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Seasonal and annual variations in physiological and biochemical responses from transplanted marine bioindicator species *Mytilus* spp. during a long term field exposure experiment

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Abstract

In a pilot field study the long term response of transplanted bioindicator organisms *Mytilus* spp. was analyzed on the basis of physiological indices and biochemical measurements related to the energy budget. Three different time series with deployment times of eight to twelve months were compared according to seasonality and repeatability of the responses. Test organisms were incubated at a coastal station in the anthropogenically impacted estuary of the river Elbe and at an North Sea station located in vicinity to the Island of Helgoland in the German Bight . The stations differ in their hydrological as well as chemical characteristics. They can be discriminated by statistical factor analysis based on the measured biochemical parameter. Levels of all energy budget biomarker varied between seasons; however, the degree of variation of the specific response was differently expressed. The mussels deployed at Helgoland showed a reproducible high condition index in each sampling series and an oscillating gonadosomatic index representing the reproduction cycle. The lowest available energy was recorded in mussels at the estuarine sampling station compared to the off-shore station. This may be caused by the energetically costly maintenance of osmotic balance and consequently result in a lower amount of energy available for defense against chemical stress, growth and reproduction.

key words:

environmental biomonitoring, mussel, condition index, biomarker, energy budget

1. Introduction

The assessment of environmental pollution is a considerable and ongoing challenge since the variability, number and amount of potential hazardous chemicals of industrial use is tremendous (Lepom et al., 2009) (Thomaidis et al., 2013). In the European Marine Strategy Framework Directive (MSFD) one criterion for a good environmental status is that “the concentrations of contaminants are at levels not giving rise to pollution effects”. The concentration of contaminants in the marine environment and their effects need to be assessed taking into account the impacts and threats to the ecosystem (Hagger et al., 2008). Therefore monitoring approaches should have an integrative character combining chemical and ecological aspects with abiotic and biotic parameters (Schettino et al., 2012). Regular monitoring

programs rely on the availability of efficient and robust tools and technologies able to deliver appropriate and reliable data (Allan et al., 2006; Brooks et al., 2009; Galloway et al., 2004). Principles and standards for an environmental monitoring under the requirements of the MSFD were provided by the OSPAR Guidance for an integrated assessment of biological effects (Board, 2011). Bivalves belong to the first choice species as bioindicators for environmental and chemical stress because these are sentinel benthic organisms living as filter – feeders and exposed to different environmental compartments. Due to their wide distribution, resistance to variable environmental conditions and ability of bioaccumulation, they are prolific tools for biomonitoring of chemical pollution. Traditionally, wild mussel populations were applied for environmental monitoring (Roberts, 1976; Cantillo, 1998; Bricker et al., 2014;; Paraskevopoulou et al., 2014). However, the utilization of transplanted mussels enables the compensation of the biological diversity and scarcity of natural mussel populations, which often complicates the final data interpretation as well as the wide-spread application of such approaches. The deployment of transplanted mussels in an *in situ* experiment provides important information on the bioavailability of contaminants and associated biological effects. Furthermore, it combines the advantages of environmental realism and semi-controlled experimental conditions (Salazar and Salazar, 1995). The usefulness of applying caged mussels for biomonitoring purposes have been shown in several studies (Bodin et al., 2004; Hunt and Slone, 2010). A further successful example for caged mussel experiment is described by Tsangaris et al. (2010) to distinguish polluted from less-polluted sites at the Greek Mediterranean coastline (Tsangaris et al., 2010). A long-term biomonitoring study with transplanted *Perna perna* integrating data on bioaccumulation of different classes of pollutants with data on biomarker related to different defense mechanisms pointed out differences between sampling stations, seasons and critical areas (Pereira et al., 2012). The targeted placement of mussel cages at gas or oil platforms or other contamination hot spots enables the monitoring of potential sources of hazardous substances where no natural populations are available (Beyer et al., 2013; Brooks et al., 2015). Even short term exposure experiments with transplanted mussels as bioindicators were performed to assess the water quality by separating impacted areas based on physicochemical and biochemical parameter (Giarratano et al.). In the North Sea, the pilot study performed in the BECPELAG project is an excellent example for the methodological performance and the integrative character of such caged

mussel approach (Hylland et al., 2006). A comprehensive and integrative monitoring approach measuring a multitude of biological responses in transplanted mussels in the Baltic Sea is described in Turja et al. (2014). The recommended core set of biomarkers indicating the biological effects of contaminants includes several parameters on different levels of biological organization from subcellular and tissue responses up to whole organism effects (Cajaraville et al., 2000; Sarkar et al., 2006; Thain et al., 2008). Such a suit of potential biomarker in different levels of biological organization is appreciated because it intensifies the information content regarding the biological effects of contaminants; however, the methods for regular monitoring should be as reliable and time-saving as possible and furthermore avoid sacrificing many organisms. Therefore, methods and assessment criteria have to be optimized in order to obtain reliable and comparable data and make low-cost tools and technologies available for regular monitoring purposes. Furthermore the application of assays requiring living animals like stress on stress or fresh hemolymph cells e.g. for Lysosomal stability is often not appropriate for regular monitoring. One main aspect of the present study was to apply comparably low effort parameter which must not necessarily be measured after sampling and to test their power in relation to conventional indices. Furthermore the application of assays requiring living animals like stress on stress or fresh hemolymph cells e.g. for Lysosomal stability is often not appropriate for regular monitoring approaches. The Condition Index (ratio of tissue to shell) and Gonadosomatic Index (ratio of gonads to tissue) are well established as standard parameter (Andral et al., 2004; Lucas and Beninger, 1985). The biochemical analysis of the energy budget is a sensitive methodology used to evaluate the energetic status (Smolders et al., 2004). It is suitable for mussels as well as for other species (De Coen and Janssen, 2003; Erk et al., 2008; Gomes et al., 2015; Moolman et al., 2007; Olsen et al., 2008; Verslycke et al., 2004). The measurement of the total amount of available energy utilized for growth, reproduction and maintenance may provide information on the general environmental stress level and on the organisms overall condition and response to toxic stress. Living in suboptimal environments costs metabolic resources which are quantified as carbohydrate, lipid and protein content in the digestive glands of transplanted mussels. This approach has been proposed as alternative to the measurement of the Scope for Growth (De Coen and Janssen, 2003; Verslycke et al., 2004).

The aim of the present study was the implementation of a long-term field experiment with transplanted mussels. It was conducted in order to analyze the repeatability and robustness of such kind of biomonitoring experiment with mussels exposed for a long period covering different seasons by evaluating physiological and bioenergetics biomarker every six weeks. It can be judged as feasibility study to investigate an effective long term sampling strategy and set of physiological and biochemical measurements.

2. Material and Methods

2.1 Field exposure and sampling

Cohorts of mussels of the same origin (obtained from commercial fisheries at the Island of Sylt, Germany) were deployed in cages at two different field stations; one located at the Island of Helgoland (54.18; 7. 88), German Bight and the other at the estuary of the river Elbe in Cuxhaven (53.88; 8.70), Germany, from June 2011 to January 2012 (series a: eight months), April 2012 to April 2013 (series b: twelve months) and from May 2013 to May 2014 (series c: twelve months). In order to achieve synchronization of the data, the sampling timing was summarized as start, summer with two time point T1 and T2, autumn, winter (T1 and T2), and spring (T1 and T2). The exact sampling timing of each series was summarized in table 1. Every series started with a new batch of adult mussels (60.3 +/-2.9 mm length). Oceanographic data such as Sea Surface Temperature (SST) and Salinity were continuously recorded using the Coastal Observing System for Northern and Arctic Seas COSYNA powered by the Helmholtz-Zentrum Geesthacht Centre of Materials and Coastal Research. Mussel sampling occurred every 6-7 weeks. The sampling at Helgoland was done by the Center of Scientific Diving, Alfred Wegener Institute for Polar- and Marine Research Bremerhaven, Station Helgoland: After recovery of the samples from the submerged station, the mussels were placed in filtered Helgoland seawater over night in a flow-through tank to allow their depuration and shipped to Cuxhaven under cooled conditions (transportation time approximately 8h). The cohort of mussels at Cuxhaven was accessible via an elevator construction, and both sample groups were transported to the laboratory simultaneously in cooled container in order to minimize stress -related effects. The organisms obtained at the Station

Cuxhaven were placed in aerated Cuxhaven water, which was purified by centrifugation in order to allow a depuration over night as it was done for the Helgoland mussels.

2.2 Trace element determination in whole body mussel tissue

The analysis of accumulated trace elements was performed as described previously (Helmholz et al, 2015), Opening and tissue removal from the shells were done with a cleaned ceramic knife to avoid any contamination of the tissue After the opening of the shell the inside of the shell as well as the whole soft tissue were flushed with MilliQ water to remove any remaining particles or sea water residues. Composite samples of the whole wet tissue (3 mussels per sample and three replicates s per sampling campaign and station) were homogenized and freeze dried. For trace element analysis an aliquot of approximately 200 mg dry material was digested by microwave accelerated digestion (CEM, Kamp-Lintfort, Germany) using a mixture of 5 mL HNO_3, 2 mL HCl and 1 mL of H_2O_2.. The samples were measured using inductively coupled plasma tandem mass spectrometry (Agilent 8800). An external calibration was performed for quantification, which utilizes different multielement solutions to cover the targeted analyte range.

2.3 Physiological parameter

Composite samples, each randomly composed of ten organisms, were used for the measurement of the physiological parameters Condition Index (CI tissue dry weight/shell dry weight) and Gonadosomatic Index (GSI sum of mantle wet weight/tissue wet weight x 100). The whole tissue and shells were dried by lyophilization for five days and weighted for CI calculation. Further ten randomly retained animals were used for the determination of biochemical parameter and the GSI. The gills, digestive glands and mantle tissues were prepared in this order, weighed and stored discretely in cryostat vials.

Sex determination was performed according to Jabbar and Davies (Jabbar and Davies, 1987) by heating a small piece (0.3 x 0.3 cm up to 0.5 x 0.5 cm) of mantle tissue in 20% trichloracetic acid (TCA) solution (2 mL) and 0.5 mL of 0.75% thiobarbituric acid (TBA) at 100°C for 15 – 20 min. The development of yellow and pink color indicates male and female individuals respectively. For some mussels the color

development was not clear due to a poor gonad development or reproduction status. Questionable results were not included in the assessment.

2.4 Biochemical parameter

2.4.1 Protein, carbohydrate and lipid determination for the quantification of available energy (Eav)

In principle, the procedure for the determination of the basic nutrients (carbohydrate, lipid, protein) as major metabolic resources was based on the method described by Smolders at al. (2004) for Zebra mussels and Erk et al. (2011) for Mediterranean mussels.

The digestive glands of each second sampling campaign were thawed on ice and homogenized with 100 mM 2-Amino-2-hydroxymethyl-propane-1,3-diol (Tris) buffer pH 8.6. The ratio of gland tissue to buffer was 100 mg tissue in 1 mL buffer. Tissue homogenization was achieved by extensive mixing (Ultra-Turrax® IKA Labortechnik, Staufen, Germany) for three times 1 min with 30 sec break on ice. Aliquots (three per nutrient) measuring 200 µL were taken in order to ensure equivalent replicate measurements. Homogenates were stored at -80°C until nutrient determination. Prior to each analysis, the samples were slowly thawed on ice.

The protein content of gland homogenates were measured after centrifugation 10 min 2000 rpm at 4°C in the resulting supernatant using Bovine serum albumin (BSA ACS chemicals Inc. USA) as protein standard in the range of 0.025 mg/mL to 0.4 mg/mL (Bradford, 1976).

The carbohydrate content was analyzed by the phenol-sulphoric acid method described by Dubois et al. (1995) modified as a micro format. Carbohydrates were measured in the homogenate after protein precipitation using 15% TCA in a first and 5% TCA in a second step. Glucose (Sigma-Aldrich Chemie GmbH, Munich, Germany) was applied as standard in the range of 0.031 – 0.75 mg/mL. The development of an orange color was determined photometrically at a wavelength of 490 nm.

Lipid determination was a two-step procedure, starting with the liquid-liquid extraction of the tissue homogenate with chloroform:methanol:water (2:2:1 v:v:v) (Bligh and Dyer, 1959). The second step was the detection by adding concentrated sulphoric acid and heating for 25 min at 200°C. The brownish color was measured spectrophotometrically at a wavelength of 340 nm against triplamitine (Sigma-

Aldrich Chemie GmbH, Munich, Germany) as standard (0.25 – 4.0 mg/mL) (Marsh and Weinstein, 1966).

The measured values (mg/g_{ww}) were converted into energy equivalents in order to calculate the available energy (Eav). According to Gnaiger (1983) the specific enthalpy of combustion is 17.5 kJ/g for carbohydrates, 39.5 kJ/g for lipids and 24.0 kJ/g for proteins (Gnaiger, 1983).

2.4.2 Enzyme detection

The activity of the mitochondrial electron transport system (MET) was measured photometrically by reducing the tetrazolium dye (2-(4-Iodophenyl) 3-(4-nitrophenyl) 5-phenyl 2H-tetrazolium chlorid (synonym: p-iodonitrotetrazolium violet INT; Sigma-Aldrich Chemie GmbH, Munich, Germany) following the protocol of Owens and King (1975) with modifications of de Coen and Janssen (1997). The fresh homogenate was diluted 1:1 with 0.2 M phosphate buffer containing 150 µM magnesium sulphate, 0.3% poly vinyl pyrrolidone and 0.4% Triton X-100 and frozen at -80°C until use. Aliquots were prepared for two replicative measurements. After thawing and centrifugation of this mixture at 4°C at 2000 rpm for 10 min, three replicates measuring 60 µL were mixed with 180 µL ETS substrate buffer (0.1 M phosphate buffer pH 8.5) containing 50 mM Tris, 0.2% Triton X-100 and the co-substrates 0.25 mM NADPH and 1.7 mM NADH were freshly supplemented. The reaction was started by adding 60 µL 0.2% INT solution and the absorbance change was observed immediately at 30°C over a period of 20 min at a wavelength of 490 nm.

A stoichiometric ratio of 2 mol formed formazan corresponding to 1 mol consumed oxygen was presumed for the calculation of oxygen consumption. The MET activity was transformed into energetic equivalents using an average oxyenthalpic equivalent of 480 kJ/mol oxygen (Gnaiger, 1983).

2.5. Statistical evaluation

The statistical evaluation was performed by using the excel Add In WinSTAT® version 2012.1. Ten biological replicates were measured in at least two independent experiments with 3 (9 for lipid) replicative technical measurements per nutrient. Normality of the data was checked by Kolmogorov-Smirnov-Test and in the case of non-normal distribution the non-parametrical Mann–Whitney (U) - test

was applied. Statistical differences in biological responses between sampling season were analyzed with ANOVA (F) followed by post-hoc Scheffés test method, whereas the variance homogeneity was checked using the Bartlett test. In order to analyze significant differences between the stations, all data of each series per parameter were summarized without taking into account the primary samples. Factor analysis was conducted in order to show the arrangement of parameters and sample groups. Collinearity between biological parameter was determined conducting the Pearson correlation analysis. In order to visualize the parameter in a comparable way e.g. by star plots the data were transformed into standard normal distribution ($\mu = 0$; $\sigma = 1$). Therefore mean and standard deviation were calculated for all values measured at both stations for each parameter separately. The values were normalized by subtracting the mean and dividing by the standard deviation (Beliaeff and Burgeot, 2002).

3. Results

3.1 Temporal dynamics in salinity and temperature at the stations

Salinity and sea surface temperature (10 m depth) were continuously recorded in the framework of the COSYNA project powered by the Helmholtz-Zentrum Geesthacht. Although there was patchy information for the Helgoland station during the „series c“, the main sequences can be compared. The salinity at the station Helgoland was constant at 33 without any drastic variances throughout and within the three series (supplemental material). Opposite to the stable conditions at Helgoland, the near-shore station at the Elbe estuary in Cuxhaven was influenced by the tidal rhythm with a daily salinity range of 15-25. Despite of a pronounced low salinity phase dropping down to 5 caused by an Elbe flood in June – July 2014 during „series c“, this normal range (15-25) was relatively constant. There were no differences in air exposure and UV radiation since both mussel cage holder were submersed continuously (figure 1 photograph)

The temperature profiles of the two stations differed slightly (supplemental material). At the Helgoland station, the temperature curve was smoother, meaning that it was on average two degrees warmer during the winter period and colder during the summer. The temperature increase in spring occurred later than in Cuxhaven as well as did the decrease in autumn.

3.2 Chemical contamination

The multi-element analysis of whole tissue preparations showed a broad spectrum of enriched elements. A summary of the whole dried tissue concentration of selected priority and environmentally relevant toxic elements is given in table 2. The body burden concentration of the final campaign of each series showed a very consistent element pattern and the absolute amounts were in the same order of magnitude or approximate. These results support the very stable level of sediment contamination of the North Sea with inorganic hazardous substances.

The elements arsenic, cadmium, chromium, molybdenum, nickel, lead, selenium and zinc were enriched in the whole dry tissue samples of mussels exposed at Cuxhaven in comparison to the start and Helgoland samples. The tissue of mussels exposed at the Helgoland North Sea station showed a lower element content except of selenium that was enriched in the Helgoland samples compared to the start values.

3.3 Physiological and biochemical Parameter

3.3.1 Sex determination

The photometrical method introduced by Jabber and Davies (1987) resulted in a clear development of both a yellow and a pink color and therefore a defined sex determination for most of the cases (97%). It was an easy and fast procedure. Female organism dominated the sample composition in each of the three sampling seasons and at both stations (figure 2). There was no significant difference between the series, seasons and stations. There were no remarkable correlations or significant differences in the physiological and biochemical values with respect to the sex of the analyzed mussels. Therefore the sex was not taken into account for the statistical analysis of other physiological and biochemical indices.

3.3.2 Physiological indices

The CI and GSI are well established as parameter to compare and analyze the development and fitness of transplanted mussels used as bioindicator for environmental and chemical stress. Both indices of all three sampling series were summarized in table 3.

Although the stocking material of „series a“ had an elevated CI compared to the material in „series b and c”, the CI courses over all sampling periods were in principle similar. The mussels hatched at Helgoland showed a slight increase of the meat-to-shell weight-ratio over the summer season followed by a decrease during autumn and winter. There was no pronounced recreation during spring („series b and c”) until the final sampling in April/May. For the mussels hatched at the Cuxhaven station, a continuous decrease of the CI was observed in every sampling series. The CI values differ significantly between the seasons autumn compared to spring („series b“) and summer/autumn to spring („series c“) for the mussels at Cuxhaven. There were no significant seasonal variations in the CI values observed for the mussels at Helgoland.

A relatively high GSI was already measured in the mussel cohort of „series a“ at the beginning of the caging experiment without a significant reduction or increase over the year. During „series b and c”, the mussels at the Helgoland station expressed a reproduction cycle with a maximum in autumn („series c“) and winter („series b“) and a minimum after the spawning in early spring. Opposite to this regular behavior, the mussels at Cuxhaven responded with a rapid decrease of the GSI after the deployment and a stable but very low GSI level throughout the whole exposition experiment. Due to the high biological variance, a significant change of the GSI within the annual terms cannot be pointed out for both stations. Because a correlation of physiological and biochemical parameter was supposed, the wet weights of the digestive glands were utilized as physiological fitness parameter. Due to the high biological variance within the ten randomly chosen individuals, as indicated by the error bars in figure 4, it was problematic to point out statistically sound trends and variances. There were no significant seasonal and spatial differences within the first series. In „series b“ a significant reduction of the digestive gland weight until autumn was observed with a slight recreation during winter and spring. This recreation in form of gland weight increase was not observed in „series c“. The mussels at Helgoland expressed a pronounced increase in the gland weight during winter and spring in „series b“ in contrast to the significant tissue weight loss in „series c“ from autumn to the winter-January sampling.

3.3.3 Energy-budget-related parameter

The concentrations of the basic nutrients (carbohydrate, lipid, protein) in the gland tissue and their sum parameter the available energy were documented in figure 3 and table 4. The higher values of all Helgoland samples in each sampling series were most obviously. The carbohydrate values express a pronounced and reproducible seasonality with a high level in the summer/autumn season followed by nutrient dissimilation over winter to spring. This trend could be observed at both stations.

Such a reproducible trend could not be seen for the lipid values. In „series a“, there was a significant lipid accumulation at both stations. In the following series, the values vary in the Helgoland samples („series b“) with maxima in autumn and a spring recreation. This remarkable accumulation of lipids until autumn could not be detected in the “series c”. Although less pronounced, there was also a lipid accumulation until autumn in the Cuxhaven samples combined with a significant loss over the starvation period in winter („series a and c”).

The protein concentration of the digestive glands seemed to be balanced since there was no significant seasonality in the Helgoland samples in „series b“ and “series c” but a significant increase in the autumn samples during the outstanding first series. In contrast, a significant variance in protein values was not observed in the digestive glands of the mussels transplanted to Cuxhaven during „series a“. However, in the following „series b and c” a significant loss over the exposition period was observed.

Since the Eav reflected the trends of all three nutrients, these values built a comprehensive picture over all three sampling series with slight variances in the absolute values. However, in general the organisms at Helgoland were able to store energy which allows a survival and continuance over an extended field experiment period. In opposite, the organisms at Cuxhaven feed more or less on the starting basis without a recreation. Due to this an extension of the experiment over 12 month will not be possible.

3.2.4 Activity of the mitochondrial electron transport system

The activities of the mitochondrial electron transport enzyme complexes, indicating the oxygen consumption as parameter for the respiratory capacity, were summarized in table 5. The values measured in samples from the different years could not be merged. Even the stock material of every series, the primary samples, expressed different measurements and consequently the MET values of the exposed samples were different. A statistical comparison of the seasonal and station variance was performed;

however, the picture was indifferent and no clear trend can be pointed out by comparing the series profiles. In the samples of “series a” a less active cellular respiration can be measured at both stations in the autumn samples. In “series b” the lowest MET values were measured in the winter samples and the opposite trend was seen in “series c” with small values in summer and spring. In "series a” the stations differ significantly in the autumn sampling, in „series b“ in the summer and winter sampling and in „series c“ in all sampling seasons. In most of the cases, the cellular respiration indicating a higher metabolic rate was elevated in the Cuxhaven samples despite of the summer, autumn and spring samples in the last year.

Due to this inconsistency, the MET activities were not considered as a sound option for the identification of a key factor indicating chemical and environmental stress in field studies.

3.3 Correlations and distinctions

Comparing the absolute values of the three sampling series, the first “series a” seemed to be quite different. Regardless, the data were incorporated into the statistical correlation and factor analysis. These analysis have been performed with and without “series a” and no differences were apparent in the principle statements and achievements.

Despite of the MET values, all main physiological and biochemical parameters were included in the factor analysis. The definite separation of the near-shore station in the Elbe estuary at Cuxhaven and the North Sea station at the Island of Helgoland became obvious in figure 5. The percentage of explained variance was 45% with factor 1 (30%) and factor 2 (15%). The most noticeable separation was observed for the Eav parameter, whereas the gland weight seems to be less significant.

All parameters indicated a significantly improved fitness of the mussels exposed at the Helgoland station as shown by the z-normalized values. This kind of normalization enables the direct comparison of the measured parameter since the absolute mean is reduced to zero. The individual raw values were transformed to z-scores in order to display values of different magnitudes in one diagram. The z-score is negative when the raw score is below the mean and positive when above. Especially the star plots (figure 5) showed the reduction of the fitness parameter of the mussels deployed at the Cuxhaven station in comparison to mussels transplanted to Helgoland at first sight. Additionally the summarized z-scores

in the Box-Whisker Plot (supplement) indicated the broad distribution of the data and verified the importance of a high sample size.

A summary of links between the parameter was given in table 6. Inter-correlation of the biochemical parameters have been found for both stations as supposed, since the energy values of carbohydrates, lipids and proteins were utilized to calculate the available energy. Furthermore, Eav, protein and carbohydrate concentration of the digestive gland tissue showed a correlation to the CI values. A weak correlation can also be assumed for both physiological indices GSI and CI. The latter correlations originate of results yielding from the Cuxhaven group.

4. Discussion

The development of continuous and robust biomonitoring approaches considering seasonal and habitat impacts is an ongoing issue in order to achieve the objectives of national and international regulatory settings for assessing the environmental and chemical status of marine environments (Brenner et al., 2014). Latest and recent literature prove the suitability of active methodologies with the utilization of transplanted organisms - especially bivalves - rather than passive methodologies requiring the occurrence of indigenous species (Besse et al., 2012).

However, long term transplantation studies ranging over a year with replicative experiments under comparable conditions at the same fixed stations are rare. Among the multitude of potential molecular, cellular and organismal signals indicating environmental stress biomarkers of exposure, response and susceptibility can be distinguished (Livingstone et al., 2000). The selected physiological indices and the energy-budget-related responses belong to the general marker, which are related to most of types of environmental stress independently of certain pollution. They are supposed to allow a quantitative measure of animal performance or fitness and were selected due to the fact that the two stations differ in their hydrodynamic as well as chemical characteristics (Helmholz et al., 2015). The 8 and 12 month exposure led to a balanced whole tissue contamination level which is comparable between the series and therefore it was not a major factor for the explanation of annual differences of physiological responses. However the remarkable enrichment of inorganic contaminants in the Cuxhaven samples was a potential cause for spatial variances.

The condition index is one of the most relevant indices for mussel fitness integrating pollution-induced variations as well as feeding, assimilation and energy expenditure, and there are contrary observations available in the literature regarding the responses on environmental and chemical stress (Fang et al., 2009). In the present study, the CI can only be used to distinguish between the two stations but did not indicate any seasonal differences for the North Sea station. However, a supposed reduction of the CI over the year was proven for the mussels from the tidal and industrial impacted station near Cuxhaven. Comparing the scientific findings with literature data created an indifferent picture. (Signa et al., 2015) observed a pronounced reduction of the CI at the impacted station but no seasonality during January until July in caged mussels at two stations of South Italy. (Lundebye et al., 1997) found no CI variations for natural mussel from different stations and no correlations with other stress protein levels. Contrary, the fundamental work of Andral et al. (2004) on transplanted mussels recommended the CI as important pollution marker. (Bodin et al., 2004) showed a clear seasonality with a maximum in the late spring/early summer in Mediterranean mussels whereas (Cravo et al., 2009) detected no difference in the CI values between summer and winter in natural mussel populations at the south of Portugal. Regardless of these opposite findings, utilizing the CI can be recommended in the context of further biomarkers due to the valuable information on animal physiological status, the robust methodology of measurement and the option to use the freeze-dried meat for further purposes like chemical analysis.

Since the reproductive cycle and the gender were seen as confounding biotic factors for the assessment of contamination data, it was recommended to use only mature animals with sampling outside the reproductive period and only males (Besse et al., 2012). Practically, this was difficult to manage while sampling over several months and without analysis of sex prior to sample preparation for the selected biochemical measurements. Although different stock material was used for each of the three series, the female mussels dominated the cohort of caged mussels. In general, the impact of the sex on the biomarker response in environmental monitoring studies is poorly understood or is not detected in most of the environmental studies. (Richir and Gobert, 2014) analyzed the bioaccumulation of trace elements and found increased tissue concentrations in female organisms prior to spawning. This might be explained by the more pronounced assimilation of reserves in females during the pre-spawning period. Regarding the correlation to specific biomarker (Bebianno et al., 2000) could not detect an impact of

sex on the concentrations of metallothioneins as well did (Zilberberg et al., 2011) for a map-kinase. Corresponding to these findings, there was no impact of sex on the measured parameter in the present study. However, specific biomarkers of exposure to organic contaminants with endocrine disrupting activity seem to have a correlation to the gender of bivalves (Ortiz-Zarragoitia and Cajaraville, 2006). A proteomic study on the protein expression as response to brominated flame retardants clearly showed a differentiated protein expression in male and female organisms (Ji et al., 2014).

Gametogenesis is an energy demanding process. During the annual reproductive cycle, nutrients are stored when food supplies are abundant and gonad activity is minimal. The annual cycle of the Helgoland sample group in „series b“ reflected the natural cycle of gametogenesis and reproduction with a minimum in June/July and a maximum during winter (Gosling, 2003). Since a pronounced reproduction cycle was only observed in mussels exposed at Helgoland, it can be assumed that the nutrition status in the Cuxhaven mussels is not sufficient for a successful and numerous reproduction.

Due to the high degree of variance within the organisms per sampling it was not possible to detect significant differences between seasons. As seen for the CI, only the two stations can be distinguished.

The enzyme activity of the mitochondrial electron transport system is not a very common potential biomarker in mussels. This multienzyme-complex consists of several enzymes situated in the inner membrane of mitochondria responsible for the electron transport and finally establishing the electrochemical gradient as a prerequisite for the ATP synthesis as major molecular energy storage. The content and activity of these enzymes represent the respiratory requirements of the mussels. A systematic evaluation of the MET thought in gonad homogenates was performed by Gagne et al. (2008) who detected a significant temperature dependency, impacted by different sites but not by gender. Opposite to the present findings, the MET values are characterized as stable parameter of high variance. A negative correlation to the CI and GSI was detected and it was also suitable to distinguish differently impacted stations. Such systematic responses in the MET values of caged mussels were not detected in the BECPELAG project when monitoring two pollution gradients (Smolders et al., 2006). A multifactorial study with natural mussels sampled at different stations of Halifax harbor showed a gender and sampling site correlation of MET values. Interestingly the electron transport activity was characterized as one of the most important biomarker (Yeats et al., 2008). Natural mussel population of

an estuarine and a coastal station showed also a different activity of the mitochondrial electron transport (Erk et al., 2011). Significant regional differences have also been detected in the present study. However, the seasonal response was indifferent comparing the three different twelve – months - series. The activity of the MET complex depends on the oxygen availability, nutrients and temperature additionally to contaminant concentrations (Gagne et al., 2008). Due to these multifactorial inconsistencies, the application of MET measurements representing the respiratory activity is more suitable for short time experiments with defined incubation conditions rather than for field studies.

In the present study, the available energy was estimated as the most important factor for differentiating the sampling stations and also the seasonality was consistent with high levels in autumn. Since the available energy was a sum parameter of the nutrients, the carbohydrate content reflected the seasonality best, whereas the protein and the lipid content contribute to the energy budget by their high tissue concentration and the high enthalpy of combustion, respectively.

The maximum of carbohydrate concentrations in late summer/autumn and the lipid decrease have also been detected by Peteiro et al. (2007); however, the low protein concentration in September cannot be confirmed. The study on Zebra mussels by Smolders et al. (2004) showed that carbohydrate, protein and lipid contents were sensitive endpoints for a fast detection of chemical stressors and according to de Coen et al. (2003), the lipid reserve was the most critical one. Contrarily, in the study with transplanted mussels performed by Brooks et al. (2015), no significant differences of the nutrient components were found within the stations profile. Likewise to the present experiments, the major contribution of protein to the available energy was pointed out (Brooks et al., 2015). In a waste water study, significant differences between different treatment groups were not found for the major types of nutritional molecules (Beyer et al., 2013). Since the assessment of the energy resources is a promising tool for environmental monitoring also in other marine organisms, further studies and field experiments for the measurement of base line, general stress and pollution response levels will be a valuable contribution to investigation of novel biomonitoring approaches.

5. Conclusion

In the present study, we assessed the seasonality and repeatability of common physiological stress indices and biomarkers related to the energy budget of *Mytilus* spp. transplanted as active sampler in order to evaluate their applicability for environmental monitoring. Three years of continuous sampling resulted in a complex pattern of responses, and absolute baseline levels for the selected biomarker were difficult to define. Insofar, the detected biological response should be assessed in relation to the physiological condition of the stock cohorts. According to the presented results and experiences, at least a three point sampling over one year can be recommended. The different responses after a high-consumption phase (summer T2), after starvation (winter_T2) and after a potential recreation (spring_T2) should be observed. The parameters expressing the energy budget were very appropriate as indicator of general environmental stress at differently impacted habitats.

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List of tables and figures:

Picture of the station in Cuxhaven (river Elbe estuary); the mussels were deployed in cages made of titanium, continuously submerged, and sampling was realized with an elevator construction.

Figure 2:

Abundance and distribution of female and male individuals between series and stations

Figure 3:

Content of nutrients in the homogenates of digestive glands of transplanted mussels A: carbohydrate and B: lipid in $mg*g^{-1}_{ww}$ wet weight; mean and SD (error bars), n=10

Figure 4:

Factor analysis of the main measured parameter for the differentiation of the stations Helgoland and Cuxhaven

Figure 5:

Star plots representing the z-normalized values of main parameters measured in each series a, b and c and for the main sampling times summer_T1, autumn, winter_T2 and spring_T2 (black line: Cuxhaven; grey line: Helgoland)

Table 1: Sampling timing of each series

	Series a	Series b	Series c
Start	01/06/11	19/04/12	17/05/13
Summer_T1	19/07/11	05/06/12	11/07/13
Summer_T2	01/09/11	26/07/12	29/08/13
Autumn	19/10/11	11/09/12	17/10/13
Winter_T1	01/12/11	08/11/12	03/12/13
Winter_T2	29/01/12	30/01/13	28/01/14
Spring_T1		20/03/13	12/03/14
Spring_T2		30/04/13	21/05/14

Table 2:

Element concentrations measured in dry whole body tissue; Mean (SD) n=9

	Series a			Series b			Series c		
Element (mg/kg$_{dw}$)	**Start**	**Cuxhaven Winter T2**	**Helgoland Winter T2**	**Start**	**Cuxhaven Spring T2**	**Helgoland Spring T2**	**Start**	**Cuxhaven Spring T2**	**Helgoland Spring T2**
Ag	0.025 (0.002)	0.126 (0.019)	0.060 (0.017)	0.055 (0.006)	0.116 (0.011)	0.095 (0.001)	0.106 (0.022)	0.110 (0.001)	0.110 (0.001)
Al	337.330 (29.151)	269.900 (60.859)	39.760 (10.363)	284.366 (13.796)	416.827 (1.534)	42.163 (8.409)	422.167 (175.120)	523.619 (128.659)	100.030 (21.970)
As	11.440 (0.561)	14.840 (0.941)	12.6855 (1.159)	17.263 (1.545)	17.928 (0.470)	16.016 (0.500)	15.328 (0.711)	19.459 (0.800)	16.379 (0.328)
Cd	0.406 (0.018)	3.700 (0.019)	0.710 (0.036)	0.633 (0.080)	3.550 (0.520)	0.851 (0.045)	0.721 (0.021)	3.173 (0.076)	0.918 (0.033)

Co	0.749 (0.055)	1.040 (0.189)	0.592 (0.069)	1.432 (0.141)	1.426 (0.070)	0.802 (0.064)	0.974 (0.089)	1.719 (0.225)	0.962 (0.069)
Cr	1.004 (0.035)	1.357 (0.209)	0.342 (0.013)	0.802 (0.212)	1.973 (0.466)	0.340 (0.039)	1.381 (0.160)	1.976 (0.049)	0.667 (0.057)
Cu	8.272 (0.307)	11.056 (0.219)	7.294 (0.512)	5.945 (0.596)	11.746 (0.328)	7.667 (0.451)	6.067 (0.897)	14.682 (1.009)	6.677 (0.025)
Mo	0.819 (0.078)	2.119 (0.074)	0.970 (0.147)	0.955 (0.122)	1.374 (0.052)	0.936 (0.111)	0.815 (0.157)	2.757 (0.197)	2.667 (0.078)
Ni	2.007 (0.174)	4.197 (0.540)	1.232 (0.158)	2.882 (0.264)	6.229 (0.355)	1.397 (0.241)	2.442 (0.132)	6.225 (1.041)	1.714 (0.221)
Pb	1.201 (0.158)	2.112 (0.506)	1.027 (0.244)	1.276 (0.143)	3.096 (0.226)	1.209 (0.163)	1.605 (0.260)	2.798 (0.003)	1.424 (0.122)
Se	3.159 (0.046)	8.049 (1.359)	5.005 (0.447)	2.262 (0.200)	8.032 (0.772)	5.790 (0.123)	4.261 (0.158)	8.896 (0.423)	5.552 (0.215)
Zn	73.340 (2.938)	194.758 (49.308)	98.006 (12.393) c	100.217 (8.580)	161.439 (31.351)	109.949 (16.620)	91.412 (1.440)	192.894 (22.896)	142.647 (11.210)

Table 3: Condition index CI and Gonadosomatic index GSI of mussels transplanted at a coastal station in Cuxhaven and a North Sea station at Helgoland. The values represent three independent sampling series. The start value refers to both stations. Mean (SD) n=10, n.d. not determined; * significant difference between Cux and Hel; # significant differences compared to summer $p<0.05$ Mann – Whitney (U)-test

Index	CI							
	Series a		Series b		Series c		Series a	
Station	Cux	Hel	Cux	Hel	Cux	Hel	Cux	Hel
Start	0.200 (0.042)	0.200 (0.042)	0.127 (0.023)	0.127 (0.023)	0.140 (0.032)	0.150 (0.046)	20.32 (5.62)	20.32 (5.62)
Summer_T1	0.179 (0.038)	0.226 (0.031)	0.132 # (0.042)	0.161 (0.034)	0.099 # (0.031)	0.171 * (0.040)	21.47 (6.46)	26.15 (9.90)
Summer_T2	0.174 (0.032)	0.241 * (0.041)	0.117 (0.031)	n.d.	n.d.	0.178 (0.029)	21.03 (7.21)	25.93 (5.63)
Autumn	0.181 (0.040)	0.219 (0.056)	0.093 (0.030)	0.166 * (0.041)	0.107 # (0.041)	0.163 * (0.052)	22.94 (3.30)	25.92 (6.78)
Winter_T1	0.132 (0.037)	0.195 * (0.030)	n.d.	n.d.	0.087 (0.039)	0.155 * (0.067)	22.14 (4.90)	23.94 (4.80)
Winter_T2	0.145 (0.018)	0.174 * (0.016)	0.069 # (0.029)	0.174 * (0.035)	0.075 (0.029)	0.129 * *(0.050)	21.54 (7.22)	25.11 (8.35)
Spring_T1	n.d	n.d.	0.077 # (0.019)	0.156 * (0.052)	0.067 (0.016)	0.157 * (0.042)	n.d	n.d.
Spring_T2	n.d.	n.d.	0.058 # (0.010)	0.146 * (0.054)	0.048 # (0.020)	0.123 * (0.025)	n.d.	n.d.

Table 4: Protein content and available Energy (Eav) calculated as sum of the energy equivalents of carbohydrate. lipid and protein content of the digestive glands in transplanted mussels. mean (SD); n = 10

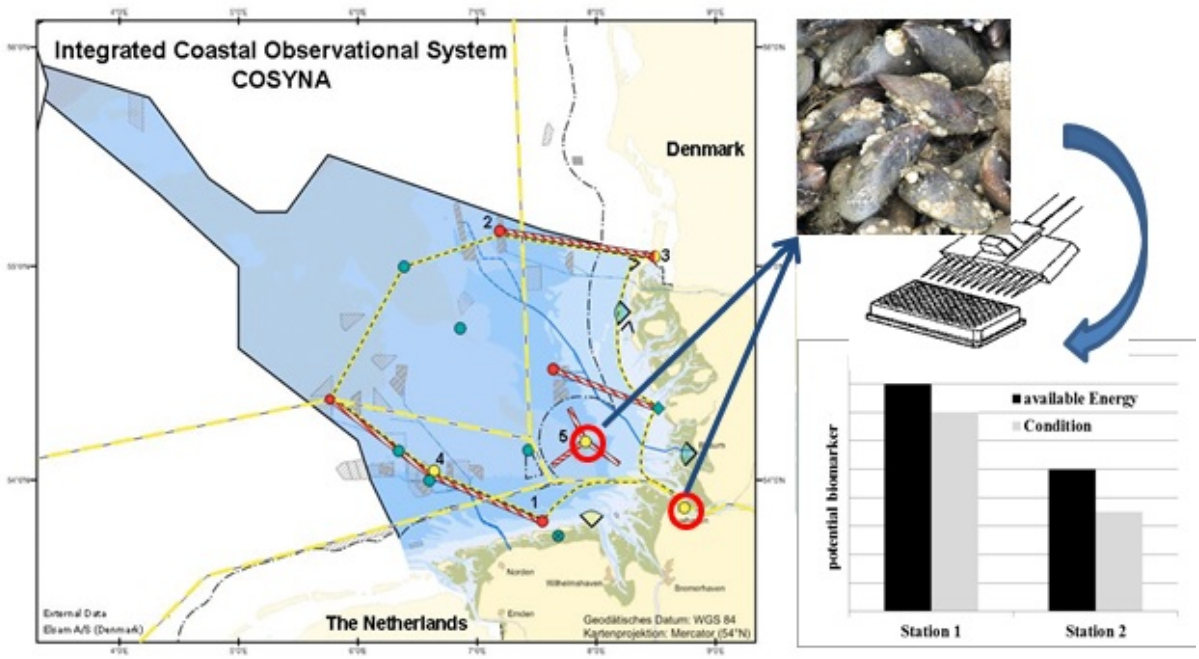
sampling		Protein [mgg^{-1}_{ww}] Cuxhaven	Protein [mgg^{-1}_{ww}] Helgoland	Eav [Jmg^{-1}_{ww}] Cuxhaven	Eav [Jmg^{-1}_{ww}] Helgoland
Series a	Start	71.71 (9.34)		3040 (267)	
	Summer_T1	78.33 (16.40)	63.71 (4.23)	2712 (523)	2790 (369)
	Autumn	90.88 (17.67)	109.61 (10.46)	3086 (483)	3859 (298)
	Winter_T2	76.56 (9.52)	78.74 (13.73)	2401 (252)	2749 (435)
Series b	Start	75.24 (9.49)		2605 (417)	
	Summer_T1	71.42 (9.16)	85.61 (8.16)	2302(352)	2804 (249)
	Autumn	63.45 (12.31)	82.45 (15.79)	2132 (522)	3197 (546)
	Winter_T2	68.01 (9.65)	77.79 (10.31)	2009 (284)	2582 (330)
	Spring_T2	48.23 (8.61)	73.37 (10.70)	1443 (264)	2642 (381)
Series c	Start	84.79 (9.41)		3724 (308)	
	Summer_T1	63.64 (6.92)	71.62 (8.60)	2272 (377)	2866 (260)
	Autumn	69.43 (10.37)	77.34 (9.19)	2517 (508)	2996 (283)
	Winter_T2	57.61 (9.43)	76.48 (12.62)	1872 (333)	2657 (499)
	Spring_T2	50.14 (4.23)	66.68 (9.38)	1592 (135)	2198 (265)

Table 5: Activity of the mitochondrial electron transport system measured in digestive glands [$J*g_{ww}^{-1}*h^{-1}$]; mean (SD) n=10, * comparison between stations *p<0.05* Mann-Whitney (U)-Test# comparison between summer samples and the other seasons *p<0.05* Mann-Whitney (U)-Test; n.d. not determined

Season	Start	Summer_T1		Autumn		Winter_T2		Spring_T2	
Station		Cux	Hel	Cux	Hel	Cux	Hel	Cux	Hel
Series a	9.71	9.95	9.74	8.57*#	7.73*#	9.67#	9.38#	n.d.	n.d.
	(0.57)	(0.35)	(0.49)	(0.47)	(0.84)	(0.32)	(0.47)	n.d.	n.d.
Series b	5.92	8.71*	7.46*	6.88#	7.25	6.41*#	5.34*#	n.d.	n.d.
	(0.75)	(0.40)	(0.42)	(0.70)	(0.47)	(0.34)	(0.37)	n.d.	n.d.
Series c	4.20	4.50*	5.16*	5.14*#	6.16*#	6.58*#	6.08*#	4.39*	5.36*
	(0.51)	(0.44)	(0.43)	(0.69)	(0.39)	(0.22)	(0.29)	(0.84)	(0.23)

Table 6: Pearson correlation factor showing the relationship of main physiological and biochemical parameter

	CI	GSI	gland	carb	lipid	protein	Eav
CI	1	**0.517**	0.284	**0.588**	0.391	**0.514**	**0.615**
GSI	**0.517**	1	0.332	0.475	0.326	0.376	0.475
gland	0.284	0.332	1	0.282	0.285	0.133	0.255
carb	**0.588**	0.475	0.282	1	0.505	0.429	**0.720**
lipid	0.391	0.326	0.285	0.505	1	0.464	**0.748**
protein	**0.514**	0.376	0.133	0.429	0.464	1	**0.893**
Eav	**0.615**	0.475	0.255	**0.720**	**0.748**	**0.893**	1



Graphical Abstract

Figure 2

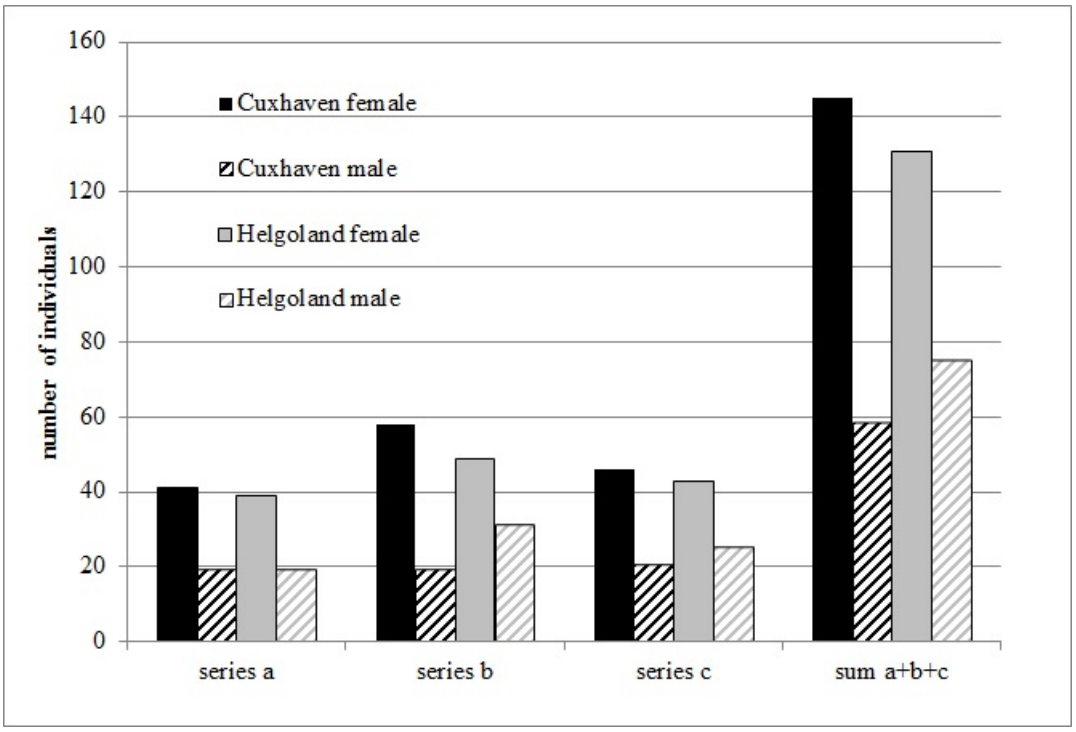

160
140
120
100
80
60
40
20
0
number of individuals
Cuxhaven female
Cuxhaven male
Helgoland female
Helgoland male
series a
series b
series c
sum a+b+c

Figure 2

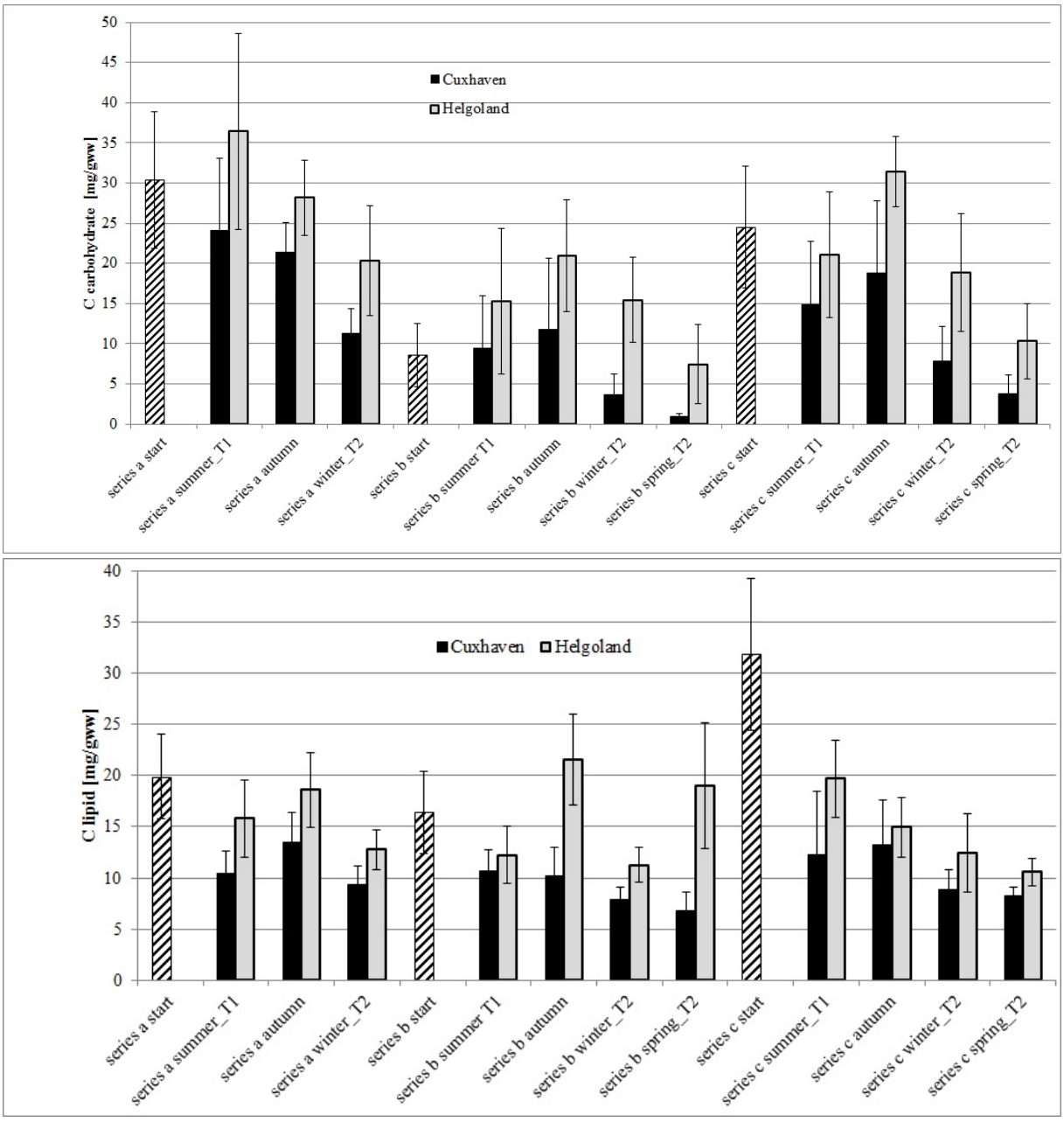


Figure 3 a and b

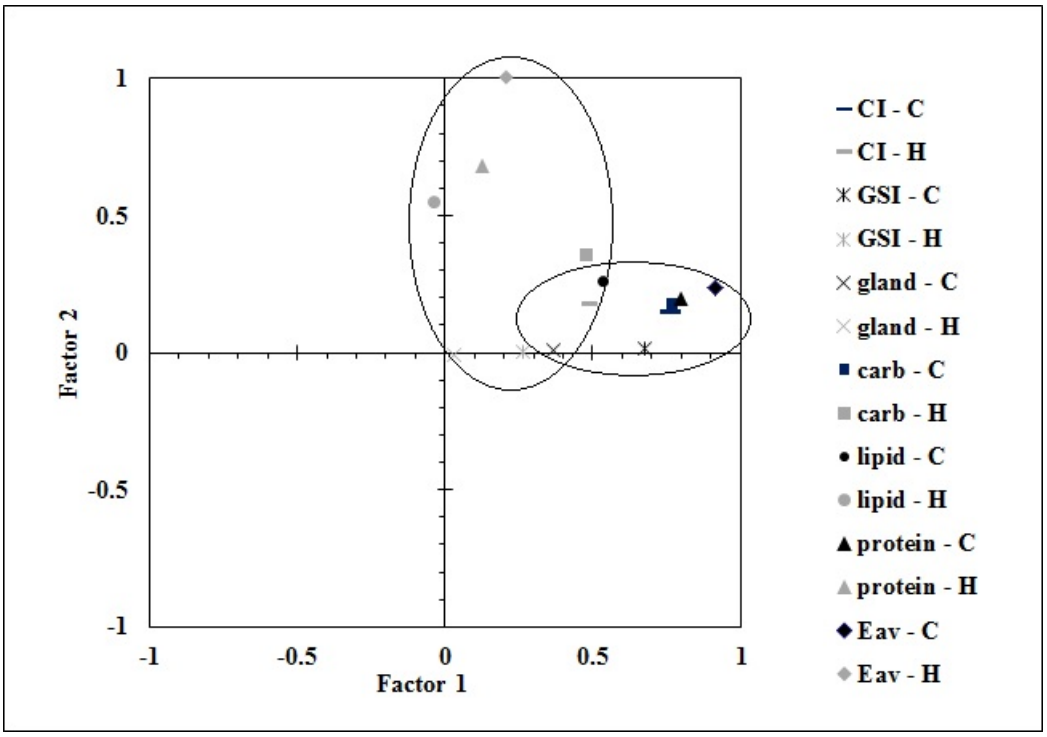

1
0.5
0
-0.5
-1
Factor 2
-1
-0.5
0
0.5
1
Factor 1
CI - C
CI - H
GSI - C
GSI - H
gland - C
gland - H
carb - C
carb - H
lipid - C
lipid - H
protein - C
protein - H
Eav - C
Eav - H

Figure 4

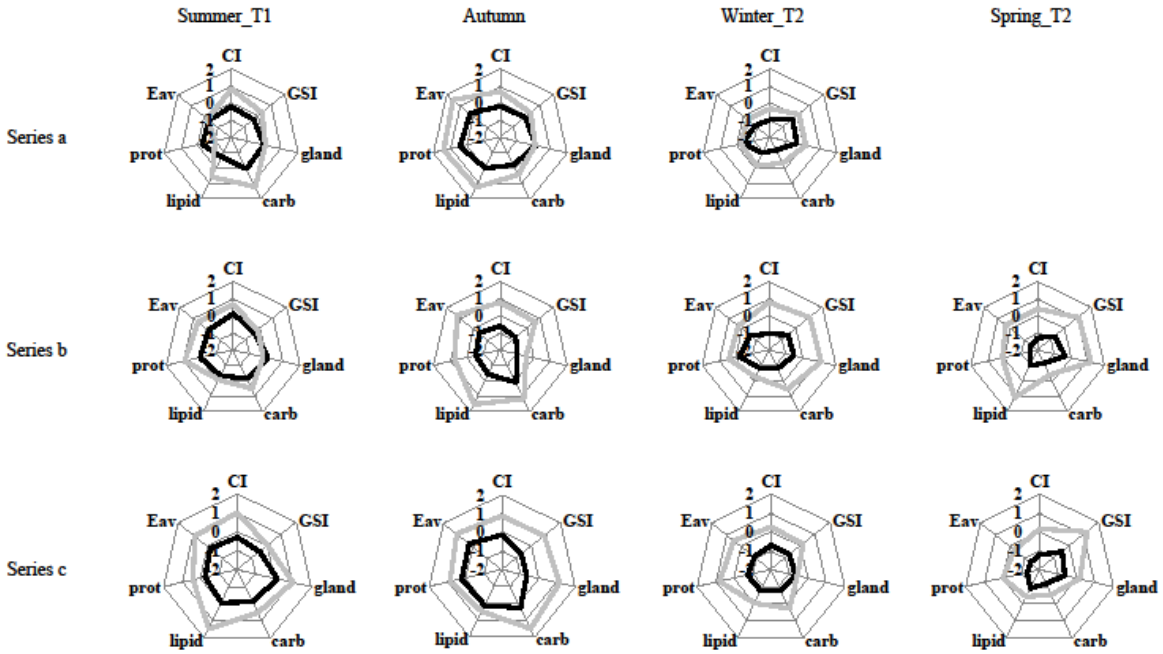
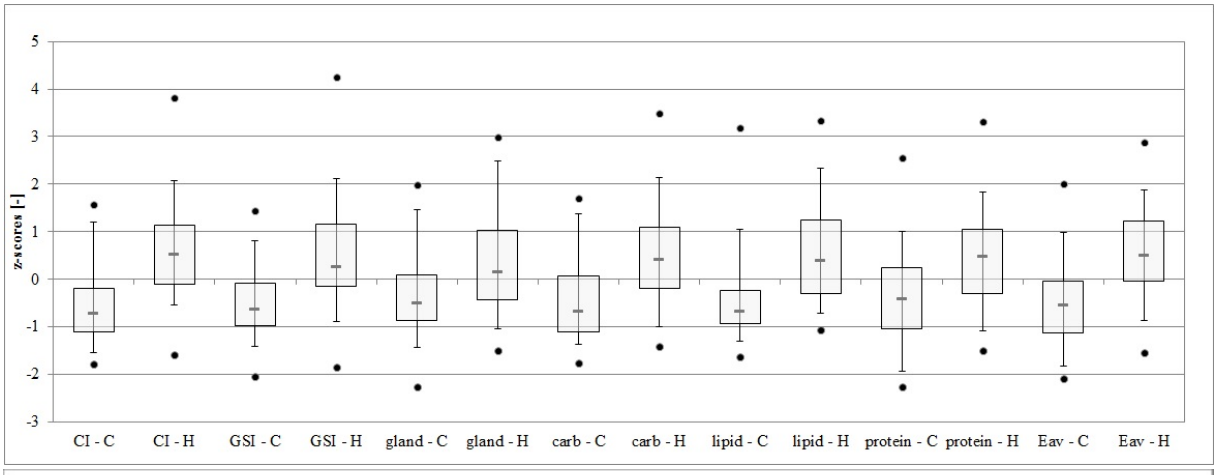
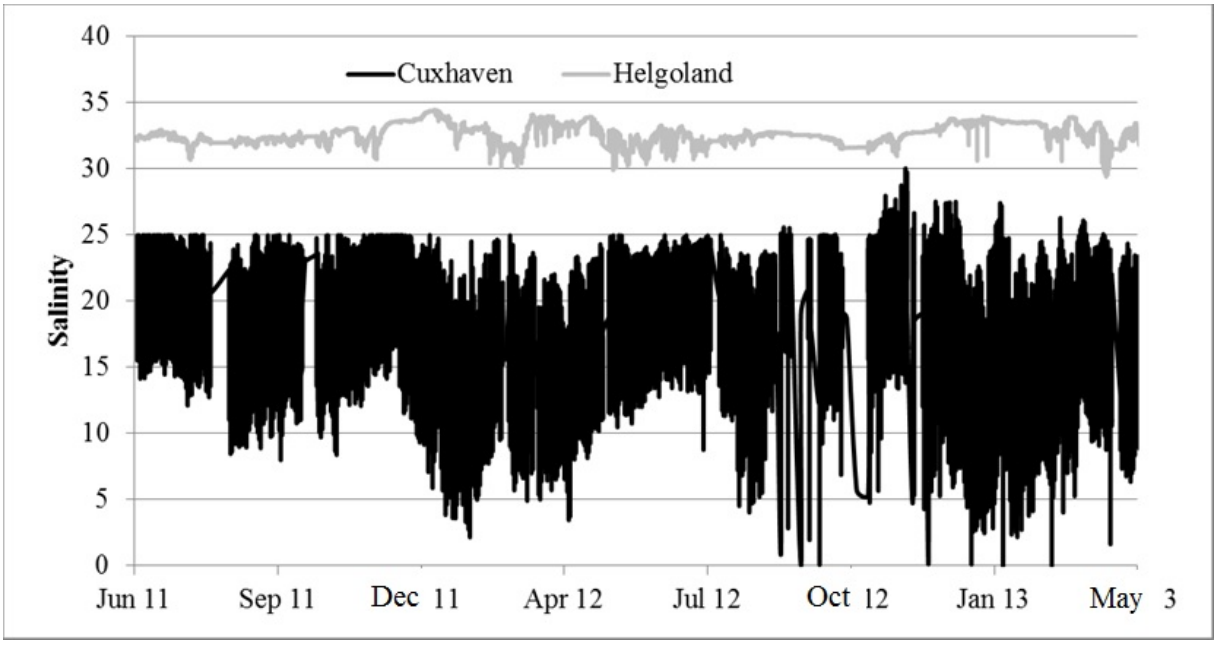
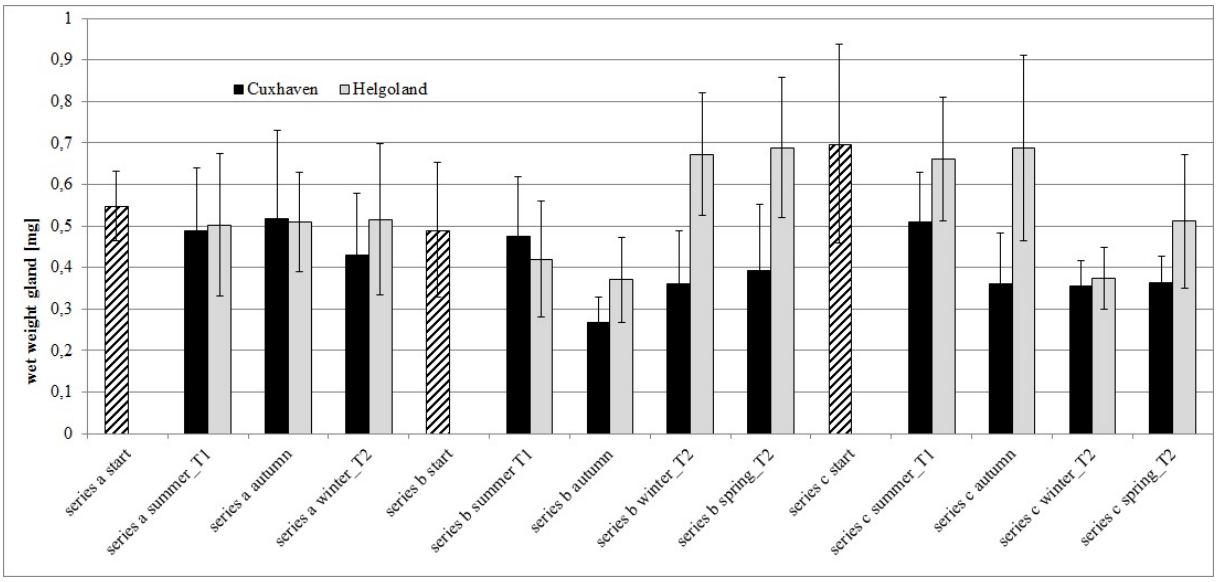

Summer_T1
Autumn
Winter_T2
Spring_T2
Series a
Series b
Series c
CI
GSI
gland
carb
lipid
prot
Eav

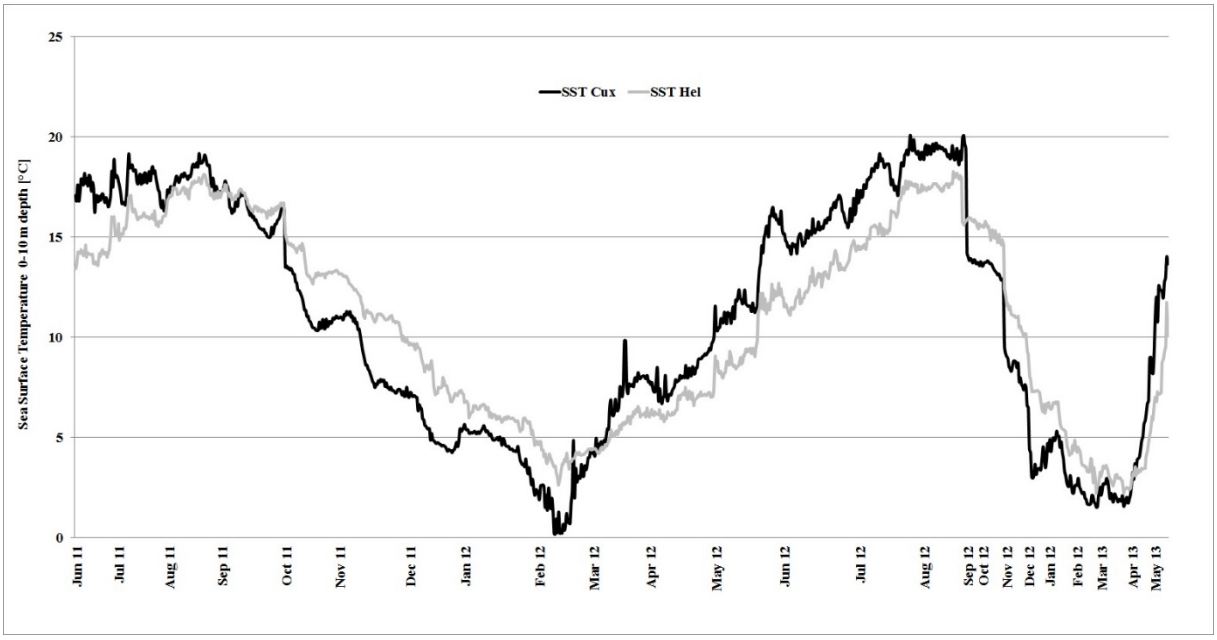
Figure 5

Supplement


z-scores [-]
5
4
3
2
1
0
-1
-2
-3
CI - C
CI - H
GSI - C
GSI - H
gland - C
gland - H
carb - C
carb - H
lipid - C
lipid - H
protein - C
protein - H
Eav - C
Eav - H


Cuxhaven
Helgoland
Salinity
40
35
30
25
20
15
10
5
0
Jun 11
Sep 11
Dec 11
Apr 12
Jul 12
Oct 12
Jan 13
May 3


wet weight gland [mg]
Cuxhaven
Helgoland
series a start
series a summer_T1
series a autumn
series a winter_T2
series b start
series b summer T1
series b autumn
series b winter_T2
series b spring_T2
series c start
series c summer_T1
series c autumn
series c winter_T2
series c spring_T2


Sea Surface Temperature 0-10 m depth [°C]
SST Cux
SST Hel
Jun 11
Jul 11
Aug 11
Sep 11
Oct 11
Nov 11
Dec 11
Jan 12
Feb 12
Mar 12
Apr 12
May 12
Jun 12
Jul 12
Aug 12
Sep 12
Oct 12
Nov 12
Dec 12
Jan 12
Feb 12
Mar 13
Apr 13
May 13