite (C1, C2, C3, C4, and C5) and adult stages (A) were identified under the microscope.

For the Pärnu Bay (part of the Gulf of Riga) and Bothnian Bay populations all the experiments were performed at 20°C, at four different salinities (0.5, 5, 10 and 15). For each area 10 clutches (from 10 different females) were used. Each clutch was split in 8 groups of eggs (3-7 eggs/group), which were exposed individually to the different salinity conditions. For each salinity treatment 2 groups of eggs were used from all the 10 clutches. After hatching development stage of individuals were checked daily until maturity/death. Hatching success, survival and time to metamorphosis/ adulthood was recorded and means for each group were calculated.

Results

Effects of temperature and salinity on development

Development time was influenced by both temperature (Welch's ANOVA, $p < 0.001$, $F_{(1, 183)} = 64$) and salinity (Welch's ANOVA, $p < 0.001$, $F_{(1, 68)} = 61$). An increase in temperature from 15 to 20°C led to faster development and adulthood at 20°C was reached 2.8 days earlier at 6 ppt (ANOVA, $p < 0.001$, $F_{(1, 141)} = 47$) and 1.5 days earlier at 2ppt compared to 15°C (ANOVA, $p=0.001$, $F_{(1, 55)} = 12$) (Fig. 1). A decrease in salinity from 6 to 2 ppt caused a delay in development, which was 3.3 days later at 15°C (Welch's ANOVA, $p<0.001$, $F_{(1, 59)} = 50$) and 2 days later at 20°C (Welch's ANOVA, $p=0.02$, $F_{(1, 16)} = 6$). The combined effect of salinity and temperature resulted in the fastest development at T20/S6 (11.8 days) and the slowest at T15/S2 (16.6 days) (Welch's ANOVA, $p < 0.001$, $F_{(1, 48)} = 123$). Furthermore, there was no significantly different in the development of the copepods reared at T15/S6 and T20/S2 (Welch's ANOVA, $p=0.6$, $F_{(1, 17)} = 0$). Development time variability among individuals was high, with some individuals having faster development at 15°C compared to others at 20°C. Also, despite the general trend, some individuals developed faster at the lower salinity. The offspring of some females had faster development at T15/S6 (ANOVA, $p < 0.001$, $F_{(4, 58)} = 6$) and T15/S2 (ANOVA, $p < 0.01$, $F_{(6, 24)} = 4$) (Fig.2.).

For two of our treatments, males completed development earlier than females. The difference was 1.8 days at T15/S2 (ANOVA, $p= 0.02$, $F_{(1, 39)} = 5$) and 1 day at T20/S6 (ANOVA, $p <0.001$, $F_{(1, 78)} = 13$).

Larval phase was shorter than juvenile phase at T15/S6 (Welch's ANOVA, $p< 0.001$, $F_{(1, 84)} = 137$), T15/S2 (ANOVA, $p< 0.001$, $F_{(1, 80)} = 22$) and T20/S6 (Welch's ANOVA, $p< 0.001$, $F_{(1, 151)} =$

199), while at T20/S2 the difference was not significant (ANOVA, p= 0.14, F $_{(1,\ 30)}$ = 2). Temperature increase caused a shortening of both phases at 6 ppt (Welch's ANOVA, p< 0.001, F $_{(1,\ 166)}$ = 59 for nauplius; p< 0.001, F $_{(1,\ 189)}$ = 33 for copepodite), while at 2 ppt only the nauplia phase was affected. Salinity decrease had the opposite effect and individuals needed more time to complete each phase (Welch's ANOVA, p< 0.001, F $_{(1,\ 80)}$ = 31 for nauplius; p < 0.001, F $_{(1,\ 59)}$ = 54 for copepodite) at both salinities. For the individuals from Pärnu bay (part of the Gulf of Riga), the development was significantly influenced by salinity (ANOVA, p< 0.001, F $_{(3,\ 33)}$ =16). While at the higher salinities (5, 10, 15) development was completed in 10, 11 and respectively 12 days, a significant slowdown was encountered at 0.5 and maturity was reached in 18 days (Tukey, p< 0.001) (Fig.1.). These differences are explained by the significant effect of salinity on the duration of the juvenile phase (ANOVA, p< 0.001, F $_{(3,\ 33)}$ =12), which was longer at 0.5 ppt compared to the higher salinities. Larval phase was unaffected by the salinities changes (ANOVA, p=0.2, F $_{(3,\ 37)}$ =1). Bothnian Bay individuals had a development time of ~14 days at both 10 and 15 ppt (ANOVA, p= 0.9, F $_{(1,\ 2)}$ = 0). However, the decrease in salinity from 15 to 10 caused a shortening of the larval phase (ANOVA, p< 0.001, F $_{(1,\ 14)}$ =11), while the juvenile phase was not affected (Welch's ANOVA, p=0.6, F $_{(1,\ 2)}$ =0). Individuals from Bothnian Bay had longer larval phase at both 10 (ANOVA, p< 0.01, F $_{(1,\ 13)}$ =16) and 15 ppt (ANOVA, p< 0.01, F $_{(1,\ 14)}$ =25).

Copepods from Pärnu Bay (part of the Gulf of Riga) reared at 0.5 ppt had longer juvenile phase (ANOVA, p=0.04, F $_{(1,\ 7)}$ =6), while for the Bothnian Bay population at 15 pp the opposite situation was found (Welch's ANOVA, p=0.02, F $_{(1,\ 2)}$ =45).

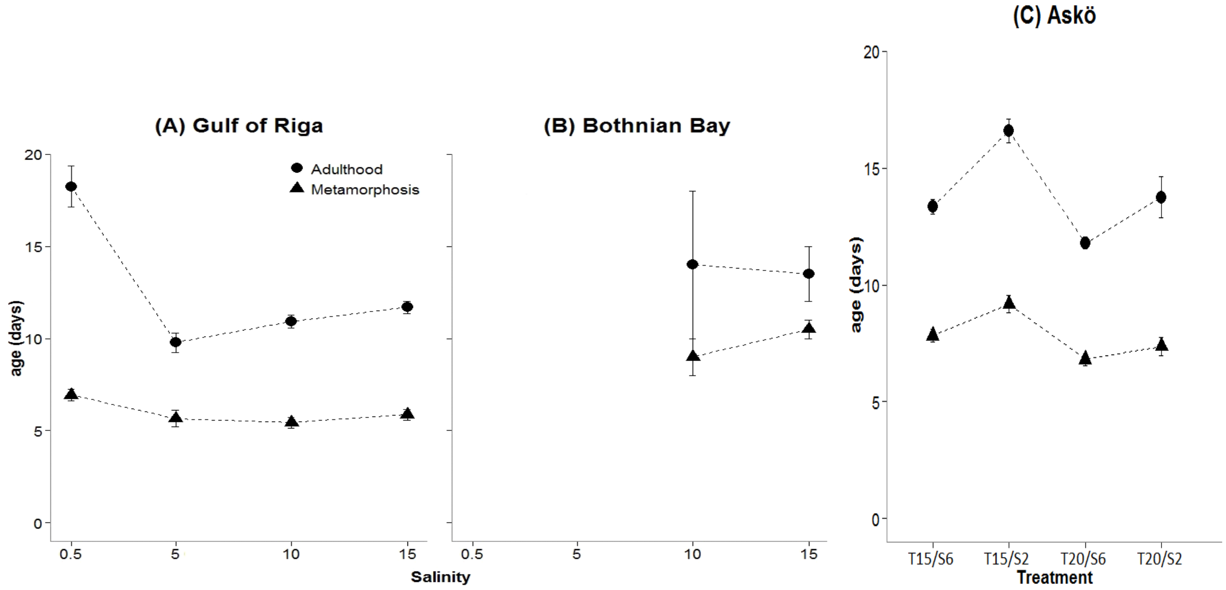

Fig.1. Development time to metamorphosis and adulthood in response to different conditions. A, B Age (days) of the individuals from Gulf of Riga and Bothnian Bay at the time of metamorphosis and maturity as function of salinity. Values are means of replicate clutches ± SE. C, Age of the individuals from Askö at the time of metamorphosis and maturity as function of salinity and temperature (two different temperatures and two salinities). Values are means of individuals from all clutches ± SE.

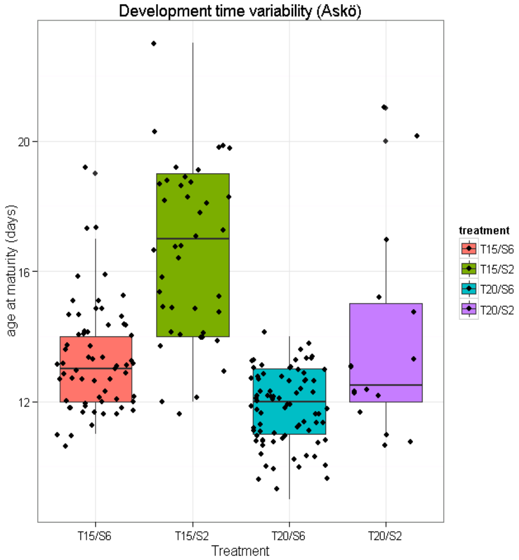

Fig.2. Variability in reaching adulthood for copepods from Askö area. Values represent development time for each individual reared at the four experimental treatments.

Hatching success

The mean clutch size was ~21 (eggs/female/clutch) and was not affected neither by temperature (ANOVA, p=0.2, $F_{(1, 135)}$ = 0) nor by salinity (ANOVA, p=0.6, $F_{(1, 135)}$ = 1).

At T15/S6, five females released the eggs individually (during 6 consecutive clutches), which is unusual for an egg-bearing species. Because those eggs did not hatch during the next 6 days, hatching success was considered 0. In general eggs hatched directly from the clutch attached to the female, or after the release of the entire clutch. In some occasions eggs were observed to be degraded soon after clutch extrusion and no hatching occurred. This observation was mostly made at T20/S2. 0 values were excluded from the analysis.

At 15°C salinity decrease had a negative effect on hatching success, which dropped from 94% to 54% (Welch's ANOVA, p<0.001, $F_{(1, 31)}$ =39) (Fig.3.). At 20°C instead no significant effect was found. Temperature rise had same negative effect at 6 ppt (Welch's ANOVA, p=0.001, $F_{(1, 37)}$ =12). At T20/S6 the eggs produced by some females had higher hatching success compared to others (ANOVA, p=0.01, $F_{(7, 14)}$ =5).

For the Gulf of Riga and Bothnian Bay population, hatching success was not influenced by salinity changes (ANOVA, p= 0.3, $F_{(3, 156}$ = 1). However, the difference between the areas were significant (Welch's ANOVA, p<0.001, $F_{(1, 127)}$ =71) and the eggs originating from the Gulf of Riga had higher hatching success than the ones from the Bothnian Bay at all four salinities (Fig.3.).

For both areas a maternal effect was revealed, the eggs of some females having higher hatching success compered to others (Gulf of Riga: ANOVA, p<0.001, $F_{(9, 70)}$ =19; Bothnian Bay: ANOVA, p<0.001, $F_{(9, 70)}$ =7).

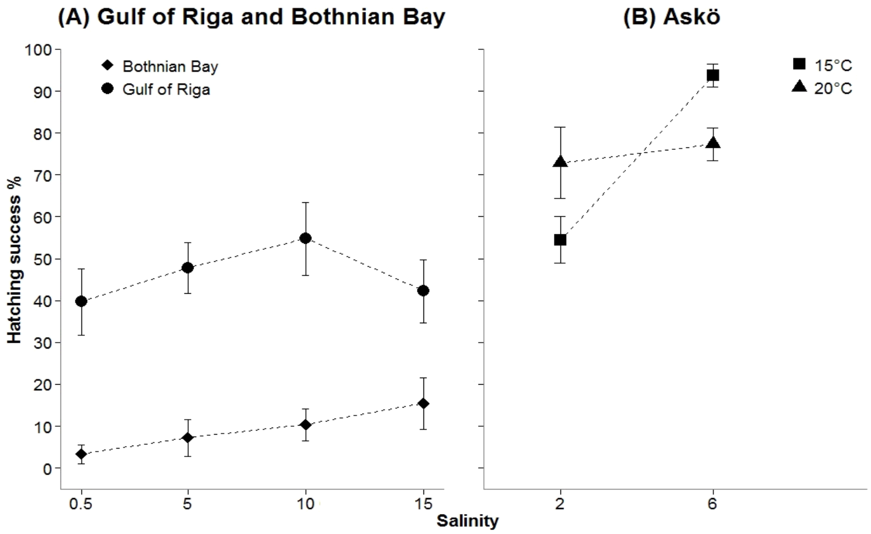

Fig.3. Hatching success in terms of percentage of total number of eggs that hatched per replicate cluch. A) Hatching success of the clutches originating from Gulf of Riga and Bothnian Bay as a function of salinity. B) Hatching success of the clutches originating from Askö as function of salinity and temperature. Values are means of replicate clutches ± SE.

Survival

For the Askö population, salinity decrease negatively influenced the survival of the copepods at 15°C (ANOVA, $p<0.001$, $F_{(1,35)}= 29$) as well as 20°C (ANOVA, $p<0.001$, $F_{(1,30)}= 18$) (Fig.4.). Temperature increase had the same effect at 2 ppt (ANOVA, $p=0.02$, $F_{(1,41)}= 6$), but no significant influence at 6 ppt. The differences in survival were significant between T15/S6 and T20/S2, as well as between T20/S6 and T15/S2. The survival was higher among the copepodite stages than nauplii at T15/S6 (ANOVA, $p= 0.02$, $F_{(1, 8)} = 7$) and T20/S6 (ANOVA, $p= 0.001$, $F_{(1, 18)} = 13$). The percentage of individuals that survived to adulthood was higher for the Gulf of Riga area (ANOVA, $p< 0.01$, $F_{(1,76)}= 8$) (Fig.4.). Salinity has shown a significant influence for this area (Welch's ANOVA, $p< 0.01$, $F_{(3,33)}= 6$), with lower survival at 0.5 ppt (15%) compared to 10 (56%) and 15 (63%) (Tukey, $p<0.01$). No significant difference was found in survival of the copepods from Bothnian Bay. Within all treatments there was no significant difference between the survival of naupliar and copepodite stages. Nauplii as well as copepodites from the Gulf of Riga had higher survival than the ones from Bothian Bay (Welch's ANOVA; nauplii: $p< 0.001$, $F_{(1,118)}= 50$; copepodites: $p< 0.001$, $F_{(1,112)}= 44$) at all four salinities. Salinity had no effect on nauplii survival for none of the areas, while the copepodites from Gulf of Riga had higher survival at 10 and 15 compared to 0.5 ppt (Tukey, $p<0.01$).

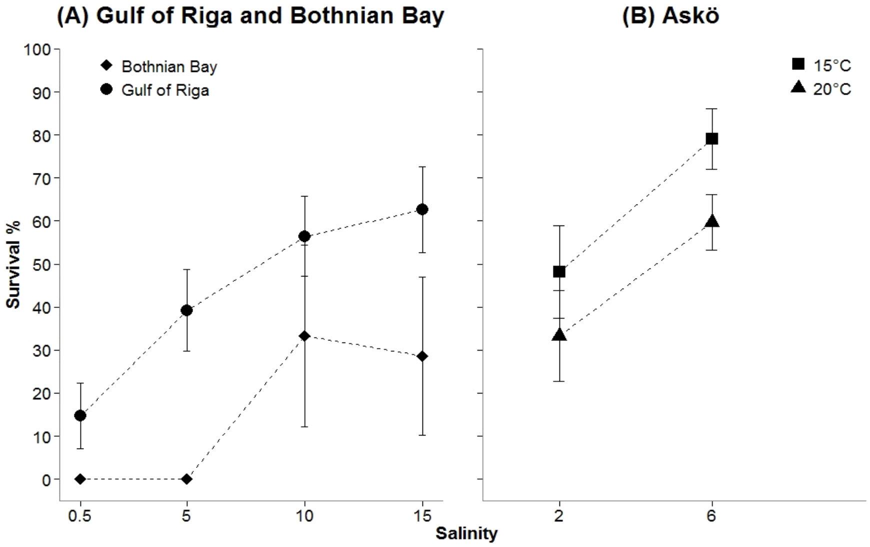

Fig.4. Survivorship in terms of percentage of total number of nauplii that survived to adulthood. A) Survival of individuals from Gulf of Riga and Bothnian Bay as a function of salinity. B) Survival of individuals from Askö as function of salinity and temperature. Values are means of replicate clutches ± SE.

Discussion

Using *E. affinis* individuals from three different areas (Askö, Pärnu Bay (part of the Gulf of Riga) and Bothnian Bay) our study reveals how survival, development time and hatching success are affected by temperature, salinity and the interaction between these two environmental factors. Our results revealed the most beneficial and detrimental conditions, and the manner in which stress manifested on our endpoints. Depending on the degree of stress to which they were subjected, the optimum condition proved to be T15/S6, while the combination of high temperature with low salinity the most detrimental (T20/S2).

Assessing the survival to adulthood is a good method to evaluate copepods ability to cope with different environmental conditions. Highest survival (76%) of the Askö copepods at T15/S6 is an expected result considering that temperatures around 15°C are optimal for *E.affinis* and 6 ppt is the common salinity in this area. Lower survival found for the other treatments demonstrates that individuals were under osmotic (T15/S2), thermal stress (T20/S6) or both (T20/S2). Osmotic and heat shock has been shown for *E. affinis* and are associated with changes in protein expression. Even though copepods can adapt to osmotic stress by osmoregulation it is costly in terms of energy and dependent on the degree of stress. Considering this, our findings for the survival analysis suggest that at T15/S2 and T20/S6 the degree of stress was intermediate between the lowest (T15/S6) and highest (T20/S2).

Low survival was found for both Bothnian Bay and Gulf of Riga populations. Anyhow it was higher for individuals from the latter one, and was influenced by salinity changes. These results are in accordance with local environmental conditions in the two areas. Bohnian Bay is characterized by low salinity around 3 ppt, while in the Gulf of Riga the most encountered salinities are between 5 and 6.5 ppt. No individuals from Bothnian Bay survived at 0.5, suggesting that this population might be already at the lower tolerance limit for low salinities. High temperature of 20°C was another stress factor, leading to no survivors at 5 ppt as well. Even though the survival was low, individuals from Gulf of Riga coped better at the lower salinities. For this area higher survival would be expected at 5 ppt, our results suggesting that temperature had a strong negative effect.

The interaction between temperature and salinity defines copepods fitness, and this is also revealed by our results. For the Askö population temperature increase from 15 to 20°C had no significant effect on survival at the optimum salinity (6ppt), but caused a major drop when combined with osmotic stress (2 ppt). Reared at 20°C, copepods from Gulf of Riga and Bothnian Bay had higher survival at 10 and 15 ppt compared to the lower salinities. This is in accordance with previous studies on *E. affinis*, which emphasized that tolerance for low and high salinities is decreasing as temperature increases. Yet the role of acclimation in survival at different salinities should not be disregarded. Acclimation to gradually increased salinities can lead to reduced mortality, especially when the test salinity approaches the final acclimation salinity. Thus our finding might have been different if we had used acclimation to gradually decreased salinities combined with gradually increased temperatures.

The different treatments used in the present study proved to have an effect on post-embryonic development. An increase in temperature from 15°C to 20°C induced a more rapid development of the copepods from the Askö area. At the lower salinity (2 ppt) the difference is bigger, suggesting osmotic stress. These changes coincide with adjustments in the length of both nauplius and copepodite phase. Decrease salinity from 6 to 2 ppt had the opposite effect causing a slowdown in their development. Larger differences at 15°C were expected considering that for this population T15/S6 was optimal regarding both factors and only osmotic stress was imposed by T15/S2. At 20°C both treatments caused thermal discomfort with the addition of osmotic stress at T20/S2, leading to smaller differences. At T15/S2 slower metabolic rate (compared to 20°C) combined with the allocation of more energy for osmoregulation (due to low salinity) has lead to the slowest development among our treatments. To the opposite, the shortest generation time was found at T20/S6, due to increased metabolic rate and osmotic comfort. Hence our results regarding the Askö population point out that *E. affinis* post-embryonic development is determined by the interaction between temperature and salinity, which can impose high degree of stress.

For the Gulf of Riga and Bothnian Bay populations salinity also proved to influence the development. At higher salinities individuals from Gulf of Riga had a faster development compared to the lowest. Even though the survival was not the highest, at 5 ppt the survivors had the most rapid development indicating that this salinity was in their comfort zone. Osmotic stress at 0.5 ppt caused a significant delay in development, despite the high temperature. An increase in salinity from 10 to 15 had no effect on the development time of the Bohnian Bay copepods, but influenced the larval phase, which was shorter at the former treatment. This suggests that the degree of stress was higher at 15 ppt, considering the low salinities that characterized this area.

In general, our results revealed faster development compared to other studies that used approximately same condition. Reared at 5.5 ppt, individuals collected from the Archipelago Sea (Northern Baltic) had a mean generation tine of 18.5 days at 15°C and 15.5 at 20°C [59]. For *E. affinis* from the Seine estuary the development time was 16.7 days when reared at T15/S5 [43]. At T13/S5 individuals from St Lawrence and Lake Michigan needed 27 and 22.5 days respectively to reach adulthood. Chesapeake Bay population had also slower development at T14/S5 (15 days) compared to our T15/S6 treatment. These differences show *E. affinis* capability to adapt to cooler or warmer conditions. The different clades inhabiting the European and North American estuaries also need to be considered.

Appendix IV

Effects of natural environmental conditions and shipping on the distribution of the invasive round goby

J. Kotta; H. Ojaveer, K. Nurkse (UT-EMI, P6), R. Puntila (SYKE, P7)

Paper by Kotta et al. is attached and serves as appendix 4

Kotta, J.; Ojaveer, H.; Puntila, R.; Nurkse, K. 2016. Shipping and natural environmental conditions determine the distribution of the invasive non-indigenous round goby *Neogobius melanostomus* in a regional sea. Estuarine Coastal and Shelf Science, 169, 15-24.

Introduction

Introductions of non-indigenous species (NIS) are considered a major threat to aquatic ecosystems worldwide. While it is valuable to know the distributions and ranges of NIS, predictive spatial models along different environmental gradients are more useful for management of these species. In this study we modelled how external drivers and local environmental conditions contribute to the spatial distribution of an invasive species using the distribution of the round goby *Neogobius melanostomus* in the Baltic Sea as an example. Using the collected distribution data, an updated map on the species distribution and its invasion progress in the Baltic Sea was produced.

Conclusions and future perspectives

The current range of the round goby observations is extensive, covering all major sub-basins of the Baltic Sea. The most recent observations appeared in the northern regions (Northern Baltic Proper, the Gulf of Bothnia and the Gulf of Finland) and on the eastern and western coasts of southern Sweden. Modelling results show that the distribution of the round goby is primarily related to local abiotic hydrological conditions (wave exposure). Furthermore, the probability of round goby occurrence was very high in areas in close proximity to large cargo ports. This links patterns of the round goby distribution in the Baltic Sea to shipping traffic and suggests that human factors together with natural environmental conditions are responsible for the spread of NIS at a regional sea scale.

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Shipping and natural environmental conditions determine the distribution of the invasive non-indigenous round goby *Neogobius melanostomus* in a regional sea

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ABSTRACT

Introductions of non-indigenous species (NIS) are considered a major threat to aquatic ecosystems worldwide. While it is valuable to know the distributions and ranges of NIS, predictive spatial models along different environmental gradients are more useful for management of these species. In this study we modelled how external drivers and local environmental conditions contribute to the spatial distribution of an invasive species using the distribution of the round goby *Neogobius melanostomus* in the Baltic Sea as an example. Using the collected distribution data, an updated map on the species distribution and its invasion progress in the Baltic Sea was produced. The current range of the round goby observations is extensive, covering all major sub-basins of the Baltic Sea. The most recent observations appeared in the northern regions (Northern Baltic Proper, the Gulf of Bothnia and the Gulf of Finland) and on the eastern and western coasts of southern Sweden. Modelling results show that the distribution of the round goby is primarily related to local abiotic hydrological conditions (wave exposure). Furthermore, the probability of round goby occurrence was very high in areas in close proximity to large cargo ports. This links patterns of the round goby distribution in the Baltic Sea to shipping traffic and suggests that human factors together with natural environmental conditions are responsible for the spread of NIS at a regional sea scale.

1. Introduction

Humans have greatly accelerated the pace of interregional migration of species globally. In aquatic environments, this occurs mainly by transporting species in ballast water, on the hulls of ships, or by releasing exotic aquarium species (Carlton and Geller, 1993). When species are released into new environments their establishment success is affected by the intensity of the propagule pressure for a given species (*e.g.*, Lockwood et al., 2009; Simberloff, 2009; Wonham et al., 2013) and suitability of the habitat in relation to the species' physiological tolerances (Lynch and Gabriel, 1987). This explains why coastal areas of enclosed seas and estuaries, characterized by intense transoceanic shipping and the presence of a wide range of environmental conditions, are some of the most highly invaded environments in the world (Carlton and Geller, 1993).

Accumulating evidence on successful invasion events, as well as, failures of eradication of non-indigenous species (NIS) from the invaded ecosystems, highlight the need for predictive tools for evaluating the risks of invasions at specific locations. The relationship between the number of organisms initially released into the environment and the risk of a successful invasion is theoretically understood (Drake, 2004; Courchamp et al., 2008). However, these models often fail to predict species distributions (*e.g.*, Taylor and Hastings, 2005). In the real world, species invasions often stem from large-scale and repeated releases (Wonham, 2008). These processes are potentially characterized by vector-scale models, which also match the scale at which many preventive regulations are being developed (IMO, 2004; Albert et al., 2013; Lee et al., 2013).

Practical challenges in measuring the propagule pressure

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associated with an invasion vector exist and proxy variables are widely used in analyses to overcome such difficulties (Lockwood et al., 2009; Simberloff, 2009; Haydar and Wolff, 2011). The expected shape of the risk–release relationship is not clear, and both linear and nonlinear models have been applied to empirical data (Ricciardi, 2006; Reusser et al., 2013). Because of this variability, machine learning techniques are a useful method to empirically determine the shape and strength of risk-release relationships, as they do not limit the outcome to pre-determined data models but rather use an algorithm to learn the relationship between the response and its predictors Thereby, machine learning techniques can fit a diverse array of functional response curves (Hastie et al., 2009).

The round goby, *Neogobius melanostomus* (Pallas 1814), is a successful and widespread invader worldwide and is considered one of the most invasive NIS in the Baltic Sea (Kornis et al., 2012). It was first observed in the Baltic Sea in Poland in 1991 (Skóra and Stolarski, 1993) and later recorded in several other areas of the Baltic Sea (AquaNIS, 2014). Round goby is a territorial, aggressive, and voracious generalist benthivore reported to prefer bivalves when they are available (Marsden et al., 1997; Kornis et al., 2012). As shipping is likely behind the invasion of the round goby (Sapota and Skóra, 2005; Kornis et al., 2012), useful proxies for propagule pressure include: distance to harbour, historic records of vessel traffic, tonnage and ballast volume of ships. These proxies are easy to define and therefore have been widely used in earlier bioinvasion studies (Jazdzewski et al., 2005; Ricciardi, 2006; Lo et al., 2012; Chan et al., 2013).

Apart from propagule pressure, extensive knowledge on environmental tolerances of the species is needed in order to model their distribution in the invaded ecosystem. Even though a region may have a high probability for invasion, local biotic and abiotic characteristics determine the success of establishment and reproduction (Lynch and Gabriel, 1987; Roura-Pascual et al., 2011). Assessing environmental factors related to the presence of the round goby therefore requires knowledge of the prevailing physical and chemical conditions in the invaded locations. Once these optimal conditions are empirically documented, the relationships can be used to predict the probability of the presence of the round goby along measured environmental gradients. Although considered one of the most invasive NIS in the Baltic Sea and worldwide, development of a spatial predictive model for the round goby had previously been hampered by a lack of information about its current distribution and environmental preferences in the invaded ecosystems (Shearer and Grodowitz, 2010; Ojaveer and Kotta, 2014).

In contrast to many native species, successful NIS often tolerate broad ranges of environmental conditions and can even adapt life history strategies to local conditions in the invaded environments (*e.g.*, reproduction, see Platt and Jeschke, 2014). Anthropogenic transfer processes infer a bottleneck on NIS, which assures that only the hardiest individuals arrive to the new location. Therefore, surviving individuals will theoretically be better suited for establishment and further expansion (*sensu* Blackburn et al., 2011). Similarly, the round goby has shown great establishment success in several invaded ecosystems due to favourable environments and several species-specific traits (Charlebois et al., 2001). The secondary spread of NIS within a new ecosystem may occur through a combination of natural dispersal and anthropogenic transport mechanisms (Minchin et al., 2009). When water bodies are interconnected, secondary dispersal of NIS may effectively take place as active migration and/or movement by water currents (*e.g.*, Minchin et al., 2009). Human-induced dispersal mechanisms can also contribute to their secondary spread. The larvae and early juveniles of the round goby, similar to several demersal fish species, undergo diel vertical migration and therefore nocturnal ballasting can result in the transport of larval and young round gobies (Hensler and Jude, 2007; Hayden and Miner, 2009). Moreover, the gobiidae are known to lay eggs on hulls or within sea-chests (Wonham et al., 2000; Jude et al., 1995) and their pelvic fins reduce maintenance costs while carried within ships' ballast water (French and Jude, 2001). Such a combination of characteristics may explain why this group of fishes have been generally more successful over other fishes by shipping transport. However, distribution and spread of NIS may not be fully predictable as each invasion has a strong stochastic element. Moreover, if the establishment process of NIS is still in progress, *i.e.*, the species has not yet filled all of their potential niche space, then relationships between the environment and species distribution pattern may not emerge.

In the current study we modelled how external drivers and local environmental conditions relate to the probability of occurrence of the round goby. We analysed whether areas where the round goby has been observed in the Baltic Sea share certain specific abiotic characters or whether the current distribution of the round goby is largely uncoupled from its abiotic environment and is primarily defined by the intensity of propagule pressure *i.e.* shipping. We expect that large-scale environmental stresses and disturbances, such as climatically driven changes in seawater temperature or wave exposure, can synchronize population changes over wide geographical areas, as they have a potential to affect recruitment or mortality of the round goby and its prey. Nevertheless, as the round goby is territorial with limited swimming range (Ray and Corkum, 2001), we also expect that shipping intensity increases the probability of occurrence of the species. We also aim to show how and to what degree the distribution pattern of the round goby is explained by eutrophication. We expect that eutrophication, one of the key disturbances in the Baltic Sea, plays an important role in dispersal of the round goby by increasing nutrient loads and, therefore, promoting higher invertebrate abundances including the most preferred food items of the round goby (Kotta et al., 2009; Järv et al., 2011), ultimately resulting in an increase in the probability of occurrence of the fish. Although, the roles of facilitation and inhibition by resident fauna are dominant themes in the invasion literature (*e.g.*, Elton, 2000; Gurevitch et al., 2011), apart from eutrophication-induced effects of the prey, we focused our scope to propagule pressure and abiotic drivers. This is because the round goby has been ranked among the most aggressive demersal fishes in the Baltic Sea range and competition and predation by native fish species only marginally impact the gobies' densities and their spread (Marsden et al., 1997; Järv et al., 2011).

2. Material and methods

2.1. The study area

The Baltic Sea is an example of an environment where biological invasions are becoming increasingly widespread, posing a serious threat to biodiversity and ecosystem (Olenin et al., 2007; Zaiko et al., 2011; Ojaveer and Kotta, 2014). As a typical representative of a temperate semi-enclosed brackish sea, it has extensive coastal areas characterized by basin-scale gradients of temperature, salinity, and oxygen content (Segerstråle, 1957). Ever-increasing maritime shipping and other invasion vectors maintain the elevated propagule pressure of non-indigenous species into the Baltic Sea (HELCOM, 2010). On top of this, a majority of the projected climate change scenarios suggest extreme shifts in the Baltic Sea environment (BACC, 2008), which will further destabilize local environment and create space for novel non-indigenous species. These conditions, together with spatially variable and relatively low overall species richness (Ojaveer et al., 2010), broadly define the 'invasion environment' of the Baltic Sea favourable for both new arrivals as well as secondary spread of already existing non-indigenous species (NIS).

2.2. Round goby distribution data

As only one country has marine alien species monitoring program in place in the Baltic Sea (ICES, 2012), the knowledge of the current distribution range of the round goby is scattered and incomplete. In the current study all the existing information on the round goby in the Baltic Sea basin was systematically reviewed and recorded as "presence" along with the observation year (from 1990 to 2014) and formatted into a geo-referenced distribution dataset. Data for round goby observations were obtained from various sources: literature (*e.g.* annual reports of the Working Group on Introductions and Transfers of Marine Organisms of the International Council for the Exploration of the Sea (ICES WGITMO); Wandzel, 2000; Bacevičius, 2003; Corkum et al., 2004; Sapota, 2004; Ojaveer, 2006; Rakauskas et al., 2008; Kornis et al., 2012; Rakauskas et al., 2013; Azour et al., 2015); public web pages presenting round goby observations (ArtDatabanken, 2014; Finnish Alien Species Database, 2014; Fischfauna-Online, 2015); authors own data, originating both from coastal fish monitoring programs as well as contacts with local professional and recreational fishermen (mainly from Estonia, Lithuania and Latvia).

Since coastal fish monitoring efforts and methods vary between locations, estimating round goby abundances was impossible. Nevertheless, the gobies' observations covered broad ranges of environmental gradients and spanned vast areas across the geographic space (*e.g.*, port and remote areas). A comparison of the distribution of goby locations to the distribution of background locations in environmental space showed that these statistical distributions were similar; thus, sampling bias was not a concern.

Maps presenting round goby invasion in the Baltic Sea were created using QGIS software (Quantum GIS Development Team, 2014). All round goby observations ($n = 333$) where used in the distribution map and in the MaxEnt model (see the modelling chapter below).

2.3. Supporting environmental data

The round gobies' distribution dataset was supplemented with the key environmental data potentially impacting the establishment and spread of this invasive fish species (Table 1). All environmental variables were continuous. Investigated variables included the following proxies of propagule pressure: shipping intensity (Density of ships equipped with Automatic Identification System, monthly average per pixel of 2200 × 2200 m size; HELCOM, 2014), amount of annual cargo traffic at a nearest port (tons; HELCOM, 2014) and distance to nearest port (km). Variables characterizing the tolerance of round goby to abiotic environment included vertically aggregated mean seawater temperature (°C), maximum salinity (psu), stratification (mean difference in water density between surface and bottom layers i.e. mixing intensity, kg m^{-3}), exposure to waves ($m^2\ s^{-1}$) and depth (m). As a proxy for eutrophication the surface water, chlorophyll *a* (chl *a*, mg m^{-3}) and water attenuation coefficient (K_d) were used.

Shipping intensity data were obtained from the HELCOM data service. The raw AIS data were averaged over months and then the resulting layer was converted into a raster image. Similarly, information on ports and associated cargos was extracted from the HELCOM portal at http://maps.helcom.fi/website/mapservice/index.html.

The values of water temperature, salinity and stratification were obtained from the hydrodynamical model calculations from April to August 2005–2012. The calculations were based on the COHERENS model, which is a primitive equation ocean circulation model. It was formulated with spherical coordinates on a 10 × 10 min horizontal grid and 30 vertical sigma layers. The model was forced with hourly meteorological fields of 2 m air temperature, wind speed, wind stress vector, cloud cover and relative humidity. The meteorological fields were obtained from an operational atmospheric model. The model was validated against water level, temperature, salinity and water velocity measurements from the study area (Bendtsen et al., 2009).

The Simplified Wave Model method was used to calculate the wave exposure for mean wind conditions represented by the ten year period between 1 January 1997 and 31 December 2006 (Isaeus, 2004). A nested-grids technique was used to take into account long distance effects on the local wave exposure regime. The resulting grids had a resolution of 25 m. In the modelling the shoreline was divided into suitable calculation areas and fetch and wave exposure grids were calculated. Subsequently the separate grids were integrated into a seamless description of wave exposure along the study area. This method results in a pattern where the fetch values are smoothed out to the sides, and around island and skerries in a similar way that refraction and diffraction make waves deflect around islands. The depth raster was obtained from the database of the Estonian Marine Institute (version 2014).

As a proxy for eutrophication the MERIS satellite derived water transparency (K_d) and water chlorophyll *a* (chl *a*) values were used. The frequency of satellite observations was generally every second day over the whole ice-free period (years 2009–2014). However, several observations were discarded due to cloudiness. The spatial resolution of satellite data was 300 m. False zeroes, for example resulting from cloudiness, were removed from the data prior to the statistical analysis.

2.4. Modelling

In locations where species data have been collected systematically, for example through biological monitoring, both presence

Table 1
Environmental variables used in the MaxEnt models.

No	Variable	Unit	Function in model	Type of data	Years of collection	Spatial resolution
1	Shipping intensity	coefficient	Propagule pressure	Continuous	2014	2200 m
2	Cargo traffic	tons	Propagule pressure	Continuous	2014	Not relevant
3	Distance to port	km	Propagule pressure	Continuous	2014	Not relevant
4	Mean temperature	°C	Abiotic environment	Continuous	2005–2012	1000 m
5	Maximum salinity	psu	Abiotic environment	Continuous	2005–2013	1000 m
6	Mixing intensity	kg m^{-3}	Abiotic environment	Continuous	2005–2014	1000 m
7	Exposure to waves	$m^2\ s^{-1}$	Abiotic environment	Continuous	1997–2006	25 m
8	Depth	m	Abiotic environment	Continuous	2014	50 m
9	Chlorophyll *a*	mg m^{-3}	Eutrophication	Continuous	2009–2014	300 m
10	K_d	coefficient	Eutrophication	Continuous	2009–2014	300 m

and absence of species at each site have been recorded. However, in most locations round goby observations were collected non-systematically and available as presence-only records and traditional modelling tools could not be used. In order to maximize the utility of the database, presence-only species distribution modelling was used instead.

In this study the contribution of each environmental variable on the probability of occurrence of round goby in the Baltic Sea range was explored using the MaxEnt method. MaxEnt is a machine learning algorithm for modelling species distributions from presence-only species records. In brief, MaxEnt seeks what makes the environment of the occurrence localities of a species different from the environment in the whole geographical region of interest. Based on the observed mismatch a species' distribution is defined. More specifically, MaxEnt model minimizes the relative entropy between two probability densities (one estimated from the presence data and one, from the landscape) defined in covariate space. When doing so the model compares the density of covariates in the region to the density of covariates occupied by the species and such comparison informs us what environmental variables are important and estimates the relative suitability of one location vs. another. The null model for the raw distribution is uniform distribution over the landscape, since without any data we would have no reason to think the species would prefer any location to another. MaxEnt's predictive performance is consistently competitive with the highest performing methods. Since becoming available in 2004, it has been utilized extensively for finding correlates of species occurrences, mapping current distributions, and predicting to new times and places across many ecological, evolutionary, conservation and biosecurity applications (Elith et al., 2006).

Multicollinearity can be an issue with MaxEnt when answering if and when environmental variables are of ecological interest. Thus, prior to modelling, a correlation analysis was conducted for environmental variables and the final MaxEnt models included variables that were not significantly correlated with each other (at $p < 0.05$). Among the studied environmental variables only the proxies of propagule pressure correlated (between shipping intensity and distance to nearest harbour $r = -0.29$; $p < 0.001$). Thus, in order to avoid multicollinearity issue and to assess their usefulness in predicting the distribution of round goby, separate models were run for each of the shipping proxies.

In this study MaxEnt models were fitted as combinations of basic functions and features. MaxEnt had six feature classes: linear, product, quadratic, hinge, threshold and categorical. Products were all possible pairwise combinations of covariates, allowing simple interactions to be fitted. Threshold features allowed a “step” in the fitted function; hinge features were similar except they allowed a change in gradient of the response. Many threshold or hinge features were fitted for one covariate, giving a potentially complex function.

Segment-based (non-gridded) data were modelled using SWD (samples-with-data) format in MaxEnt for both presence and background sites (*i.e.*, the whole Baltic Sea). A 10-fold cross-validation was used to obtain out-of-sample estimates of predictive performance and estimates of uncertainty around fitted functions. In order to reduce model overfitting, a balance between accurate prediction (model fit) and generality (model complexity) was sought by maximizing the penalized maximum likelihood function, *i.e.*, the gain function. When doing so, regularization or the LASSO penalty was applied by exploring a range of regularization parameter values and choosing a value that maximizes measures of fit on a cross-validation data set. The LASSO penalty is based on the rationale that features with larger variance should incur a larger penalty and, thus be less likely to be included in the model (Hastie et al., 2009). For model validation a random selection of 25% of the overall localities of round gobies were used. The percent contributions of individual variables to the final model were identified with jackknife tests. The jackknife test evaluates how each variable contributes to the “gain” of the MaxEnt's model (*i.e.*, improvement in penalized average log likelihood compared to null model) (Elith et al., 2011). A variety of error measures can be calculated when comparing modelled and observational data. In particular, the use of threshold-independent receiver operating characteristic (ROC) plots has received considerable attention. A ROC plot is obtained by plotting all true positive fraction (i.e. correctly classified values) on the y-axis against their equivalent false positive fraction for all available thresholds on the x-axis. The area under the ROC function (AUC) is usually taken to be an important index because it provides a single measure of overall accuracy that is not dependent upon a particular threshold. The value of the AUC is between 0.5 and 1.0 with $AUC = 1.0$ indicating that the model has a perfect match and $AUC = 0.5$ indicating that model is no better than random (Fielding and Bell, 1997).

3. Results

The current range of round goby observations in the Baltic Sea is extensive (Fig. 1). Since the first observation in 1990, the species has been detected in all major sub-basins. It appears that during the first decade of invasion, the distribution area was mostly confined to the Gulf of Gdansk area, while further spread to the south-western Baltic was observed during the first half of the 2000s. The most recent observations appear in the northern regions (Northern Baltic Proper, the Gulf of Bothnia and the Gulf of Finland) and on the eastern and western coasts of southern Sweden. Currently the northernmost observations are from Bothnian Bay (Raahe, Finland in 2012).

MaxEnt models explained a majority of the round goby distribution, inferring that selected variables were largely responsible for observed pattern in species presence. The cross-validated AUC for the model was estimated at 0.978 indicating that the model has almost a perfect match. However, the AUC plot for MaxEnt involves the fraction of the total study area predicted present instead of the more standard omission rate, *i.e.*, the fraction of absences predicted present. Thus, the presented AUC value is not directly comparable to a standard ROC/AUC approaches involving specificity and sensitivity.

The model suggested that both local hydrography and propagule pressure (measured as shipping activity) largely determine the distribution of the round goby in the Baltic Sea. It appeared that the round goby has an affinity towards locations characterized by low exposure to waves, low salinity, high temperature and high vertical mixing of the water column. In addition, reduced distance to a nearest port and elevated amount of cargo traffic at the port increased the probability of the round goby occurring at a location (Figs. 2 and 3). Although all proxies of propagule pressure contributed to the MaxEnt models, distance to nearest port had the highest predictive power (Table 2). When cargo traffic or shipping intensity was used as a proxy for propagule pressure, exposure to waves was even more important in the MaxEnt models.

Jackknife test showed that exposure to waves contributed over 60% of the model variability *i.e.*, this variable contained information to the largest extent that was not present in other variables. Distance to a nearest port explained 17.6% of model variability, whereas other variables contributed only marginally to the model. Interestingly, eutrophication-related variables such as water chlorophyll *a* level and water transparency as well as water depth did not significantly change the probability of occurrence of the round goby and had only minimal impacts to the final model (contribution below 1.4%). Removal of exposure to waves significantly

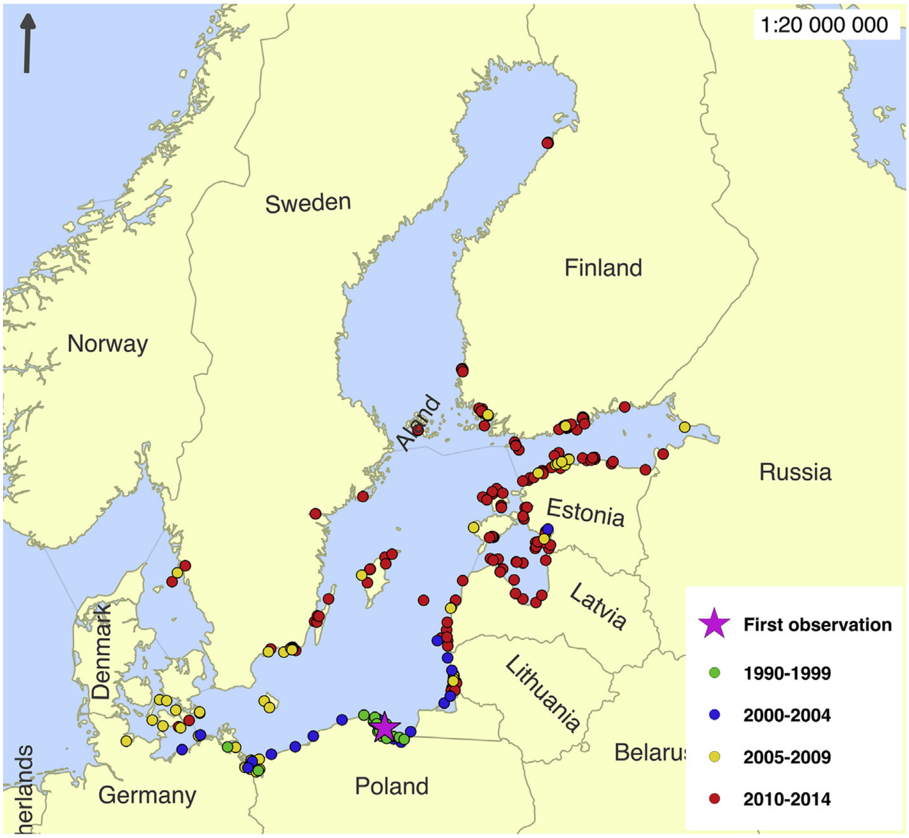

Fig. 1. Observations of the round goby in the Baltic Sea.

reduced the overall predictive performance of models and increased the contribution of depth in the MaxEnt models (Table 2), demonstrating that coastal topography and wave climate play a major role in driving the current distribution of the round goby.

4. Discussion

Since the range of an invasive species is an important predictor of their large scale impacts (Parker et al., 1999) and the best predictor of range size is time after invasion (Byers et al., 2015), one of the most urgent challenges in bioinvasion science is to accurately predict distribution and potential spread of NIS in order to inform stakeholders on invasion risks and suggest management actions. However, as each invasion has a strong stochastic element and NIS may spread far beyond their native niches (Parravicini et al., 2015), development of such predictive models is still hampered by our limited knowledge on the relative contribution of mechanisms behind each bioinvasion, and the roles of the environment modulating species establishment and their further spread (Roura-Pascual et al., 2011). When NIS has already established in the recipient ecosystem, we can learn greatly from the species–environment relationships and estimate to what extent the invasion success is related to the intensity of propagule pressure and/or species tolerance to specific set of local abiotic characters.

The MaxEnt models used in this study performed very well (AUC between 0.978 and 0.980) indicating a significant role of the selected environmental variables to the spatial distribution of the round goby. As the environmental proxies were carefully selected from the literature, the final models describe the best physiological requirements, potential niche space and ecology of the species. Importantly, the model demonstrated that only a handful of environmental drivers are needed to accurately predict the occurrences of the round goby. This suggests that there are very few factors influencing the round goby dispersal in the Baltic Sea.

Among natural drivers, exposure to waves was by far the most important variable defining the environmental envelope of the round goby, low exposure sites being characterized by higher probability of occurrence of the species. This result suggests an affinity of the round goby to sheltered and moderately exposed areas. Round goby is an extremely sedentary species (Ray and Corkum, 2001) and highly exposed areas with a narrow macroalgal belt lacking habitat stability, provide only a limited amount of suitable habitat for the species. In such habitats food is not limiting, as exposed reefs of the Baltic Sea are often covered by a dense population of bivalves (Kotta et al., 2013) indicating a tradeoff between suitable habitat and availability of preferred prey.

As aquatic pollution may increase the relative success of invasive species (Crooks et al., 2010) and, specifically, the round goby has been found to be tolerant to contamination (McCallum et al., 2014), our results indicate that preference of coastal areas by the species might be simply related to the fact that coastal areas are under higher anthropogenic impact than offshore areas. Also, it should be mentioned that our data mostly originate from fishers in the period of spawning and feeding time, when the species is present in the coastal areas. In the cold season, the fish has also been observed in deep offshore areas both in its native and invaded areas (Kostyuchenko, 1969; Walsh et al., 2007). The observed exposure–occurrence relationship may also apply elsewhere, as to date the round goby has also failed to establish in the exposed areas of the Great Lakes (Grigorovich et al., 2003b; Daniel Heath, Personal Communication).

In the current study, the probability of round goby occurrence

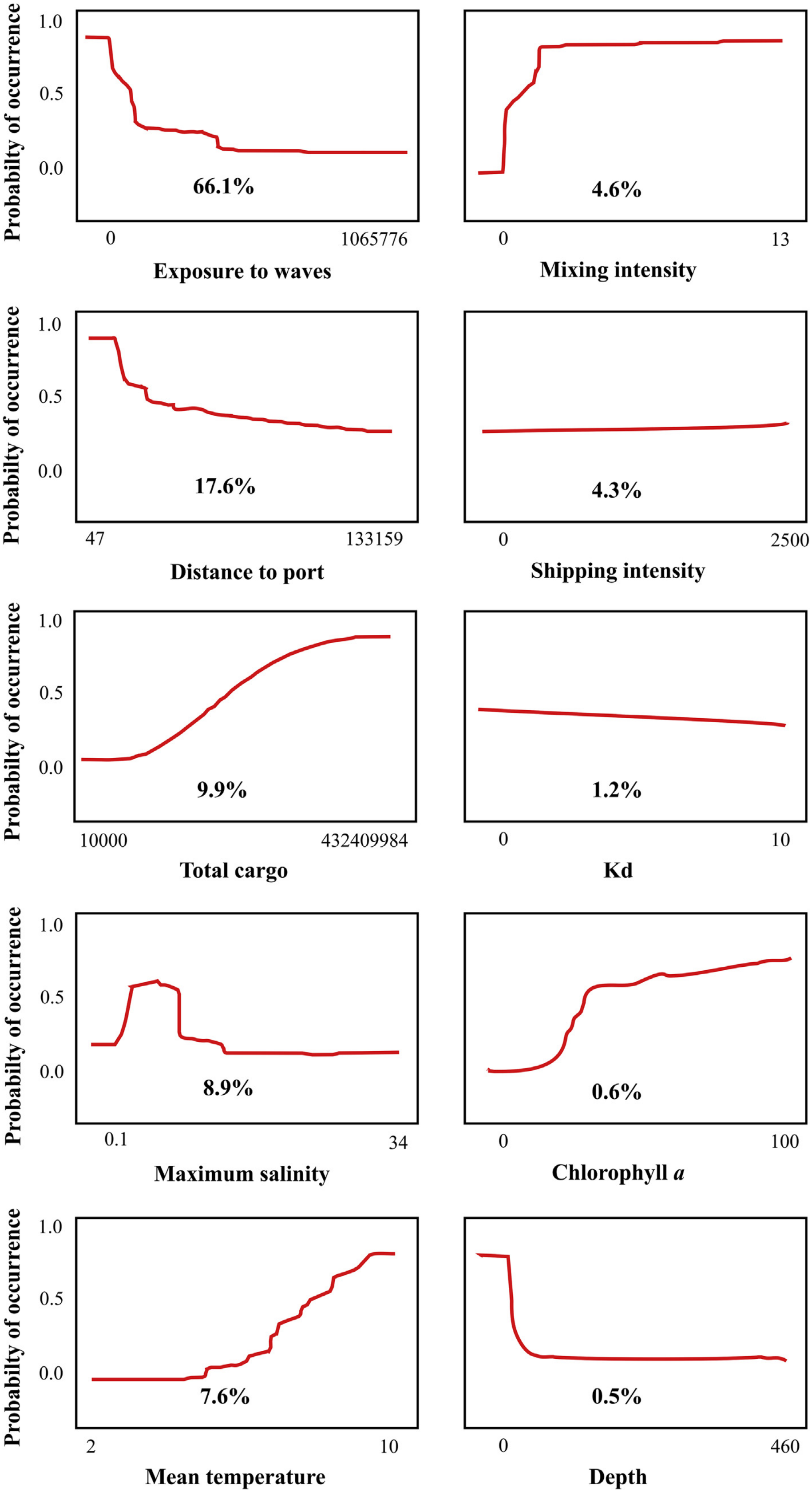

Fig. 2. Dependence plots showing how each environmental variable separately affects the MaxEnt prediction i.e. each of the following curves represents a different model using only the corresponding variable. The separate contribution of each variable is shown in each graph (%). The y-axes indicate logistic output.

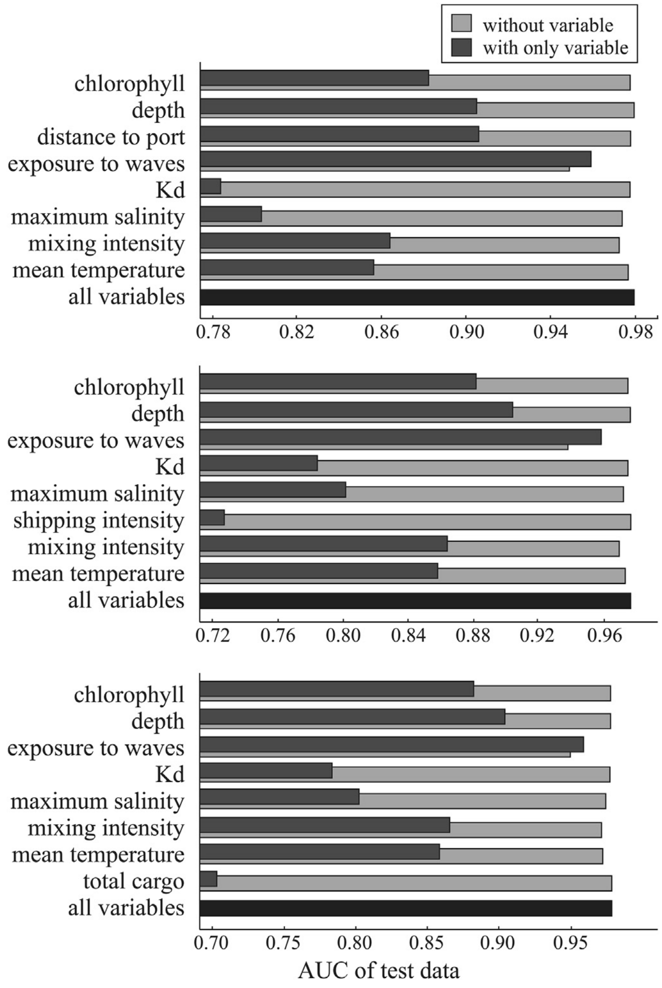

Fig. 3. Results of the jackknife test of variable importance in the MaxEnt models. In these tests test gain was used. In this analysis the environmental variable that decreases the gain the most when it is omitted has the most information that is not present in the other variables.

Table 2
Results of MaxEnt models run separately for each three shipping proxies: distance to port, total cargo and shipping intensity. Separate models were run to avoid multicollinearity issue in the MaxEnt models as shipping intensity and distance to nearest harbour were weakly intercorrelated ($r = -0.29$; $p < 0.001$). Models' descriptive power and percent contributions of each variable to the relevant MaxEnt models are shown. The AUC is a measure of overall model accuracy with the values above 0.9 suggesting almost a perfect match of all three models. Unregulized test gain is a measure of goodness of fit of models. It represents the presence likelihood of training records in comparison with background records. Gain is not regularized/compensated for the number of terms in the model.

Model descriptive power/variable	1	2	3
Test data AUC	0.980	0.978	0.978
Unregulized test gain	3.525	3.513	3.511
Distance to port	17.6		
Total cargo		9.9	
Shipping intensity			4.3
Exposure to waves	61.9	67.7	68.8
Salinity maximum	8.4	8.4	10
Temperature mean	6.1	7.8	8.8
Mixing intensity	4.1	4.3	5.5
Depth	0.4	0.6	0.6
K_d	1.3	0.8	1.4
Chlorophyll *a*	0.3	0.6	0.8

was significantly higher in close proximity to ports characterized by elevated cargo amounts. This clearly links patterns of the round goby distribution to the cargo traffic and is in line with evidence from elsewhere suggesting that the human factor (national wealth and human population density) is a significant predictor in the majority of models when analysed jointly with climate, geography and land cover (Pyšek et al., 2010). When cargo tonnage or shipping intensity was used as a proxy for propagule pressure, exposure to waves gained importance in the MaxEnt models. This may indicate that proximity to ports was the best proxy for describing propagule pressure.

The North American Great Lakes also host very dense populations of the round goby. Since its apparent arrival via ballast water in 1990 (Jude et al., 1992) the species is widely distributed across all of the five lakes and is spreading rapidly into adjacent tributaries (Jude, 2001; Kornis and Vander Zanden, 2010). Substantial genetic variation, multiple founding sources, large numbers of propagules, and a unique population structure is likely behind this ecological success story (Brown and Stepien, 2009).

Recent genetic analyses demonstrated that a combination of short-distance diffusion and long-distance dispersal contributes to the current distribution of the round goby in the lakes and rivers of its introduced North American ranges (Bronnenhuber et al., 2011). This evidence also suggests that commercial shipping potentially promotes frequent long distance spread of the round goby in these habitats (LaRue et al., 2011). Although different in methodologies, the North American studies and our paper have independently come to a similar conclusion, and jointly suggest that the spread of the round goby can largely benefit from shipping.

There are several examples outside of the Baltic Sea to suggest that shipping is a likely pathway for the invasion of gobies. In their review, Wonham et al. (2000) concluded that gobies (family Gobiidae) were the most commonly found fish in ballast tanks and they also dominate among fishes introduced via ballast water. There are several reasons why gobies have been more successful over other fishes by shipping transport. Specifically, gobies are known to be resilient enough to survive ocean crossings in ballast tanks (*e.g.*, Carlton, 1985, 1987) owing to the existence of a specialised lateral-line system (Jude, 1997) and tolerance of a wide range of habitat conditions (Kornis et al., 2012). The crevicolous nature of gobies when seeking refuge and laying eggs may predispose them to ballast-water transport, particularly due to the ballast-intake grates (Hoese, 1973; Carlton, 1985). Gobies may also lay eggs in small holes and thereby use ship hulls as a transport vector (Wonham et al., 2000). The recent cases of ships' ballast water transfers (as larvae or juveniles) include the introduction of the Australian bridled goby, *Arenigobius bifrenatus* to New Zealand (Willis et al., 1999) and the streaked goby *Acentrogobius pflaumii* into southwestern Australia (Maddern and Morrison, 2009).

Earlier theoretical models have shown that relationship between species release and establishment can potentially have only two shapes: hyperbolic or sigmoid (Wonham, 2008). MaxEnt models in this study demonstrated a sigmoid curve between the amount of cargo and the probability of occurrence of the round goby. This implies a clear Allee effect, *i.e.*, invading goby individuals interact positively creating an accelerating phase of a sigmoid curve (Allee, 1931). Previous studies have demonstrated either absence or presence of risk–release relationships (*e.g.*, Grigorovich et al., 2003a; Ricciardi, 2006; Costello et al., 2007). Such discrepancies may simply suggest that for some ecosystems or species, post-release processes have an overwhelming role over propagule pressure, whereas, for other ecosystems or species, propagule pressure is primarily limiting the spread of non-indigenous species. Alternatively, the selected proxies of propagule pressure may mismatch the measured species occurrences in space, time, or taxonomic resolution (Wonham, 2008). However, the MaxEnt models in this study suggested that both distance to ports and net mass of cargo are good proxies of propagule supply of the round goby and therefore can be used in a scientifically based management tools also when modelling other shipping related NIS distributions.

Temperature and salinity regime contributed only marginally to the model variance implying that the round goby has low sensitivity to environmental extremes, potentially due to either large variation in between-individual environmental optima and/or broad within-individual plasticity (Roughgarden, 1972; Abedikova, 1980; Kornis et al., 2013). Similarly, elevated chlorophyll *a* level, used as a proxy of eutrophication measure, did not yield to higher probability of occurrence of the round goby. It is likely that at the initial stage of invasion, food is not limiting the spread of this invasive fish. In general, clams and mussels constitute the majority of benthic biomass in the coastal areas of the Baltic Sea and due to a very low natural richness of epibenthic predators this food source is in excess for novel invasive species, such as the round goby (Kotta et al., 2008). Moreover, the diet of round gobies is not limited to bivalve prey. In areas where bivalves are less abundant, gobies easily consume other available prey species such as barnacles, gastropods, and chironomids (Riikka Puntila, Unpublished Data). Similar results have been observed in other areas for similar species, such as the shimofuri goby *Tridentiger bifasciatus*, who appeared to be a generalist predator in the invaded San Francisco Estuary by consuming seasonally most abundant benthic invertebrate prey (Matern and Brown, 2005).

Management of marine NIS should be primarily focussed on managing invasion vectors and pathways, as eradication of the already invaded NIS has been proven mostly impossible in the marine environment (Ojaveer et al., 2014; Lehtiniemi et al., 2015 and references therein). Amongst invasive fish, information of their transport and release are the least investigated aspects and therefore research on the transport and dispersal should be prioritized (García-Berthou, 2007). Our results indicate, that the combination of long-distance dispersal (evidenced as shipping as a significant factor in the MaxEnt models) and short-distance spread from the shipping hotspots explain the current pattern of round goby observations in the Baltic Sea. The round goby is classically considered as a demersal fish throughout its life cycle. However, recent evidence suggest that this might not be completely true, as diurnal vertical migration of both fish larvae and early juveniles was observed occurring in the pelagic zone during the night (Hayden and Miner, 2009; Hensler and Jude, 2007). This is important especially from management perspective: if ships were ballasting their tanks near the surface only during daylight, it may have reduced further spread of the fish in the Baltic Sea and elsewhere.

The round goby has high potential for secondary spread. In the Great Lakes the round goby was initially expected to remain within rocky habitats but in just 5 years after the first appearance, the invasive fish colonized all the lakes, with the exception of a large part of Lake Superior (U.S. Geological Survey, 2015), and is currently expanding its distribution upstream in adjacent rivers (Bronnenhuber et al., 2011). The range expansion has been much slower in the Baltic Sea where a pan-Baltic spread was reached in about two decades. The round goby has invaded the Baltic Sea probably already in late 1980's, but only very recently significantly expanded its range in several localities, mostly to port and harbour areas. To date, there are still several ecologically suitable areas which have remained uncolonized (*e.g.*, west coast of the Baltic Sea) or where the abundance remains relatively low. Such slow colonization could be attributed either to a broad range of environmental conditions of the Baltic Sea or a low genetic diversity of the round goby, described from haplotype analysis (Grigorovich et al., 2003a). Puck Bay in the south-eastern Baltic Sea was suggested to be the primary invasion site in the Baltic Sea (Björklund and Almqvist, 2010). However, due to our limited knowledge it cannot be concluded whether there has been only one primary invasion or multiple invasions from different source populations as has taken place in the Great Lakes (Brown and Stepien, 2009; Björklund and Almqvist, 2010). Nevertheless, temperate high-productivity brackish water seems to be a very favourable habitat for the round goby as the species exhibits longer lifespan and larger individual sizes in the Baltic Sea compared to their native distribution area (Sokołowska and Fey, 2011). Only the northernmost areas of the Baltic Sea, like Bothnian Bay, might likely pose difficulties for the round goby due to too extreme thermal conditions. However, our MaxEnt models did not indicate this restriction (see also Fig. 1).

In conclusion, the models used in this study provide valuable insights to roles of different environmental variables determining the round goby's distribution in the Baltic Sea. Potentially the models can be applied to predict future distribution trends of this species if used with caution. The models demonstrate clearly that the spatial distribution of the round goby in the Baltic Sea is a function of shipping intensity (distance to port, cargo traffic) and abiotic hydroclimatic environment (wave exposure). Although high frequency of release does not necessarily lead to successful invasions, the round goby seems not to have major environmental constraints in the Baltic Sea. This points to an obvious need for effective management measures of the Baltic shipping, including performing relevant risk assessments in intra-Baltic shipping (*e.g.*, David et al., 2013).

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References

Abedikova, T.M., 1980. Some regularities in the growth of sturgeons Acipenseridae in the Sea of Asov, USSR. Vopr. Ikhtiologiy 20, 473–480 (in Russian).

Albert, R.J., Lishman, J.M., Saxena, J.R., 2013. Ballast water regulations and the move toward concentration-based numeric discharge limits. Ecol. Appl. 23, 289–300. http://dx.doi.org/10.1890/12-0669.1.

Allee, W.C., 1931. Animal Aggregations: a Study in General Sociology. University of Chicago Press, Chicago.

AquaNIS, 2014. Information System on Aquatic Non-indigenous and Cryptogenic Species. WWW Page. http://www.corpi.ku.lt/databases/aquanis.

ArtDatabanken, 2014. WWW Page, http://www.slu.se/artdatabanken.

Azour, F., van Deurs, M., Behrens, J., Carl, H., Hüssy, K., Greisen, K., Ebert, R., Møller, P.R., 2015. Invasion rate and population characteristics of the round goby Neogobius melanostomus: effects of density and invasion history. Aquat. Biol. 24, 41–52. http://dx.doi.org/10.3354/ab00634.

BACC, 2008. Assessment of climate change for the Baltic Sea basin. In: Regional

Climate Studies. Springer, p. 474.

Bacevičius, E., 2003. Notes on round goby, a newcomer in the Baltic Sea and the Curonian Lagoon. J. Nat. 3, 38–40. http://dx.doi.org/10.2478/v10043-008-0030-z.

Bendtsen, J., Gustafsson, K.E., Söderkvist, J., Hansen, J.L.S., 2009. Ventilation of bottom water in the North Sea-Baltic Sea transition zone. J. Mar. Syst. 75, 138–149. http://dx.doi.org/10.1016/j.jmarsys.2008.08.006.

Björklund, M., Almqvist, G., 2010. Rapid spatial genetic differentiation in an invasive species, the round goby *Neogobius melanostomus* in the Baltic Sea. Biol. Invasions 12, 2609–2618. http://dx.doi.org/10.1007/s10530-009-9669-z.

Blackburn, T.M., Pyšek, P., Bacher, S., Carlton, J.T., Duncan, R.P., Jarošík, V., Wilson, J.R.U., Richardson, D.M., 2011. A proposed unified framework for biological invasions. Trends Ecol. Evol. 26, 333–339. http://dx.doi.org/10.1016/j.tree.2011.03.023.

Bronnenhuber, J.E., Dufour, B.A., Higgs, D.M., Heath, D.D., 2011. Dispersal strategies, secondary range expansion and invasion genetics of the nonindigenous round goby, *Neogobius melanostomus*, in Great Lakes tributaries. Mol. Ecol. 20, 1845–1859. http://dx.doi.org/10.1111/j.1365-294X.2011.05030.x.

Brown, J.E., Stepien, C.A., 2009. Invasion genetics of the Eurasian round goby in North America: tracing sources and spread patterns. Mol. Ecol. 18, 64–79. http://dx.doi.org/10.1111/j.1365-294X.2008.04014.x.

Byers, J.E., Smith, R.S., Pringle, J.M., Clark, G.F., Gribben, P.E., Hewitt, C.L., Inglis, G.J., Johnston, E.L., Ruiz, G.M., Stachowicz, J.J., Bishop, M.J., 2015. Invasion expansion: time since introduction best predicts global ranges of marine invaders. Sci. Rep. 5, 12436.

Carlton, J.T., 1985. Transoceanic and interoceanic dispersal of coastal marine organisms: the biology of ballast water. Oceanogr. Mar. Biol. – An Annu. Rev. 23, 313–371.

Carlton, J.T., 1987. Patterns of transoceanic marine biological invasions in the Pacific Ocean. Bull. Mar. Sci. 41, 452–465.

Carlton, J.T., Geller, J.B., 1993. Ecological roulette: the global transport of nonindigenous marine organisms. Science 261, 78–82. http://dx.doi.org/10.1126/science.261.5117.78.

Chan, F.T., Bailey, S.A., Wiley, C.J., MacIsaac, H.J., 2013. Relative risk assessment for ballast-mediated invasions at Canadian Arctic ports. Biol. Invasions 15, 295–308. http://dx.doi.org/10.1007/s10530-012-0284-z.

Charlebois, P.M., Corkum, L.D., Jude, D.J., Knight, C., 2001. The round goby (*Neogobius melanostomus*) invasion: current research and future needs. J. Gt. Lakes Res. 27, 263–266. http://dx.doi.org/10.1016/S0380-1330(01)70641-7.

Corkum, L.D., Sapota, M.R., Skora, K.E., 2004. The round goby, *Neogobius melanostomus*, a fish invader on both sides of the Atlantic Ocean. Biol. Invasions 6, 173–181. http://dx.doi.org/10.1023/B: BINV.0000022136.43502.db.

Costello, C., Drake, J.M., Lodge, D.M., 2007. Evaluating an invasive species policy: ballast water exchange in the Great Lakes. Ecol. Appl. 17, 655–662. http://dx.doi.org/10.1890/06-0190.

Courchamp, F., Berec, L., Gascoigne, J., 2008. Allee effects in ecology and conservation. Environ. Conserv. 36, 80–85. http://dx.doi.org/10.1017/S0376892909005384.

Crooks, J.A., Chang, A.L., Ruiz, G.M., 2010. Aquatic pollution increases the relative success of invasive species. Biol. Invasions 13, 165–176. http://dx.doi.org/10.1007/s10530-010-9799-3.

David, M., Gollasch, S., Leppäkoski, E., 2013. Risk assessment for exemptions from ballast water management-the Baltic Sea case study. Mar. Pollut. Bull. 75, 205–217. http://dx.doi.org/10.1016/j.marpolbul.2013.07.031.

Drake, J.M., 2004. Allee effects and the risk of biological invasion. Risk Anal. 24, 795–802. http://dx.doi.org/10.1111/j.0272-4332.2004.00479.x.

Elith, J., Graham, C.H., Anderson, R.P., Dudík, M., Ferrier, S., Guisan, A., Hijmans, R.J., Huettmann, F., Leathwick, J.R., Lehmann, A., Li, J., Lohmann, L.G., Loiselle, B.A., Manion, G., Moritz, C., Nakamura, M., Nakazawa, Y., Overton, Jacob McC., Peterson, A.T., Phillips, S.J., Richardson, K., Scachetti-Pereira, R., Schapire, R.E., Soberón, J., Williams, S., Wisz, M.S., Zimmermann, N.E., 2006. Novel methods improve prediction of species' distributions from occurrence data. Ecography 29, 129–151. http://dx.doi.org/10.1111/j.2006.0906-7590.04596.x.

Elith, J., Phillips, S.J., Hastie, T., Dudik, M., Chee, Y.E., Yates, C.J., 2011. A statistical explanation of MaxEnt for ecologists. Divers. Distrib. 17, 43–57. http://dx.doi.org/10.1111/j.1472-4642.2010.00725.x.

Elton, C.S., 2000. The Ecology of Invasions by Animals and Plants. University of Chicago Press.

Fielding, A.H., Bell, J.F., 1997. A review of methods for the assessment of prediction errors in conservation presence/absence models. Environ. Conserv. 24, 38–49.

Finnish Alien Species Database, 2014. WWW Page, http://www.riistakala.info/alien_species/index.html.

Fischfauna-Online Bremen University of Applied Sciences, 2015. Fischfauna-Online. http://www.fischfauna-online.de.

French III, J.R.P., Jude, D.J., 2001. Diets and diet overlap of nonindigenous gobies and small benthic native fishes co-inhabiting the St. Clair River, Michigan. J. Gt. Lakes Res. 27, 300–311.

García-Berthou, E., 2007. The characteristics of invasive fishes: what has been learned so far? J. Fish Biol. 71, 33–55. http://dx.doi.org/10.1111/j.1095-8649.2007.01668.x.

Grigorovich, I.A., Colautti, R.I., Mills, E.L., Holeck, K., Ballert, A.G., MacIsaac, H.J., 2003a. Ballast-mediated animal introductions in the Laurentian Great Lakes: retrospective and prospective analyses. Can. J. Fish. Aquat. Sci. 60, 740–756. http://dx.doi.org/10.1139/f03-053.

Grigorovich, I.A., Korniushin, A.V., Gray, D.K., Duggan, I.C., Robert, I., Macisaac, H.J., 2003b. Lake Superior: an invasion coldspot? Hydrobiologia 499, 191–210. http://dx.doi.org/10.1023/A:1026335300403.

Gurevitch, J., Fox, G.A., Wardle, G.M., Taub, D., 2011. Emergent insights from the synthesis of conceptual frameworks for biological invasions. Ecol. Lett. 14, 407–418. http://dx.doi.org/10.1111/j.1461-0248.2011.01594.x.

Hastie, T., Tibshirani, R., Friedman, J., Hastie, T., Friedman, J., Tibshirani, R., 2009. The Elements of Statistical Learning. Springer.

Haydar, D., Wolff, W.J., 2011. Predicting invasion patterns in coastal ecosystems: relationship between vector strength and vector tempo. Mar. Ecol. Prog. Ser. 431, 1–10. http://dx.doi.org/10.3354/meps09170.

Hayden, T.A., Miner, J.G., 2009. Rapid dispersal and establishment of a benthic Ponto-Caspian goby in Lake Erie: diel vertical migration of early juvenile round goby. Biol. Invasions 11, 1767–1776. http://dx.doi.org/10.1007/s10530-008-9356-5.

HELCOM, 2010. Maritime Activities in the Baltic Sea - an Integrated Thematic Assessment on Maritime Activities and Response to Pollution at Sea in the Baltic Sea Region. In: Baltic Sea Environmental Proceedings.

HELCOM, 2014. WWW Page, http://maps.helcom.fi/website/mapservice/index.html.

Hensler, S.R., Jude, D.J., 2007. Diel vertical migration of round goby larvae in the Great Lakes. J. Gt. Lakes Res. 33, 295–302. http://dx.doi.org/10.3394/0380-1330(2007)33[295:DVMORG]2.0.CO;2.

Hoese, D.F., 1973. The introduction of the gobiid fishes *Acanthogobius flavimanus* and *Tridentiger trigonocephalus* into Australia. Koolewong 2, 3–5.

ICES, 2012. Report of the ICES Working Group on Introduction and Transfers of Marine Organisms (WGITMO). ICES CM 2012/ACOM:31. 14–16 March 2012 Lisbon, Portugal.

IMO, 2004. International Convention for the Control and Management of Ships' Ballast Water and Sediments (BWM). London, UK.

Isaeus, M., 2004. Factors Structuring Fucus Communities at Open and Complex Coastlines in the Baltic Sea. Meta. Stockholm University.

Järv, L., Kotta, J., Kotta, I., Raid, T., 2011. Linking the structure of benthic invertebrate communities and the diet of native and invasive fish species in a brackish water ecosystem. Ann. Zool. Fenn. 48, 129–141. http://dx.doi.org/10.5735/086.048.0301.

Jazdzewski, K., Konopacka, A., Grabowski, M., 2005. Native and alien malacostracan crustacea along the Polish Baltic Sea coast in the twentieth century. Oceanol. Hydrobiol. Stud. 34, 175–193.

Jude, D.J., Reider, R.H., Smith, G.R., 1992. Establishment of Gobiidae in the Great Lakes basin. Can. J. Fish. Aquat. Sci. 49, 416–421.

Jude, D.J., 1997. Round gobies: cyberfish of the third millennium. Gt. Lakes Res. Rev. 3, 27–34.

Jude, D.J., 2001. Round and tubenose gobies: 10 years with the latest Great Lakes phantom menace. Dreissena 11, 1–14.

Jude, D.J., Janssen, J., Crawford, G., 1995. Ecology, distribution, and impact of the newly introduced round and tubenose gobies on the biota of the St. Clair and Detroit Rivers. In: Munawar, M., Edsall, T., Leach, J. (Eds.), The Lake Huron Ecosystem: Ecology, Fisheries and Management. SPB Academic Publishing, Amsterdam, The Netherlands, pp. 447–460.

Kornis, M.S., Mercado-Silva, N., Vander Zanden, M.J., 2012. Twenty years of invasion: a review of round goby *Neogobius melanostomus* biology, spread and ecological implications. J. Fish Biol. 80, 235–285. http://dx.doi.org/10.1111/j.1095-8649.2011.03157.x.

Kornis, M.S., Sharma, S., Vander Zanden, M.J., 2013. Invasion success and impact of an invasive fish, round goby, in Great Lakes tributaries. Divers. Distrib. 19, 184–198. http://dx.doi.org/10.1111/ddi.12001.

Kornis, M.S., Vander Zanden, M.J., 2010. Forecasting the distribution of the invasive round goby (*Neogobius melanostomus*) in Wisconsin tributaries to Lake Michigan. Can. J. Fish. Aquat. Sci. 67, 553–562.

Kostyuchenko, A.A., 1969. Regularity of distribution and migrations of the round goby in the Sea of Azov. In: Trudy Azov.-Chernom. Nauchn.-Issled. Rybn. Khoz. Okeanogr (Proceedings of the Commission for Complex Investigation of the Caspian Sea), vol. 26, pp. 14–19 (in Russian).

Kotta, J., Herkül, K., Kotta, I., Orav-Kotta, H., Lauringson, V., 2009. Effects of the suspension feeding mussel *Mytilus trossulus* on a brackish water macroalgal and associated invertebrate community. Mar. Ecol. 30, 56–64. http://dx.doi.org/10.1111/j.1439-0485.2009.00303.x.

Kotta, J., Kutser, T., Teeveer, K., Vahtmäe, E., Pärnoja, M., 2013. Predicting species cover of marine macrophyte and invertebrate species combining hyperspectral remote sensing, machine learning and regression techniques. PLoS One 8, e63946. http://dx.doi.org/10.1371/journal.pone.0063946.

Kotta, J., Lauringson, V., Martin, G., Simm, M., Kotta, I., Herkül, K., Ojaveer, H., 2008. Gulf of Riga and Pärnu Bay. In: Schiewer, U. (Ed.), Ecology of Baltic Coastal Waters. Springer-Verlag, Berlin Heidelberg, pp. 217–243.

LaRue, E.A., Ruetz III, C.R., Stacey, M.B., Thum, R.A., 2011. Population genetic structure of the round goby in Lake Michigan: implications for dispersal of invasive species. Hydrobiologia 663, 71–82.

Lee, H., Reusser, D.A., Frazier, M., 2013. Approaches to setting organism-based ballast water discharge standards. Ecol. Appl. 23, 301–310. http://dx.doi.org/10.1890/11-1638.1.

Lehtiniemi, M., Ojaveer, H., David, M., Galil, B., Gollasch, S., McKenzie, C., Minchin, D., Occhipinti-Ambrogi, A., Olenin, S., Pederson, J., 2015. Dose of truth—monitoring marine non-indigenous species to serve legislative requirements. Mar. Policy 54, 26–35. http://dx.doi.org/10.1016/j.marpol.2014.12.015.

Lo, V.B., Levings, C.D., Chan, K., 2012. Quantifying potential propagule pressure of aquatic invasive species from the commercial shipping industry in Canada. Mar. Pollut. Bull. 64, 295–302. http://dx.doi.org/10.1016/j.marpolbul.2011.11.016.

Lockwood, J.L., Cassey, P., Blackburn, T.M., 2009. The more you introduce the more you get: the role of colonization pressure and propagule pressure in invasion ecology. Divers. Distrib. 15, 904–910. http://dx.doi.org/10.1111/j.1472-4642.2009.00594.x.

Lynch, M., Gabriel, W., 1987. Environmental tolerance. Am. Nat. 129, 283. http://dx.doi.org/10.1086/284635.

Maddern, M.G., Morrison, S., 2009. Introduction of the streaked goby *Acentrogobius pflaumii* (Bleeker 1853) (Pisces: Gobiidae) into southwestern Australia. Aust. Zool. 35, 96–99.

Marsden, J.E., Charlebois, P., Wolfe, K., Jude, D.J., Rudnicka, S., 1997. The round goby, Neogobius melanostomus (Pallas): a review of European and North American literature. In: The Round Goby Conference, p. 60.

Matern, S.A., Brown, L.R., 2005. Invaders eating invaders: exploitation of novel alien prey by the alien shimofuri goby in the San Francisco Estuary, California. Biol. Invasions 7, 497–507.

McCallum, E., Charney, R., Marenette, J., Young, J.M., Koops, M., Earn, D.D., Bolker, B., Balshine, S., 2014. Persistence of an invasive fish (*Neogobius melanostomus*) in a contaminated ecosystem. Biol. Invasions 16, 2449–2461. http://dx.doi.org/10.1007/s10530-014-0677-2.

Minchin, D., Gollasch, S., Cohen, A.N., Hewitt, C.L., Olenin, S., 2009. Characterizing vectors of marine invasion. In: Rilov, G., Crooks, J.A. (Eds.), Biological Invasions in Marine Ecosystems. Springer, pp. 109–116.

Ojaveer, H., 2006. The round goby *Neogobius melanostomus* is colonising the NE Baltic Sea. Aquat. Invasions 1, 44–45. http://dx.doi.org/10.3391/ai.2006.1.1.11.

Ojaveer, H., Galil, B.S., Minchin, D., Olenin, S., Amorim, A., Canning-Clode, J., Chainho, P., Copp, G.H., Gollasch, S., Jelmert, A., Lehtiniemi, M., McKenzie, C., Mikuš, J., Miossec, L., Occhipinti-Ambrogi, A., Pećarević, M., Pederson, J., Quilez-Badia, G., Wijsman, J.W.M., Zenetos, A., 2014. Ten recommendations for advancing the assessment and management of non-indigenous species in marine ecosystems. Mar. Policy 44, 8–13. http://dx.doi.org/10.1016/j.marpol.2013.08.019.

Ojaveer, H., Jaanus, A., Mackenzie, B.R., Martin, G., Olenin, S., Radziejewska, T., Telesh, I., Zettler, M.L., Zaiko, A., 2010. Status of biodiversity in the Baltic Sea. PLoS One 5. http://dx.doi.org/10.1371/journal.pone.0012467.

Ojaveer, H., Kotta, J., 2014. Ecosystem impacts of the widespread non-indigenous species in the Baltic Sea: literature survey evidences major limitations in knowledge. Hydrobiologia. http://dx.doi.org/10.1007/s10750-014-2080-5.

Olenin, S., Minchin, D., Daunys, D., 2007. Assessment of biopollution in aquatic ecosystems. Mar. Pollut. Bull. 55, 379–394. http://dx.doi.org/10.1016/j.marpolbul.2007.01.010.

Parker, I.M., Simberloff, D., Lonsdale, W.M., Goodell, K., Wonham, M., Kareiva, P.M., Williamson, M.H., Van Holle, B., Moyile, P.B., Byers, J.E., Goldwasser, L., 1999. Impact: toward a framework for understanding the ecological effects of invaders. Biol. Invasions 1, 3–19.

Parravicini, V., Azzurro, E., Kulbicki, M., Belmaker, J., 2015. Niche shift can impair the ability to predict invasion risk in the marine realm: an illustration using Mediterranean fish invaders. Ecol. Lett. 18, 246–253. http://dx.doi.org/10.1111/ele.12401.

Platt, V., Jeschke, J.M., 2014. Are exotic species red queens? Ethol. Ecol. Evol. 1–11. http://dx.doi.org/10.1080/03949370.2014.898699.

Pyšek, P., Jarošík, V., Hulme, P.E., Kühn, I., Wild, J., Arianoutsou, M., Bacher, S., Chiron, F., Didžiulis, V., Essl, F., 2010. Disentangling the role of environmental and human pressures on biological invasions across Europe. Proc. Natl. Acad. Sci. 107, 12157–12162.

Quantum GIS Development Team, 2014. Quantum GIS Geographic Information System (Open Source Geospatial Foundation Project).

Rakauskas, V., Bacevičius, E., Pūtys, Ž., Ložys, L., Arbačiauskas, K., 2008. Expansion, feeding and parasites of the round goby, *Neogobius melanostomus* (Pallas, 1811), a recent invader in the Curonian Lagoon, Lithuania. Acta Zool. Litu. 18, 180–190. http://dx.doi.org/10.2478/v10043-008-0030-z.

Rakauskas, V., Pūtys, Ž., Dainys, J., Lesutienė, J., Ložpys, L., Arbačiauskas, K., 2013. Increasing population of the invader round goby, *Neogobius melanostomus* (Actinopterygii: Perciformes: Gobiidae), and its trophic role in the Curonian Lagoon, SE Baltic Sea. Acta Ichthyol. Piscat. 43, 95–108. http://dx.doi.org/10.3750/AIP2013.43.2.02.

Ray, W.J., Corkum, L.D., 2001. Habitat and site affinity of the round goby. J. Gt. Lakes Res. 27, 329–334. http://dx.doi.org/10.1016/S0380-1330(01)70648-X.

Reusser, D.A., Lee, H., Frazier, M., Ruiz, G.M., Fofonoff, P.W., Minton, M.S., Miller, A.W., 2013. Per capita invasion probabilities: an empirical model to predict rates of invasion via ballast water. Ecol. Appl. 23, 321–330. http://dx.doi.org/10.1890/11-1637.1.

Ricciardi, A., 2006. Patterns of invasion in the Laurentian Great Lakes in relation to changes in vector activity. Divers. Distrib. 12, 425–433. http://dx.doi.org/10.1111/j.1366-9516.2006.00262.x.

Roughgarden, J., 1972. Evolution of niche width. Am. Nat. 106, 683–718.

Roura-Pascual, N., Hui, C., Ikeda, T., Leday, G., Richardson, D.M., Carpintero, S., Espadaler, X., Gómez, C., Guénard, B., Hartley, S., Krushelnycky, P., Lester, P.J., McGeoch, M.A., Menke, S.B., Pedersen, J.S., Pitt, J.P.W., Reyes, J., Sanders, N.J., Suarez, A.V., Touyama, Y., Ward, D., Ward, P.S., Worner, S.P., 2011. Relative roles of climatic suitability and anthropogenic influence in determining the pattern of spread in a global invader. Proc. Natl. Acad. Sci. U. S. A. 108, 220–225. http://dx.doi.org/10.1073/pnas.1011723108.

Sapota, M.R., 2004. The round goby (*Neogobius melanostomus*) in the Gulf of Gdansk – a species introduction into the Baltic Sea. Hydrobiologia 514, 219–224. http://dx.doi.org/10.1007/978-94-017-0920-0_20.

Sapota, M.R., Skóra, K.E., 2005. Spread of alien (non-indigenous) fish species *Neogobius melanostomus* in the Gulf of Gdansk (south Baltic). Biol. Invasions 7, 157–164. http://dx.doi.org/10.1007/s10530-004-9035-0.

Segerstråle, S.G., 1957. Baltic sea. Geol. Soc. Am. Memoirs 67, 751–800.

Shearer, J.F., Grodowitz, M.J., 2010. Impact of Data Availability on Site Assessment and Predictive Behavior of Aquatic Invasive Species. No. ERDC/TN-ANSRP-10–1. Engineer Research and Development Center Vicksburg MS Environmental Lab.

Simberloff, D., 2009. The role of propagule pressure in biological invasions. Annu. Rev. Ecol. Evol. Syst. 40, 81–102. http://dx.doi.org/10.1146/annurev.ecolsys.110308.120304.

Skóra, K.E., Stolarski, J., 1993. *Neogobius melanostomus* (Pallas 1811) a new immigrant species in Baltic Sea. In: Proceedings of the Second International Estuary Symposium Held in Gdańsk, pp. 18–22.

Sokołowska, E., Fey, D.P., 2011. Age and growth of the round goby *Neogobius melanostomus* in the Gulf of Gdańsk several years after invasion. Is the Baltic Sea a new promised land? J. Fish Biol. 78, 1993–2009. http://dx.doi.org/10.1111/j.1095-8649.2011.02986.x.

Taylor, C.M., Hastings, A., 2005. Allee effects in biological invasions. Ecol. Lett. 8, 895–908. http://dx.doi.org/10.1111/j.1461-0248.2005.00787.x.

U.S. Geological Survey, 2015. Nonindigenous Aquatic Species Database. Florida, Gainesville. WWW Page. http://nas.er.usgs.gov.

Walsh, M.G., Dittman, D.E., O'Gorman, R., 2007. Occurrence and food habits of the round goby in the profundal zone of Southwestern Lake Ontario. J. Gt. Lakes Res. 33, 83–92. http://dx.doi.org/10.3394/0380-1330(2007)33[83:OAFHOT]2.0.CO;2.

Wandzel, T., 2000. Round goby, *Neogobius melanostomus* Pallas, 1811 in the catches of R/V Baltica. Acta Ichthyol. Piscat. 30, 81–91.

Willis, T.J., Saunders, J.E.H., Blackwood, D.L., Archer, J.E., 1999. First New Zealand record of the Australian bridled goby, *Arenigobius bifrenatus* (Pisces: Gobiidae). N. Z. J. Mar. Freshw. Res. 33, 189–192.

Wonham, M.E.A., 2008. Modelling the relation between propagule pressure and invasion risk to inform policy and management. Vasa 23, 1691–1706. http://dx.doi.org/10.1890/12-1985.1.

Wonham, M., Byers, J.E., Grosholz, E.D., Leung, B., 2013. Modeling the relationship between invasion risk and propagule pressure to inform invasion policy. Ecol. Appl. 23, 1691–1706.

Wonham, M.J., Carlton, J.T., Ruiz, G.M., Smith, L.D., 2000. Fish and ship: relating dispersal frequency to success in biological invasions. Mar. Biol. 136, 1111–1121.

Zaiko, A., Lehtiniemi, M., Narščius, A., Olenin, S., 2011. Assessment of bioinvasion impacts on a regional scale: a comparative approach. Biol. Invasions 13, 1739–1765. http://dx.doi.org/10.1007/s10530-010-9928-z.

APPENDIX V

Salinity tolerance of round goby

Jane W. Behrens (DTU Aqua, P2))

Behrens JW, van Deurs M, Christensen AF. Aerobic scope and blood plasma osmolality predict secondary dispersal potential of an invasive fish. Submitted

Introduction

Non-indigenous species (NIS) can have strong impacts on marine biodiversity and ecosystem structure and function. Hence, predicting secondary dispersal patterns is pivotal. Once introduced into a new region, secondary dispersal of NIS depends on a suite of ecological factors such as presence of predators, competitors, and parasites, yet with the most fundamental constraints on the distribution arising from the organism's physiological limitations in relation to the ambient environment. Predicting dispersal however remains a challenge. In fish, aerobic scope (AS, the difference between maximum and standard metabolic rate) has been linked to fitness parameters such as growth, dominance, feeding, and swimming performance. However, little is known about the link between AS and the dispersal potential of invasive species in novel environments.

The round goby *Neogobius melanostomus* (Pallas, 1814) is one of the most wide-ranging invasive fish species in Europe and North America, thriving in both brackish and fresh water. It has however hitherto remained unclear if it will endure high saline conditions. Nevertheless, the species is currently spreading at 30 km year^{-1} into a steep salinity gradient segregating the brackish Baltic Sea from the oceanic North Sea. To evaluate the potential for secondary dispersal into more saline water bodies, we examined AS and blood plasma osmolality in relation to salinities of 0, 10, 15, 25 and 30 PSU.

Results

The combined effects of salinity on SMR and MMR resulted in the highest AS at 10 PSU and lowest at 30 PSU (reduced by 31% as compared to optimal conditions). There was a negative relationship between AS and osmotic potential ([ambient osmolality-blood plasma osmolality]) and a positive relationship between AS and survival. Blood plasma osmolality was not affected by ambient salinities from 0 to 25 PSU; only at 30 PSU could a negative effect be detected. Overall, there was pronounced inter-individual variation, especially at the higher salinities and some individuals were able to completely offset the negative impact of high salinity on their performance.

Conclusions and future perspectives

These results suggests that round goby has the capacity to occupy full oceanic environments and we predict that the species will continue to spread into the Baltic Sea-North sea transition zone, although the depressed physiological performance at high salinities might reduce its competitive potential as it approaches the North Sea. However, a pronounced high inter-individual variation in physiological performance at the highest salinities suggests that some individuals are able to offset the negative effects of high salinity, thus increasing the dispersal potential at the invasion front.

Using AS as a tool to make early predictions of dispersal potential and future ‘area of impact’ is a great asset in relation to taking appropriate management actions.

Appendix VI

Salinity and temperature effects on egg development in round goby - Hand line survey in the Western Baltic

Jan Niemax, Axel Temming, Jens Peter Herrmann, Sven Matern (University of Hamburg, P3)

Introduction

Since 1990 round goby *Neogobius melanostomus* has managed to successfully establish within most parts of the Baltic Sea. Within the first 15 years of invasion the dispersal was limited to areas with brackish waters. In the past decade the spreading exploded and included more and more areas with higher salinities (compare map in Kotta, Nurkse, Puntila, & Ojaveer, 2015, Appendix IV). However, the physiological limits, especially in relation to reproduction, are not well understood. As a first step to estimate the potential spreading of round goby in saltier waters of the Western Baltic and the Kattegat, experiments were performed to test egg development and hatching success in different combinations of salinity and temperature. The aim is to test to what extent combinations of salinity and temperature act as a barrier to further spreading of the round goby into the Belt areas and the Kattegat.

Methods

Round gobies *Neogobius melanostomus* were caught in Oktober 2014 by angling in the Travemünde Bight. The gobies were maintained in tanks with an artificial seawater circulation system. Salinity was adjusted to 16 psu, temperature was about 15 °C and light was set to 14 hours daylight. To initiate spawning, the gobies were exposed to an artificial seasonal cycle by variation of day length and light intensity as well as temperature. Temperature was reduced down to 8 °C and day length to 8 hours with a light intensity of 60% within 16 days. These conditions were maintained for 47 days, afterwards day length and temperature increased again for 17 days until reaching summer conditions of 16 hours daylight at 100% and 16°C.

For mating the fish were split up into groups of 3 females and one male to prevent territorial fights of males for shelters. Each tank was equipped with a shelter. The shelters were constructed with exchangeable glas ceilings. Thus removing of clutches was possible without opening the shelters. Shelters were checked daily for new clutches. If spawning occured, the glas ceiling was removed and placed in a tank with predefined salinity and temperature conditions (see Tab. 1). For each combination at least 3 replicates were performed. The clutches were photographed on Monday, Wednesday and Friday. The 20°C eggs were photographed every day. Subsequently the eggs were counted based on the pictures and categorized into 4 stages, good, bad, blackeye, hatched.

1) ”Good” refers to egg in good condition, but without black eyes of a larvae visible. 2) ”Bad” means the egg is in a bad condition characterized by a dissolving yolk within the egg and a blurry appearance, a development is not expected. 3)”Blackeye” is characterized by little black spots visible inside the egg, which during the development are recognizable as part if the developing eyes.

4) ”Hatched” was assumed if an egg had a blackeye stage before and only the hull was left.

Results

Note: Results have to be considered as preliminary as further analyses are ongoing.

Development time until the blackeye stage

The developing time from fertilization up to the first appearance of the blackeye stage decreases with temperature and increases with salinity. The fastest development up to blackeye stage occurred at 20°C and 5 psu within 4 days. An elongated time was measured at 10°C and 20 psu, with up to 30 days (Tab. 1).

Table 1. Time (days) from fertilization up to the first appearance of the blackeye stage in relation of salinity and temperature

Mean time in days till black eye stage				
sal | Temp	5°C	10°C	15°C	20°C
5	unsuccessful	22	9	4
10	unsuccessful	24	9	5
15	not tested	22	10	5
20	not tested	30	10	6

Hatching success

We found that round goby was able to develop successfully until hatching within a wide temperature and salinity range. Successful hatching occurred in all combinations of 10,15,20 °C with salinities of 5,10,15 and 20 psu, with one exception. At a temperature of 10°C and a salinity of 20 psu hatching did not take place (Tab. 2). The mortality of eggs through the different treatments is highly variable. In general the lowest mortality rates or vice versa the highest hatching success was observed at 15°C. With increasing or decreasing temperature from 15°C the success was reduced. Higher salinities always led to higher mortality rates (see Fig. 1+2). In 5 treatments we located one replicate that performed noteworthyly better than the rest.

Table 2. Hatching success (at least one larvae hatched successfully) dependent on salinity and temperature

Hatching				
sal | Temp	5°C	10°C	15°C	20°C
5	unsuccessful	successful	successful	successful
10	unsuccessful	successful	successful	successful
15	not tested	successful	successful	successful
20	not tested	unsuccessful	successful	successful

Here the results of two treatments (5 + 20 psu, 15°C) are shown as an example (Fig.1 + 2). It appeared that at 5 psu first hatching was observed 23-27 days past spawning and finished nearly simultaneously in all replicates after 33-34 days while most eggs actually hatched around day 32. The overall hatching success varied marginally between clutches. Hatching at 20 psu also started around day 23-37, but needed slightly more time until all eggs were hatched. The time varied within the replicates from 35 – 43 days after spawning. The data of this trial revealed that the time to hatch is relatively constant across all replicates. Similar patterns were found in the other treatments.

The major influence of salinity became apparent by a closer look at the survival of eggs. Whereas at 5 psu the mortality is about 6% or less it ranges from 57% up to 71% at 20 psu.

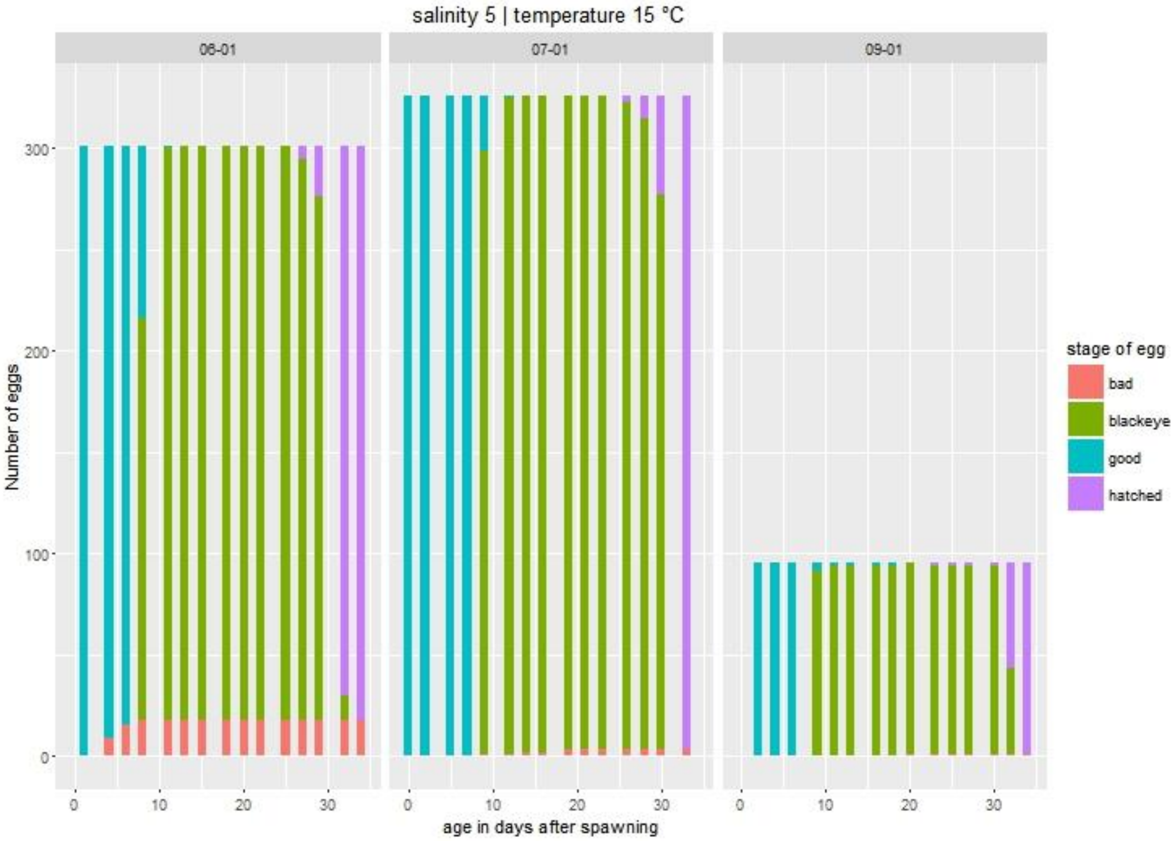


Fig.1: Hatching time and success at 5psu and 15°C. Shown are the numbers of eggs at each stage over time in days after spawning for 3 replicates.

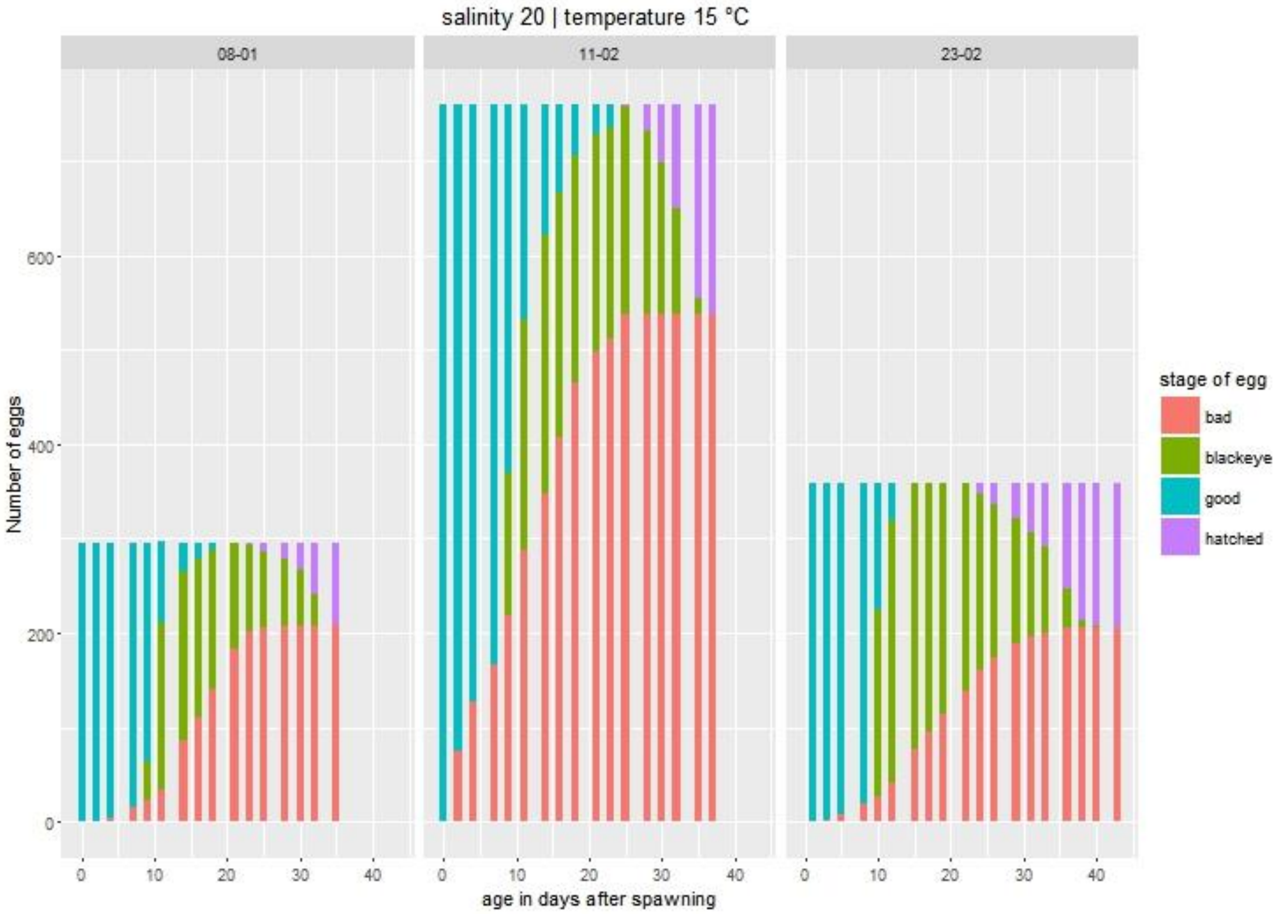


Fig.2: : Hatching time and success at 20 psu and 15°C. Shown is the numbers of eggs at each stage over time in days after spawning for 3 replicates.

Conclusions and future perspectives

The results show that round goby *Neogobius melanostomus* is able to cope with a wide range of salinities. Nevertheless higher salinities have a strong impact on the amount of successfully hatched larvae. The high variability between replicates within 5 treatments indicates that a pronounced maternal effect on hatching success exists. (A cross-breeding experiment investigating effects of possible adaptation to certain environments is planned for summer 2016 by the University of Gothenburg in cooperation with University of Hamburg.)

Considering these results, the endpoint of round goby dispersal is probably not reached, although it will maybe progress slower caused by a reduced reproduction success. The areas in which round goby find suitable conditions for a successful breeding are obviously substantially wider than their current expansion. Recent investigation by Jane Behrens show that adult round goby are able to resist high salinities up to nearly oceanic environments (see results of P2 (DTU Aqua) in Appendix V) These findings indicate that expanding the reproduction experiments up to 30 psu is advisable. Furthermore, experiments are in preparation that simulate ship-based travel of eggs, assuming that round goby uses a ship hull to spawn. Eggs are going to be exposed to oceanic salinities for about 1 week and then moved to brackish conditions, similar to a harbor in a river mouth. The results probably explain a vector of round goby dispersal

Hand line survey in the Western Baltic

Introduction

The spreading of round goby along the Western part of the German Baltic coast has not been investigated before. The salinity in this region varies from around 20 psu in the Flensborg Fjord mouth down to roughly 13 psu in the Trave estuary. A hand line survey for round goby was conducted along the Baltic coast from Lübeck to Flensburg.

Methods

The angling took place at eleven different locations along the coastline of the Western Baltic Sea in Schleswig Holstein, Germany. Sampling occurred between 11-Aug 2014 and the 2-Oct 2014.

Most sampling sites were located in or close to sport boat harbours and all sampling sites were characterized by anthropogenic structures like quay walls and pontoons.

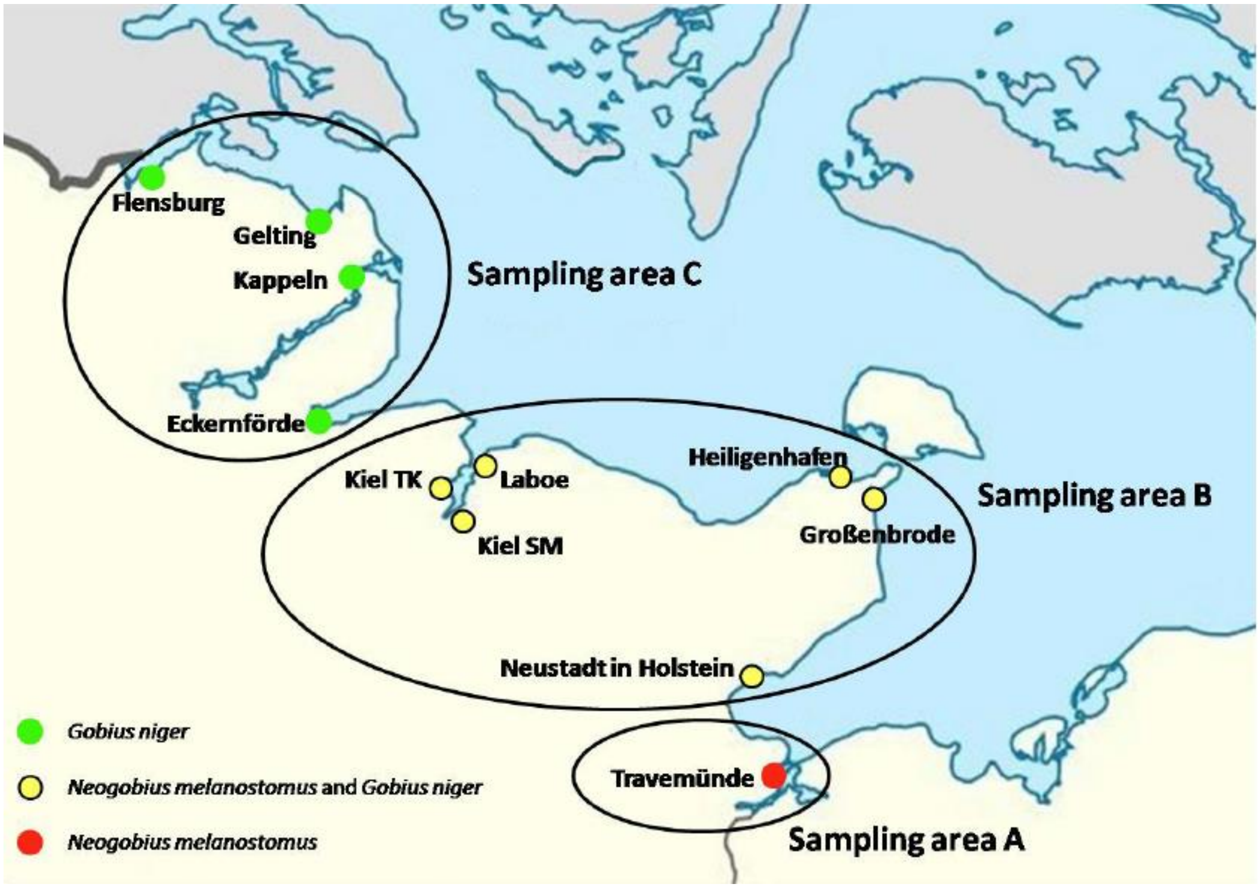


Fig. 3: **Map of the sampling sites and the sampling areas**, which were defined by the distribution of *Neogobius melanostomus* and *Gobius niger* at the different sampling sites (map modified from www.esys.de).

Results

A distribution boundary of round goby was found in the area of Kiel Bight. Further was a co-occurrence of the black goby (*Gobius niger*) found in areas with moderate round goby abundances (sampling area B, Fig. 3). Black goby was absent at the location with the highest densities of round goby (*Neogobius melanostomus*) in Travemünde (sampling area A, Fig. 3). North of the Kiel Fjord (sampling area C, Fig. 3) only black gobies were caught, with highest CPUE in the Flensburg Fjord (Tab. 3.2). Highest salinity was measured at the sampling site Eckernförde with 18 psu (Tab 3.2). Lowest salinities occurred at the sampling site of Travemünde with 13.8 psu (Tab 3.1). The salinity at the sampling site in Flensborg was about 14.8 psu. The sex ratio was 45.2 % male in Kiel and 61.4 % male in Travemünde. Thus the ratio of females is higher at the invasion front.

Conclusions and future perspectives

The hand line survey demonstrated the present occurrance of round goby at the Baltic coastline of Schleswig Holstein. This revealed that the actual position of the invasion front can be found in the area of Kiel Bight. Nevertheless that salinity in Flensborg was in a range similar to those in sampling area B (Fig.3) but no round gobies were caught. One explanation might be that the higher salinity in the Eckernförde bight acts as barrier. Furthermore there is much less ship traffic reaching the Flensborg bight (Fig. 4). In a recent study dispersal of round goby was found to be closely linked to shipping intensity (Kotta et al., 2015) see Appendix IV. However the expansion of colonised area by round goby across the status quo is likely. As mentionted above aduld gobies are able to resist considerably higher salinities than measured in Eckernförde.

According to our results round and black goby do not compete for food. Black goby condition is also not significant different in areas with presence or absence of round goby (unpublished Data, S. Matern, University Hamburg). Therefore the absence of Black goby in Travemünde is likely caused by habitat competition, e.g. suitable spawning substrates. Aggressive behaviour was already identified to be the main factor explaining competition in the Greate Lakes (Bergstrom & Mensinger, 2009). Potentially, these aggressive behavior enables round goby to outcompete black goby in the Western Baltic. A strong impact on resident juvenile flounder and turbot was recently reported (Ustups et al., 2016) Although males are more active swimmers and show more exploratory behavior (Marentette et al., 2011) more females were found in relation to males at the “invasion front” than at the “older” population. This result is confirmed by a study from the Danube River (Brandner, Cerwenka, Schliewen, & Geist, 2013). Thus the continuation of the hand line survey at the German Baltic coastline to investigate the further dispersal of round goby and its effects on black goby is planned for 2017.

Tab. 3.1: **General information on the catch composition**: Goby ratio = percentage of round goby of total gobiid catch.

Sampling site	**TM**	**NS**	**GB**	**HH**	**LA**	**LA**	**SM**	**TK**
Sampling area	**A**	**B**						
Sampling date	15-Sep-	16-Sep-2014	4-Sep-2014	5-Sep-	13-Aug-2014	2-Sep-	17-Sep-	11-Aug-
Fishing time [h]	6	6	6	6	6	-	2	6
Number of Angler	4	4	4	4	4	2	2	4
Salinity [PSU]	13.8	15.7	16.6	14.4	15.8	-	17.2	14.6
Surface water temperature [°C]	17.9	17.5	-	17.6	23.0	-	17.0	22.1
Number of Gobies	347	114	29	107	139	17	47	92
Neogobius melanostomus	347	14	5	51	3	1	17	3
Gobius niger	0	100	24	56	136	16	30	89
Goby ratio	1.00	0.12	0.17	0.48	0.02	0.06	0.36	0.03
CPUE *N. melanostomus*	14.50	0.58	0.21	2.13	0.13	-	4.25	0.13
CPUE *G. niger*	0.00	4.17	1.00	2.33	5.67	-	7.50	3.71
Bycatch								
CPUE bycatch	0.13	0.17	0.17	0.67	0.17	-	0	0.42
Pomatoschistus sp. Perca	2		4	15				
Gasterosteus	1	2						
aculeatus				1	3			9
Belone belone		2						
Zoarces viviparus					1			
								1

Tab. 3.2: **General information on the catch composition**: Goby ratio = percentage of round goby of total gobiid catch.

Sampling site	**TK**	**TK**	**EF**	**EF**	**KA**	**GE**	**GE**	**FB**	**FB**
Sampling area	**B**		**C**						
Sampling date	2-Sep-	17-Sep-	14-Aug-2014	11-Sep-2014	2-Oct-2014	12-Aug-2014	11-Sep-	15-Aug-2014	11-Sep-
Fishing time [h]	-	2	6	-	2.5	6	-	6	-
Number of Angler	2	2	4	2	3	4	2	4	2
Salinity [PSU]	-	12.9	18	-	-	15	-	14.8	-
Surface water temperature [°C]	-	17.0	17.5	-	17.4	23.3	-	19.9	-
Number of Gobies	24	24	98	22	34	155	20	364	20
Neogobius melanostomus	4	7	0	0	0	0	0	0	0
Gobius niger	20	20	98	20	32	155	20	364	20
Goby ratio	0.17	0.26	0.00	0.00	0.00	0.00	0.00	0.00	0.00
CPUE *N. melanostomus*	-	1.75	0.00	-	-	0.00	-	0.00	-
CPUE *G. niger*	-	5.00	4.08	-	-	6.46	-	15.17	-
Bycatch									
CPUE bycatch	-	3.00	0.25	-	0.40	0.13	-	0	-
Pomatoschistus sp.			2	2	2				
Perca fluviatilis			4			1			
Gasterosteus aculeatus		12							
Belone belone						2			
Zoarces viviparus									
Myoxocephalus scorpius					1				

Abbreviations:

EF: Eckernförde | FB: Flensburg | GB: Großenbrode | GE: Gelting | HH: Heiligenhafen | KA:|Kappeln | Kiel SM: Kiel / Schwentinemündung | Kiel TK: Kiel / Tiessenkai | LA: Laboe | NS: Neustadt in Holstein | SM: Kiel / Schwentinemündung |TK : Kiel / Tiessenkai | TM: Travemünde

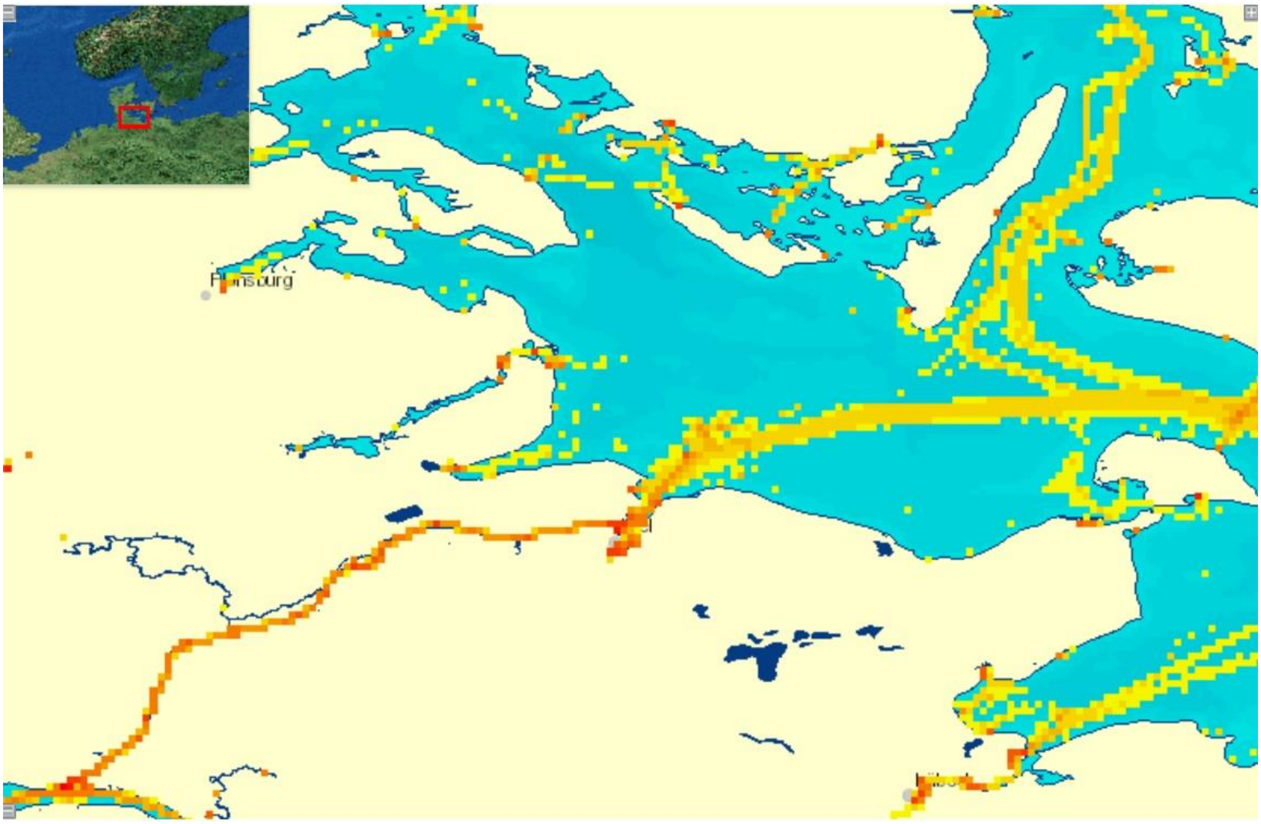


Fig. 4: **AIS based vessel density** per km² in year 2014 (https://www.geoseaportal.de/gdi-bsh-portal/ui)

References

Bergstrom, M. a., & Mensinger, A. F. (2009). Interspecific Resource Competition between the Invasive Round Goby and Three Native Species: Logperch, Slimy Sculpin, and Spoonhead Sculpin. *Transactions of the American Fisheries Society*, *138*(5), 1009–1017. http://doi.org/10.1577/T08-095.1

Brandner, J., Cerwenka, A. F., Schliewen, U. K., & Geist, J. (2013). Bigger Is Better: Characteristics of Round Gobies Forming an Invasion Front in the Danube River. *PLoS ONE*, *8*(9). http://doi.org/10.1371/journal.pone.0073036

Kotta, J., Nurkse, K., Puntila, R., & Ojaveer, H. (2015). Shipping and natural environmental conditions determine the distribution of the invasive non-indigenous round goby Neogobius melanostomus in a regional sea. *Estuarine, Coastal and Shelf Science*, (December). http://doi.org/10.1016/j.ecss.2015.11.029

Marentette, J. R., Wang, G., Tong, S., Sopinka, N. M., Taves, M. D., Koops, M. A., & Balshine, S. (2011). Laboratory and field evidence of sex-biased movement in the invasive round goby. *Behavioral Ecology and Sociobiology*, *65*(12), 2239–2249. http://doi.org/10.1007/s00265-011-1233-z

Skora, K. E., & Stolarski, J. (1993). New fish species in the Gulf of Gdansk, Neogobius sp. (cf. Neogobius melanostomus (Pallas 1811)). *Bulletin of the Sea Fisheries Institute*, *1*(128), 83–84.

Ustups, D., Bergström, U., Florin, A. B., Kruze, E., Zilniece, D., Elferts, D., … Uzars, D. (2016). Diet overlap between juvenile flatfish and the invasive round goby in the central Baltic Sea. *Journal of Sea Research*, *107*, 121–129. http://doi.org/10.1016/j.seares.2015.06.021

Appendix VIIa

Temperature and size-dependent functional response of sprat, *Sprattus sprattus* L.

R. Kulke; J-P. Herrmann, S. Kolodzey, L. Meskendahl, A. Temming (University of Hamburg, P3)

Growth and recruitment success of sprat depends strongly on water temperature and food availability (Peck et al. 2013). Despite the great economic and ecological significance of sprat, relationships between feeding rates and temperature were not investigated before. The present results serve as basis for estimations on food densities required for optimal growth and good recruitment success.

MATERIAL AND METHODS

Capture and maintenance of experimental fish

Young-of-the-year sprat were caught in September 2013 and 2014 in the Harbour of Wendtorf (Baltic Sea, 54°41'N; 8°36'8E) with a hand-operated dip-net (area: $4 m^2$; mesh size 6mm) and then transported in a 700-1000L box with aerated sea water to the aquarium facilities of the Institute of Hydrobiology and Fishery Science at the University of Hamburg. Prior to experiments, sprat were maintained in groups of 1000-2000 individuals in circular tanks (1,000L). Tanks were supplied with continuous flow of mechanically and biologically filtered, artificial sea water (Aqua medic) from the recirculation system. Before the experiments, sprat were slowly acclimated to laboratory conditions (Temperature = 12.0 ± 0.1 °C; Salinity = 16 PSU). Fish were maintained under a 13 L:11 D light regime and were fed an artificial pellet diet (Marico advance 0.5-0.8 mm, Coppens International bv) and live *Artemia salina* nauplii (SEPArt-Cysts, INVE Aquaculture).

Prey type

Non-evasive *Artemia salina* nauplii (771 ± 90 µm total length; N = 316; 0.0016 ± 0.0004 mg dry weight; N = 181) with low escape responses were used to determine the maximum feeding rate of sprat at different water temperatures and fish sizes. A detailed description of the rearing conditions for *A. salina* can be found in Brachvogel et al. 2013.

Experimental Tank

The experimental tank was separated into two parts, the fish chamber (square-shaped, 486L) where the sprat were kept and the collecting chamber (324L) where all prey items lost through the water over-flow (2.8 $L min^{-1}$) were collected (Figure 1). Feeding behaviour of sprat during experiments was recorded by an underwater camera (GoPro Hero3). The fish and collecting chambers were connected by a gentle circular water flow supplied with filtered (20 µm pore diameter) water from the recirculation system. An aerated *S*-shaped PVC-panel in the fish chamber caused a gentle vertical circular water flow within this chamber and promoted a homogeneous distribution of prey items. Prey items lost via the over-flow were collected every 10 min (time intervals = 0–10, 10–20, 20–30, etc.) in a 100-µm mesh-bottomed cup. To determine the actual prey concentration in the fish chamber all collected prey items from every time intervals were counted under a binocular microscope.

Experimental set-up

A set of experimental tanks was constructed to perform simultaneous experiments with similar sized sprat. Prior to the experiment fish were not fed for 24 hours. At the start of an experiment, a certain amount of prey was added to the experimental tank (fish chamber) to achieve an initial target prey concentration of 100-300 L^{-1}. During an experiment the prey concentration decreased exponentially due to feeding activity and the water overflow of the fish chamber. Experiments lasted until no feeding fish were observed on the screen (duration of experiment between 1.5-3 hours). After each experiment sprat were removed and killed rapidly by an overdose of anaesthetic (MS222). For every single fish, the biometric data was obtained and the stomach content was analysed in order to determine the percent of feeding fish.

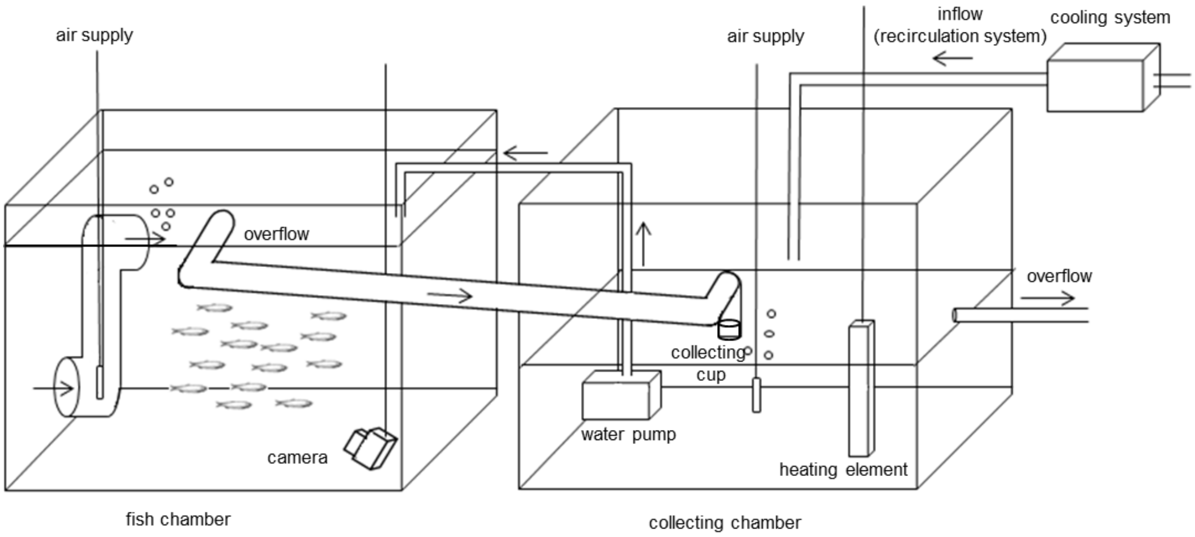


Figure 1: Diagram of experimental arrangement.

Temperature effect

The temperature effects on the functional response were investigated with sprat (87.2 ± 6.6 mm total length) at five temperatures: 5, 8, 12, 16 and 20°C (±0.2°C). Three to four experiments were performed with different fish groups of 20-30 individuals per temperature. Sprat were acclimatized slowly from an ambient temperature of 12°C to the target temperature in the experimental tank within one to four weeks (maximum change of water temperature per day = 0.6°C). The sprat for the 5°C experiments were kept separately in a storage tank.

Fish size effect

The fish size effect on the functional response was investigated with three different sprat size classes (87.2 ± 6.6, 63.0 ± 5.3 and 37.1 ± 3.2 mm total length) at 16°C. The data of the largest size class originated from the temperature effect experiments of the present study and the data for the mid-sized fish (63 mm) were adopted from Brachvogel et al. 2013. The experimental set-up was similar for all size classes besides tank dimensions: experimental tank was smaller (fish chamber = 239L and collecting chamber = 162L) and thus experiments were performed with less individuals of 10-20. The use of a larger experimental tank for the largest size class (87.2 mm) allowed normal feeding behaviour of sprat.

Data analyses

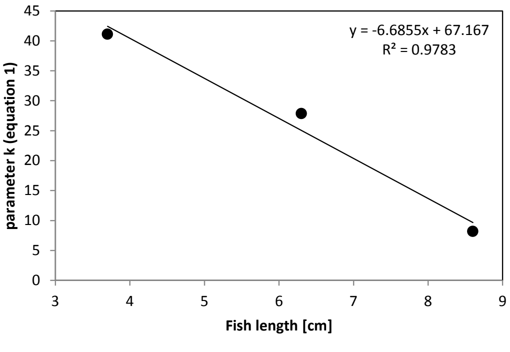
The prey concentration (Ct) for a 10-min time interval was calculated from Ct = Nt/FR, where Nt is the total number of prey items (N) in the collection-cup divided by the length of the time interval, and FR is the overflow rate (2.8-3.8 Lmin^{-1}). The recorded videos were played with half-speed to determine the average biting rate for each 10-min time interval of an experiment. In each 10-min time interval 10–20 individual fish were tracked. Fish were selected randomly. Each individual fish was tracked for 10–60 s, and its biting rate (biting acts s^{-1}) was determined visually. It was considered that only one prey item was consumed per biting act.

Model fitting

We analysed the feeding responses of sprat in relation to different prey concentrations of *Artemia salina* nauplii using nonlinear models where both temperature and fish-size-effects were included. The analysis was performed using *nls*-function in R (R Core Team 2014; Version 3.1.1). Model diagnostics followed descriptions in Ritz and Streibig (2008). A Michaelis-Menten-model, which is mathematically equivalent to Holling´s disc equation model (1959), was used to represent the expected biting rate (BR; s^{-1}) as a function of prey concentration (conc; L^{-1}):

$$\mathrm{BR} = BRmax * \mathrm{conc} \, / \, (k + \mathrm{conc}) \qquad (1)$$

Three different models were used where temperature (T; °C) and length effects (cm TL) for the two parameters *k* and *BRmax* were included. Models were compared via AIC following an IT-Approach (Burnham and Anderson 2004; Mazerolle 2006). As a first step, we applied equation (1) to all data measured at 16°C grouped per fish size and separately to all data measured for 8.7 cm fish grouped per temperature. This allowed visualising trends of *BRmax* and *k* with either temperature or fish size. The parameter *k* decreased linear with fish size, whereas *BRmax* increased with fish size (Figure 2), but not clearly in a linear way. Both parameters increased with temperature in a nonlinear function (Figure 3). Thus, temperature and length effects were included as a nonlinear model adopted from Lefébure et al. (2014) for *BRmax* in two of the tested models, whereas length effects were model to be linear. Finally, a set of three models was compared and the model with the highest probability of being the best was chosen as final model.



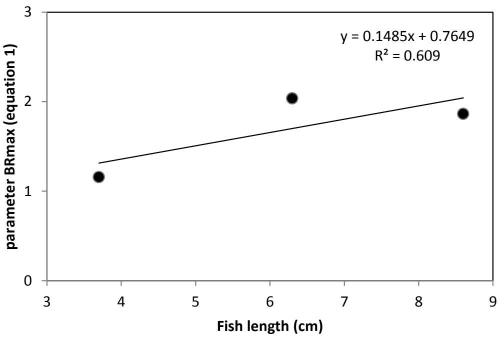


Figure 2. Change of the two parameters (BRmax and k) from equation 1 with fish size [cm total length].

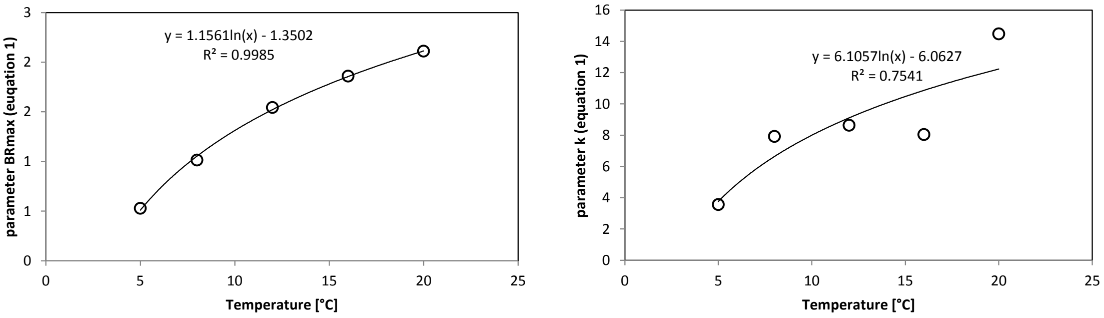


Figure 3. Change of the two parameters (BRmax and k) from equation 1 with water temperature [°C].

RESULTS

The feeding rates for all size classes of sprat and for each experimental temperature followed Holling's type II functional response curve. The frequency of feeding fish was high, but decreased slightly from 98.7% at 20°C to 84.5% 8°C (Figure 4). Only at 5°C much less fish were feeding (26.3%). Feeding rates of sprat on *A. salina* increased with increasing temperatures (Figure 5). Of the three tested models to explain functional response of sprat in relation to temperature and fish length, we finally chose Model 3 as the model with the highest probability of being the best (Table 1). Model 1 assumes linear relationships of *BRmax* and *k* for length and temperature, the other two models include a nonlinear relationship for *BRmax*. As Model 3 has the lowest AIC and lowest number of parameters, we suggest using this formula for further applications. Using our final model 3 (Table 1), we calculated three different Q_{10} values for the different fish sizes. Small fish with 3 cm TL have a Q_{10} of 3.3, larger fish with 6 cm TL have a Q_{10} of 1.8 and the largest fish have a Q_{10} of 1.6. There was a clear size effect for the functional response of sprat preying on *A. salina* nauplii. BRmax increased with increasing fish size whereas the parameter k decreased with increasing fish size (Figure 2). The difference was especially pronounced between 3.7 cm fish and the larger ones. The smallest fish had much lower maximum biting rates than fish of 6.3 or 8.7 cm total length.

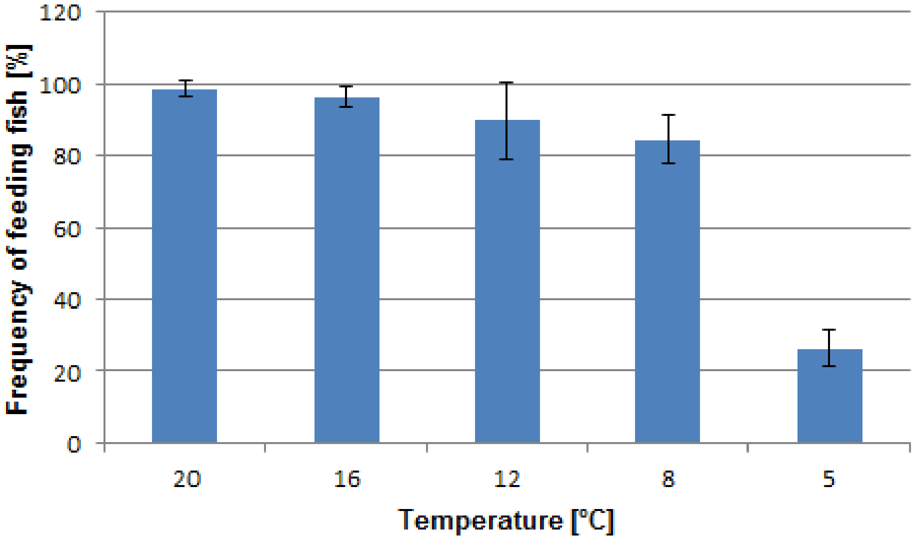


Figure 4: Frequency of feeding sprat [%] per temperature [°C] when preying on *A. salina.*

Table 1: Set of fitted models to explain functional response (BR; s^{-1}) of sprat preying on *A. salina* nauplii at various concentrations (conc; L^{-1}) in relation to different temperatures (T; °C) and fish lengths (L; cm total length)

Model	Formula	*P*	Estimate	AIC
1	*BR* = $\frac{((a\_BRmax*\,T\,-\,c\_Brmax\,*\,L\,+\,b\_BRmax)\,*\,conc))}{((a\_k\,*T\,-c\_k*L+b\_k)\,+\,conc))}$	*a_BRmax* *b_BRmax* *c_BRmax* *a_k* *b_k* *c_k*	0.116 -1.318 -0.158 0.372 26.041 2.292	-434
2	*BR* = $\frac{((-a\_BRmax+b1*\log(T)+b2*\,\log(L))\,*\,conc))}{((a\_k\,*T\,-c\_k*L+b\_k)\,+\,conc))}$	*a_BRmax* *b1* *b2* *a_k* *b_k* *c_k*	3.249 1.115 0.938 0.029 33.433 2.617	-507
3	*BR* = $\frac{((-a\_BRmax+b1*\log(T)+b2*\,\log(L))\,*\,conc))}{((-c\_k*L+b\_k)\,+\,conc))}$	*a_BRmax* *b1* *b2* *b_k* *c_k*	3.324 1.111 0.937 33.946 2.626	-509

Note: *P* = parameter; AIC = Akaike´s information criterion; log = natural logarithm

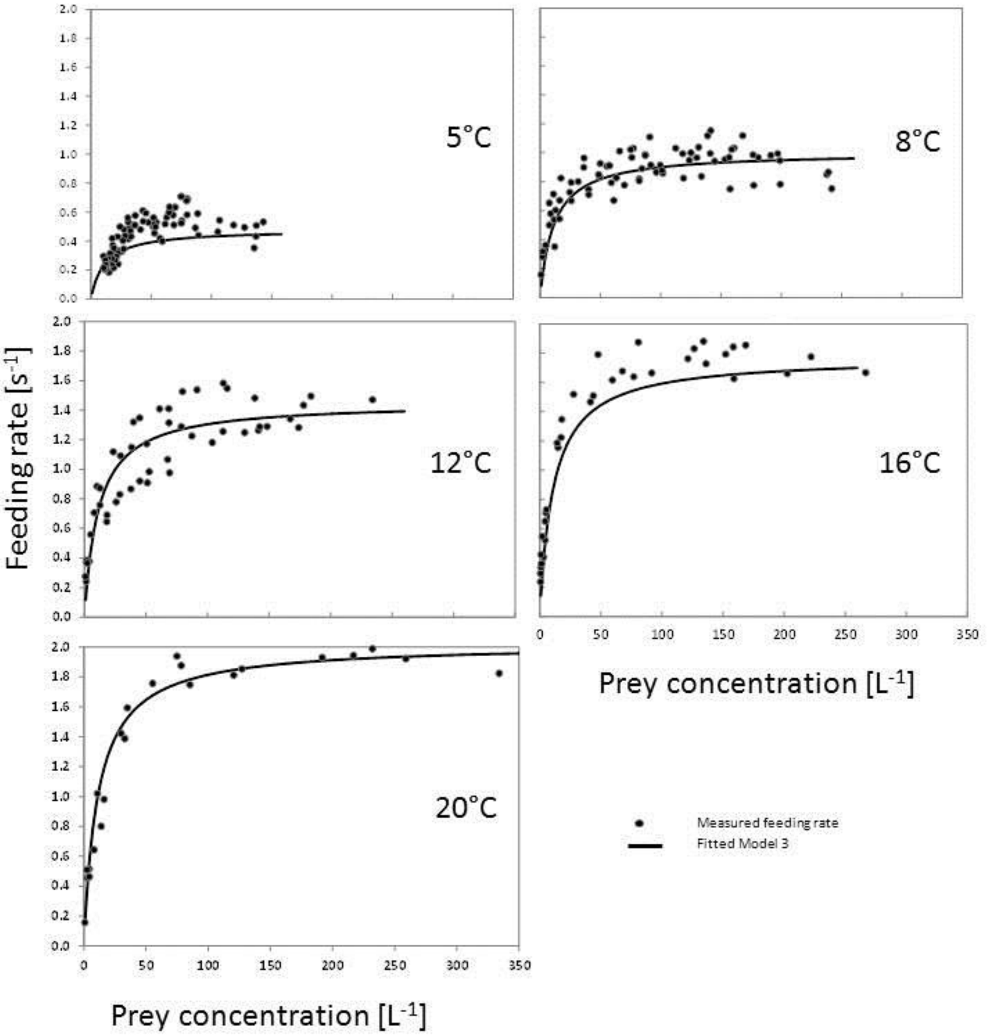


Figure 5. Measured (dots) and modelled (lines) feeding rates of sprat (~ 8.7 cm total length) feeding on different concentrations of *A. salina* nauplii at five temperatures. For details on Model 3 see Table 1 and text.

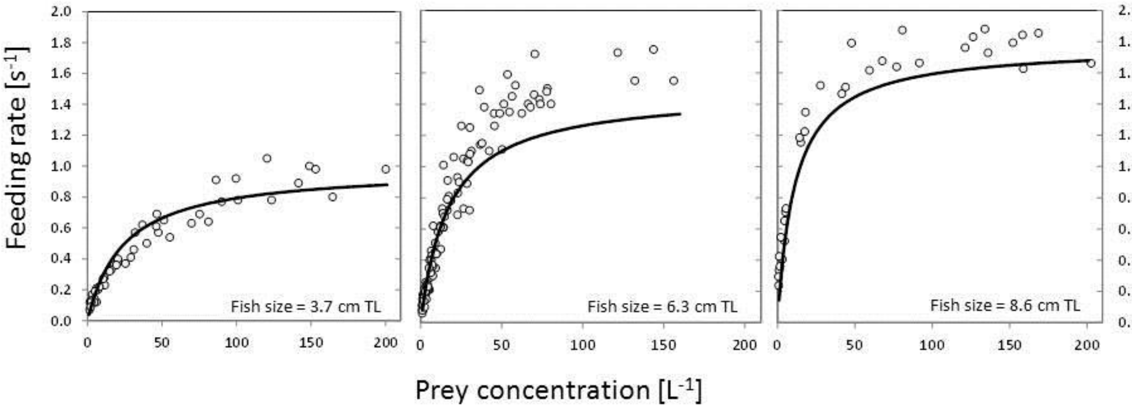


Figure 6. Measured (dots) and modelled (lines) feeding rates of sprat at 16°C feeding on different concentrations of *A. salina* nauplii separated by fish size. For details on Model 3 see Table 1 and text.

DISCUSSION

The present results indicate strong relationships between functional response and temperature in sprat. Studies on roach and perch have demonstrated strong effects of temperature on feeding rates (Q_{10} = 3.1-3.7; Linløkken et al. 2010). These values are similar to those found in the present study for our smallest fish size class (Q_{10} = 3.3). However, larger fish had (according to our final model), much lower Q_{10} values. Small sprat exhibits other environmental conditions than larger fish, which undertake daily vertical migrations associated with rapid changes in temperature. Juvenile fish, however, are mainly abundant in the coastal zones or upper water layers with higher and more constant temperatures. Thus, they are able to feed faster at higher temperatures, whereas larger individuals (adults) may reach their maximum possible biting rates at even lower temperatures.

The proportion of feeding sprat was high at the temperature range from 20°C to 8°C (> 80%; Figure 2). This corresponds to field observations made in spring and summer (Bernreuther 2007). However, at 5°C only 26.3% of sprat were feeding in our study. Stomach data from March 2013 (western Baltic Sea) revealed a similar rate of 25.24% at 1.07 ± 0.28 °C (unpublished data). This indicates that sprat is able to feed over the entire year even at relatively low temperatures, but benefits from higher water temperatures above 8°C.

Feeding rates of sprat increased with body size in the present study, which is consistent with previous findings for other fishes (Breck and Gitter 1983; Hjelm and Persson 2001; Persson 1987; Persson et al. 1998). Larger sprat can pronounce higher absolute swimming speeds than smaller ones, so that they are able to reach the next prey item much faster than smaller sprat. In addition, the visual faculty of larger sprat might be better than for small juveniles due to higher development of their retina. Thus, larger fish might detect prey items better even at a higher distance than smaller sprat. The size-dependent development of fish retina was demonstrated for the bluegill (*Lepomis macrochirus*) by Hairstone et al. (1982). Blaxter and Jones (1967) also found for herring (*Clupea harengus*) a positive correlation between the retinal development and body size. Another possible reason for the higher feeding rates is that handling time decreases exponentially with increasing fish size and then remains constant above a given fish size (Werner et al. 1981, Mittelbach 1981, Gill and Hart 1994).

REFERENCES

Bernreuther M. 2007. Investigations on the feeding ecology of Baltic Sea herring (*Clupea harengus* L.) and sprat (*Sprattus sprattus* L.). Dissertation, Universität Hamburg

Blaxter J.H.S. and Jones M.P. 1967. The development of the retina and retinomotor responses in the herring. Journal of the marine biological Association UK 47: 677-697.

Brachvogel R., Meskendahl, L., Herrmann, J.-P., and Temming, A. 2013. Functional responses of juvenile herring and sprat in relation to different prey types. Marine Biology 160:465-478.

Breck J.E. and Gitter M.J. 1983. Effect of fish size on the reactive distance of bluegill (*Lepomis macrochirus*) sunfish. Canadian Journal of Fisheries and Aquatic Sciences 40: 162–167.

Burnham K.P. & Anderson D.R.S. 2004. Multimodel inference. Understanding AIC and BIC in Model Selection. Sociological Methods & Research, Vol. 33 (2), 261-304.

Gill A.B. and Hart P.J.B. 1994. Feeding behaviour and prey choice on the threespine stickleback: the interacting effects of prey size, fish size and stomach fullness. Animal Behavior 47: 921-932.

Hairston N.G., Li K.T. and Easter S.S. 1982. Fish vision and the detection of planktonic prey. Science. New Series 218(4578): 1240-1242.

Hjelm J. and Persson L., 2001. Size-dependent attack rate and handling capacity: inter-cohort competition in a zooplanktivorous fish. Oikos 95, 520–532.

Lefébure R., Larsson S. and Byström P. 2014. Temperature and size-dependent attack rates of the three-spined stickleback (Gasterosteus aculeatus); are sticklebacks in the Baltic Sea resource-limited? Journal of Experimental Marine Biology and Ecology 451: 82-90.

Linløkken, A.N., Bergman, E. and Greenberg, L. 2010. Effect of temperature and roach *Rutilus rutilus* group size on swimming speed and prey capture rate of perch *Perca fluviatilis* and *R. rutilus*. J. Fish Biol.76: 900-912.

Mazerolle, M.J. 2006. Improving data analysis in herpetology: using Akaike´s Information Criterion (AIC) to assess the strength of biological hypotheses. Amphibia-Reptilia 27: 169-180.

Mittelbach, G.G. 1981. Foraging efficiency and body size: A study of optimal diet and habitat use by Bluegills. Ecology 62(5): 1370-1386.

Persson L., 1987. The effects of resource availability and distribution on size class interactions in perch, Perca fluviatilis. Oikos 48: 148–160.

Persson L., Leonardsson K., De Roos A.M., Gyllenberg M. and Christensen B. 1998. Ontogenetic scaling of foraging rates and the dynamics of a size-structured consumer–resource model. Theor. Popul. Biol. 54, 270–293.

Ritz, C., and Streibig, J.C. 2008. Nonlinear regression with R., Series Use R!, *Edited by* R. Gentleman, K. Hornik, and G. Parmigiani, Springer, Berlin.

R Core Team (2014) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL http://www.R-project.org/

Werner E.E, Mittelbach G.G. and Hall D.J. 1981. Foraging profitability and the role of experience in habitat use by bluegill sunfish. Ecology 62: 116-125.

Appendix VIIb

Another critical period: the importance of timing of the early juvenile stage for the potential growth performance in Baltic sprat

Günther, C., Temming, A., Meskendahl L., Kulke R., Herrmann JP. (University of Hamburg, P3)

Abstract

Most recruitment determining mechanisms in marine fish species act in the earliest life-stages, when mortality is highest. In Baltic sprat, a clupeid schooling fish with an extended spawning season, extensive research effort was invested to uncover processes in the larval stage that regulate year-class strength. However, the large amount of recruitment variability is still unexplained. Previous studies showed that the amount of larvae is unrelated to the number of YoY-recruits, highlighting the importance of the post-larval life-stage as a critical period modulating year-class-strength. In order to detect recruitment regulating mechanisms in the post-larval life-stages of Baltic sprat we performed a simulation study on growth and food demand of YoY-sprat wrapping up a comprehensive database on otolith-derived growth rates and experimental investigations on feeding habits. We assumed a spawning time from February to August and modeled growth of various seasonal cohorts in relation to temperature. In a first simulation approach, we converted the daily length increases into the equivalent required amount of energy, applying a bio-energetic budget approach. We found that seasonal cohorts originating from the first half of the spawning season have a high total energy demand in the juvenile stage in contrast to later spawned cohorts despite a similar growth performance of early and late born sprat. Later spawned cohorts experience comparatively lower temperatures as early juveniles which reduces their metabolic costs in an already food demanding life stage. In a second simulation approach we calculated maintenance concentrations for seasonal cohorts applying a length and temperature dependent relationship between prey concentrations and snatching rates. We found that cohorts from the first half of the spawning season, which have the potential to grow fast, soon rely on high maintenance rations to fulfill the metabolic demands of a large body size. In contrast, later cohorts with a similar growth potential reach large body sizes accompanied by high maintenance rations later in the season. Thus, the risk of starvation and depletion of energy reserves before the onset of winter is higher for earlier than for later born cohorts. A high cumulative energy demand and the disadvantage of being too large too early in the season, have the potential to decrease survival rates of early born cohorts and thus underline the importance of the right timing of the post-larval, early juvenile stage. Our results are supported by previous studies showing that successful YoY-recruits of Baltic sprat stem from the second half of the spawning season only.

Introduction

Recruitment in Baltic sprat in highly variable (ICES 2015) and not well understood. Previous studies have pointed out that mechanisms influencing year-class strength take place after the larval phase in the early juvenile stage (Köster et al. 2003, Baumann et al. 2006) However, this life-stage was not adequately sampled during last projects (STORE, GLOBEC) which investigated the population dynamics of Baltic sprat focusing on offshore areas and basins where spawning of sprat takes place. The reason for that can be twofold: First, young sprat are too large to be sampled by standard plankton nets (e.g. Bongo nets) but too small to be caught by the fishing net and second, main nursery habitats of young sprat are probably located in shallow coastal habitats which were not in the focus of sprat recruitment research so far. Despite an expanded spawning season of sprat from February to August, recruits stem from eggs spawned late in the season (~July, Baumann et al. 2008) when maximal spawning effort has already taken place (~May, Voss et al. 2012). Baumann et al. (2006) found a high correlation ($r^2 > 0.7$) between recruitment strength of sprat and surface temperatures in August in the central Baltic Sea They suggest that late cohorts benefit from high summer temperatures in the larval stage resulting in fast growth rates of these cohorts and a low cumulative mortality. However, biological mechanisms behind these correlations are not understood and the fate of early cohorts remained uncertain. In a further study analyzing the temporal origin of post-larval metamorphosing sprat sampled in near-shore areas in summer time, Günther (2008) found individuals originating from the first half of the spawning season with first feeding dates in April. However, most recruits sampled in autumn of the same year were summer born and thus originate from later spawned eggs. Thus, mechanisms acting in the shallow coastal zones of the Baltic Sea during the post-larval early juvenile stage may modulate the survival pattern and can broaden our understanding why early cohorts disappear while late cohorts survive until autumn. During the post-larval stage, sprat exhibit strong growth rates while experiencing the highest temperatures during their whole development. At the end of the larval stage, sprat start to increase their body weight and height during metamorphosis (Günther et al. 2012) and then increase their length and fat reserves during the juvenile stage. To maintain high growth rates, food demand is steadily increasing as sprat become larger: A 50 mm sprat consumes ten times more than a 30 mm individual under *ad libitum* conditions in the laboratory (Günther et al. 2015). Furthermore, the required food needed to growth at high temperatures (16°C) is 30% higher than feeding at lower temperatures (22°C). Thus, the requirements of the nursery habitats concerning food availability are high and may be critical for the growth performance in the juvenile life-stage.

In this study, we investigated physiological boundaries that might emerge in juvenile nursery habitats and can have consequences for growth performance, survival and recruitment of Baltic sprat. To investigate the fate of different seasonal cohorts originating from the extended spawning season, we used two different simulation approaches modeling the growth of seasonal cohorts as a function of temperature. In a backwards approach (1) we reconstructed the energy demand of seasonal cohorts. In a forward approach (2), we calculated the maintenance and optimum ration young sprat need during the seasons. To validate the model results we analyzed four different years (2002, 2003, 2006, and 2007), where previous studies uncovered the temporal origin of autumn survivors by the microstructure analysis of otoliths (Baumann et al 2008, Günther et al 2012).

Material & Methods

General ideas of simulation approaches

Both simulation approaches performed in this study are based on similar assumptions (Figure 1). In the first approach (1), we simulated the length growth as a function of temperature and deduced the food demand (backwards approach) and in a second approach

(2), we assumed a food concentration and calculated the theoretical length growth (forward approach). In the backwards approach (1) seasonal cohorts start to develop as an egg according to a spawning period. Eggs develop to larvae, and larvae grow in length and become juveniles. The simulated daily length growth is than converted into a mean energy demand in Joule and to a corresponding number of *Acartia sp*. assuming a bio-energetic budget equation. In the forwards approach (2), the simulation of length growth starts with 30 mm, e.g. in the post-larval stage. Every day, a length growth increment is added to the length of a cohort. This growth increment is calculated assuming a certain plankton concentration. Using a relationship between plankton concentration and snatching rate at different length and temperatures (Kulke et al. 2016), we calculated the number of prey items (*Acartia sp*.) that can theoretically be consumed per day. The daily food amount is than converted to the daily length increment assuming the same bio-energetic budget equation as in the backwards simulation approach (1). In the best case (optimal growth) the daily length increment corresponds to the growth performance reconstructed from otoliths of successful recruits. The prey concentration when growth of a cohort is next to zero is defined as the maintenance concentration needed to keep up metabolic functions. This simulation is performed with various plankton concentrations to estimate optimum/maintenance rations for a large range of length classes and calendar days. We performed both simulation approaches applying surface temperature profiles from the years 2002, 2003, 2006 and 2007 measured at Kiel Lighthouse in the Western Baltic Sea. For these four years, microstructure otolith data of YoY-sprat exist documenting which seasonal cohorts were “successful” and

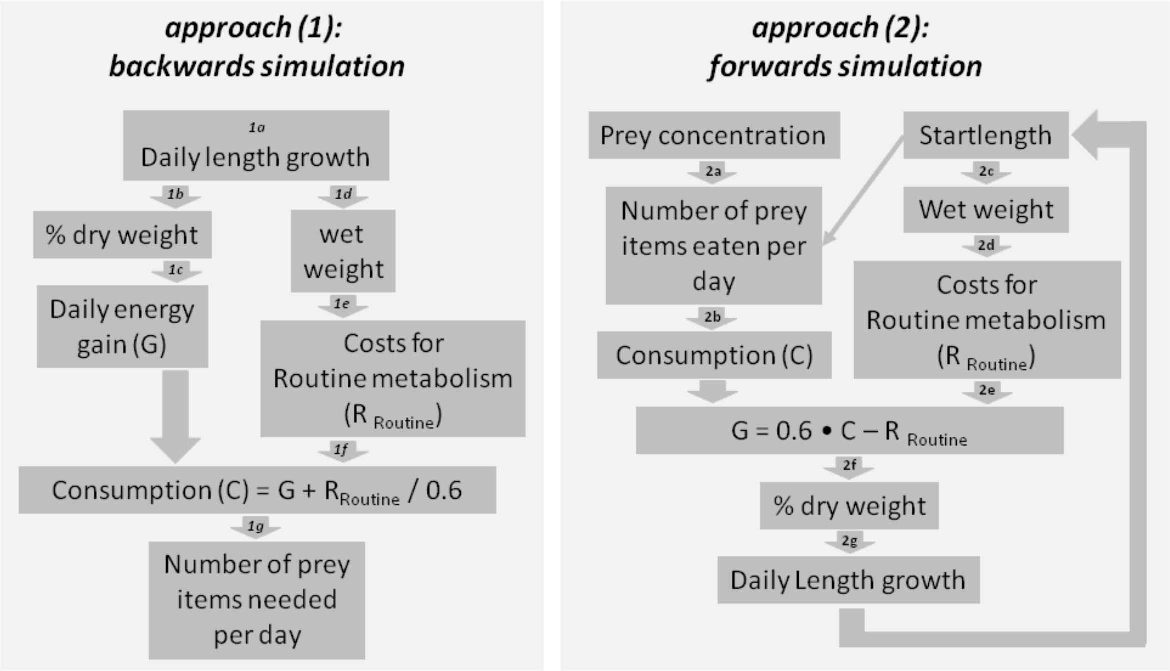


Figure 1: Schematic description and calculation steps of both simulation approaches; numbers and letters indicate different calculation steps (see text).

Backwards simulation

Early development and length growth

The first step of this approach was the simulation of length growth of different seasonal cohorts (step 1a, Figure 1). We simulated the growth of 19 seasonal cohorts assuming a spawning period from February/March to the middle of August. All cohorts start with a temporal distance of 10 days as recently spawned eggs. Egg and early larval development start was modeled as a function of temperature according to Petereit et al. (2008). The fractional contribution of daily egg/larval development was calculated based on the respective temperature of the day. The larvae start to feed and start to grow with a length of 5mm standard length according to Alshuth et al. (1988). Functions for length growth of larval and juvenile sprat in relation to temperature were derived from otolith-based back-calculated length growth rates at corresponding temperatures measured in the field. Hereby, we used otolith data on daily increment growth of more than 800 fishes sampled in the Baltic Sea during previous studies comprising YoY-sprat from 2002 and 2003 from Baumann et al (2008) as well as 2006 and 2007 from Günther et al. (2012). Daily otolith growth was converted to length growth using a non-linear back-calculation method taking into account the reduction of length growth during metamorphosis from larvae to juveniles (Günther et al. 2012). We established linear functions of daily length growth rates in relations to temperature measured in the field for 5 mm intervals resulting in 4 equations for the larval stage (5-25 mm), 5 equations for the early juvenile phase (25-50 mm)and six equations for the juvenile stage (50-80 mm)(Table 1).

Table 1: Simulation settings for development and growth of seasonal cohorts of YoY-sprat (calculation step 1a); T: temperature

Parameter/setting and description	Unit	Equation	Source
GR_{egg}, Egg developement	% of total development	$GR_{egg} = (3.9255*T^{0.6296})*0.01$	Petereit *et al*. 2008
GR_{yolk}, Yolk sac development	% of total development	$GR_{egg} = 1.7621*T^{0.7688}*0.01*4$	Petereit *et al.* 2008
$GR_{larvae\ I}$, length growth 5-10mm	mm	$GR_{larvae\ I} = 0.0055*T+0.5949$	this study
$GR_{larvae\ II}$, length growth 10-15mm	mm	$GR_{larvae\ II} = 0.0304*T+0.1409$	this study
$GR_{larvae\ III}$, length growth 15-20mm	mm	$GR_{larvae\ III} = 0.0235*T+0.1342$	this study
$GR_{larvae\ IV}$, length growth 20-25mm	mm	$GR_{larvae\ IV} = 0.0095*T+0.2113$	this study
$GR_{early\ juv\ I}$, length growth 25-30mm	mm	$GR_{early\ juv\ I} = 0.0332*T+0.1819$	this study
$GR_{early\ juv\ II}$, length growth 30-35mm	mm	$GR_{early\ juv\ II} = 0.0306*T+0.1415$	this study
$GR_{early\ juv\ III}$, length growth 35-40mm	mm	$GR_{early\ juv\ III} = 0.0361*T+0.175$	this study
$GR_{early\ juv\ IV}$, length growth 40-45mm	mm	$GR_{early\ juv\ IV} = 0.0256*T+0.4438$	this study
$GR_{early\ juv\ V}$, length growth 45-50mm	mm	$GR_{early\ juv\ V} = 0.0217*T+0.5494$	this study
$GR_{juv\ I}$, length growth 50-55mm	mm	$GR_{juv\ I} = 0.0219*T+0.5129$	this study
$GR_{juv\ II}$, length growth 55-60mm	mm	$GR_{juv\ II} = 0.0278*T+0.3387$	this study
$GR_{juv\ III}$, length growth 60-65mm	mm	$GR_{juv\ III} = 0.0368*T+0.0753$	this study
$GR_{juv\ IV}$, length growth 65-70mm	mm	$GR_{juv\ IV} = 0.0431*T-0.134$	this study
$GR_{juv\ V}$, length growth 70-75mm	mm	$GR_{juv\ V} = 0.0355*T-0.971$	this study
$GR_{juv\ VI}$, length growth 75-80mm	mm	$GR_{juv\ VI} = 0.0299*T-0.0864$	this study

Consumption and number of prey items

We converted the daily length increase of each cohort into daily amounts of energy needed to perform the simulated growth and followed hereby roughly the protocol described in Günther et al (2015).The general equation of this conversion is the energy balance equation of Winberg (1960):

$$C = G + R_{Routine} + R_{feedact} + R_{SDA} + E + F \qquad (1)$$

Here the consumption (C) is the sum of growth (G), losses for excretion (E) and faeces (F), and metabolic costs for routine respiration ($R_{Routine}$), feeding activity ($R_{feedact}$) and specific dynamic action (R_{SDA}). All addends of equation (1) were transferred in Joule. The terms E, F,

$R_{feedact}$ and R_{SDA} were assumed to be 10 % of the total consumption which denotes a conservative estimation of conversion efficiency. For the term G, the length of a cohort at a specific day was converted into percent dry weight of wet weight (step 1b) following a segmented regression on % dry weight versus standard length (Table 2). This regression based on a comprehensive dataset of early juveniles (Günther et al. 2008) and juveniles sampled during July to August in 2006. The water content (reciprocal value of %DW) was converted into an energy content (step 1c) following the equation published in Günther et al (2015) (see Table 2). The energy content was multiplied with a length specific dry weight (Table 2) to calculate the energy content of an individual. By using the difference between two lengths of consecutive days the energy gain of a specific day was received which was used as term G in equation (1). Costs for the routine metabolism (term $R_{Routine}$) were calculated in three steps: We converted length into wet weight (step 1d, Figure 1) using a simple allometric function (Table 2). According to Meskendahl et al. (2010) we calculated the metabolic costs for the routine metabolism (step 1e in Figure 1, Table 2) and used an oxy-caloric factor for the conversion into Joule published by Elliott & Davison (1975) (step 1f in Figure 1, Table 2). Using the daily energy gain and the costs for routine metabolism, we calculated the energy which is needed to be consumed daily by a single sprat from a seasonal cohort using equation (1). Furthermore, we assumed that sprat is feeding on *Acartia* which is an abundant copepod in the western Baltic Sea and converted the consumption into individuals consumed per day.

Table 2: Equations and formulas needed for the conversion of length growth into consumption (backwards approach (1)); DW:dry weight; WW: wet weight; SL: standard length; WC: water content; T:temperature; TL: total length; conc: concentration of *Artemia salina*.

Parameter/setting and description	Unit	Equation	Calculation step (Figure 1)	Source
%DW <49mm; % dry weight of wet weight		%DW=0.107*SL+14	1b, 2f	Günther 2008;
%DW >49 & < 75mm; % dry weight of wet weight		%DW=0.433*SL-2.5312	1b, 2f	Günther 2008
%DW >=75mm; % dry weight of wet weight		%DW=0.1*SL+20.8	1b, 2f	Herrmann et al *unpublished data*
EC; Energy content	$J*g^{-1}$	EC =-28964*WC+46153	1c, 2g	Günther et al 2015
DW; dry weight	G	$DW = 5E\text{-}08*SL^{3.9673}$	1c, 2g	Günther et al. 2008
WW; wet weight	G	$WW=1R\text{-}06*SL^{3.5599}$	1d, 2c	Günther et al 2008
$R_{routine}$; Costs for Routine metabolism	$mgO_2\ h^{-1}$	$R_{routine}=0.074*WW^{1.077}*e^{0.08*T}$	1e, 2d	Meskendahl et al 2010
$R_{routine}$; Conversion into Joule	$J*d^{-1}$	$R_{routine}=R_{routine}[mgO_2\ h^{-1}]*13.72\ J\ mgO_2^{-1}*24h$	1f, 2d	Elliott & Davison 1975
EC prey; energy	$J*Ind^{-1}$	EC prey=0.17	1g, 2b	Kerambrun

content of *Acartia sp.*				1987
SR; Snatching rate	Ind*sec^{-1}	SR=((3.23791+1.11154*log(T)+0.93713*log(TL))*conc)/((-2.62572*TL+33.94642)+conc)	2b	Kulke et al. 2016.

Forwards simulation

In the forward simulation, seasonal cohorts start to grow beginning with a length of 30 mm according to model assumptions, temperature and prey concentration. Each simulation day, a daily length growth increment is calculated and added to the start length at the corresponding day. The resulting length is the start length at the following simulation day.

The central step in the forward approach is the conversion of the prevailing prey concentration into the number of prey items that can theoretically be consumed by a fish (Figure 1, step 2a). The equation used here (Table 2) bases on a comprehensive experimental study from Kulke et al. (2016), where the feeding/snatching rate of young sprat was estimated as a function of prey concentration (*Artemia salina*) for a length range from 4-8 cm at 5, 8, 12, 16, and 20°C. In general, Kulke et al. (2016) showed that snatching rate increases with length and temperature. Assuming that feeding takes place during daylight, we calculated the energy amount that can be consumed assuming that young sprat feed on *Acartia* and that snatching rates for *Acartia* are the same as for *Artemia salina*. (Figure 1, step 2b). In the reverse way to the backwards simulation approach, we used the daily consumption to calculate the energy gain accounting for a temperature dependent routine metabolism (Figure 1, step 2e) and assuming the same bio-energetic budget than in approach (1) (Figure 1).

Lastly, the daily energy increase was converted into the daily length growth increment. Therefore, the start length at the corresponding simulation day was translated into percent dry weight and total energy content analog to simulation approach (1) (Figure 1, step 2f and 2g; Table 2). The energy gain calculated in the steps 2a – 2e, was then added to the total energy content. Finally, the energy content was re-converted into length to estimate the daily length growth increment. Daily length increase was limited according to growth rates back-calculated from otoliths of autumn caught survivors.

Simulation runs were performed for different prey concentrations (0.01-10 Ind•L^{-1} by 0.01 Ind•L^{-1} steps; n =100) which were kept constant over the season. Length, day and prey concentration for each cohort were extracted when growth was almost zero which corresponds to the maintenance ration. The forwards simulation was restricted to the year 2003, which is the year with highest recruitment of the four years examined with the backwards approach.

Results

Backwards simulation

The simulation of length growth shows that cohorts from the middle of the spawning season (May) were largest 150 days after spawning (Figure 2e-h) irrespective of the simulation year despite distinct differences in input temperature profiles (Figure 2a-d). Lowest length growth can be observed in cohorts that started at the beginning and at the end of the spawning season. However, these cohorts were judged as unsuccessful as they could not be sampled in late summer and autumn (black lines in Figure 2). In contrast, successful cohorts (red lines in Figure 2) exhibit various growth performances including the best ones (cohorts from May) in 2002 and 2007. Interestingly, the main cohort of survivors (thick red line, Figure 2) is not the cohort with the largest length growth potential. The number of prey items needed to accomplish these length growth performances varies strongly between seasonal cohorts (Figure 2i-l). Except of the first cohorts, early cohorts including those spawned in May need the highest prey numbers per day. This is especially the case for larger individuals. Irrespective of the simulation year, later cohorts like the main cohort of survivors, need a lower number of prey items while performing similar length growth compared to the best growing cohort from May.

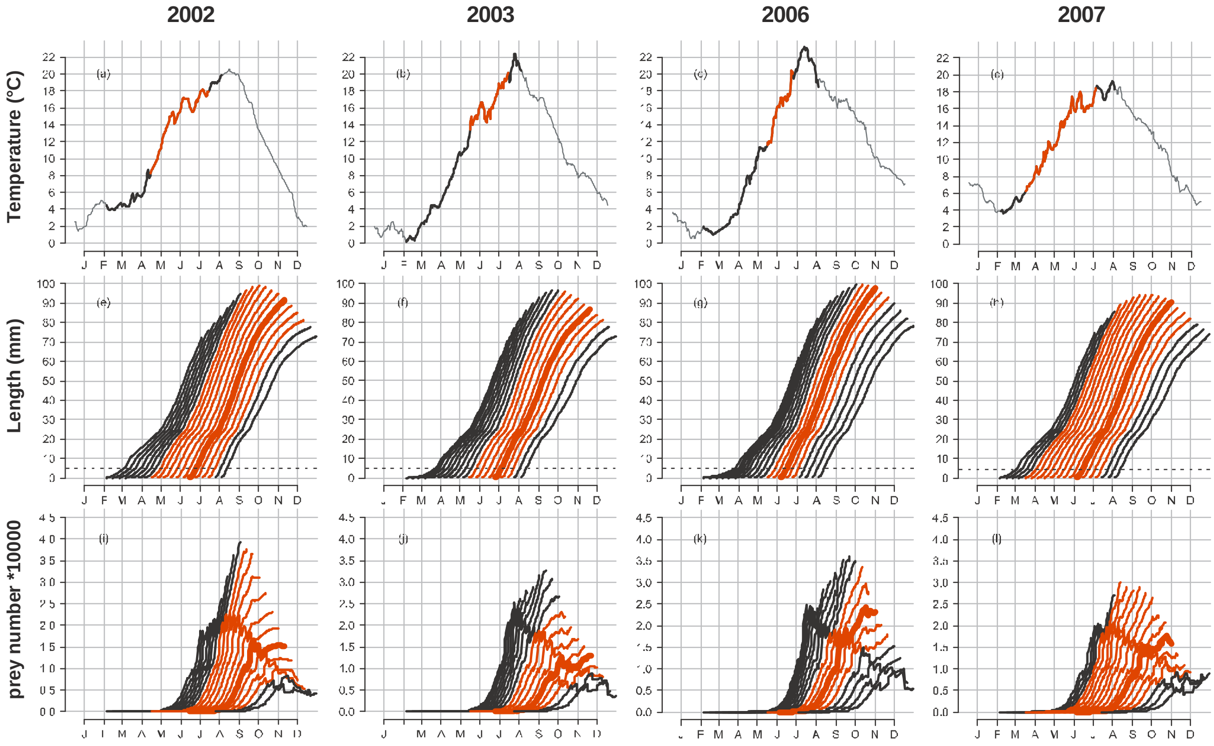


Figure 2: Results of backward simulation; a-d: surface temperature profiles measured at Kiel Lighthouse in the corresponding simulation years; e-h: simulated length growth of seasonal cohorts for 150 days; dashed horizontal line marks the 5 mm limit when length growth started (all length lower than 5mm are artificial and display the fractional development rather than length); i-l: daily consumed number of prey items ingested per sprat assuming feeding on Acartia spp. In the first 150 days. Red lines represent cohorts that exhibit the day of first increment formation at the same time than YoY-survivors which were sampled in late summer and autumn. The fat red line indicates the main cohort of survivors. Black lines indicate cohorts resulting from the extended spawning season that have not been sampled in autumn.

Forwards simulation

The prey concentration needed for maintenance, i.e. balancing metabolic costs without growing, is determined by length and season. The concentration for maintenance of young sprat increased with increasing length. For instance, in September 2003 (Figure 3), individuals with a length of about 50 mm could balance their metabolic costs with a constant prey concentration of 0.5 Ind/L^{-1}. With a length of about 60 mm, the concentration doubles and exceeds 2.0 Ind/ L^{-1} when sprat reach a length of 70 mm. With progressing season, the prey concentration needed to fulfill metabolic demands increases. Following the length growth trajectories generated with the backwards simulation, the earliest cohorts need the lowest prey concentrations to raise maintenance costs at distinct sizes.

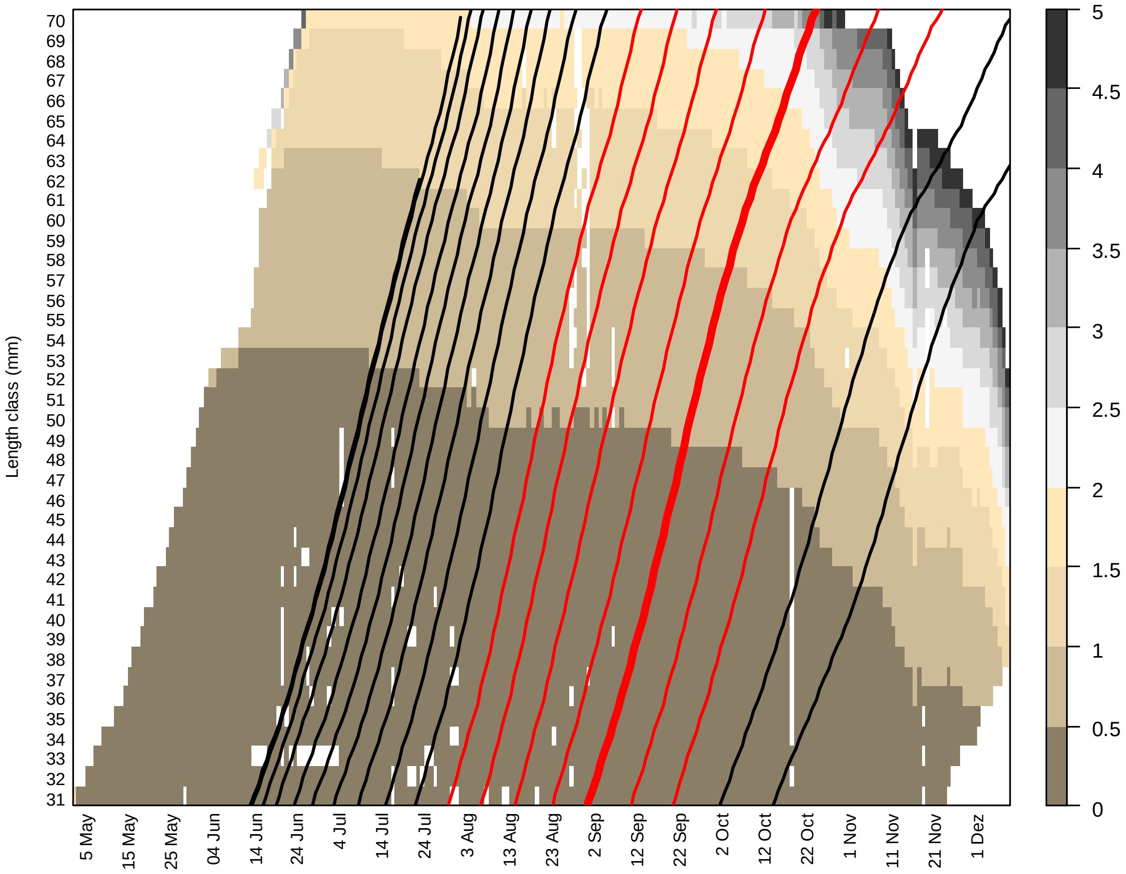


Figure 3: Maintenance ration for different length classes throughout the summer in 2003 according to the forward simulation approach. Colors of areas indicate prey concentrations (Ind*L^{-1}) needed to balance metabolic costs. Lines indicate growth trajectories of seasonal cohorts generated with the backwards simulation; Red lines represent cohorts that exhibit the day of first increment formation at the same time than YoY-survivors which were sampled autumn. The fat red line indicates the main cohort of survivors. Black lines indicate cohorts resulting from the extended spawning season that have not been sampled in autumn.

Discussion

Total energy demand of seasonal cohorts

The backwards simulation approach generates two major results: (1) the total demand of prey items is lower for later seasonal cohorts that stem from the second half of the spawning season and (2) in four years when daily increments on otoliths of YoY-sprat were analyzed (2002, 2003, 2006, 2007), most individuals that survived until autumn/late summer originate from the second half of the spawning season.

We assume that later cohorts benefit due to a comparatively lower temperature in the juvenile stage which reduces the metabolic costs in contrast to earlier cohorts, exhibiting higher temperatures. Consequently, the timing of the juvenile stage mainly influences the food demand. Thus, later cohorts have not only a benefit in the larval stage (Baumann et al. 2008), but also in the juvenile stage. That might be an explanation for the second finding of the backwards simulation approach that more individuals of later cohorts survive until late summer and autumn. We found the highest growth potential in earlier cohorts. However, earlier cohorts have a higher energy demand and are thus more sensitive to growth reduction and starvation. If food availability in the early juvenile phase of earlier cohorts limits growth, the cumulative total mortality pressure on this seasonal cohort increases which might influence survival rates. In other words, low growth rates in the case of food deprivation elongates the stage duration and thus increases the risk of mortality, assuming that mortality decreases with increasing length. Such a selective survival for fast growing individuals may explain why the early cohort e.g. the seasonal cohort from May, is not the main cohort of survivors despite showing the highest growth potential. In contrast, the later cohorts have a slightly lower growth potential but are more robust to decreases in food availability because lower temperatures in the juvenile stage of these seasonal cohorts lead to a lower total energy demand to fulfill a just slightly lower growth performance.

Maintenance rations of early and late born sprat

The high total energy demand of earlier cohorts calculated in the backwards simulation implies that earlier cohorts need a higher amount of food. However, the prey concentration for maintenance is similar for early and late spawned cohorts. The reason for that is that early cohorts experience higher temperatures when they become larger. This increases snatching rates enabling these cohorts to eat more prey items at a similar prey concentration. Thus, maintenance concentrations of seasonal cohorts do not reflect the higher total demand of energy for earlier spawned cohorts. The strongest determinants for the concentration necessary to achieve maintenance ration are fish size and season (interaction between temperature and day length). In summer, when early cohorts enter the juvenile stage, necessary concentrations for larger individuals are smallest. In contrast, later spawned cohorts need larger concentrations at the same length as season progresses. At a first glance, the lower concentration needed for early cohorts in contrast to later cohorts are contradicting to the hypothesis that later cohorts have an advantage. However, individuals that are large early in the season rely on high prey concentrations for fulfill their maintenance for the rest of the growing season. In other words, the risk to starve before winter time increases. Individuals that are born later in the season need a slightly higher prey concentration for maintenance. However the period they need to bypass before winter is reduced and thus, the risk to starve before winter. Being too large too early in the season is unprofitable, which might explain why the share of early cohorts in autumn caught survivors is of minor importance.

Summary

The results of both simulation approaches underline the advantages of later born individuals in the juvenile life-stage of Baltic sprat. On the one hand the total energy demand is lower for late seasonal cohorts. On the other hand, early cohorts might suffer from the fact that they achieve large body size early in the season and rely on high prey concentrations for the rest of the growing season. Thus, early cohorts suffer during the juvenile stage in summer and their probability to survive until autumn is reduced. The interaction between temperature in nurseries and food demand of juvenile sprat determines the temporal origin of cohorts dominating autumn recruits.

References

Alshuth S (1988) Daily Growth Increments on Otoliths of Laboratory-Reared Sprat, Sprattus-Sprattus L, Larvae. Meeresforschung-Reports on Marine Research 32:23-29

Baumann H, Hinrichsen HH, Möllmann C, Köster FW, Malzahn AM, Temming A (2006) Recruitment variability in Baltic Sea sprat (*Sprattus sprattus*) is tightly coupled to temperature and transport patterns affecting the larval and early juvenile stages. Can J Fish Aquat Sci 63:2191-2201

Baumann H, Voss R, Hinrichsen HH, Mohrholz V, Schmidt JO, Temming A (2008) Investigating the selective survival of summer-over spring-born sprat, Sprattus sprattus, in the Baltic Sea. Fish Res 91:1-14

Elliott JM, Davison W (1975) Energy Equivalents of Oxygen-Consumption in Animal Energetics. Oecologia 19:195-201

Günther C (2008) Untersuchungen zum Sommerwachstum juveniler Sprotten (Sprattus sprattus) in der westlichen Ostsee. Universität Hamburg,

Günther C, Temming A, Baumann H, Huwer B, Möllmann C, Clemmesen C, Herrmann J-P (2012) A novel length back-calculation approach accounting for ontogenetic changes in the fish length – otolith size relationship during the early life of sprat (Sprattus sprattus). Can J Fish Aquat Sci 69:1214-1229

Günther CC, Herrmann JP, Temming A (2015) Laboratory calibration of optimal growth to deduce in situ feeding conditions of early juvenile sprat Sprattus sprattus from otoliths. Mar Ecol Prog Ser 525:199-215

ICES (2015) Report of the Baltic Fisheries Assessment Working Group (WGBFAS).

Kerambrun P (1987) Elementary Chemical-Composition (C,H,N) and Energy Equivalent of Acartia-Clausi (Crustacea, Copepoda), an Important Species in the Bioenergetics of the Coastal Ecosystems of the Northwest Mediterranean. Mar Biol 95:115-121

Köster FW, Hinrichsen HH, Schnack D, St John MA, Mackenzie BR, Tomkiewicz J, Möllmann C, Kraus G, Plikshs M, Makarchouk A, Aro E (2003) Recruitment of Baltic cod and sprat stocks: identification of critical life stages and incorporation of environmental variability into stock-recruitment relationships. Sci Mar 67:129-154

Kulke R, Herrmann J, Meskendahl L, Temming A (2016) Temperature and size-dependent functional response of sprat, Sprattus sprattus L. . Bio C3 Project Appendix

Meskendahl L, Herrmann JP, Temming A (2010) Effects of temperature and body mass on metabolic rates of sprat, Sprattus sprattus L. Mar Biol 157:1917-1927

Petereit C, Haslob H, Kraus G, Clemmesen C (2008) The influence of temperature on the development of Baltic Sea sprat (*Sprattus sprattus*) eggs and yolk sac larvae. Mar Biol 154:295-306

Voss R, Peck MA, Hinrichsen H-H, Clemmesen C, Baumann H, Stepputtis D, Bernreuther M, Schmidt JO, Temming A, Köster FW (2012) Recruitment processes in Baltic sprat – A re-evaluation of GLOBEC Germany hypotheses. Prog Oceanogr

Winberg GG (1960) Rate of metabolism and food requirements of fishes. Fisheries Research Board of Canada 194

APPENDIX VIII

Fish egg - buoyancy– experimental and field approach

Petereit, C., (GEOMAR, P1) and Huwer, B., (DTU-Aqua, P2)

[Report section includes results of joined activities and contributions from Hans-Harald Hinrichsen, Burkhard von Dewitz (both P1), Sofia Nyberg and Anders Nissling in the frame of collaborative experiments with the BONUS INSPIRE project]

Introduction

What are potential drivers of change which may impact survival, development duration or (spatial) distribution of early life stages of cod and other commercially important key species? To address some of these complex processes we have selected, (partly preliminary) results from three case-studies. The objective of the first study is **to compare the potential dimensions of egg buoyancy differences over several spawning seasons to assess general patterns**. The objective of the second study was to **investigate the importance of different oxygen and salinity conditions in relation to the available habitat determined by the eggs´ specific density.** This is particularly interesting by comparing different hydrographic conditions which have recently been changed due to the Major Baltic Inflow (MBI) end of 2014. The third case study is an example how to **use field-derived egg diameter data in combination with buoyancy data and to link this with stock characteristics to improve the current estimation method of a spawning stock biomass**.

Methods:

Egg sampling:

Eggs were caught by ‘Helgoländer Larven Netz’ (HLN, 143 cm diameter, 500 µm mesh size) towed vertically (0.2 knots) from ~7 m above the bottom to the surface (Petereit et al., 2009). Alive eggs were immediately sorted by species from the plankton samples with 3 mm diameter pipettes and staged under a stereomicroscope (WILD M3Z) at 5-9°C in the temperature controlled laboratory on RV ALKOR (Petereit et al., 2009). Egg diameter was measured with the internal scale in the stereomicroscope to the nearest scale bar below (0.06 mm) at 240x magnification. Accordingly, eggs were divided into small (1.09-1.26 mm), medium (1.27-1.38mm) and large (1.39 -1.46 mm) diameter size classes. Only alive eggs with clear and sharp developing cell structures were used for measurements in the density gradients. Egg number varied among samples, 10-15 eggs per size class was envisaged, but sometimes sample sizes of eggs were lower due to low abundance in the haul.

Egg strip spawning and artificial fertilization:

Fish in spawning conditions was caught by scientific trawling on RV ALKOR (Young fish trawl; haul duration 30 minutes with 3 knots trawling speed).. The selection of running ripe females and males from the catch including the fertilization was completed within 30 min after the haul was on deck. Unfertilized eggs were strip spawned into 1l PVC cylindrical cones filled with artificial seawater of 20 psu between 6 and 9°C. Spermatozoa of three males (2-3 drops per male) were activated with artificial seawater (20 psu, 6-9°C) and poured into the cone to fertilize the eggs. The detailed procedure of individual selection and fertilization process was described previously in Petereit et al. (2014). After 60min fertilization period, all positively buoyant eggs were removed from the cone and transferred into fresh seawater. Unfertilized or bad-quality eggs sank within the cone and could easily be discarded. A first visual check was performed to estimate egg batch quality before the eggs were stored in 500ml PVC containers for 24h at 6°C in a scientific refrigerator. The next day, 30 eggs were size-measured under a stereomicroscope (WILD M3Z including scale bar), a picture was taken to justify egg quality of each batch and the eggs were inserted into the density gradient.

Egg density measurements:

We used density gradient columns similar to the setup described by Coombs (1981) and Coombs et al. (1985). This general method has been regularly applied to measure fish egg density (neutral egg buoyancy) for various Baltic fish species including cod, sprat and flatfish from many areas of the Baltic Sea (Nissling et al., 1994, 2002; Petereit et al., 2009; 2014).

A minimum of 3–5 calibrated glass floats (Spartel, UK) assured the determination of accurate linear calibration curves ($r^2 > 0.99$) for each salinity gradient, which were used to convert the measured heights of the inserted eggs in the column into the respective density values. As density relations are known to be highly temperature sensitive, all measurements were conducted in a temperature controlled climate cabinet with temperature being controlled in addition at the surface of each column after each measurement. The adjusted temperatures differed between the cruises since 2009 but ranged from 5-9°C and were chosen according to the tolerable temperature for all three species (Westernhagen von, 1970; Nissling, 2004; Petereit, 2004; Petereit et al., 2008). Only eggs with regular cell divisions were inserted into the salinity gradient to assure optimal egg quality. After a settling time of 60 min, the position of every single egg in the salinity gradient column was recorded to calculate the respective density value.

Abiotic environment measurements:

The depth at which each egg would have had potential density equilibrium was calculated from temperature, salinity, oxygen, and sigma-t profiles measured using a CTD (ADM-company) at each station.

More detailed methods and results for the different case studies

Inter annual pattern in egg buoyancy & potential drivers of change - Egg size

One case study is the continuation of previously initiated work by Petereit et al. (2009) who had analyzed live- fish egg samples from the Baltic Sea and measured their densities. The specific egg density determines the specific neutral buoyancy level of an egg. On this level, eggs float, develop and get transported by underlying currents. Thus, these density layers define the abiotic environment (temperature, salinity, oxygen, pH) experienced by the eggs. Petereit et al. (2009) reported for one of their analyzed cruises a significant effect of egg size on neutral egg buoyancy during the peak spawning season in May/June 2008. This resulted in a significantly different depth distribution between different egg size classes. The largest egg size class experienced significantly warmer (+1°C) and less (~0.5 psu) saline conditions compared to the smaller size classes. The magnitude of these differences is important if the physiological tolerance limits of the eggs are close to the *in situ* measured values. In sprat, successful egg survival (hatching) potential is reduced below 5°C and mortality increases significantly below 4°C (Nissling, 2004; Petereit et al., 2008).

For this case study, most data were available for sprat (*Sprattus sprattus L.*), a clupeid fish characterized by an undetermined batch spawning mode. Eggs had been previously sampled during scientific cruises in April from 2009-2013 on several stations during fish early spawning season in the Bornholm Basin, Baltic Sea (Fig. 1A). This existing time series could now be continued in the frame of the BIO-C3 project during the April cruises in 2014 and 2015.

Results

Data from the 6-year time series (data analysis from up to four different stations) confirm a positive relation between egg size and egg buoyancy for sprat (Fig. 1B) during April spawning seasons. Large eggs float higher in the water column compared to small eggs. The egg density was significantly different between size categories (p<0,0001 ANOVA, F:2;527=204). Inter-annual variation was detectable but in different magnitudes between stations (Fig.1 C). Highly significant differences could be observed, e.g. in the density distribution of the medium egg size category for 2009 and 2012 compared to the years 2014 and 2015 on both stations BB23 and BB06 (ANOVA, F:3;81=23,1; Tukey HSD p<0,001 & F:5;79=9,0; TUKEY HSD; p<0,001).

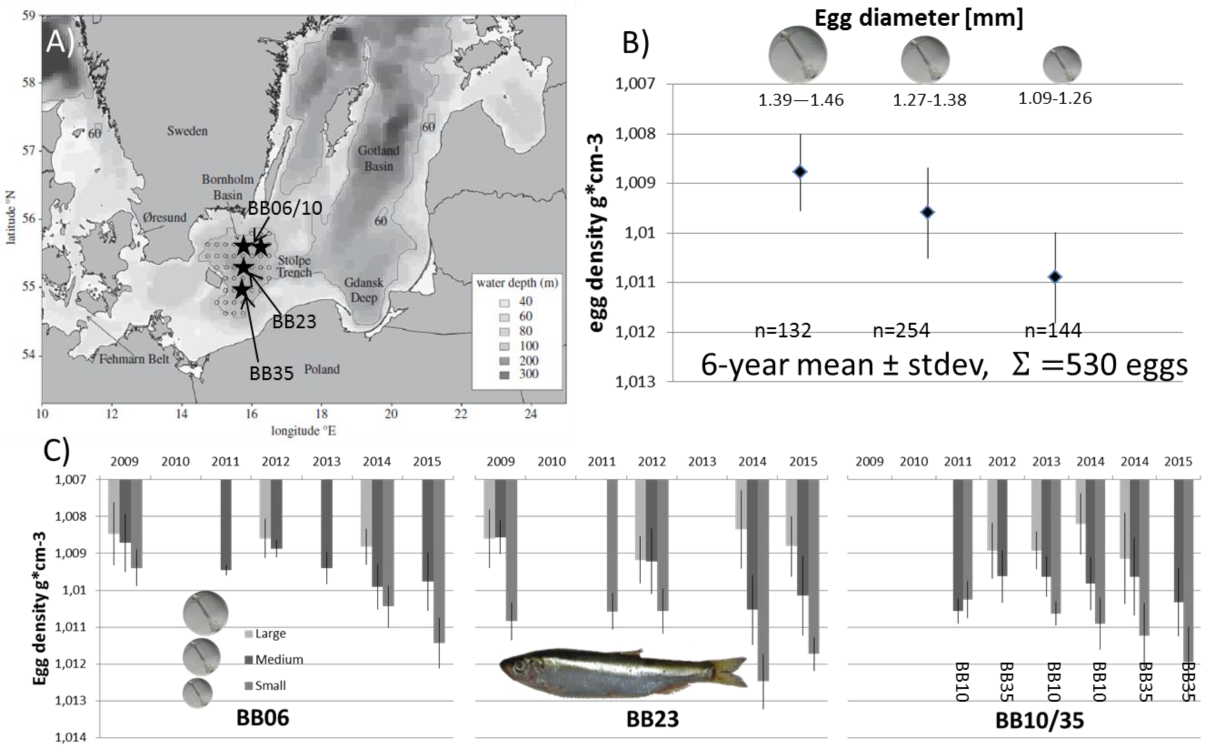


Fig. 1: A) Map of the four sampling stations in the Bornholm Basin (BB). B) Overall mean (±stdev) values of sprat egg density of the 6-year time period in relation to egg diameter. C) Station and year-specific mean (±stdev) of egg density distributions.

The inter-annual variation in the specific density layer was lowest for the largest diameter sprat eggs, thus reflecting rather stable density conditions (Fig. 2A). Medium-sized eggs showed more variation, however levels of the standard deviation do not indicate significant annual changes on the mean density layer (Fig. 2A). The egg density trend of the smallest egg size class was the most variable one. Egg density increased in 2014 and 2015 compared to previous years, although with large variation in 2014. In 2015, the highest significant mean value in the egg density time series could be measured.

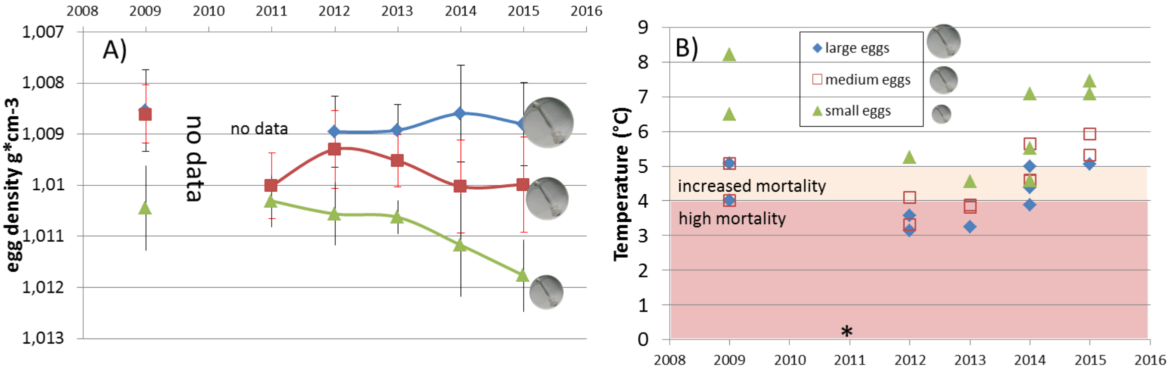


Fig. 2 A) Egg diameter specific mean (± stdev) sprat egg density characteristics. Data points are connected by interpolating lines. No data are available in the large egg diameter category in 2011. B) Temperature on the mean egg density levels for the three diameter categories. Temperature threshold levels reducing (<5°C; light red) or highly significantly reducing (<4°C; dark red) the probability for hatching of viable larvae are taken from Nissling (2004) and Petereit et al. (2008). * no accurate CTD data available in 2011.

Based on CTD data on the respective station, the abiotic parameters on the mean density levels could be determined (Fig. 2B; Salinity and oxygen not shown). Large sprat eggs experience a colder environment compared to medium or small-sized eggs during April (Fig. 2B). The temperature levels are close to critical (below 5°C) or very serious low levels undercutting 4°C. The smallest egg diameter class exceeded the most critical temperature threshold levels which resulted in more or less suitable temperature conditions in all analyzed years. The years 2012 and 2013 were in general characterized by low April water temperature and 2014 and 2015 by significantly higher temperatures. The oxygen conditions in the analyzed density layers were also variable between years – however, if considering the threshold level of 2mg/l oxygen (Nissling et al., 2003), all egg diameter classes in all years and stations experienced values above that level. In April 2015, independent of size, all sprat eggs were above the critical threshold. This might be directly caused by the improved hydrographical condition due to the major Baltic inflow in December 2014 (Mohrholz et al., 2015), but needs further to be investigated.

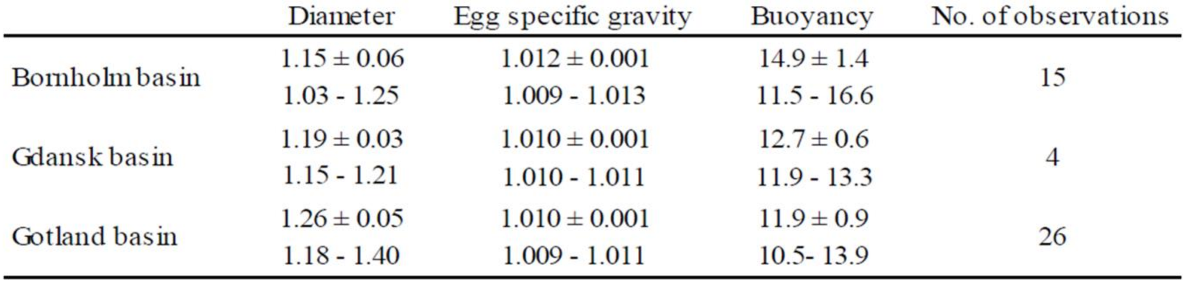
Inter annual pattern in egg buoyancy & potential drivers of change - Oxygen and Salinity

The second case study concentrates in particular on the modified hydrographic condition due to the major Baltic inflow (MBI) of high-saline and oxygen-rich North Sea water (Mohrholz et al., 2015) between the two spawning seasons 2014 and 2015 in the central Baltic Sea and its consequence for the survival probability of fish eggs. The objective was to **investigate the importance of different oxygen and salinity conditions in relation to the available habitat determined by the eggs´ specific density.** Flounder (*Platichthys flesus*) were chosen as model species and individuals were strip-spawned and eggs artificially fertilized onboard during two cruises with RV ALKOR in April 2014 and 2015. In addition potential differences between individuals from the three ICES subdivisions (Bornholm Basin (SD25), Gdansk Deep (SD26) and southern Gotland Basin (SD28)) were considered. Survival probability of the egg batches was judged based on threshold levels of oxygen concentration above or below 1.5 ml/l and temperature above or below 2°C. This case-study was performed in close collaboration with the BONUS INSPIRE project (Dr. Anders Nissling) and most parts of this investigation have been extracted from the jointly supervised Bachelor thesis of Sofia Nyberg (2015).

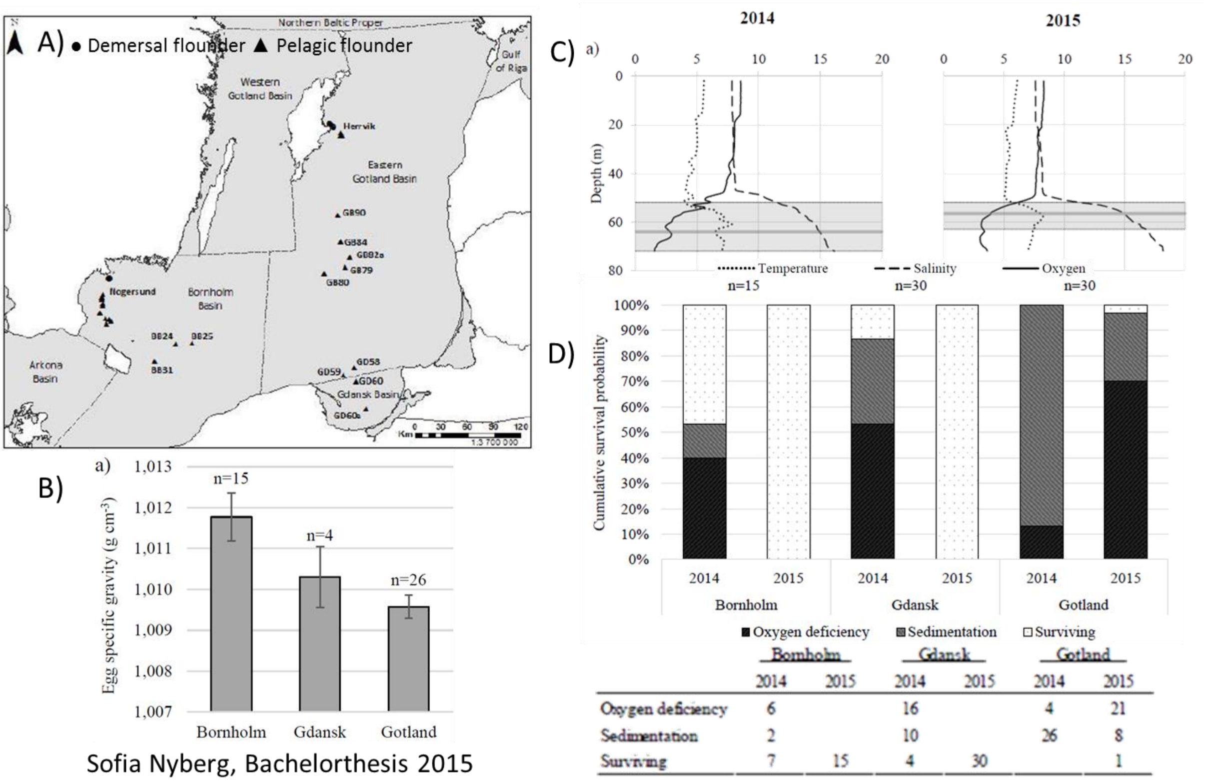
Results

A total of 45 individual flounder batches from three ICES subdivisions could be analyzed during the two cruises (Tab. 1). In addition, 26 batches had been retrieved from other (gillnet) fishing activities performed in the frame of another project (BONUS INSPIRE). Stations are indicated in the map (Fig. 3A). The general egg characteristics diameter, egg specific gravity and buoyancy of the pelagic flounder eggs are presented in Tab 1.

Tab.1: Diameter (mm), egg specific gravity (g*cm-3) and buoyancy (psu) of pelagic flounder eggs obtained in the Bornholm Basin, Gdansk Deep and Gotland Basin respectively, stated in mean±standard deviation in the upper row and range in the row below.



	Diameter	Egg specific gravity	Buoyancy	No. of observations
Bornholm basin	1.15 ± 0.06	1.012 ± 0.001	14.9 ± 1.4	15
	1.03 - 1.25	1.009 - 1.013	11.5 - 16.6	
Gdansk basin	1.19 ± 0.03	1.010 ± 0.001	12.7 ± 0.6	4
	1.15 - 1.21	1.010 - 1.011	11.9 - 13.3	
Gotland basin	1.26 ± 0.05	1.010 ± 0.001	11.9 ± 0.9	26
	1.18 - 1.40	1.009 - 1.011	10.5- 13.9	



	Bornholm 2014	Bornholm 2015	Gdansk 2014	Gdansk 2015	Gotland 2014	Gotland 2015
Oxygen deficiency	6		16		4	21
Sedimentation	2		10		26	8
Surviving	7	15	4	30		1

Sofia Nyberg, Bachelorthesis 2015

Fig. 3: A) Sampling and study area. B) Results of egg specific gravity measurements of Flounder batches during 2014 and 2015 cruises. C) Hydrographic profiles of a deep Bornholm Basin station in 2014 and 2015 with flounder egg layer indicated as grey shaded area. D) Resulting cumulative survival probability of flounder eggs per subdivision and year derived by comparing measured egg batch density and CTD derived environmental data using survival threshold levels for density (salinity) and oxygen. Figures compiled from Nyberg (2015).

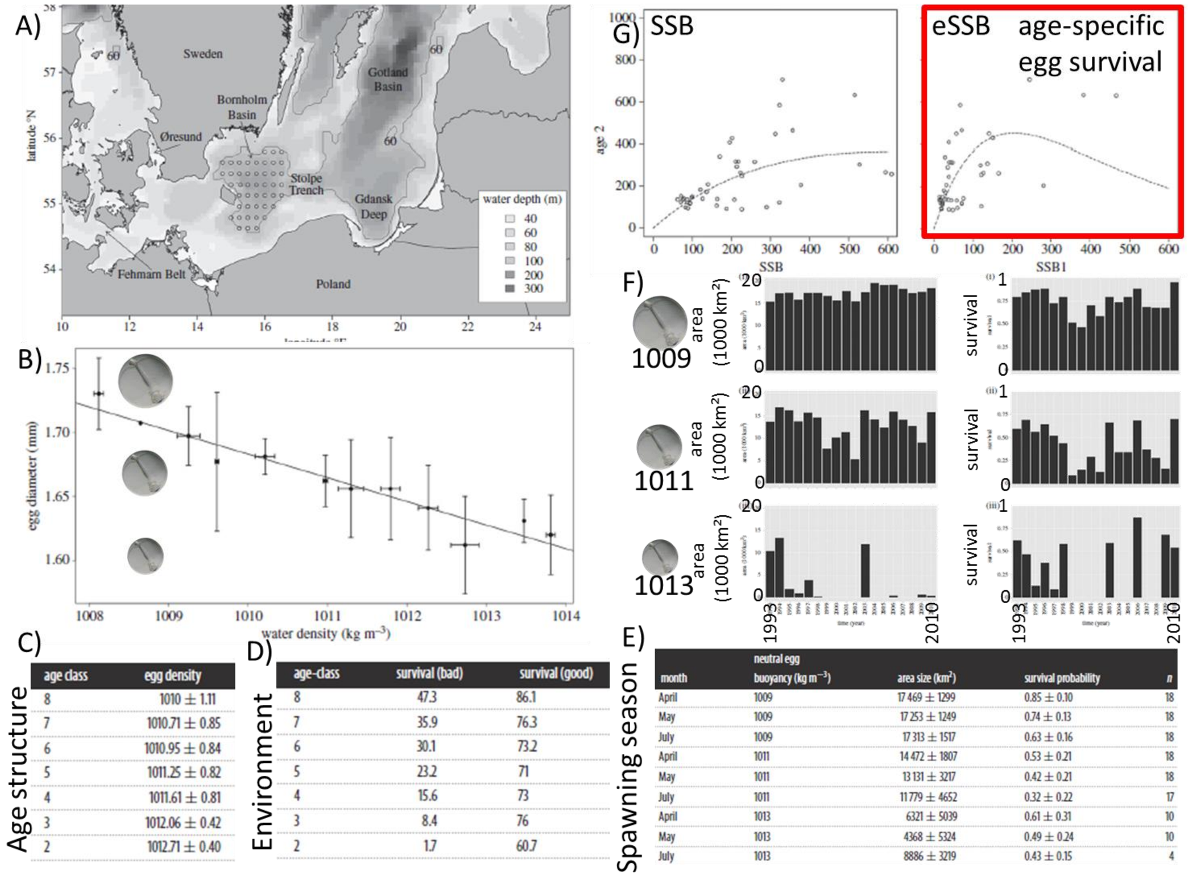
The measurements of the egg specific gravity (density) showed statistical differences between the Bornholm Basin and the Gdansk/Gotland areas (Fig. 3B; ANOVA, $F_{2:42}=39.12$, $p<0.01$). The specific gravity of flounder eggs from the Bornholm basin were higher (1.012 ± 0.001) than for eggs obtained in the Gdansk (1.010 ± 0.005) and Gotland (1.010 ± 0.001) basins, thus they achieve neutral buoyancy at higher salinities. Batches from Gdansk Deep and Gotland Basin were not statistically different (Fig. 3B; t-test, $t_{28} = 1.99$, $p>0.05$). Due to the MBI in 2014, the hydrographic profiles had changed between years as shown by an example from the Bornholm Basin (Fig. 3C). Accordingly, the cumulative survival probabilities of egg batches might have changed in those areas. The overall results indicated highly enhanced survival probabilities in Bornholm Basin 2015 ($p<0.01$), as egg survival increased from 47% in 2014 to 100% the following year (Fig. 3D). In Gdansk Deep the situation was similar, and survival probability increased from 13% to 100% ($p<0.01$) (Fig. 3D). In Gotland Basin no difference in survival probability was identified, although the dominant cause of mortality shifted from sedimentation, i.e. due to low salinity conditions in 2014, to oxygen deficiency in 2015 ($p<0,01$) (Fig. 3D).

Patterns and drivers of change: modeled combination between egg size, egg density and oxygen

The third case study is an example how to **use field-derived egg diameter data in combination with buoyancy data and to link this with stock characteristics to improve the current estimation method of a spawning stock biomass**. The newly established relationship between egg diameter and buoyancy (floating depth) allowed quantifying the number of effective spawners which were able to successfully reproduce under ambient and hydrographic conditions. This study used eastern Baltic cod (*Gadus morhua*) eggs sampled during 8 years in the central Bornholm Basin (Fig. 4A).

Results

For the time period 1993-2010, large variations in the horizontal extent of spawning habitat (1000-20000 km²) and oxygen-dependent egg-survival (10-80%) was observed (Fig. 4F). The novel concept of an effective spawning stock biomass (eSSB) takes into account offspring that survive depending on the spawning stock age/size structure, if reproductive success is related to egg buoyancy and the extent of hypoxic areas. Effective spawning stock biomass reflected the role of environmental conditions for Baltic cod recruitment better than the spawning stock biomass alone, highlighting the importance of including environmental information in ecosystem-based management approaches. Presented results are extracted from the publication by Hinrichsen et al. (2016).



age class	egg density
8	1010 ± 1.11
7	1010.71 ± 0.85
6	1010.95 ± 0.84
5	1011.25 ± 0.82
4	1011.61 ± 0.81
3	1012.06 ± 0.42
2	1012.71 ± 0.40

age-class	survival (bad)	survival (good)
8	47.3	86.1
7	35.9	76.3
6	30.1	73.2
5	23.2	71
4	15.6	73
3	8.4	76
2	1.7	60.7

month	neutral egg buoyancy (kg m^{-3})	area size (km^2)	survival probability	*n*
April	1009	17 469 ± 1299	0.85 ± 0.10	18
May	1009	17 253 ± 1249	0.74 ± 0.13	18
July	1009	17 313 ± 1517	0.63 ± 0.16	18
April	1011	14 472 ± 1807	0.53 ± 0.21	18
May	1011	13 131 ± 3217	0.42 ± 0.21	18
July	1011	11 779 ± 4652	0.32 ± 0.22	17
April	1013	6321 ± 5039	0.61 ± 0.31	10
May	1013	4368 ± 5324	0.49 ± 0.24	10
July	1013	8886 ± 3219	0.43 ± 0.15	4

Fig. 4: A) Sampling area including station grid in the Bornholm Basin. B) Multi opening closing net derived cod egg diameter to density relationship (2002-2010). C) Age structure derived mean egg densities according to Vallin and Nissling (2000). D) Age class dependent

egg survival potential assessed for different environmental conditions (inflow: good conditions; stagnation: bad conditions). E) Seasonal change in available suitable spawning area including calculated survival probability (1993-2010). F) Time series of spawning area extension and corresponding cod-oxygen related egg survival for different density levels (1009 kg*m-3 ~ 11psu; 1011kg*m-3 ~13,5 psu; 1013kg * m-3 ~ 16 psu at 5°C). G) Ricker stock recruitment curves without and including environmental information through the implemented age-specific egg survival coefficients. Figures are (partly modified) taken from Hinrichsen et al. (2016).

Conclusions and future perspectives

Interestingly, the driver “egg-size” may influence egg survival of different species in different directions. This was shown in our three case studies with cod, flounder and sprat. Egg size is influenced by stock structure. It can be influenced by spawning state e.g. reflected by batch number in season or by nutritional state in undetermined batch-spawners like sprat.

In sprat, egg survival is reduced due to critical temperature conditions, which act as the main driver. Being a “large” egg early (April) in the spawning season might not be optimal for sprat; being “small” at that time however, increases thermal survival conditions in a still tolerable environment concerning oxygen. This size-pattern turns into the other direction in the continuation of the spawning season in May/June. During late spring and early summer, the temperature of the water column above the halocline increases and the larger eggs now experience the warmer conditions (Nissling et al., 2003; Petereit et al., 2009) and thus are outside of critical tolerance limits.

For flounder spawning pelagic eggs, the key driver is likely adequate salinity (acting through appropriate water density characteristics) to sustain floating during the egg development in the water column. MBI events as observed late 2014 have the potential to improve conditions in terms of salinity and also oxygen. However, this effect may be only spatially or partly sufficient. Egg survival was significantly improved (to 100%!) in the Bornholm Basin and Gdansk Deep 2015 and the proportion of egg batches floating in the water column of the Gotland Basin at least doubled. Yet, oxygen threshold tolerance limits were not met. Therefore, effectively egg survival condition did not improve. Only the source of egg mortality changed from sedimentation to hypoxia.

In cod, oxygen related egg survival is one of the key drivers determining egg abundances in different years. The new approach presented combines directly cod age structure with mean egg diameter and resulting mean density characteristics. This relationship is used as a feedback loop to model the number of effectively contributing females under known water density characteristics including the application of the well-accepted physiological tolerance limits for especially oxygen, temperature and salinity.

Of course, more research is necessary to (re)assess and evaluate the relation between female size and egg size in the course of the batch cycle. This is of particular importance, due to the increasing evidence of a significant change in food composition of cod and proportion of rather young and smaller sized fish in recent years compared to the reference period in the end of 1990th , from which the basic experimental relationships have been taken (Vallin and Nissling, 2000). Compared to the case study on sprat, being a “small egg” seems not to be beneficial even early during the spawning season. Only for large eggs, abiotic conditions seem to sustain continuously sufficient over the analyzed time series to survive and develop successfully to viable larvae.

In conclusion, species specific drivers become evident which may also change during spawning season and year. This implies the need for a thorough and continuous effort to monitor changes in the egg density characteristics of the three ecologically and economically important species. This knowledge may aid to detect and understand peculiarities or changes in their stock structures.

References

Coombs, S.H., 1981. A density-gradient column for determining the specific gravity of fish eggs, with particular reference to eggs from the mackerel *Scomber scombrus*. Marine Biology 63, 101–106.

Coombs, S.H., Fosh, C.A., Keen, M.A., 1985. The buoyancy and vertical distribution of eggs of sprat (*Sprattus sprattus*) and pilchard (*Sardina pilchardus*). Journal of the Marine Biological Association of the United Kingdom 65, 461–474.

Hinrichsen, H.-H, von Dewitz, B, Dierking, J, Haslob, H, Makarchouk, A, Petereit, C, Voss R. (2016): Oxygen depletion in coastal seas and the effective spawning stock biomass of an exploited fish species. R. Soc. open sci. 3: 150338. http://dx.doi.org/10.1098/rsos.150338

Mohrholz, V., Naumann, M., Nausch, G., Krüger, S., Gräwe, U. (2015): Fresh oxygen for the Baltic Sea — An exceptional saline inflow after a decade of stagnation. J Mar Sys 148:152–166.

Nissling, A., Kryvi, H., Vallin, L., (1994): Variation in egg buoyancy of Baltic cod (*Gadus morhua*) and its implications for egg survival in prevailing conditions in the Baltic Sea. Marine Ecology Progress Series 110, 67–74.

Nissling, A., Westin, L., Hjerne, O. (2002): Reproductive success in relation to salinity for three flatfish species, dab (*Limanda limanda*), plaice (*Pleuronectes platessa*) and flounder (*Pleuronectes flesus*), in the brackish water Baltic Sea. ICES Journal of Marine Science 59, 93–108.

Nissling, A., Müller, A., Hinrichsen, H.-H. (2003): Specific gravity and vertical distribution of sprat eggs in the Baltic Sea. J Fish Biol 63: 80-299.

Nissling, A. (2004): Effects of temperature on egg and larval survival of cod (*Gadus morhus*) and sprat (*Sprattus sprattus*) in the Baltic Sea- Implications for stock management. Hydrobiologia 514: 115-123.

Nyberg, S., (2015): Egg buoyancy and survival probabilities of Baltic Flounder (*Platichthys flesus*) – Differences between spawning areas and inter-annual variation in conditions for reproduction. Bachelor Thesis Uppsala University, 24p.

Petereit, C. (2004) Experimente zum Temperatureinfluss auf frühe Entwicklungsstadien des Ostseedorsches *Gadus morhua*. Diploma thesis, University of Kiel, Germany.

Petereit, C., H. Haslob, G. Kraus, Clemmesen, C. (2008): The influence of temperature on the development of Baltic Sea sprat (*Sprattus sprattus*) eggs and yolk sac larvae. Mar. Biol., 154: 295-306.

Petereit, C., Hinrichsen, H.-H., Voss, R., Clemmesen, C., Haslob, H., Freese, M., Kraus, G., (2009): The influence of different salinity conditions on egg buoyancy and development and yolk sac larval survival and morphometric traits of Baltic Sea sprat (*Sprattus sprattus balticus* Schneider). In: Clemmesen, C., Malzahn, A.M., Peck, M.A., Schnack, D. (Eds.), Advances in Early Life History Study of Fish. Sci Mar 73(S1), 59–72.

Petereit, C., Hinrichsen, H.-H., Franke, A., Köster, F.W. (2014): Floating along buoyancy levels: dispersal and survival in western Baltic fish eggs. Prog. Oceanogr. 122, 131–152. (doi:10.1016/j.pocean.2014.01.001)

Vallin, L., Nissling, A. (2000): Maternal effects on egg size and egg buoyancy of Baltic cod, *Gadus morhua*: implications for stock structure effects on recruitment. Fish. Res. 49, 21–37. (doi:10.1016/S0165-7836(00)00194-6)

Von Westernhagen, H. (1970): Erbrütung von Dorsch (*Gadus morhua*), Flunder (*Pleuronectes flesus*) und Scholle (*Pleuronectes platessa*) unter kombinierten Temperatur- und Salzgehaltsbedingungen. Helgol wiss Meeresunters 21:21–102

Appendix IX

Cod larvae – populations across geographical scales – experiments

Catriona Clemmesen, Felix Mittermayer, Martina Stiasny (GEOMAR, P1)

Introduction

Through the increased usage of fossil fuels and changed land use the concentration of atmospheric carbon dioxide has been steadily rising since the onset of the industrial revolution. A portion of this CO_2 is dissolving into the oceans causing a decrease in pH, e.g. acidifying a habitat that covers 2/3 of this planet. This process has been coined “the other CO_2 problem” or ocean acidification (OA)(Doney, Fabry, Feely, & Kleypas, 2009).

Even though adult fish are able to tolerate CO_2 concentrations of up to 16.000µatm (Ishimatsu, Hayashi, & Kikkawa, 2008), their early life stages have been shown to be more vulnerable as they often lack the regulatory acid-base mechanisms needed to negotiate a high CO_2 environment (Falk-Petersen, 2005). The detrimental effects of OA on larval fish include changed sensory competence, behaviour, otoliths, development and tissue structure (Bignami, Enochs, Manzello, Sponaugle, & Cowen, 2013; Checkley et al., 2009; Dixson, Munday, & Jones, 2010; A. Y. Frommel et al., 2012; Andrea Y. Frommel et al., 2014; R. Maneja et al., 2013; Munday et al., 2009, 2012). However a number of studies have found no detrimental effects of OA on larval fish survival (Munday et al., 2009, 2015). This can partly be explained by these species natural environment or life history strategies and traits. In the case of cod (*Gadus morhua*) it remains uncertain if larvae from different populations are in the same way affected by acidification. One study has shown that in respect to hatching success, mortality, development and otolith size cod larvae from the Baltic appear to be robust to OA (Frommel et al 2013a) while others, namely Norwegian coastal cod have shown negative effects on growth patterns and development (Frommel et al. 2013b). These experiments were performed to establish how newly hatched, first feeding and developing larvae from different cod populations, namely Western Baltic and Barents Sea cope with end of the century ocean acidification levels. The aim is to derive as many variables, under comparable settings, from these experiments as possible; these include mortality, growth, behaviour, bone and tissue development, otoliths, lipid analysis, genetics and genomics.

Three experiments were performed, one at the Sven Lovén Centre in Kristineberg, Sweden in 2013 and two at the Centre for Marine Aquaculture, Tromsø, Norway in 2014 and 2015. At the first location the larvae were fed natural plankton from the adjacent fjord, while in Tromsø in 2014 two variants of aquaculture feeding protocols were applied. In 2015 adult cod where kept under control and treatment for 20 weeks prior to spawning to investigate potential effects of parental acclimatization, e.g. transgenerational effects.

Methods

Cod from the Western Baltic population were caught in the Øresund (55°58’N, 12°38’E) in March 2013 and from the Barents Sea (approx. 70°15’N, 19°00’E) in March 2014. The fish from the Øresund were stripped spawned and their spawn was transported to the Sven Lovén Centre, Kristineberg, while the life fish from the Barents Sea were transferred to the

Centre for Marine Aquaculture, Tromsø and kept in large tanks under ambient lighting to initiate spawning and they were allowed to spawn naturally, the resulting eggs were collected from the tanks. Fish for the 2015 experiment were caught in November 2014 in the Barents Sea (approx. 70°15’N, 19°00’E) and transferred to the National Cod Breeding Centre, Tromsø. All fish were tagged using floy-tags to allow individual identification and were randomly distributed between the control (ambient 424.0 ± 5.2 uatm) and end-of-century CO_2 treatment (892.4 ± 22.2 uatm) into large rearing tanks. This broodstock was fed ad libitum with frozen capelin (*Mallotus villosus*) and the lighting regime was matched to the outside to stimulate the gonadal development and ripening. In March fish were strip spawned to create families and the resulting eggs were supplemented with natural spawn collected from the rearing tank.

Water for the increased CO_2 treatments were produced by controlling the pH values in header tanks with pH sensors connected to a pH control computer system (IKS Aquastar), correct values for the end-of-century treatment were achieved through an automatically opening and closing of a magnetic valve steered from the pH control unit when the water in the pH in the header tank was too low or too high. Opening of the magnetic valve resulted in a pulse of CO_2 gas to be injected into the header tank, this in combination with the large volume of the header tank ensured a thorough mixing and equilibration of CO_2 before the water would flow into the rearing tanks thereby assuring constant conditions in the rearing tanks, while ambient water passed the header tanks at the same flow rate but without the addition of CO_2. Additionally every day the pH was checked manually in the rearing tanks with a separate pH probe (WTW pH/Cond 340i/3320). According to the Best Practices Guide (Riebesell, Fabry, Hansson, & Gattuso, 2010) carbon chemistry of the water, including DIC and alkalinity, were sampled at the beginning and the end of the experiment for the Western Baltic cod experiment and in weekly intervals for the Barents Sea cod experiment.

In all experiments the eggs were transferred and incubated in ambient pCO_2 (426 ± 47 µatm CO_2 Western Baltic and 503 ± 89 µatm CO_2 Barents Sea 2014 and 452.5 ± 55.5 µatm CO_2 Barents Sea 2015) and at end-of-century pCO_2 (1033 ± 255 µatm CO_2 Western Baltic and 1179 ± 87 CO_2 µatm Barents Sea 2014 and 1017.6 ± 105.8 CO_2 µatm Barents Sea 2015). Eggs and larvae from the Western Baltic were kept at 7 °C and under an ambient light regime that was matched to the outside sun rise and fall weekly while eggs and larvae from the Barents Sea (2014 and 2015) were kept at 6 °C which were later increased to 10 °C and under constant light, which reflects the ambient light levels. Once the majority of Barents Sea larvae had hatched they were redistributed at same densities to twelve 180 l larvae tanks, which were divided among the two CO_2 treatments and in 2014 even under two fed treatments. After hatching larvae from the Western Baltic were fed with natural plankton from the Gullmars Fjord under green water conditions with *Nannochloropsis* thus recreating natural food levels, while larvae from the Barents Sea were initially given *Nannochloropsis* followed by *Brachionus* and later *Artemia* nauplii. The latter, being a standard aquaculture protocol, was applied in two intensities, one in which the fish were fed ad libitum and the other were the number of feedings per day was reduced to less than half. The aim of the latter feeding regimes was to investigate, whether increased feeding rates can mediate the adverse effects of ocean acidification. In 2015 only the latter feeding regime was used. All feeding regimes are listed in Table 1a and 1b.

Tab. 1: a) Feeding densities for the Western Baltic population and b) Barents Sea populations

a	Western Baltic Natural Plankton			
dph	*Nannochloropsis* added	First Daily Feeding (prey org ml^{-1} feeding^{-1})	Second Daily Feeding (prey org ml^{-1} feeding^{-1})	Third Daily Feeding (prey org ml^{-1} feeding^{-1})
1	yes	0	0	0
2	yes	0	0	0
3	yes	0	0	0
4	yes	0	0	0
5	yes	0.18	0	0
6	yes	0	0	0
7	yes	0.34	0	0
8	yes	0.51	0	0
9	yes	0.20	0.18	0
10	yes	0.18	0.34	0
11	yes	0.70	0	0
12	yes	0.70	0.67	0
13	yes	0.37	0.34	0
14	yes	0.43	0.48	0
15	yes	0.65	0.55	0.44
16	yes	0.18	0.40	0
17	yes	0.12	0.17	0.20
18	yes	0.24	0.34	0
19	yes	0.13	0.19	0
20	yes	0.28	0.15	0.18
21	yes	0.11	0.08	0
22	yes	0.17	0.12	0.15
23	yes	0.21	0.08	0
24	yes	0.12	0	0
25	yes	0.27	0.86	0

b) Aquaculture Low Food Aquaculture High Food

DPH	Greenwater/ Nannochloropsis	Rotatoria per Tank per Day(mill.)	Number of daily feedings of Rotatoria	Artemia per Tank per Day(mill.)	Number of daily feedings of Artemia	Greenwater/ Nannochloropsis	Rotatoria per Tank per Day(mill.)	Number of daily feedings of Rotatoria	Artemia per Tank per Day(mill.)	Number of daily feedings of Artemia
1	yes	4,27	7			yes	4,27	7		
2	yes	4,27	7			yes	4,27	7		
3	yes	4,27	7			yes	4,27	7		
4	yes	4,27	7			yes	4,27	7		
5	yes	7,35	7			yes	7,35	7		
6	yes	7,35	7			yes	7,35	7		
7	yes	7,35	7			yes	7,35	7		
8	yes	7,35	7			yes	7,35	7		
9	yes	7,35	7			yes	7,35	7		
10	yes	7,35	7			yes	7,35	7		
11	yes	7,35	7			yes	7,35	7		
12	yes	7,35	7			yes	7,35	7		
13		7,35	7				7,35	7		
14		3,15	3				7,35	7		
15		3,15	3				7,35	7		
16		3,15	3				7,35	7		
17		3,15	3				7,35	7		
18		3,15	3				7,35	7		
19		3,15	3				7,35	7		
20		3,15	3				7,35	7		
21		3,15	3				7,35	7		
22		3,15	3				7,35	7		
23		3,15	3				7,35	7		
24		3,15	3				7,35	7		
25		2,28	3	0,51	3		5,35	7	1,19	7
26		2,28	3	0,51	3		5,35	7	1,19	7
27		2,28	3	0,51	3		5,35	7	1,19	7
28		2,28	3	0,51	3		5,35	7	1,19	7
29		2,28	3	0,51	3		5,35	7	1,19	7
30				0,53	1				0,80	3
31				0,53	1				0,80	3
32				0,53	1				0,80	3
33				0,53	1				0,80	3
34				0,53	1				0,80	3

Survival of larvae from the Western Baltic population was measured daily counting the dead larvae collected from each tank, in combination with the initial number of larvae per tank (~800) a daily mortality rate was back calculated. In case of the Barents Sea population mortality was calculated by density estimates, which were obtained by taking 5 samples of 0.8 l volume using a pipe from the whole water column of rearing tank. The samples were taken after an even larvae distribution was achieved by increasing the aeration, subsequently the number of larvae in every subsample was counted and the larval density calculated for every rearing tank. These samplings were started at 8 days post hatching (dph) and then repeated every 4 to 5 days. The mean mortality coefficients were calculated after non-linear curve fitting of a negative exponential function for each replicate tank in both experiments. A comparison of the mortality rates between the treatments was done using a t-test (Western Baltic stock) and a two-way ANOVA (Barents Sea stock) after homogeneity of variance was achieved through appropriate data transformations.

Samples for individual analysis of the larvae such as growth and development were obtained by randomly scoping out batches of larvae from the tanks after increasing the aeration to homogenize the distribution of larvae. These larvae were euthanized with MS-222 before being preserved individually and/or in batches in a variety of, for the different analysis, appropriate solutions and temperatures.
Larvae for growth analysis were frozen in seawater at -80 °C by batches of ten from each replicate. Prior to photographing individually under a stereomicroscope all larvae were thawed on ice. Subsequently the standard length was measured using IMAGE-Tool. After the picture was taken larvae were freeze-dried for 16h and consequently weighed to the nearest µg. Through the whole process the larvae were stored in individually labelled tube on ice for short times and at -80 °C for any longer periods of time and for later down-stream analyses such as RNA/DNA quantification. The standard length of the larvae were measured from the tip of the lower jaw (where applicable) through the centre of the eye, along the vertebrae's upper edge, ending at the vertebrae tip.

Further details regarding sampling, methods and carbon chemistry analysis will be available in the individual publications.

Results

The experimentally estimated daily mortality rates were consistent among all stocks and feeding conditions, the end-of-century CO_2 treatment resulted in an approximately doubling of mortality (Fig1). The daily mortality increased from 9.2 to 20.4% in the Western Baltic (T-test, t=-3.749, df=2.41, *p*=0.024) and from 7 to 13% in the Barents Sea 2014 population (Two-way ANOVA F=8.434, df= 1, *p*=0.023). Food density had no detectable effect on the mortality rate of the Barents Sea population, neither as main effect nor in interaction with the acidification treatment.

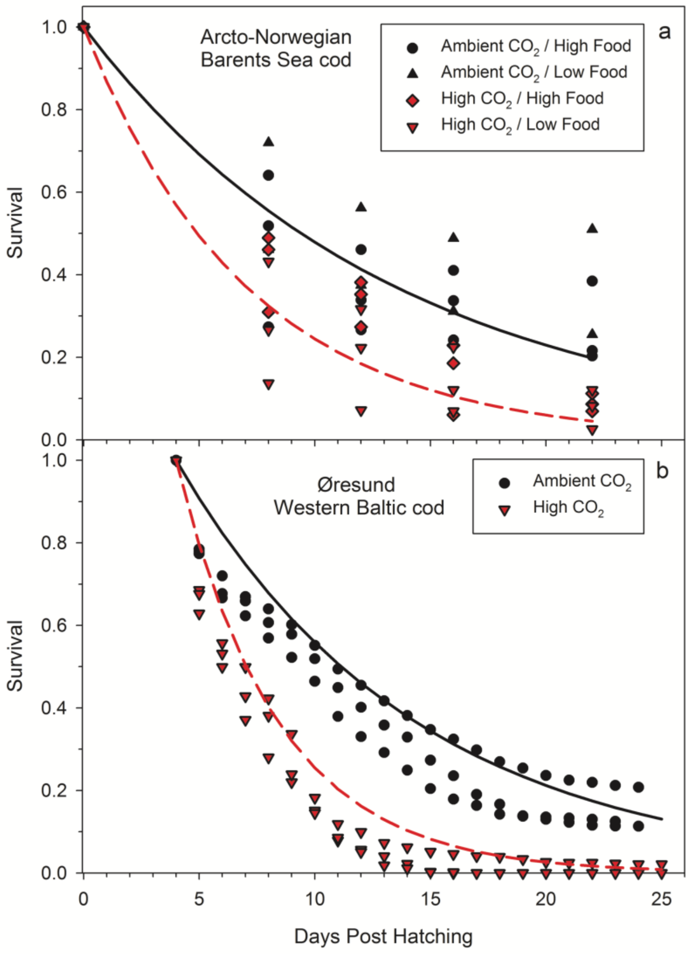


Fig 1: Effect of increased CO_2 on early life survival of *Gadus morhua* from a) Barents Sea cod b) Western Baltic cod. Each symbol represents the value of one replicate tank. Lines depict the number of survivors according to the fitted negative exponential function.

In 2014 the ocean acidification treatment resulted in longer (Fig. 2a) and heavier (Fig. 2b) larvae under the reduced feeding regime, these effects are only visible at and after 31 dph.

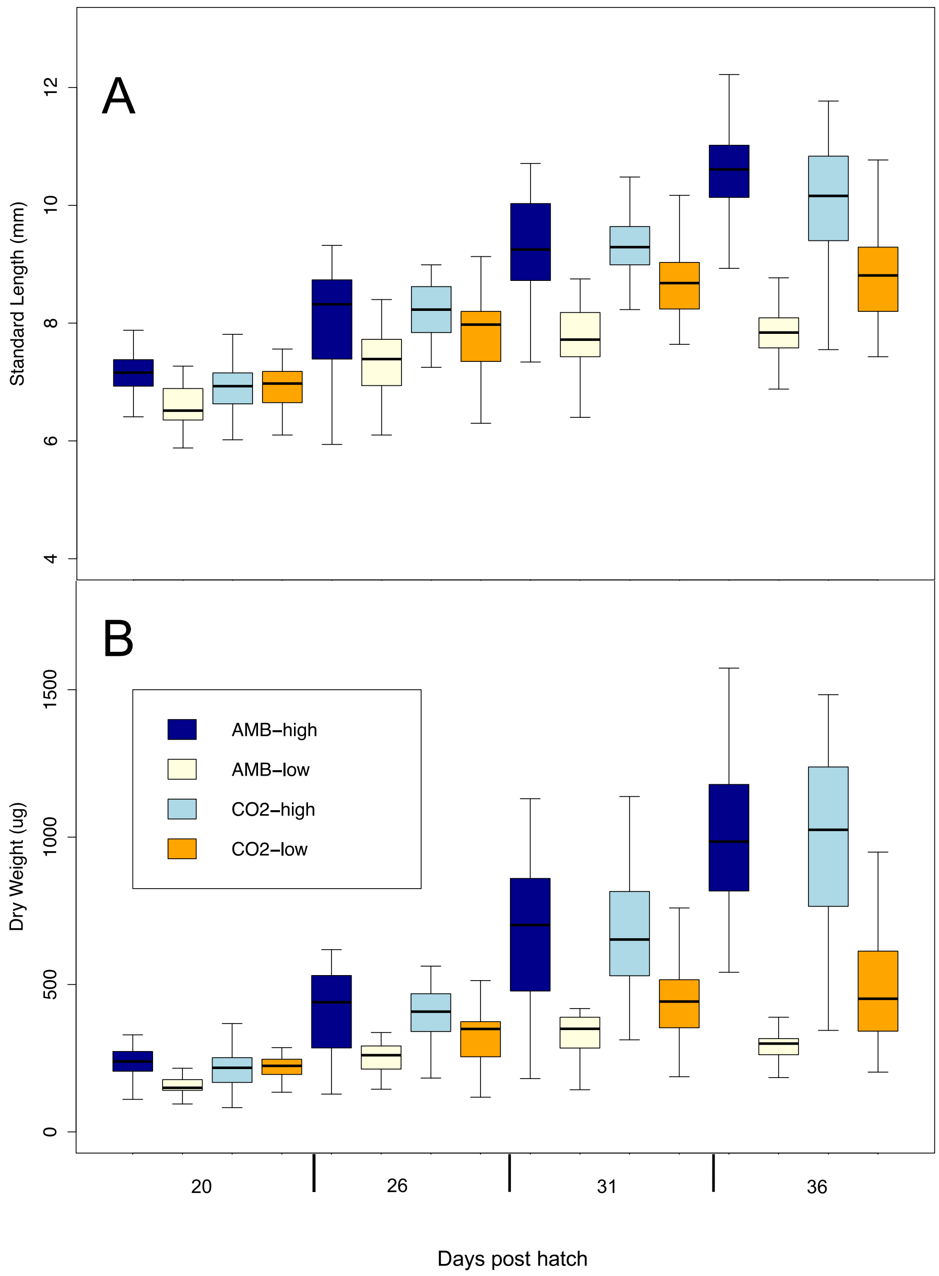


Fig 2; Effect of increased CO_2 on early life growth of *Gadus morhua* from the Barents Sea in 2014 under different feeding regimes at days 20, 26 ,31 and 36 post hatching a) in standard length (mm) and b) in dry weight (µg). Boxplots represent median, 25 and 75 percent quartiles and the total range. High and low present the two different feeding regimes used.

The growth pattern in the 2015 experiment, in which larvae were kept under the low food regime, shows no effect of direct exposure of ocean acidification on the larvae (Fig. 3). However parental acclimatization had a significant effect on the growth of the larvae at day 34 both in terms of standard length and dry weight while the direct exposure of eggs and larvae had no apparent effect (Tab. 2, Fig. 4)

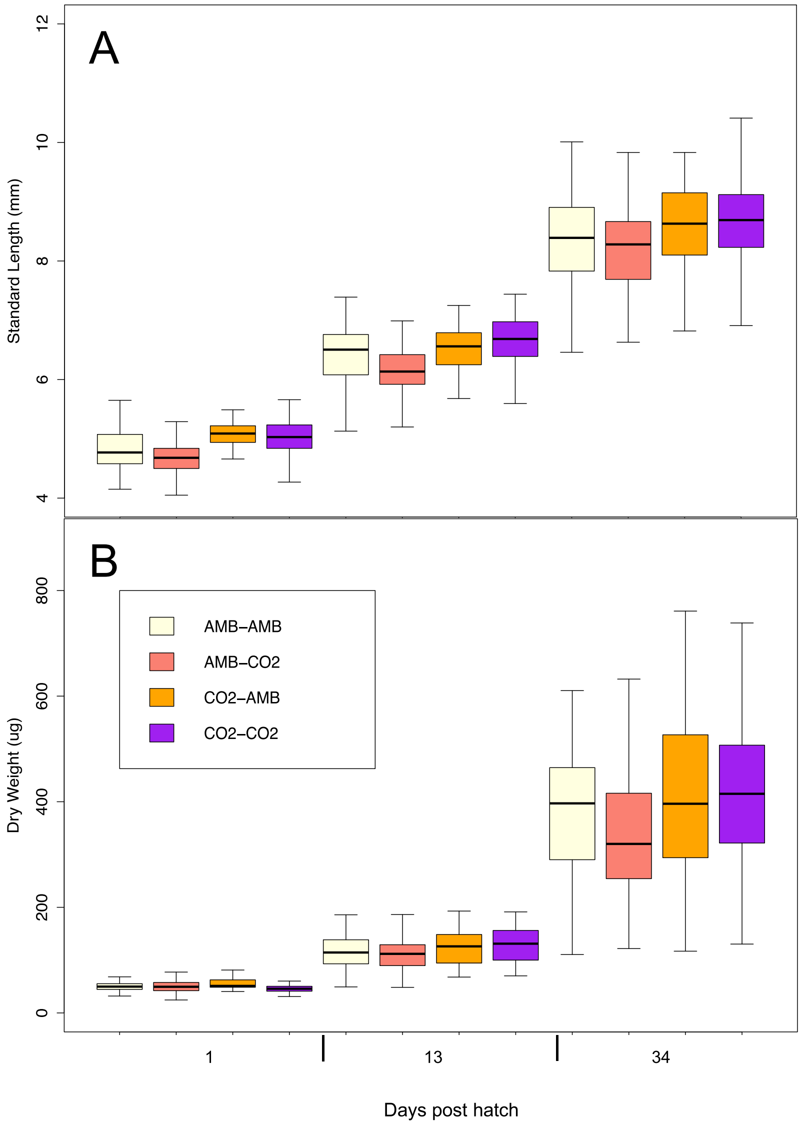


Fig. 3: Effect of increased CO_2 on early life growth of *Gadus morhua* from the Barents Sea in 2015 under different parental acclimatization (Present-day and end-of-century pCO_2) at days 1, 13 and 34 post hatching a) in standard length (mm) and b) in dry weight (µg). Boxplots represent median, 25 and 75 percent quartiles and the total range.

Tab. 2: Statistical summary Two-Way ANOVA a) standard length and b) dry weight

a) Two-way ANOVA	Standard Length		
	DF	F	p-value
Parental Treatment	1	7.165	0.0132
Larvae Treatment	1	0.094	0.7624
Parental x Larvae Treatment	1	0.046	0.6442
Residuals	24		

b) Two-way ANOVA	sqrt Dry Weight		
	DF	F	p-value
Parental Treatment	1	5.446	0.0283
Larvae Treatment	1	0.028	0.08681
Parental x Larvae Treatment	1	0.921	0.3469
Residuals	24		

Additional analyses and statistics will be provided in the respective publication.

Conclusions and future perspectives

The experiments performed for this study clearly show that larvae of Western Baltic and coastal Barents Sea cod in 2014 are impacted by near future levels of ocean acidification. While some results are easy and directly to interpret, namely the massively increased daily mortalities, other variables such as the growth patterns need to be analyzed in more detail to understand the underlying physiological and genomic mechanisms as well as their implications, may they be ecological or socio-economic.

Our findings additionally suggest that even at *ad libitum* feeding, e.g. a richer energy budget that would allow for more efficient acid-base regulation, cod larvae cannot mediate the adverse effects of ocean acidification. This strengthens the hypothesis that larvae will be negatively affected by ocean acidification independent from food availability in a patchy or match-mismatch environment. However this apparently does not hold true for growth patterns. At high fed availability, larvae in ambient and end-of-century treatment show no apparent differences in standard length and dry weight by the end of the experiment (36 dph).

Additionally, our experiments suggest that offspring of brood stock from the Barents Sea that has been exposed to end-of-century $p\mathrm{CO}_2$ levels grow longer and heavier in both ambient and end-of-century larvae treatment by day 34 past hatching compared to offspring from brood stock kept at ambient $p\mathrm{CO}_2$ levels.

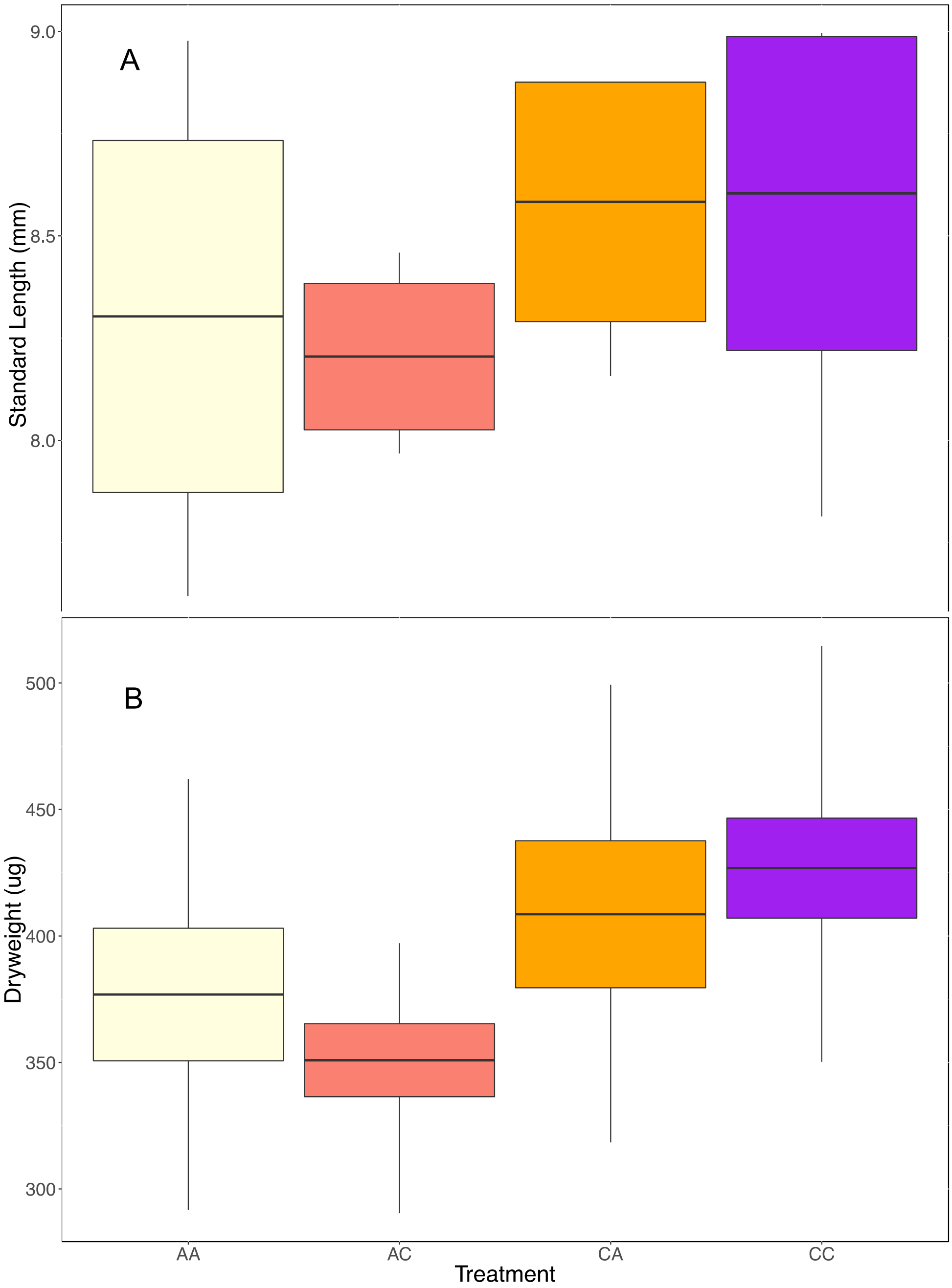


Fig. 4: Effects of parental and larval exposure to increased pCO_2 on *Gadus morhua* offspring a) standard length (mm) and b) dry weight (µg) at 34 days post hatch. Boxplots represent mean, SEM and range.

Further our results highlight the importance of investigating several responses to changes in the physiological environment in the same experiment, as in our case mortality and growth. Looked at separately the growth patterns of the high CO_2 treatment could be interpreted as ocean acidification having a positive effect on cod larvae through increased growth; yet if one includes the detrimental increase in mortality it will most likely have several effects on the population dynamics of both cod populations. Once the remaining samples for growth analysis are measured the changed growth patterns and their causes will be examined by their lipid content, as it has been show in earlier experiments that larvae under high CO_2 treatments deposit lipid vacuoles in certain organs, which, at a later point, lead the increased mortality (Frommel et al., 2012). Additionally samples for histology and bone ossification are currently being processed to address the question, whether the changed growth patterns are only caused by increased growth and/or by increased developmental speed.

In the near future we plan to deepen our investigation in the growth, condition and developmental patterns between the different populations and treatments by analyzing the RNA/DNA ratio of larvae at critical stages in their development. However, the main focus will be put on the transcriptomic analysis of the 2015 experiment to evaluate if the observed parental effects in growth are reflected in changed gene expression patterns. In which case it can be assumed that some epigenetic mechanisms (Metzger & Schulte, 2016) are involved e.g. a case for transgenerational adaptation. Additionally we have developed and prepared as microsatellite toolkit to perform a parentage analysis with the aim of addressing family specific mortality, e.g. to estimate if offspring from certain families have higher or lower than average mortality rates and thus suggesting that not all parts of these populations react similarly to changes in their habitat's carbon chemistry. Additionally the results from mortality, growth and respiration will be included in recruitment, socio-economic and individual-based-models to address, the often called for (Lam, Cheung, & Sumaila, 2014; MacNeil et al., 2010) ecological and economic implications of the effects of ocean acidification on different cod populations.

References

Bignami, S., Enochs, I. C., Manzello, D. P., Sponaugle, S., & Cowen, R. K. (2013). Ocean acidification alters the otoliths of a pantropical fish species with implications for sensory function. *Proceedings of the National Academy of Sciences of the United States of America*, *110*(18), 7366–70. http://doi.org/10.1073/pnas.1301365110

Checkley, D. M., Dickson, A. G., Takahashi, M., Radich, J. A., Eisenkolb, N., & Asch, R. (2009). Elevated CO_2 enhances otolith growth in young fish. *Science (New York, N.Y.)*, *324*, 1683. http://doi.org/10.1126/science.1169806

Dixson, D. L., Munday, P. L., & Jones, G. P. (2010). Ocean acidification disrupts the innate ability of fish to detect predator olfactory cues. *Ecology Letters*, *13*(1), 68–75. http://doi.org/10.1111/j.1461-0248.2009.01400.x

Doney, S. C., Fabry, V. J., Feely, R. a, & Kleypas, J. a. (2009). Ocean acidification: the other CO_2 problem. *Annual Review of Marine Science*, *1*, 169–92. http://doi.org/10.1146/annurev.marine.010908.163834

Falk-Petersen, I. B. (2005). Comparative organ differentiation during early life stages of marine fish. *Fish and Shellfish Immunology*. http://doi.org/10.1016/j.fsi.2005.03.006

Frommel, A. Y., Maneja, R., Lowe, D., Malzahn, A. M., Geffen, A. J., Folkvord, A., … Clemmesen, C. (2011). Severe tissue damage in Atlantic cod larvae under increasing ocean acidification. *Nature Climate Change*, *2*(1), 42–46. http://doi.org/10.1038/nclimate1324

Frommel, A. Y., Maneja, R., Lowe, D., Malzahn, A. M., Geffen, A. J., Folkvord, A., … Clemmesen, C. (2012). Severe tissue damage in Atlantic cod larvae under increasing ocean acidification. *Nature Climate Change*, *2*(1), 42–46.

Frommel, A. Y., Maneja, R., Lowe, D., Pascoe, C. K., Geffen, A. J., Folkvord, A., … Clemmesen, C. (2014). Organ damage in Atlantic herring larvae as a result of ocean acidification. *Ecological Applications*, *24*(5), 1131–1143. http://doi.org/10.1890/13-0297.1

Frommel, A. Y., Schubert, A., Piatkowski, U., & Clemmesen, C. (2013). Egg and early larval stages of Baltic cod, *Gadus morhua*, are robust to high levels of ocean acidification. *Marine Biology*, *160*(8), 1825–1834. http://doi.org/10.1007/s00227-011-1876-3

Ishimatsu, A., Hayashi, M., & Kikkawa, T. (2008). Fishes in high-CO_2, acidified oceans. *Marine Ecology Progress Series*, *373*(1), 295–302. http://doi.org/10.3354/meps07823

Lam, V. W. Y., Cheung, W. W. L., & Sumaila, U. R. (2014). Marine capture fisheries in the Arctic: winners or losers under climate change and ocean acidification? *Fish and Fisheries*. http://doi.org/10.1111/faf.12106

MacNeil, M. A., Graham, N. a J., Cinner, J. E., Dulvy, N. K., Loring, P. a, Jennings, S., … McClanahan, T. R. (2010). Transitional states in marine fisheries: adapting to predicted global change. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, *365*(1558), 3753–63. http://doi.org/10.1098/rstb.2010.0289

Maneja, R., Frommel, A., Geffen, A., Folkvord, A., Piatkowski, U., Chang, M., & Clemmesen, C. (2013). Effects of ocean acidification on the calcification of otoliths of larval Atlantic cod *Gadus morhua*. *Marine Ecology Progress Series*, *477*, 251–258. http://doi.org/10.3354/meps10146

Maneja, R. H., Frommel, A. Y., Browman, H. I., Clemmesen, C., Geffen, A. J., Folkvord, A., Skiftesvik, A. B. (2012). The swimming kinematics of larval Atlantic cod, Gadus morhua L., are resilient to elevated seawater pCO_2. *Marine Biology*, *160*(8), 1963–1972. http://doi.org/10.1007/s00227-012-2054-y

Metzger, D. C. H., & Schulte, P. M. (2016). Epigenomics in marine fishes. *Marine Genomics*. http://doi.org/10.1016/j.margen.2016.01.004

Munday, P. L., Dixson, D. L., Donelson, J. M., Jones, G. P., Pratchett, M. S., Devitsina, G. V, & Døving, K. B. (2009). Ocean acidification impairs olfactory discrimination and homing ability of a marine fish. *Proceedings of the National Academy of Sciences of the United States of America*, *106*(6), 1848–52. http://doi.org/10.1073/pnas.0809996106

Munday, P. L., Pratchett, M. S., Dixson, D. L., Donelson, J. M., Endo, G. G. K., Reynolds, A. D., & Knuckey, R. (2012). Elevated CO_2 affects the behavior of an ecologically and economically important coral reef fish. *Marine Biology*, *160*(8), 2137–2144. http://doi.org/10.1007/s00227-012-2111-6

Munday, P. L., Watson, S.-A., Parsons, D. M., King, A., Barr, N. G., Mcleod, I. M., … Pether, S. M. J. (2015). Effects of elevated CO_2 on early life history development of the yellowtail kingfish, *Seriola lalandi* , a large pelagic fish. *ICES Journal of Marine Science: Journal Du Conseil*, (January 2016), fsv210. http://doi.org/10.1093/icesjms/fsv210

Riebesell, U., Fabry, V. J., Hansson, L., & Gattuso, J.-P. (2010). *Guide to Best Practices in Ocean Acidification Research and Data Reporting*. *Report of international research workshop on best practices for ocean acidification research (19-21 November 2008 in Kiel, Germany)*. http://doi.org/10.2777/58454