Understanding acclimation and adaptation to salinity changes in benthic osmoconformers

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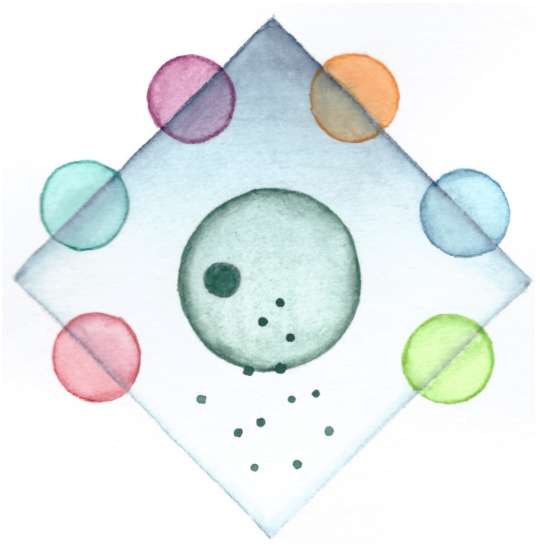

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Imke Anna Podbielski

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Summary

Changes in seawater salinity can have severe impacts of an organism's physiological condition and fitness. Thus, salinity has an essential influence on the current distribution of marine organisms.

With ongoing climate change and the associated increase in precipitation, surface run-off and melt-water, a decrease in salinity is predicted for many marine areas worldwide. The capacity for salinity tolerance will thus be a crucial factor in determining the impacts of salinity changes on individuals, populations and ecosystems. Projected salinity changes could lead to habitat loss for many marine species currently living at their distribution limit. It is thus essential to understand what limits the capacity for salinity acclimation and to assess the potential for acclimation and rapid adaptation across species.

The Baltic Sea stands out through a pronounced spatial salinity gradient that is mirrored by its fauna. The salinity gradient of the Baltic Sea can thus serve as a real-life simulation to study consequences of future desalination. The study of Baltic Sea animals may provide ideal case studies of local adaptation and physiological plasticity in terms of salinity tolerance and potential ecosystem interactions. The blue mussel is suitable model organisms for such experiments. As a reef-builder, it is a foundation species of the Baltic Sea. The *Mytilus* species complex consists of a hybrid-population of *M. edulis x trossulus* mussels. Western *M. edulis*-like populations live at high salinity conditions whereas the more salinity tolerant *M. trossulus*-like populations occur in the Eastern Baltic Sea.

The majority of marine animals are invertebrates most of which are *osmoconformers*, whose body fluid osmolality follows that of seawater. Osmoconformers use cellular volume regulation (CVR) to acclimate to changes in seawater salinity. This process utilizes cellular osmotic solutes, *osmolytes*, to adjust intracellular osmotic pressure to ambient seawater. Depletion of the organic osmolyte pool has been implicated to determine critical salinity in osmoconformers, which is further accompanied by loss of fitness (i.e. S_{crit}*-concept sensu* Podbielski et al. [262]). Yet, little is known about the role of inorganic osmolytes. Furthermore, while phenotypic biomarkers such as respiration,

growth or survival are better described [197, 171, 143], information on the transcriptomic responses to prolonged low salinity is rare.

The goal of this thesis was thus to assess the limits of salinity tolerance and its determinants on multiple levels: organismal, biochemical and transcriptomic. This project, thereby, concentrates on the process CVR in osmoconformers.

The first part of this project was a systematic review and meta-analysis of osmolytes (both organic and inorganic) utilized by osmoconforming marine invertebrates after long-term acclimation to reduced salinity. This is the first systematic review and meta-analysis on osmolyte concentrations in osmoconformers acclimated to low salinity. A total of 2389 studies was screened and search criteria produced a final reference library of 38 studies that reported osmolyte concentrations in tissues. Overall, six organic compounds and sodium were consistently reduced across phyla in response to low salinity stress. This suggests that intracellular inorganic ion concentrations are reduced in concert with organic osmolytes under long-term hypo-osmotic stress. The systematic review further revealed a shortage of studies i) that quantified intracellular ion concentrations, ii) that comprehensively analysed both osmolyte pools and iii) that investigated non-bivalve study organisms. Alanine, betaine, glycine and taurine were identified as the major organic osmolytes that are universally employed across five phyla. However, a methodological bias was revealed by the systematic review that favors the measurement of free amino acids (FAAs), thereby neglecting methylamines and methylsulfonium compounds, which can be equally important. Our meta-analysis suggests that there are common osmolyte actors employed across phyla, but no uniform concept is applicable to all. Based on these findings and shortcomings of the current literature best-practice guidelines were established for future osmolyte research to streamline experimental designs and protocols.

The suggestions derived from the best-practice guidelines were implemented in the next study. A comprehensive set of techniques was employed to measure a wide variety of inorganic and organic osmolytes (i.e. metabolomics, photometry and chromatography). This was further advanced by establishing a novel protocol for anion analysis in tissue extracts. In addition to osmolyte systems, phenotypic parameters were measured to assess fitness (i.e. survival and net growth) and capacity for CVR (i.e. water content). Further, a wider variety of organisms was chosen to counter the bivalve-bias of previous studies. The salinity tolerance of six species (mussels, snails, sea stars, sea anemones, shore sea urchins and green sea urchins) after a four-week acclimation around their low salinity threshold was investigated and the S_{crit}-concept tested.

Our experiments corroborated the importance of the organic osmolyte pool. Methylamines constituted a large portion in the organic osmolyte pool of molluscs, whereas echinoderms exclusively utilize FAAs. Inorganic osmolytes were involved in CVR in all species, except for sea urchins. This highlights the importance of this often neglected osmolyte class. The organic osmolyte pool was not completely depleted at low salin-

ity, thereby disproving the S_{crit}-concept in this case. Instead, organic and inorganic osmolytes were often stabilized at low salinity. The reduction of osmolyte pools was accompanied by a reduction of fitness and impairment of CVR. However, the interplay of parameters revealed a critical salinity range, before the actual limit is reached. Reduction of net growth and increase of water content under fine-scale monitoring indicate that the energetic trade-off for osmoregulation is aggravated until condition are not sustainable and survival is impacted.

While the first two studies investigated CVR on an organismal and biochemical level, the third study focused on changes of the transcriptomic response. Two mussel populations that are locally adapted to divergent salinity regimes (Kiel 16 vs. Usedom 7) and also differ in the salinity tolerance capacity were long-term acclimated (four weeks) to five different salinity treatments (4.5, 5, 6, 7 and 16). At the end, population-specific and conserved transcriptomic response to the low salinity was assessed. A larger number of differentially expressed genes was found in mussels from Kiel than in mussels from Usedom. Additionally, genes upregulated in the more susceptible Kiel population were downregulated in the more tolerant Usedom mussels. The common transcriptomic response to low salinity of Kiel mussels was an upregulation of solute transport and amino acid metabolism. Transcripts involved in stress response, energy storage and lipid metabolism were also enriched. Low salinity adapted Usedom mussels did not perform equally well as Kiel mussels at high salinity conditions. The results suggest that Usedom mussels are not affected by the low salinity treatment, whereas a clear stress response and a selection for more tolerant individuals in the Kiel population under low salinity stress was found.

Despite species' phenotypic plasticity, future salinity changes will likely perturb populations living close to their distribution limit. When salinity conditions are reduced, the cost of osmoregulation and subsequent energetic trade-off will not be sustainable. This could potentially lead to geographic range shift with decreasing salinity, if species are not able to adapt. In the case of the *Mytilus* species complex, a shift in allele-frequencies towards the low salinity tolerant genotype is expected. When considering other species, a selection for more tolerant phenotypes and/or genotypes is likely. Rapid adaptation might facilitate survival in lower salinity conditions if phenotypic plasticity reaches it limits.

Future salinity changes and its potential consequences underline the importance to understand salinity tolerance capacities and limits. This thesis, is an important cornerstone for future research in salinity tolerance as it employs an integrative approach to measure phenotypic plasticity towards low salinity across a wide range of species from biochemical to transcriptomic levels. The study of the capacity for acclimation and adaptation to salinity changes in marine species will thus be a highly relevant field of research in the future to predict the effects of desalination on species, populations and ecosystems.

Zusammenfassung

Änderungen des Salzgehalts des Meerwassers können schwerwiegende Auswirkungen auf die physiologische Leistungsfähigkeit von Arten haben. Daher hat der Salzgehalt des Meerwassers einen wesentlichen Einfluss auf die Verbreitungsgrenzen von Meeresorganismen.

Im Zuge des Klimawandels wird ein Anstieg der Niederschläge, erhöhter Oberflächenabfluss und vermehrtes Schmelzwasser-Aufkommen vorhergesagt. Dies wird für viele Meeresgebiete weltweit zu einer Verringerung des Salzgehalts führen. Wie stark sich diese Umweltveränderungen auf Individuen, Populationen und Ökosysteme auswirken werden, wird daher bestimmt sein von der Fähigkeit niedrigere Salzgehalte zu tolerieren. Die prognostizierten Veränderungen des Salzgehalts könnten zum Verlust von Lebensraum für viele Arten führen, welche derzeit an ihrer Verbreitungsgrenze leben. Es ist daher von entscheidender Bedeutung zu verstehen, was die Anpassungsfähigkeit an niedrige Salzgehalte limitiert. Um einschätzen zu können wie stark die Auswirkungen auf die marine Tierwelt sein werden, ist es enorm wichtig zu untersuchen wie hoch das Potenzial zur Akklimatisierung und schnellen genetischen Anpassung ist.

Die Ostsee zeichnet sich durch einen ausgeprägten horizontalen Salzgehaltsgradienten aus, der sich auch in der Verbreitung der Fauna widerspiegelt. Der Salzgehaltsgradient der Ostsee kann daher als realistische Simulation dienen, um die Folgen der künftigen Verringerung des Salzgehalts zu untersuchen. Ostsee-Tiere sind hierbei ideale Studienobjekte um die lokale Anpassung und physiologische Plastizität in Bezug auf die Salzgehaltstoleranz von Arten und deren Ökosystem-Interaktionen zu untersuchen. Die Miesmuschel ist ein geeigneter Modellorganismus für solche Experimente. Als Riffbildner ist sie eine ökologisch bedeutungsvolle Ostsee-Art. Der *Mytilus*-Artenkomplex besteht aus einer Hybrid-Population von *M. edulis x trossulus*-Muscheln. Westliche *M. edulis*-ähnliche Populationen leben unter hohen Salzgehaltsbedingungen, während die toleranteren *M. trossulus*-ähnlichen Populationen in der östlichen Ostsee vorkommen.

Die meisten Meerestiere sind Invertebraten, von denen wiederum die Mehrzahl *Osmokonformer* sind, deren Osmolalität dem Salzgehalt des Meerwassers folgt. Osmokonformer nutzen die zelluläre Volumenregulierung, um sich an Salzgehalts-Veränderungen

des Wassers anzupassen. Bei diesem Prozess werden zelluläre gelöste osmotische Substanzen, sogenannte *Osmolyte*, verwendet, um den intrazellulären osmotischen Druck an die Umgebung anzugleichen. Das sogenannte kritische Salinitätskonzept *sensu* Podbielski et al. [262]) beschreibt die völlige Entleerung des organischen Osmolyt-Pools zusammen mit einem Verlust der Fitness als Anzeichen für das Erreichen des Toleranzlimits von Osmokonformern. Dennoch ist wenig über die Rolle der anorganischen Osmolyte bekannt. Während phänotypische Indikatoren wie Respiration, Wachstum oder Überleben besser beschrieben sind [197, 171, 143], sind kaum Informationen über die Auswirkungen von lang anhaltenden niedrigen Salzgehalten auf die Genexpression vorhanden.

Ziel dieser Arbeit war es daher, die Kapazität zur Anpassung an niedrige Salzgehalte und deren Grenzen auf mehreren Ebenen zu untersuchen: auf organismischer, biochemischer und transkriptomischer Ebene. Dieses Projekt konzentriert sich dabei auf den Prozess der zellulären Volumenregulation bei Osmokonformern.

Der erste Teil dieser Arbeit war eine systematische Übersicht und Meta-Analyse von Osmolyten (sowohl organische als auch anorganische), die von osmokonformen, marinen Invertebraten nach einer Langzeitakklimatisierung an einen geringeren Salzgehalt reduziert werden. Dies ist das erste Systematische Review mit Meta-Analyse über die Osmolyt-Konzentrationen bei Osmokonformern. Insgesamt wurden 2389 Studien gesichtet, und die Suchkriterien ergaben eine endgültige Referenzbibliothek von 38 Studien, die Osmolytkonzentrationen in Geweben gemessen haben. Es wurden sechs organische Substanzen und Natrium ermittelt, deren Konzentration nach Anpassung an geringe Salzgehalte in allen Fällen reduziert wurden. Dies deutet darauf hin, dass sowohl anorganische Ionenkonzentrationen, als auch organischen Osmolyte bei langfristigem hypo-osmotischem Stress reduziert werden. Das Systematische Review ermittelte außerdem einen Mangel an Studien, die i) die intrazellulären Ionenkonzentrationen quantifizierten, ii) beide Osmolyt-Pools umfassend analysierten und iii) nicht-muschelartige Studienorganismen untersuchten. Alanin, Betain, Glycin und Taurin wurden als die wichtigsten organischen Osmolyte identifiziert. Diese werden universell in allen untersuchten Phyla verwendet (n = 5). Es wurde jedoch ein systematischer methodischer Fehler festgestellt. In vergangenen Studien wurde oftmals gezielt freie Aminosäuren gemessen. Dieser methodische Ansatz vernachlässigt jedoch Methylamine und Methylsulfoniumverbindungen. Diese Substanzen können ebenso wichtig sein wie Aminosäuren. Die Meta-Analyse deutet darauf hin, dass es Osmolyte gibt, die in allen Phyla verwendet werden. Allerdings gibt es kein einheitliches Konzept welches für alle Arten/Phyla anwendbar ist. Auf der Grundlage dieser Ergebnisse und der Unzulänglichkeiten der aktuellen Literatur wurden Best-Practice-Richtlinien für die künftige Osmolyt-Forschung aufgestellt, um die Versuchs-Designs und Protokolle zu modernisieren.

Die aus den Best-Practice-Richtlinien abgeleiteten Vorschläge wurden in der nächsten Studie umgesetzt. Eine umfassende Reihe von Techniken wurde eingesetzt, um

eine Vielzahl anorganischer und organischer Osmolyte zu messen (d. h. Metabolomik, Photometrie und Chromatographie). Darüber hinaus wurde ein neuartiges Protokoll für die Anionenanalyse in Gewebeextrakten entwickelt. Zusätzlich zu Osmolyten wurden phänotypische Parameter gemessen, um die Fitness (hier: Überleben und Wachstum) und die Kapazität zur zellulären Volumenregulation (hier: den Wassergehalt des Gewebes) zu bewerten. Darüber hinaus wurde eine größere Vielfalt von Organismen ausgewählt, um der einseitigen Ausrichtung auf Muscheln in früheren Studien entgegenzuwirken. Die Salinitätstoleranz von sechs Arten (Muscheln, Schnecken, Seesterne, Seeanemonen, Strandseeigel und Dröbachs Seeigel) wurde nach einer vierwöchigen Akklimatisierung an Salzgehalte nahe ihrer Toleranzschwelle untersucht und das kritische Salinitätskonzept getestet.

Unsere Experimente bestätigten die Bedeutung des organischen Osmolyt-Pools. Methylamine machten einen großen Teil des organischen Osmolyt-Pools von Mollusken aus, während Echinodermaten ausschließlich freie Aminosäuren verwenden. Anorganische Osmolyte waren bei allen Arten, außer bei Seeigeln, an der zellulären Volumenregulation beteiligt. Dies unterstreicht die Bedeutung dieser oft vernachlässigten Osmolyt-Gruppe. Der Pool der organischen Osmolyte war bei niedrigem Salzgehalt nicht vollständig entleert, wodurch das kritische Salinitätskonzept hier widerlegt wurde. Stattdessen erreichten organische und anorganische Osmolyt-Konzentrationen bei niedrigem Salzgehalt oft ein konstantes Niveau. Die Reduzierung der Osmolyt-Pools ging mit einer Verringerung der Fitness und einer Beeinträchtigung der zellulären Volumenregulation einher. Das Zusammenspiel der Parameter ergab jedoch einen kritischen Salzgehaltsbereich, bevor das eigentliche physiologische Limit erreicht wird. Die Verringerung des Nettowachstums und der Anstieg des Wassergehaltes deuten darauf hin, dass die Kosten für osmoregulatorische Prozesse ansteigen und andere nicht-essentielle Prozesse reduziert oder eingestellt werden, bis die Bedingungen nicht mehr nachhaltig sind und kein Überleben mehr ermöglichen.

Während die ersten beiden Studien die zelluläre Volumenregulation auf organismischer und biochemischer Ebene untersuchten, konzentrierte sich die dritte Studie auf Veränderungen im Transkriptom. Zwei Muschelpopulationen, die lokal an unterschiedliche Salinitätsregime angepasst sind (Kiel 16 vs. Usedom 7) und sich auch in der Salzgehaltstoleranz unterscheiden, wurden langfristig (vier Wochen) an fünf verschiedene Salzgehalte (4.5, 5, 6, 7 und 16) akklimatisiert. Am Ende wurde die populationsspezifische und konservierte transkriptomische Antwort auf den niedrigen Salzgehalt ermittelt. In Kieler Muscheln wurde eine größere Anzahl unterschiedlich exprimierter Gene gefunden als in Muscheln aus Usedom. Außerdem wurden Gene, die in der anfälligeren Kieler Population hochreguliert waren, in den toleranteren Usedomer Muscheln herunterreguliert. Die gemeinsame transkriptomische Reaktion der Kieler Muscheln auf den niedrigen Salzgehalt war eine Hochregulierung des Stofftransports und des Aminosäurestoffwechsels. Transkripte, die an der Stressreaktion, der Energiespei-

cherung und dem Lipidstoffwechsel beteiligt sind, wurden ebenfalls angereichert. Usedomer Miesmuscheln, die an einen niedrigen Salzgehalt angepasst sind, schneiden bei hohen Salzgehalten nicht so gut ab wie Kieler Miesmuscheln. Die Ergebnisse deuten darauf hin, dass Usedomer Miesmuscheln durch die Behandlung mit niedrigem Salzgehalt nicht beeinträchtigt werden, während in der Kieler Population bei niedrigem Salzgehalt eine deutliche Stressreaktion festgestellt wurde, welches ein Indiz für eine gerichtete Selektion auf tolerantere Individuen sein kann.

Trotz der phänotypischen Plastizität der untersuchten Arten werden künftige Änderungen des Salzgehalts wahrscheinlich Populationen beeinträchtigen, die nahe an ihrer Verbreitungsgrenze leben. Wenn die Salzgehaltsbedingungen reduziert werden, werden die Kosten der Osmoregulation und der daraus resultierende energetische Trade-Off nicht mehr tragbar sein. Ein Abnehmen des Salzgehaltes könnte daher zu einer Verschiebung des geografischen Verbreitungsgebietes mariner Arten führen, wenn diese nicht in der Lage sind sich anzupassen. Im Falle des *Mytilus*-Artenkomplexes wird eine Verschiebung der Allelhäufigkeiten in Richtung des Genotyps mit geringer Salzgehaltstoleranz erwartet. Bei den anderen Arten ist eine Selektion auf tolerantere Phänotypen und/oder Genotypen wahrscheinlich. Eine rasche Anpassung könnte das Überleben unter niedrigeren Salzgehaltsbedingungen erleichtern, wenn die phänotypische Plastizität an ihre Grenzen stößt.

Künftige Veränderungen des Salzgehalts und ihre potenziellen Folgen unterstreichen, wie wichtig es ist, die Kapazitäten und Grenzen der Salztoleranz zu verstehen. Diese Arbeit ist ein wichtiger Grundstein für die künftige Forschung auf dem Gebiet der Salzgehaltstoleranz, da sie einen integrativen Ansatz zur Messung der phänotypischen Plastizität bei einer Vielzahl von Arten von der biochemischen bis zur transkriptomischen Ebene verwendet. Die Untersuchung der Fähigkeit zur Akklimatisierung und Anpassung an Veränderungen des Salzgehalts bei marinen Arten wird in Zukunft ein äußerst wichtiges Forschungsgebiet sein, um die Auswirkungen der Verringerung des Salzgehalts des Meerwassers auf Arten, Populationen und Ökosysteme vorherzusagen.

Contents

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List of Acronyms and Abbreviations

^{1}H-NMR Proton nuclear magnetic resonance

Br^{-} Bromide ion

C Carbon

Ca^{2+} Calcium ion

CVR Cellular volume regulation

D_2O Deuteriumoxide

DEG Differentially expressed genes

DSMP Dimethylsulfoniumpropionate

DW Dry weight

ECS Extracellular space

F^{-} Fluoride ion

FAA Free amino acid

FITC Fluorescein-5-isothiocyanat

FW Fresh weight

GC-MS Gas chromatography–mass spectrometry

H Hydrogen

HPLC High-performance liquid chromatography

I Iodine

IAPSO International Association for the Physical Sciences of the Oceans

K^{+} Potassium ion

LC-MS Liquid chromatography–mass spectrometry

Mg^{2+} Magnesium ion

N Nitrogen

Na^{+} Sodium ion

NO_3^{-} Nitrate ion

O_2 Oxygen

PO_4^{3-} Phosphate ion

QAC Quaternary ammonium compound

S_{crit} Critical salinity concept

SO_4^{2-} Sulfate ion

SW Seawater

TEOS-10 Thermodynamic Equation of Seawater 2010

TMAO Trimethylaminoxid

WW Wet weight

1

Introduction

Salinity has had an essential influence both on the current distribution of aquatic organisms and on the evolution of multicellular marine organisms over 500 million years ago. The Baltic Sea stands out through a pronounced spatial salinity gradient that is mirrored by the distribution of its fauna. Here, salinity poses a limiting factor for the distribution of both marine and freshwater species. With ongoing climate change, a decrease in salinity is predicted for many coastal areas worldwide with likely consequences for the distribution of species, biodiversity and ecosystem functioning. Whether species have the

potential for acclimation or rapid adaptation to changing salinities remains unclear. The Baltic Sea, with its natural salinity gradient, can thus serve as a time machine to study consequences of future desalination [283]. Baltic Sea animals thus can serve as ideal case studies to understand local adaptation and physiological plasticity across a wide salinity gradient.

1.1 What is salinity?

Salinity is a measure of the amount of dissolved salt in a body of water and was first defined in 1902 [149]. Originally, salinity was expressed as g salt kg^{-1} water. Nowadays, salinity is used and salinity is measured on the basis of conductivity (alongside temperature and pressure) and defined in terms of a conductivity ratio compared to a seawater standard and is thus dimensionless [217]. Standard reference seawater (i.e. IAPSO Standard Seawater) was used as reference for natural seawater composition and to determine practical salinity, now this has been replaced by a new artificial seawater standard (TEOS-10) called absolute salinity, which provides the best estimate of the mass fraction of dissolved matter (g kg^{-1}) [243]. Freshwater has a salinity of 0, while the mean salinity of the ocean is 35. Seawater is a complex, concentrated solution consisting of the major ions chloride, sodium, magnesium, sulfate, calcium, potassium, bromide, fluoride and bicarbonate (Tab. 1.1). Overall, oceanic salinity varies only little from 30 in high latitude oceans to up to 40 in the open ocean [343]. Compared to oceanic waters, coastal and Arctic waters are characterized by stronger salinity fluctuation due to increased evaporation, precipitation, river and surface freshwater runoff, groundwater inflow, ice melt or ice formation [385]. The interface of the ocean with freshwater creates brackish-water environments around the world. Definitions of brackish water salinity ranges vary, but can be located at salinities $5 - 30$

[51, 15, 280, 377]. The dimension of such brackish-water habitats can vary from punctual sources such as submarine groundwater discharge to local and regional proportions like estuaries, lagoons, and polar regions or brackish-water seas such as the Baltic Sea.

Table 1.1: Reference Composition of seawater with Practical Salinity (S_P) $\equiv$ 35.000 and Reference Salinity (S_R) $\equiv$ 35.16504 g kg^{-1}. Concentrations in seawater of higher or lower salinities can be found approximately by scaling all values up or down by the same factor. Units of concentration are per kilogram of seawater. Real seawater contains additional constituents which are not included in the Reference Composition. Concentrations of these constituents do not increase or decrease with salinity but are largely controlled by biogeochemical processes. Table from: Pawlowicz [243].

Reference Composition	**mmol kg^{-1}**	**mg kg^{-1}**
Na^+	468.9675	10781.45
Mg^{2+}	52.817	1283.72
Ca^{2+}	10.282	412.08
K^+	10.2077	399.1
Sr^{2+}	0.0907	7.94
Cl^-	545.8695	19352.71
SO_4^{2-}	28.2353	2712.35
Br^-	0.8421	67.29
F^-	0.0683	1.3
HCO_3^-	1.7178	104.81
CO_3^{2-}	0.2389	14.34
$B(OH)_3$	0.3143	19.43
$B(OH)_4^-$	0.1008	7.94
CO_2	0.0097	0.43
OH^-	0.008	0.14

1.2 Salinity tolerance determines geographical distribution of species

It is estimated that the majority of marine animals (>97%) are invertebrates [204]. The majority thereof are osmoconformers [145]. Thus, osmoconformers represent the vast majority of marine species. While osmoregulators such as fish or mammals can regulate the osmotic pressure (the osmolality) of their body fluids (i.e. blood, coelomic fluid, interstitial fluid, etc.), osmoconformers are characterized by their isoosmotic body fluids.

Salinity is a crucial abiotic factor that influences aquatic organisms. The number of

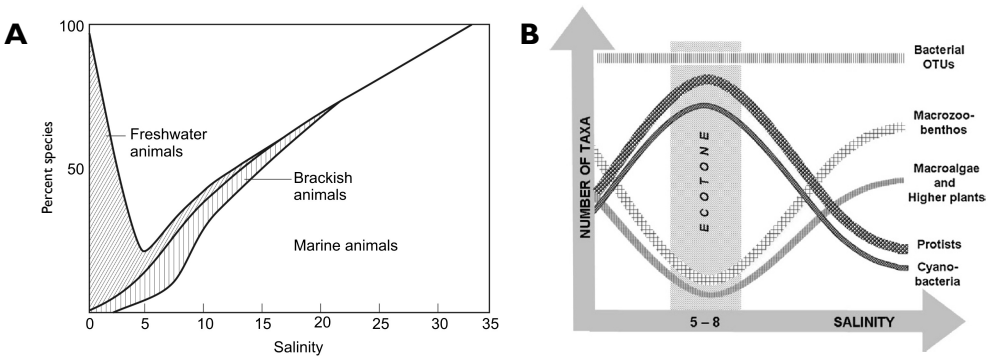

Figure 1.1: Distribution of aquatic organisms in the salinity gradient. A) A redrawn version of the Remane diagram from [377, 280]. Shown are the percentages (y-axis) of freshwater, brackish-water and marine species in relation to the total number of species and their distribution corresponding to salinity (x-axis) of a stable brackish sea. B) Distribution of benthic vs. pelagic organisms in the salinity gradient [348].

marine species has been shown to decrease with a decline in salinity until a minimum in biodiversity is reached at brackish salinities (Fig. 1.1). Salinities of 5–7 pose a physiological limit for both marine and freshwater species [377]. Genuine brackish-water species (i.e. species that exclusively occur in brackish habitats and are neither marine nor freshwater) are rare [281, 405]. However, protists were discovered to have an inversed species maximum in this brackish-water ecological niche [348] (Fig.1.1). Organisms can differ in their capacity for salinity tolerance, some can tolerate a wide spectrum of salinities down to brackish conditions - so called euryhaline species. Others are more restricted and only tolerate marine conditions - so called stenohaline species (Fig.1.2). Most marine species are stenohaline and only tolerate small deviations from marine salinity. Salinity thresholds are species-specific and can also vary between life stages. Other abiotic factors such as temperature, pH, oxygen, nutrients and toxins can affect the salinity tolerance of species (Fig.1.2) [362, 16, 261, 270].

Salinity is major factor determining species' distributions in the marine environment. As a consequence, the salinity tolerance of species shapes marine ecosystems. Depending on

the direction of the salinity gradient, species distribution can be determined horizontally (e.g. in estuaries, bays, or coastal areas) [377, 35, 128], or vertically due to density-driven stratification and to incomplete mixing (the so-called *Brackwassersubmergenz*) [282]. This happens for example in estuaries, seasonal stratification of coastal areas, or polar regions where brackish-water from melting water and precipitation is stratified atop more saline deeper waters [214, 169, 295]. The Baltic Sea stands out through its pronounced horizontal salinity gradient from marine to freshwater conditions from west to east, as well as a strong vertical gradient [282]. This is mirrored by the distribution of species [31, 405].

1.3 Salinity changes in the course of climate change

Precipitation rates are directly influenced by global warming, as increased temperatures cause increased evaporation and air water vapor [357]. An increase in precipitation frequency and intensity follows. Climate change will thus affect freshwater input and cause changes in salinity in the marine realm (Fig. 1.3). An increase in freshwater input due to higher precipitation and thus increased river input and surface run-off, as well as an increase in melt water is predicted for many oceanic and coastal regions in the course of climate change [1, 357, 218, 335, 75].

Prominent examples are higher latitudes, where an increase in melt water has been predicted and observed [228, 214, 241]. The antarctic region especially is considered to be a more stable ecosystems with respect to salinity [6]. The Antarctic fauna is relatively isolated in geographical terms and has adapted to local environmental conditions for thousands of years. Thus, slight deviations from average salinity can impact locally adapted, stenohaline zoobenthic species [57, 221].

On the opposite range, in lower latitudes, increases in extreme weather events such as

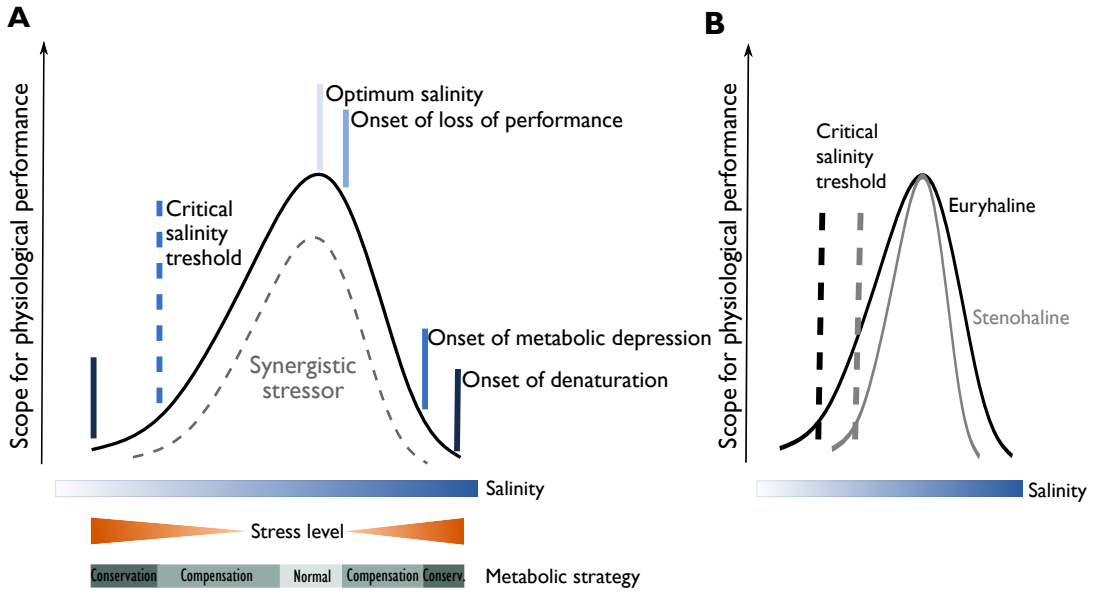

Figure 1.2: Concept of energy-limiting stress tolerance limits in euryhaline and stenohaline marine animals. A. Depicted are the optimal, critical and denaturation salinities of a marine organism, when performance decreases and tolerance becomes increasingly passive and time-limited. Position and width of the window is species-specific and can also shifts with life stage. With increasing or decreasing salinity, optimal salinity conditions shift to moderate and then extreme stress. This in turn also affects the metabolic processes. Under moderate salinity stress, the increased energy demand for osmoregulation under moderate stress can be compensated either by an increase in energy supply and/or by an energetic trade-off from non-essential processes (i.e. activity, growth, reproduction/development) that would result in a loss of physiological performance. Under more severe stress, metabolic processes are strongly reduced and aerobic metabolism is impaired. When salinity stress increases a denaturation salinity is reached that lead to unstable (denatured) protein structures [83, 34]. This hypothetical graph illustrates in which window normal physiological performance is maintained, under which conditions physiological performance is slightly impaired, but survival is maintained, before it shifts to endurance or protection under severe stress. Synergistic stressors such as heat stress, hypoxia or ocean acidification which are predicted to change alongside desalination with climate change can potentially narrow this salinity window. B. The acclimatized window is narrower for stenohaline species and wider in euryhaline species. Figure adapted from [272, 326].

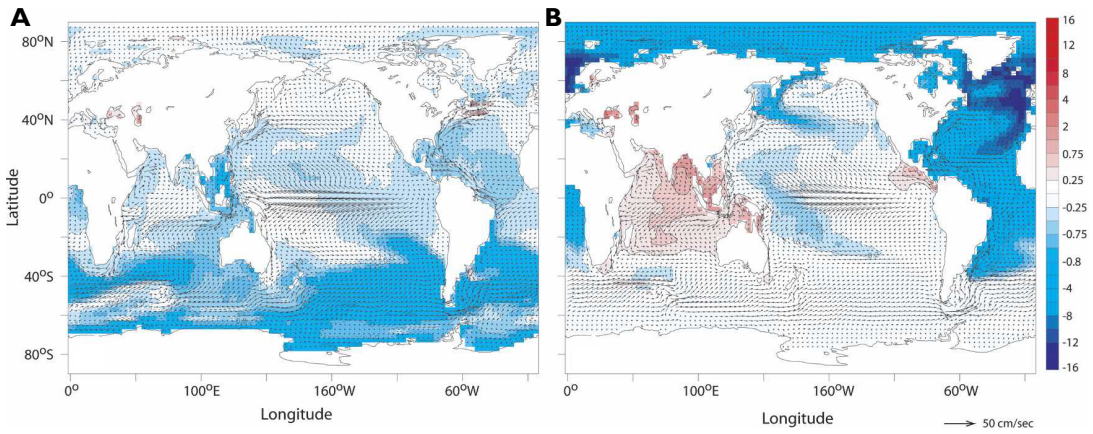

Figure 1.3: This map shows simulations of global future desalination for two freshwater input hypotheses. Depicted is the geographic distribution of surface salinity differences and the spread of the freshwater anomaly under two possible future scenarios (after 91 - 100 years) that are based on additional freshwater input events in the Antarctic region (left) and the north Atlantic region (right). Control conditions (100-year averages) are subtracted from projected salinity conditions to show the difference in surface salinity. Blue shading indicates desalination (negative values). Vectors indicate surface flow in perturbation integration. Source: [335].

floods and cyclones will likely cause large freshwater inflow events towards shallow coastal coral reefs (such as the great barrier reef) [369, 70]. Prolonged decreases in salinity have been shown to negatively affect coral fitness and promote bleaching [113, 144, 310].

Climate change scenarios for the Baltic Sea predict a basin-wide desalination of 1.5-2 by the year 2100 (Fig.1.4) [209, 107]. This will likely affect physiological performance and fitness of benthic key species, predator-prey interactions and distribution ranges of benthic key species and associated species over hundreds of kilometers [156, 367, 262]. Specifically, the *Mytilus* species complex is highlighted (Fig. 1.4). The blue mussel *M. edulis x trossulus* is a foundation species that constructs elaborate reefs and has its distribution limit at a salinity of ca. 4.5-5 [366]. Thus, the predicted desalination could cause severe geographical range shifts over hundreds of kilometers (Fig. 1.4). This in turn would cause severe changes in community structure of coastal Baltic ecosystems.

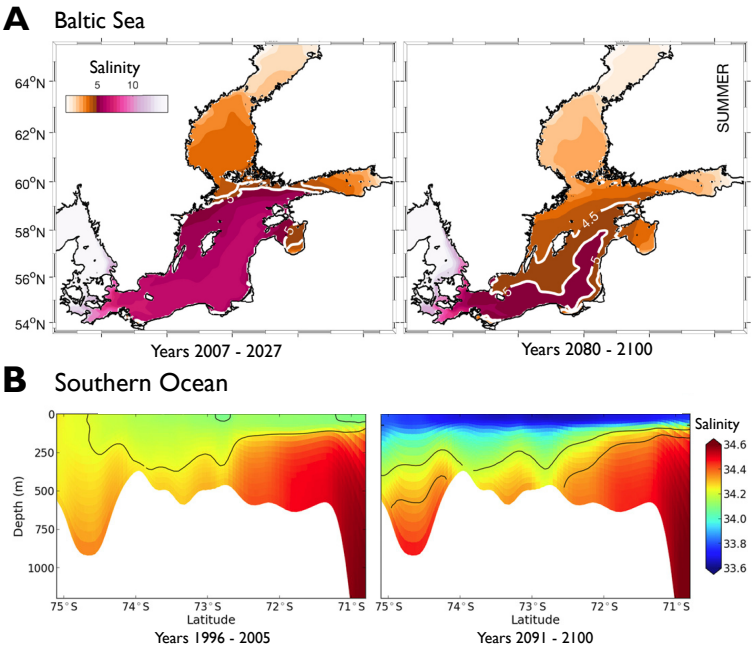

Figure 1.4: This map shows predicted future desalination using the example of the Baltic Sea (A) and Southern Ocean (B). A) Distribution of surface salinity in the Baltic Sea in current summer (2007-2027, left) and the projected desalination for the future (2080-2100, right). Salinity in the Baltic Sea is projected to decrease by 1.5-2 due to an increase in freshwater input [107]. The distribution limit of Baltic foundation species *Mytilus* sp. is depicted as two white line at a salinity of 4.5-5. Figure from Basdurak et al. (in prep.). B) Depicted is an Antarctic desalination scenario for the salinity in an interpolated cross section of the Amundsen Sea. Results are shown for the September monthly average at current conditions (left) and for the future scenario (RCP 8.5 ACCESS simulation) (right). Map from [228].

1.4 How do animals tolerate salinity changes?

When animals experience unfavorable salinity conditions the first response is usually migration into more saline waters. Less mobile species endure abrupt or transient salinity changes through shell closure, ecystment or burrowing. If these avoidance strategies are not feasible, acclimation mechanisms are initiated or differential mortality leads to selection of more salinity tolerant genotypes that can persist in the given habitat [74].

Osmolytes are defined as compounds that change in concentration when salinity changes (*sensu* [332]). They can be either of inorganic or organic nature. Osmolality is used when

referring to the number of osmoles of solute per kilogram water. Osmolality only considers solutes that contribute to a solution's osmotic pressure and varies between extra- and intracellular compartments. In extracellular fluids (i.e. blood, interstitial fluid, coelomic fluid, coelenteron fluid, etc.) of osmoconforming animals, inorganic ions are employed and their concentrations are similar to those of the surrounding seawater. The entire volume of extracellular fluids is referred to as the extracellular space (ECS). Instead of regulating extracellular fluid osmolality, osmoconformers utilize the mechanism of cellular volume regulation (CVR). Osmoconformers adjust to salinity changes by altering their intracellular osmotic pressure to that of the external medium. They thus avoid cellular swelling or shrinking due to water movement to counterbalance osmotic pressure differences. This process of CVR is facilitated by accumulation or release/catabolism of intracellular osmolytes. In intracellular fluids, inorganic ions are partly substituted by organic osmolytes. This is because high concentrations of inorganic ions are known to perturb cellular processes or structures [34]. Organic osmolytes have been attributed the major role in CVR in the past, due to their compatibility with protein function and stability (i.e. compatible osmolytes) [332].

As an immediate response to hypo-osmotic stress, cells undergo regulatory volume decrease by releasing inorganic ions from cells to prevent mechanical damage from water increase. This short-term response (minutes - hours) is followed by the reduction of organic osmolyte release or degradation (hours - days) [317]. To investigate an organism's tolerance to persistent salinity reductions they need to be fully acclimated. Appropriate acclimation times to achieve stable osmolyte pools last >14 to 28 days [260, 145].

Organic osmolytes are derived from different substance classes, namely amino acids, sugars, polyols, urea, and methylamines. Generally, osmolyte systems have been postulated to be very similar in all cells and species, only differing in relative contribution [250, 39]. Amino acids and methylamines are the prevalent osmolytes in invertebrates, but are also

used widely across phyla from bacteria, algae to mammals. However, it is not clear whether there are inter-taxonomic differences in the utilization of osmolytes within invertebrates. Differences in osmolyte composition can potentially originate from differences in diet or biosynthetic pathways. Generally, uptake from seawater, ingestion as well as biosynthesis have been shown to occur [233, 60, 62].

Organic osmolytes have not only compatible character, but often additional beneficial functions. These can be cryoprotectant effects during freezing stress, scavenging of reactive oxygen species, an increase in protein structure stabilization or cytoprotecting effects during hydrostatic stress [34, 400, 334, 323, 397]. Other compounds counteract perturbing solute effects of urea [401].

Osmolytes drive cellular volume regulation in osmoconformers. To determine an osmoconformers' low salinity threshold the *critical salinity concept (S_{crit})* was developed. It hypothesizes, that a critical salinity is reached when the organic osmolyte pool is fully depleted and fitness (i.e. reproduction, growth and survival as proxies) becomes zero [262]. So far, this concept has been successfully applied to an cnidarian and an echinoderm species [262, 308].

1.5 Salinity stress effects on the organismal, cellular and genetic level

Salinity stress affects osmoconformers on multiple levels, from the organismal to the cellular to the genetic. Duration and magnitude of salinity stress affect the extent of the physiological stress response [108].

1.5.1 Organismal level

On an organismal level, increasing salinity stress will first affect performance. When organisms are exposed to lower salinity conditions that deviate only moderately from the optimum, animals experience an increased energy demand for maintenance due to the transiently higher costs for osmoregulatory processes [290]. This means that less energy can be allocated towards storage, reproduction, growth and activity if energy input cannot compensate increased energy expenses. The increased costs for osmoregulation can cause an increase in feeding or respiration rates. In mussel neuronal tissue a strong increase in ATPase activity has been shown in response to low salinity acclimation [381]. A dynamic energy budget demonstrated a decrease in scope for growth of mussels under low salinities due to the substantial metabolic costs of osmoregulation (74-87% of total cost) [197]. Yet, a recent study showed that also biomineralization costs need to be considered and likely comprise a large portion of the overall energy consumption [297]. During extreme salinity stress, many marine invertebrates switch to metabolic depression. This can be a moderate metabolic depression that is indicated by the reduction of maintenance processes such as systemic activities (e.g. respiration and circulation) as well as energy uptake indicated by feeding rate [36, 262]. Under more severe stress metabolic depression becomes fully anaerobic [326]. This indicates a critical physiological threshold at which species try to conserve energy and overcome unfavorable periods until conditions improve. Such a state can usually not be maintained for long and is ultimately lethal (Fig. 1.2).

1.5.2 Cellular level

Generally, the identification of differentially expressed genes as a cellular response to salinity changes point towards proteins and processes that might be differentially regulated in

CVR. Biological transcriptomics are a powerful tool to better understand mechanisms in stress response. Transcriptomic analysis have proven useful in determining the impact of anthropogenic stressors on organisms as well as to detect the limiting factors of physiological processes such as biomineralization [403, 351, 190].

On a cellular level, acclimation to low salinity is achieved by an upregulation of osmoregulatory pathways. Acute salinity stress (4 hours - 8 days of exposure to novel salinity regimes) has been associated with an upregulation of key enzymes in osmolyte metabolism, ion channel activities, regulation of membrane potential and signaling pathways of osmotic stress, as well as a downregulation of amino acid and ion transporter genes [191, 409, 410, 212]. *In situ* studies that examined organisms that regularly experience mild to medium salinity changes paint a similar picture [78, 391]. Some of these genes have previously been associated with thermal or acid-base stress response [138, 2]. Generally, the response seems to be species-specific and can be tissue-specific as well [2, 184, 222]. Changes in salinity further affect protein function, protein stability, protein synthesis and translational activity [373, 34, 390, 400]. Also membrane stability, fluidity and permeability are affected by salinity stress, however studies assessing animals are rare [48, 230, 167]. Other changes can be structural adaptations, hemidesmosome-like support structures or increase of thickness of the connective tissue sheath were found on neuronal cells to reduce membrane tension [382, 321].

A further aspect of salinity stress, next to the cellular osmoregulation processes and changes in cell structure, is the cellular consequences of metabolic depression. This includes the downregulation of non-essential processes and increase in anaerobic metabolism. Hypo-osmotic stress has been shown to influence cellular metabolic pathways resulting in mobilization of sugars and lipids as energy reserves such as fatty acid beta-oxidation, or gluconeogenesis [292]. Another cellular consequence of salinity stress can be an increased

cellular immune response (involving immune genes and immuno-regulatory genes) and stress response [392, 292]. Lethal effects under severe conditions can be attributed to the permanent disruption of nervous function and cellular homeostasis [355]. Consequently, extreme salinity conditions lead to reactive oxygen species generation and activation of programmed cell death [340].

However, the majority of these studies just represent short-time response of the organisms to salinity stress. It is important to consider the time-dependent response as certain pathways can be upregulated as an immediate reaction, which will not be differentially expressed under persistent salinity reduction [199]. Up to now, there is only a small number of studies available on the effect of salinity changes on gene expression in marine animals; a fraction of those examine osmoconformers and even less use long-term acclimation (>14 days) [187, 123, 138, 292, 229]. The results of long-term studies partly match results obtained in short-term salinity studies. However, expression of genes for specific processes, such as metabolic pathways responsible for energy storage seems to differ [138, 292]. Moreover, specific osmoregulatory transporter pathways are differentially regulated [138, 292].

1.6 Salinity stress drives evolution

On a genetic level, it is still widely unknown how animals adapt to changing salinity [410]. Phenotypic plasticity as well as genetic variation are responsible for resistance to environmental stress. Yet, it is often unclear which traits are fixed and what are acclimation effects. While transcriptomic responses occur within minutes of osmoregulatory stress [365], evolution usually takes place over non-anthropological time-scales. However, selection on standing genetic variation can facilitate rapid adaptation. This has been shown for corals in response to ocean warming and acidification [30, 21]. Rapid adaptation to low

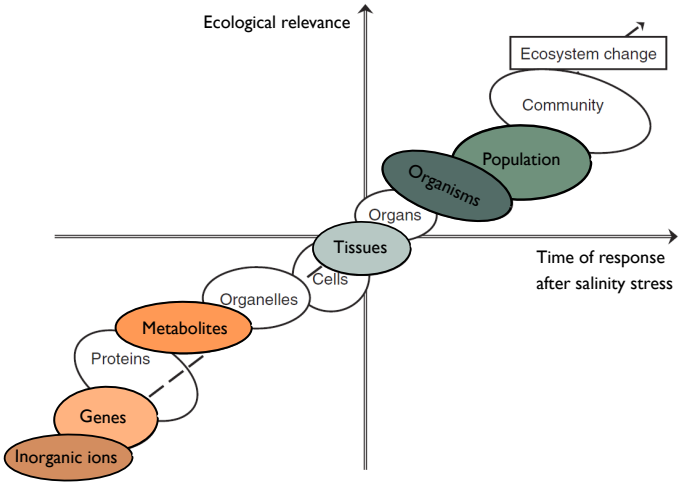

Figure 1.5: Levels of biological organization in relation to stress. This graph shows different biological levels of organization from genes (i.e. here gene expression) to community in relation to the time of response after exposure to environmental stress, such as desalination, and ecological relevance. The highlighted structures are levels covered in this study. The figure shows that there are many biological levels which are affected by salinity stress, albeit on different time scales. It further visualizes that small changes at a lower level of organization such as osmolytes and transcriptomes are interconnected to organismal responses to salinity stress and moreover that the salinity tolerance of organisms can affect entire populations and communities and ultimately lead to ecosystem changes. It is thus necessary to conduct comprehensive research on multiple levels to understand the effects of future desalination on marine organisms and ecosystems. Figure adapted from[182].

salinity has been detected in osmoregulating copepods [179, 333]. Here, an evolutionary shift at the population level was observed, that increased enzyme activity and expression of a crucial ion transport enzyme (V-type H^+ ATPase) in low salinity adapted populations [179]. Adaptation occurs through the selection of favorable phenotypes [74]. Phenotypic and molecular traits of favorable phenotypes can be heritable [73]. Tolerant phenotypes can also manifest in the population via genetic assimilation [170]. Further many environmental traits are polygenic and a cumulative result of multiple variants across many cellular pathways [20].

Today, species living at transition zones can be a good example to study natural selection. The colonization of brackish and freshwater environments by marine organisms is one of the most dramatic evolutionary transitions in the history of life [216]. Yet, it is not well documented how animals overcame this barrier. Freshwater habitats were very slowly colonized over hundreds of millions of years, a process that might have been slowed by the need to develop more complex osmoregulatory systems [216]. Documentation of genetic and physiological changes occurring in more recent invasions of neozoa or coping mechanisms in response to future desalination scenarios may help understand the processes that were involved in past colonization events [7, 99, 180].

In the Baltic Sea we find a hybrid-zone of *Mytilus* mussels in which species-specific allele-frequencies correlate with the salinity gradient. The western Baltic Sea populations have a higher fraction of *M. edulis*-like allele frequencies, whereas eastern populations are characterized by higher *M. trossulus*-like allele frequencies. *M. trossulus* are more tolerant to low salinity than *M. edulis* [311, 273]. A previous study ruled out neutral processes (such as genetic drift or neutral introgression) as a cause for this genetic difference [338]. Restricted larval dispersal was also disproven as the cause for the observed genetic differentiation [337]. Instead, it is very likely natural selection that sorts genotypes towards a *M.*

trossulus-like hybrid genome in low salinity environments [152]. Hybridization increases genetic diversity and thus may promote adaptive radiation and colonization of new habitats [312]. Understanding these processes of salinity adaption is most interesting in light of the long-term salinity changes around the world. Baltic populations present an ideal test case as to how adaptive evolution may play in the light of climate change [283]. Many of them are already locally adapted and show considerable tolerance toward environmental stressor [368].

At this point there is no study available that analysed the effect of long-term low salinity stress on *Mytilus* hybrid population gene expression of the Baltic Sea. Studying the effect of low salinity on species with different tolerance ranges may be useful to detect differences in the acclimation processes. In this respect, it is highly interesting to study two closely related populations of the same species complex, which differ in salinity tolerance capacity and where additionally salinity and species' physiological capacity has been shown to drive natural selection [152].

1.7 Research Gaps

Comparative studies and integrative studies of osmolyte systems in osmoconforming species are lacking. Literature on the role of organic and inorganic osmolytes is inconsistent and is mainly a compilation of many individual studies measuring a single substance class in a specific organism or tissue. Additionally, there is no consensus on units in osmolyte research, which renders a direct comparison of results extremely difficult as conversion is often not possible with the reported data. To date, there is no systematic literature review or meta-analysis available on effects of salinity on cellular osmolyte concentrations.

Despite the appreciation of the importance of osmolytes in CVR it remains unclear what determines successful salinity acclimation in marine invertebrate species and what differentiates euryhaline from stenohaline species. Researchers have suggested that organic osmolyte pool size, utilization of specific organic compounds, or water regulation capacity may define CVR capacity [260, 175, 258].

Even though the importance of organic osmolytes in CVR has been established, inorganic ions also can play a major part in the total osmolyte pool during long-term acclimation to low salinity [382, 187, 250]. However, quantification of the role of inorganic ions in osmoregulation has been heavily neglected in most studies. Inorganic ions are estimated to comprise >50% of intracellular total osmolality [317]. Intracellular chloride or sodium concentrations have occasionally been assessed after long-term acclimation to low salinity and were found to significantly change with salinity, hereby disproving studies that suggest a replenishment of inorganic osmolyte pool after the initial osmotic stress response [371, 250, 5]. It is unclear how modulation of intracellular ion concentration has evolved when considering the known perturbing effects of inorganic ions on protein function compared to the higher compatibility of organic osmolytes. However, not all enzymes are negatively affected by low salinity, especially those of osmoconforming species[302]. Yet, enzyme activity is very specific [11]. Some enzymes are unaffected in their activity by ionic strength of the medium [302, 11]. Other enzymes function at higher rates at low salinity in osmoconformers compared to osmoregulators [301]. Contrary to this, a reduction of enzyme activity in response to low salinity, observed in oysters, was suggested to be necessary to maintain normal metabolic function [11].

Concerning organic osmolytes, there is a methodological bias. In the past, targeted methods such as amino acid analyzers have often been employed to measure organic osmolytes, yet these methods ignore methylamines and methylated sulfonium compounds

which can comprise the majority of the organic osmolyte pool in some species [254].

In the past, inorganic anions have been titrated separately [267]. Measuring inorganic anion is thus relatively time-consuming compared to organic osmolytes. Other methods utilized ion-chromatography. However, these protocols are not sustainable, because only a small number of samples can be processed until tissue extracts clog the column and it needs replacing [274].

There is, furthermore, a general lack of long-term osmolyte studies utilizing appropriate acclimation intervals. This deficit can be due to the fact that long-term studies require time-intensive experiments. However, with respect to the projected long-term changes in salinity in many regions world-wide it is necessary to not only understand how organisms cope under fluctuating salinity conditions, but also acclimate to persisting changes in salinity. It is thus necessary to investigate the low salinity threshold of key species to discover what determines the capacity for salinity tolerance. Providing such baselines is crucial to enable prediction of how species distribution and performance might change with ongoing climate change.

1.8 Outline of the thesis

This thesis constitutes a comprehensive approach to assess the limits of tolerance to reduced salinities and its determinants on multiple levels. It focuses on benthic osmoconforming invertebrates such as mussels, snails, sea stars and sea urchins. Benthic species are especially affected by salinity change due to their low mobility. Hence, they are restricted to certain environmental conditions which they have to cope with *in situ*. Osmoconforming species are exposed more substantially to environmental salinity changes as their isoosmotic body fluids adjust to environmental salinity and thus tissues and cells

experience a wider range in osmolality as well.

The focus of this thesis was to better understand how CVR mechanisms adjust to permanent or prolonged salinity reductions.

To do this, I first performed a comprehensive literature research to conduct a meta-analysis of osmolyte composition across taxa in response to low salinity acclimation. Secondly, in order to investigate effects of low salinity on organisms and their capacity for salinity tolerance, I conducted a series of long-term (30 days) acclimation experiments to low salinity across taxa. Here, I combined biomarkers from multiple biological organizational levels and examined salinity tolerance on the biochemical, tissue and organismic level (Fig. 1.5). I assessed physiological fitness in combination with elaborate osmolyte profiles. Lastly, I conducted a focus study using two locally adapted mussel populations to assess conserved and population-specific salinity acclimation mechanisms using differential gene expression (Fig. 1.5).

The first part of this thesis established a baseline for future osmolyte research. A systematic overview of osmolyte profiles across taxa was overdue as there is a large, sometimes controversial, amount of literature on marine animal osmolytes with heterogeneous units of measurement. I conducted a systematic review on the effect of low salinity on the osmolyte concentrations across marine benthic osmoconformers. The reported results were then subjected to a meta-analysis that considers effect-size as well as experimental design. An important characteristic of this study was to include inorganic as well as organic osmolytes. The aim was to identify compounds that significantly change with salinity to distinguish true osmolytes from the vast amount of reported intracellular chemical compounds. By differentiating further between taxa, tissues and physiotypes, I aimed to identify universal and specific mechanisms of cellular volume regulation and potential research gaps. The intention was further to establish best-practice guidelines for future osmolyte research.

I investigated the following questions:

i Which inorganic and organic solutes function as osmolytes in osmoconforming species?

ii Are both, inorganic and organic osmolytes involved in salinity acclimation?

iii Are there taxonomic differences in osmolyte utilization?

iv Are there differences in utilization of osmolytes between tissue types?

v Are there differences in osmolyte use in euryhaline vs. stenohaline species?

Based on the results of the systematic literature review and the meta-analysis, the next chapter of this thesis was designed to address research gaps that were revealed previously and to expand the current knowledge on osmolytes systems. Therefore, the following experiments used an integrative approach of measuring organismal and biochemical biomarkers in response to salinity stress. In this study, I chose six species from three different taxa and five phyla (Cnidaria: Anthozoa, Echinodermata: Asteriodea Echinoidea, Mollusca: Bivalvia & Gastropoda). All of these species have an important ecological role, be it as foundation species / ecosystem engineers, keystone predators, grazers or potentially invasive species. Most of the study species are considered euryhaline, nevertheless they differ in their salinity tolerance ranges.

Using a sufficiently long acclimation time of four weeks with prior climate chamber acclimation and gradual salinity decrease, I carefully conducted six independent experiments. Species were submitted to seven different salinity treatment that clustered around the low salinity threshold. I assessed the effects of low salinity on fitness parameters, volume regulation capacity as well as a comprehensive osmolyte profile of body fluids and tissue extracts. I measured a large variety of inorganic ions and organic osmolytes with a combination of

multiple, untargeted techniques. I developed a novel protocol for the inorganic ion analysis in tissues, which is described in this chapter for the first time. Concerning organic osmolytes, I employed non-targeted metabolomics to cover all potential osmolyte classes. The chosen combination of physiological and biochemical biomarkers allowed me to test the S_{crit} concept across taxa.

The hypotheses were:

i Organic osmolyte concentrations in tissues decrease during acclimation to low salinity.

ii Organic osmolyte composition differs between taxa.

iii The inorganic osmolyte pool decreases during acclimation to low salinity. More specifically, the concentrations of the main inorganic osmolytes (i.e. sodium and chloride) decrease in tissues, while potassium and calcium remain constant.

iv A critical salinity can be determined for species across taxa and is accompanied by a reduction in survival and growth and an increase in tissue water content.

In the third part of this thesis, I continued this integrative approach to understand salinity stress acclimation responses using transcriptomics. Two locally adapted blue mussel populations from different salinity regimes were compared for differences in gene expression after acclimation to 5 salinity levels. Due to the differences in salinity tolerance, *M. trossulus*-like and *M. edulis*-like populations from the hybrid zone of the Baltic provide an ideal model system to study how bivalves adapt to hypo-osmotic conditions and differ in salinity acclimation mechanisms. A companion study has shown that these mussel populations show differences in the size of the intracellular inorganic and organic osmolyte pools, as well as differences in survival under hypo-osmotic stress [296]. Thus, the next step to un-

derstand how acclimation to low salinity works was to look at the transcriptomic responses to determine which salinity responses are universal and which are population-specific. I assumed that, in accordance with their phenotypic differences in salinity tolerance, the two *Mytilus* populations will exhibit divergent transcriptomic responses following acclimation to low salinity.

The hypotheses were:

i At low salinity mussels will upregulate processes involved in cellular volume decrease (i.e. reduction of osmolyte concentrations via release or degradation).

ii Stress response genes in the Kiel population will be upregulated at low salinities.

iii Usedom mussels (low salinity adapted) will have a lower number of differentially expressed genes than Kiel mussels (high salinity adapted) at low salinities.

iv Severely stressed organisms will be characterized by an enrichment in gene expression of stress response genes (i.e. heat-shock proteins) and pathways involved in apoptosis.

The results of the three chapters build on one another and will be synthesized in the discussion to highlight the different aspects of salinity tolerance in osmoconformers. The main goal of my thesis was to answer which mechanisms determine low salinity thresholds across taxa. Another objective was to assess which biomarkers are most useful in determining these physiological limits. It was further a goal to reveal on which levels these key regulators operate. This thesis, thus, poses an important cornerstone for future research in salinity tolerance as it employs an integrative approach to measure phenotypic plasticity towards low salinity across a wide range of species. This was done on an organismic level as well as, cellularly, on a biochemical and transcriptomical level.

2

Acclimation of marine invertebrate osmolyte systems to low salinity: a systematic review & meta-analysis

Imke Podbielski, Lara Schmittmann, Trystan Sanders, Frank Melzner

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Acclimation of marine invertebrate osmolyte systems to low salinity: a systematic review & meta-analysis

Imke Podbielski[1*], Lara Schmittmann[1], Trystan Sanders[2], Frank Melzner[1]

Affiliations

[1]Marine Ecology Research Division, GEOMAR Helmholtz Centre for Ocean Research Kiel, Kiel, Germany

[2]School of Ocean and Earth Science, National Oceanography Centre Southampton, University of Southampton, Southampton, UK

*Corresponding author: e-Mail: ipodbielski@geomar.de, Phone: (+49) 431 600 4275

Abstract

Salinity is a major environmental factor shaping the distribution and abundance of marine organisms. Climate change is predicted to alter salinity in many coastal regions, due to sea level rise, evaporation and changes in freshwater input. This exerts significant physiological stress on coastal invertebrates whose body fluid osmolality follows that of seawater (*osmoconformers*). In this study we conducted a systematic review and meta-analysis of osmolytes (both organic and inorganic) utilised by osmoconforming marine invertebrates during >14 days acclimation to reduced salinity. Of 2389 studies screened, a total of 56 studies fulfilled the search criteria. 38 studies reported tissue osmolyte. Following acclimation to reduced salinity, tissue concentrations of six organic compounds and sodium were consistently reduced across phyla. This suggests that intracellular inorganic ions are not only utilized as a rapid response system during acute exposure to low salinity stress, but also, in concert with reductions in organic osmolyte concentrations,

during longer-term acclimation. Our systematic review demonstrates that only few studies ($n = 13$) have quantified salinity induced long-term changes in intracellular ion concentrations. In addition, no study has compiled a complete intracellular osmolyte budget. Alanine, betaine, glycine and taurine were the major organic osmolytes that are universally employed across five phyla. Characterisation of organic osmolytes was heavily weighted towards free amino acids (FAAs) and derivatives - neglecting methylamines and methylsulfonium compounds, which can be as important as FAAs in modulating intracellular osmolality. As a consequence, we suggest best-practice guidelines to streamline experimental designs and protocols in osmoregulation research in order to better understand conserved mechanisms that define limits of salinity acclimation in marine invertebrates. This is the first systematic review & meta-analysis on osmolyte concentrations in osmoconformers acclimated to low salinity. It creates a valuable baseline for future research and reveals large research gaps. Our meta-analysis suggests that there are common osmolyte actors employed across phyla, but no uniform concept. In light of future salinity changes and its potential consequences, it becomes more important to understand salinity tolerance capacities and limits.

Keywords

Osmotic stress | Osmoconformer | Cellular volume regulation | Osmoregulation | Osmolytes | Ions | Salinity tolerance | Metabolomics | Free amino acids | Marine Invertebrates

2.1 Introduction

Salinity is a major abiotic factor in the aquatic environment with short-term fluctuations causing severe physiological stress in organisms [36]. An increasing num-

ber of studies predict long-term changes in salinity caused by ongoing climate change in coastal regions of the world, with potentially severe effects on ecosystems [1, 357]. This includes increasing as well as decreasing trends. Generally, higher latitudes are predicted to receive an increased freshwater input via precipitation, riverine discharge, surface run-off or meltwater [27, 221, 367]. On the other hand, subtropical regions or some estuarine regions (such as the Chesapeake Bay area) are estimated to experience an increase in salinity due to lower precipitation rates, or sea level rise and a consecutive sea water influx into previously brackish areas [75, 128]. Desalination can be as harmful to organisms as ocean warming or acidification, but has received little research attention up to now. In the Baltic Sea, widespread desalination by 1.5 is predicted by the year 2100. This would potentially render an area of $> 100.000\ km^2$ inhabitable for many marine species and would shift biomes westward [107, 262]. Shallow, coastal reefs in Australia are more likely to experience prolonged low salinity events in the future [113, 310] and Antarctic shelf systems are predicted to receive an increased input of melt-water and atmospheric precipitation causing salinity reductions by up to $0.5 - 2$ [214, 228]. Therefore, the ability of resident species to acclimate or adapt to long-term decreases in salinity is crucial for their perseverance and proliferation in the impacted areas.

Aquatic organisms employ different strategies to cope with changing salinity. Some species can avoid low salinity habitats by vertical or horizontal migration to higher saline regions. However, most benthic invertebrates in particular have a lower degree of mobility and are thus more susceptible to local salinity changes. Some species then temporarily isolate themselves from the environment during hyposaline episodes through shell closure or encystment, a state that cannot be maintained permanently. Hence, only through physiological acclimation and or adaptation to changing salinities can fitness be maintained at a high level.

There are two physiological strategies adopted by aquatic invertebrates to adjust to salinity changes in their environment. Osmoregulators are species that can maintain the osmolality of their extracellular fluids at relatively stable levels when exposed to salinity stress, primarily by means of active ion transport (e.g. the crustacean *Carcinus maenas* or the annelid *Marphysa gravelyi*) [59, 158, 291]. The extracellular fluids of osmoconformers, on the other hand, remain isosmotic with respect to ambient salinity (Tab. 2.1). This does not, however, imply that acclimation to changing salinities is a passive process: osmoconformers achieve salinity acclimation through active cellular volume regulation (CVR). When cells are acutely exposed to dilute seawater, they tend to gain water and lose solutes (they swell), the opposite happens when external osmolality increases [300, 354, 360]. Cells then respond by active CVR, thus regulatory volume decrease in the case of cell swelling, or regulatory volume increase in the case of shrinking to fully or partially restore the original cell volume. This is accomplished via adjustment of intracellular osmotically active compounds, so called osmolytes (Fig. 2.1) [132, 126, 300, 375]. Depending on the abruptness of the change in osmolality, CVR can keep pace with passive osmosis, whereas acute osmotic stress will cause volumetric changes [132]. Osmolytes, used for CVR, are inorganic or organic solutes whose concentrations are regulated during periods of osmotic stress (*sensu* Somero and Yancey 2011) (Tab. 2.1). The change of cell volume can be an additional or alternative mechanism employed to adjust to osmotic stress. An increase in cell volume can also be observed when the capability for CVR is reached or CVR is incomplete [175]. While the osmolality of extracellular fluids is exclusively driven by inorganic osmolytes, cells partially substitute inorganic with organic osmolytes during intracellular osmolality adjustment (Fig. 2.1). This is due to the fact that changing concentrations of inorganic salts strongly influence protein functioning [124, 332]. Organic osmolytes are termed compatible osmolytes, as their high intracellular concentrations do not impair protein structure

or function [124] (Tab. 2.1). Time-dependent changes in intracellular osmolyte composition usually involve an immediate reduction or rise in inorganic ions such as potassium under hypo- or hyperosmotic stress, respectively [324]. Organic osmolyte concentrations are modified minutes later [324, 365]. Rapid release or uptake of ions through ion channels can rescue cells from mechanical damage (and death) that would otherwise be caused by excessive volume change [315]. With ongoing salinity stress, inorganic ion concentrations have been suggested to be partially restored and instead organic osmolyte are reduced as osmotic replacements [324]. Upon longer time of exposure or acclimation to hyperosmotic stress, species have been shown to gradually modify the concentration of compatible organic osmolytes and the composition of organic osmolyte budgets. Subsequently, these organic osmolytes are metabolized to organic osmolytes with higher osmoprotecting abilities or replaced by osmolytes accumulated via *de novo* synthesis [279, 332]. This is a more time intensive process that can last from hours to two to four weeks depending on the magnitude of hypo- or hyperosmotic stress experienced [9].

Table 2.1: List of definitions

Term	Definition
Solute	Any dissolved compound
Osmolyte	Any inorganic or organic solute whose concentration is regulated during acclimation to an altered salinity regime
Organic osmolyte	Organic solute, usually a low molecular mass organic molecule that contributes to osmotic pressure and is regulated with changes in salinity
Compatible osmolyte	Osmolyte that contributes to osmolality yet perturbs protein function to a lesser degree than other organic solutes. Instead, compatible osmolytes often exhibit protein stabilizing attributes
Extracellular space (ECS)	ECS refers to the vascular system that contains blood, coelomic fluid or hemolymph, as well as the interstitial space filled with interstitial fluid. The latter is the extracellular space between cells. ECS contains predominantly inorganic ions as osmolytes. Extracellular fluids in most osmoconformers are very similar in composition to seawater
Cellular volume regulation (CVR)	The process of regulating solutes that contribute to osmolality, thereby influencing whether a cell has a tendency to swell (water uptake) or shrink (water loss)

Table 2.1: List of definitions

Term	Definition
Osmolality	Osmotic pressure of a solution. 1 osmole is defined as the osmotic pressure of a 1 molal solution of an ideal solute
Isoosmotic	Two fluids that are characterized by the same osmolality
Osmoconformers	Metazoans that are isoosmotic with respect to the surrounding seawater
Acclimation	Reversible process of physiological adjustment of an individual organism to changes in an environmental factor (here salinity) to enable higher fitness than in the un-acclimated state. This typically involves modifications of cellular biochemistry, membrane composition and tissue ultrastructure
Free amino acids (FAAs)	Cytosolic amino acids
Quaternary Ammonium Compounds (QACs)	Quaternary amines with the structure R_4N^+, where R can be an alkyl or an aryl group. QACs are cationic and are considered very good electrolytes.

In order to achieve long-term acclimation to reduced salinities, organic osmolyte concentrations must be reduced by discharging or catabolizing surplus osmolytes. While the immediate responses of inorganic ions to osmotic stress have been examined in a variety of studies in marine invertebrates, it is largely unexplored to what degree changes in intracellular inorganic osmolytes play a role in long-term acclimation to reduced salinity. The traditional view is that most species favour to maintain relatively constant ion concentrations while concentrations of organic osmolytes are linearly reduced, ultimately to non-detectable levels [262]. Characterisation of intracellular inorganic vs. organic osmolyte concentration adjustments in relation to salinity changes is difficult, as there is a large and variable fraction of fluids in the extracellular space (ECS, interstitial fluid and blood, coelomic fluid or hemolymph) in tissue samples (Fig. 2.1, Tab. 2.1). As a result, despite our knowledge of the general process of cellular volume regulation it remains unclear which factors determine the capacity and limits for long-term salinity tolerance in osmoconformers.

Approximately $>$ 95% of all metazoan species are invertebrates [407]. The majority of invertebrates (Cnidaria, Porifera, Ctenophora, Echinodermata, Tunicata, most Mol-

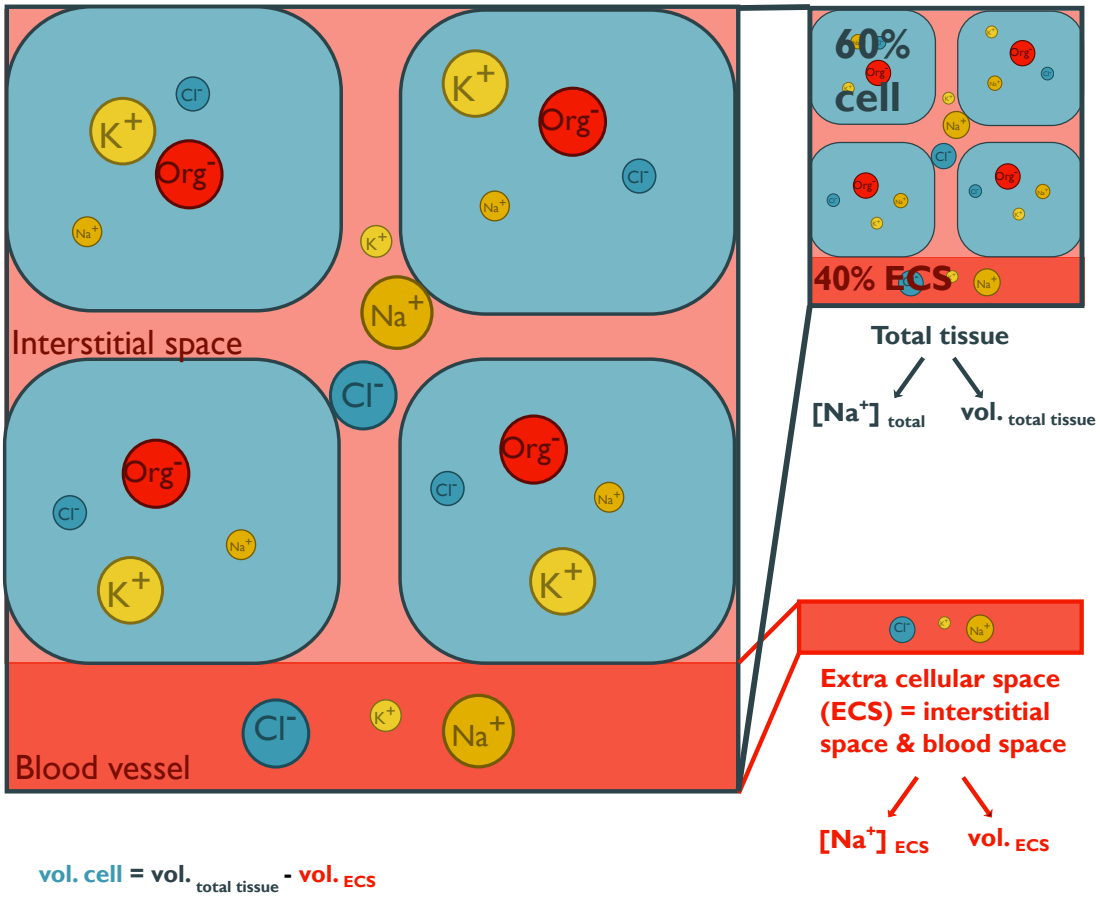

Figure 2.1: This conceptual figure shows the main components of cellular volume regulation in tissues and how intracellular solute concentrations can be estimated. It illustrates osmolyte concentrations in the extracellular space (ECS; consisting of interstitial space and blood, coelomic fluid or hemolymph space) and within cells of a generic tissue. Extracellular fluid osmolality is modulated exclusively through inorganic osmolytes, whereas cells also utilize organic osmolytes. Only the major inorganic ions are depicted, namely chloride, potassium and sodium. The abbreviation *Org* represents a variety of organic osmolytes, such as alanine, betaine, glycine, or taurine. The size of the circles represents the concentration of a specific osmolyte. A total tissue sample can contain a large proportion of the ECS that is on average 40%, with a range from $10 - 80\%$ of tissue mass. Since ion concentration in the ECS roughly represents that of the surrounding seawater and intracellular osmolality is partially accounted for by organic osmolytes, intracellular inorganic ion concentrations are lower than those in the ECS. Measurements using tissue homogenates thus overestimate cellular inorganic osmolyte concentrations and underestimate organic concentrations, if they are not corrected for the ECS contribution. Extracellular ion concentration can be measured from blood, coelomic fluid, or hemolymph samples. The ECS can be estimated via the dilution of a known volume of a radioactive, or fluorescent tracer (such as ^{14}C-inulin, or FITC-inulin) that is injected into extracellular fluid. Cell volume is estimated from the difference between tissue volume and ECS volume. Intracellular osmolyte concentration can then be calculated with the given formula.

lusca, some Annelida and Arthropoda) and few vertebrates (Myxinidae, Euchselachii and Coelacanthiformes) are osmoconformers [101, 145, 235, 399]. Despite this vast amount of osmoconforming species, it remains unclear how most osmoconformers acclimate to low salinity and whether similar osmolyte selection strategies are employed across taxa. While it has previously been shown that there are differences in organic solute utilization between plants, bacteria, invertebrates and vertebrates [146], it is unclear whether taxonomic differences in osmolyte utilization exist within osmoconforming marine animals. It has been shown that different tissues can differ in their osmolyte utilization, yet it is unclear whether these differences are systematic [9, 97, 122]. A key question that has not been addressed is whether organic osmolytes are preferentially depleted during low salinity acclimation in all invertebrate taxa or whether inorganic ion depletion occurs simultaneously.

In this review, we focus on a systematic comparison of inorganic and organic osmolytes in tissues of benthic osmoconformers. We filtered the literature for studies that have acclimated marine osmoconformers to reduced salinities for a time period of at least 14 days. Tissue organic osmolytes typically reach new steady state concentrations within such a time interval [22, 61, 193]. This screening procedure allows us to test, whether decreased inorganic ion concentrations complement reductions in organic osmolyte concentrations during long-term acclimation to reduced salinity, or whether inorganic ions are exclusively used for CVR during rapid salinity changes.

Currently, there is no systematic analysis available that has screened the available literature for factors that could define the capacity for salinity tolerance in osmoconforming organisms. In our study, we assess impacts of low salinity acclimation on osmolyte concentration of osmoconformers to obtain information on osmolyte pool size and composition. We ask the following questions:

(i) Which inorganic and organic solutes function as osmolytes in osmoconforming species?

(ii) Are both, inorganic and organic osmolytes involved in salinity acclimation?

(iii) Are there taxonomic differences in osmolyte utilization?

(iv) Are there differences in utilization of osmolytes between tissue types?

(v) Are there differences in osmolyte use in euryhaline vs. stenohaline species?

To answer these questions, we conducted an extensive systematic review of the available literature. The systematic approach follows a strict literature search and selection protocol guaranteeing an objective selection of suitable studies. With the compiled data we proceeded with a meta-analysis. The benefit of a meta-analysis over an ordinary literature review is the ability to statistically evaluate individual effect sizes by computing a summary effect for multiple studies to estimate the mean of the distribution of true effect sizes. This way, emphasis is placed on an overall perspective allowing for outliers and diverging effects. To account for the expected biological variability, we conducted categorical subgroup meta-analyses for factors such as taxonomy, tissue type and physiotype.

2.2 Methods

2.2.1 Literature search & data extraction

The literature was scanned for studies reporting inorganic and organic osmolyte concentrations of osmoconforming species in response to long-term salinity changes. To cover a wide literature base, we searched three scientific databases: ISI Web of Science, Google Scholar and Scopus. We used pre-determined, relevant keywords, their possible

variations and Boolean operators for the search strings (Appendix A.1). Additionally, hand-picked studies were added which were either already known or revealed following relevant literature trails during full-text analysis. The literature search was performed on studies collected until 30.01.2019. Marine benthic osmoconformers (free-living, non-parasitic metazoans > 1mm) were defined as our population of interest. Long-term low salinity stress (minimum 14-day exposure) was chosen as exposure variable in conjunction with marine salinity (or habitat conditions) as comparator [260]. The acclimation time here refers to the time at the final salinity treatment, excluding time for salinity adjustment. Information on the type of salinity adjustment (gradual vs. acute) was added to SI03. To explore the effect of low salinity stress on osmotically active cellular substances, we selected studies which reported inorganic ion and/or organic osmolyte concentrations in tissues and extracellular space as outcome variables. Following the initial literature search, three successional filtering steps followed: title scan, abstract scan and full-text scan (Fig. 2.2). During each step, the search results were assessed for relevance and obviously irrelevant studies were excluded, while possibly relevant studies were included in the next assessment step. If the topic of the study was unclear in anyway during the title scan an abstract was conducted to validate a potential exclusion. During full-exam we further searched the literature cited by all potentially relevant studies. Exclusion criteria were pre-defined and consistent throughout the entire filtering process. Included were studies with osmoconforming metazoan species from marine and brackish water habitats as study organisms. Partial osmoconformers were included in the study when they were reported to be isosmotic across $> 50\%$ of their habitat salinity range, but were found to regulate osmolality under severely hypoosmotic stress. Hypo- and hyperconforming species, whose osmolality is always slightly higher or lower (by $<10\ mOsm \times kg^{-1}$ in either direction) than that of the ambient medium, but changes proportionally with salinity, were also included into

our analysis. Studies were excluded when treatment salinity was unknown, when salinity was fluctuating by more than 5, or when only a single salinity treatment was reported (absence of control treatment). To guarantee complete acclimation, we restricted our analyses to experiments with a salinity acclimation duration of at least 14 days. When acclimation time was unknown, studies were excluded. For multifactorial studies, only the response to altered salinity at the control condition of the co-variable was used for our analysis. Multifactorial studies where the salinity treatment could not be differentiated from the effect of other stressors were not included. When the classification of control conditions was not clear, the ambient or mid-range level of the additional factor was used. In cases with several independent experiments within a study (i.e. different species, different populations, independent experiments using the same species), all experiments were treated as independent data if they met the overall criteria. When a large number of experiments per study would have been added using this approach, experiments were averaged if possible to avoid bias (1 study). Although incorporating multiple effect sizes from the same study decreases the independence of data points, this enabled us to explore a wider range of species and to increase our sample size. When dependent effect sizes were reported from the same experimental population and the values fell into *a priori* defined subgroups (i.e. tissue, sample type) dependent data was used, otherwise only one data point was chosen. These dependent data points were accounted for in the statistical analysis. When salinity response was recorded over a period of time with multiple sampling time points, the final time point was selected. For *before and after* study designs, the initial time point (T_0) was used as control if no control treatment was available. When an experiment reported multiple outcome variables, all responses were used in separate analyses. When an experiment reported more than one parameter for a single response variable, the most generalizing response variable was included (e.g. Total Free Amino Acids < Ninhydrin-positive Substances < Total organic

osmolytes). If response variables had an insufficient sample size ($k < 3$) for meta-analysis, they were excluded (Tab. A.1). All articles reviewed at the full-text stage are listed in Supporting Information (Tab. A.2) with reasons for exclusion. We assembled results from 68 separate experiments from a total of 56 studies (= published sources). Of these, 41 studies were revealed during the initial search, and 13 studies were identified as they were cited by studies collected via the data base searches (list of included studies and study details: Tab. A.3 & Tab. A.4). Of the 56 studies that comprised the final reference library, 38 reported osmolyte concentrations for tissues (and sometimes additionally body fluids), and 18 reported only body fluid concentrations. We extracted mean results, variance estimates and sample size for low salinity and high salinity treatments from the selected studies. Regression results reporting values for R^2 and sample size were included and converted. When a regression study lacked statistical information, but reported raw data, that was used instead. When multiple treatment levels were reported we used the lowest salinity treatment the species survived at. For the high salinity group, we used the designated control treatment, or the salinity level representing marine or habitat conditions. The high salinity treatment was chosen to never exceed normal marine conditions in order to exclude hyperosmotic stress effects. Graphical data was transferred from the primary literature using GetData Graph Digitizer (v 2.26.0.20; S. Fedorov).

2.2.2 Data analysis

The effect of low salinity stress was measured for each outcome variable for each experiment computing Hedge's g (g) as effect size (see SI 04 for all relevant formulas). The standardized mean difference is a wide-spread index used in meta-analysis. It has the advantage of being comparable even if studies use different scales [118]. There is a large variety of units to express osmolyte concentration in tissues. Although it would have been ideal to

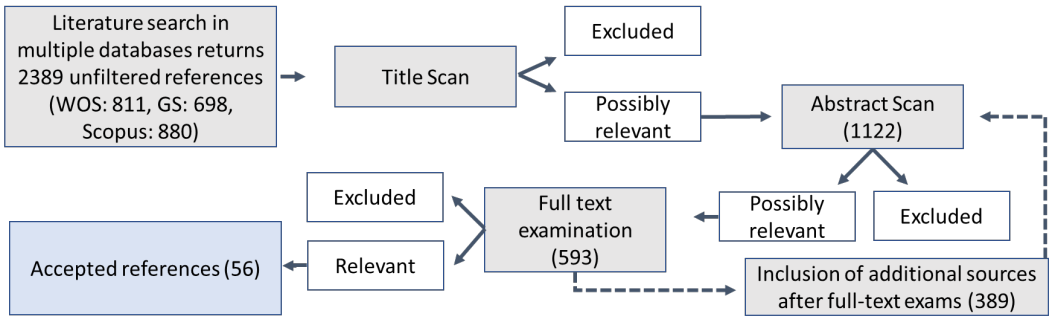

Figure 2.2: Flow chart for the systematic review process of literature retrieval and selection. Numbers indicate the number of papers per step.

convert all measurements to the same scale before comparison, many authors have not reported the data necessary to enable these conversions. Thus, we used standardized means to include as many studies as possible. Hedge's g corrects for a bias overestimating standardized mean difference for small sample sizes [117]. A Hedge's g of 0 is interpreted as the experimental treatment having no effect on the response variable, while a positive value indicates a positive effect and a negative value indicates a negative effect. Meta-analyses weigh the individual effect sizes by the inverse of the effect size variance to account for the precision of each study, hereby penalizing studies with high variance and low sample size [32]. A random effects model was used to calculate the overall mean effect for each response variable, hereby computing a weighed mean effect that accounts for sample size, within and between study variance (see Appendix A.2 for all relevant formulas). The effect size is considered significant ($\alpha = 0.05$), when the 95% confidence interval does not include zero. The Q_M value indicates how much heterogeneity is explained by the moderator and is used to test whether this proportion is significant. To compare inorganic and organic pool sizes an unweighted fixed effects meta-analysis was conducted, since most studies did not report variances. We accounted for dependent data clusters and potential underestimated variance, using robust variance estimates [119]. A robust test was used on the random

or mixed-effects model which estimates a variance-co-variance matrix of the model coefficients. This test has the advantage of adjusting for small sample sizes that improves the performance of the method when the number of clusters is small [364]. This test yields more conservative results for confidence intervals and measures of significance. It does not report on heterogeneity measures and thus results from the original model were used as indicators for residual underlying heterogeneity. For comparability between response variables the robust test was applied in all cases, even when dependent data clusters were absent.

2.2.3 Subgroup analysis

To examine the variation of effect sizes between studies we calculated the Q-statistic. A significant Q_E statistic indicates that there is significant underlying heterogeneity within the mean effect size and that other factors contribute to effect size variation. Consequently, studies were tested for differences in effect sizes in a subgroup analysis with previously defined categories. We assumed that heterogeneity in effect sizes could be due to biological differences and compared different taxonomic groups at the phylum level (Annelida, Arthropoda, Chordata, Cnidaria, Echinodermata, Mollusca, Platyhelminthes, Porifera) and down to class level where possible (Bivalvia and Gastropoda). In addition, we compared the mean effect size amongst the various sample types (i.e. whole body, gill tissue, intestinal tissue, muscle tissue, mantle tissue, heart tissue, coelomocytes). We further wanted to distinguish between different physiotypes (stenohaline, oligohaline and euryhaline). However, it was not possible to conduct a categorical mixed-effect meta-analysis assessing influence of physiotype on effect sizes due to the lack of representation of stenohaline and oligohaline organisms in literature. For the other subgroup analyses, only subgroups containing sufficient data points ($k \geq 3$) were considered. To test for differences amongst subgroups, we performed separate mixed-effect models assuming random effects within-study

variance and fixed-effects between-study variance for each category and response variable. A subgroup meta-analysis calculates a new summary mean effect size for all experiments included in the analysis as well as a mean effect size for each subgroup. Significance of the moderator variable is estimated by Q_M. The robust test was used on mixed-effects models. To avoid repeated testing of the same data which results in an increased probability of type 1 error, the analysis was limited to the *a priori* defined categories. The number of studies included in the categorical analysis might differ in the number of data points from the initial overall mean effect size analysis. Thus, the new summary effect computed by subgroup analysis might differ from the overall meta-analysis result. Lastly, the relationship between effect sizes and methodological factors was tested. Methodological factors can affect effect size and should be considered when designing experiments or evaluating literature. The magnitude of salinity stress can have an impact on effect size value. The larger the salinity stress, the more osmotic constituents are depleted. Thus, in stenohaline organisms with a smaller salinity range this will have a smaller impact than in euryhaline organisms subjected to very low salinity conditions. We quantified the differences in effect size between studies for experimental duration and magnitude of salinity stress using a continuous meta-regression approach. Additionally, acute osmotic shock can overstrain an organisms' salinity tolerance, whereas a gradual acclimation would allow the species to acclimate successfully [140, 258]. We recorded the mode of salinity adjustment where possible (instantaneous vs. gradual; Tab. A.4), however it was not defined *a priori* as a categorical moderator. Moreover, not all studies of the final data set described the time course of salinity transfer, thereby impairing statistical analysis. Despite this, an acclimation time of 14 days after salinity transfer should be sufficient for osmolyte concentrations to reach a new steady state [248].

2.2.4 Sensitivity analysis

When conducting a meta-analysis, diversity in study design across the literature can introduce potential biases. In this case, a rigorous sensitivity analysis was used to trim metadata, make study diversity transparent and assess the quality of meta-analysis results and possible publication biases (Tab.A.5& Tab.A.7). First, the data was tested for its robustness and examined for statistical outliers and influential studies. This was done using multiple visual tools such as baujat and influence plots, as well as the influence function summarizing multiple statistics. When statistical outliers were detected and there was no suspected underlying heterogeneity, these data points were omitted from the analysis (Tab. A.5). To test for publication bias we applied a series of indirect tests. To test for potential bias as a result of the lack of small studies with non-significant effects we examined symmetry of funnel plots, supported by Egger's test and completed by the *Trim and fill* procedure to adapt the model for potential asymmetry and check whether a significant effect remains valid after inclusion of imputed studies [76, 77]. However, recent research has shown that the assumptions of the small-effect study methods may be inaccurate in many cases [154, 245, 319]. Thus, Rosenberg's Fail-Safe number was applied in addition since it does not rely on funnel plot asymmetry, giving an estimate on how many studies, of the same weight (i.e. taxa, sample size) as the average of those already used studies are needed to reduce the significance of the effect size [293]. P-curve analysis has been proposed as an alternative way to assess publication bias and estimate the true effect behind the collected data [319]. Yet, p-value analysis is not robust against high heterogeneity. All results were considered with regard to publication bias, but when high heterogeneity and small effects were present more weight was put on fail-safe number results.

2.2.5 Statistics

All analyses were computed using R. Effect size calculation was done using the `esc` package [196]. Meta-analyses were performed using the `metafor` package [364]. Quality assessment was done using the latter as well as the `meta` and `dmetar` package [10, 115]. Test and model results were considered significant with a p-value < 0.05. Visualizations were conducted using R. Graphs show mean effect sizes. Error bars depict 95% confidence intervals from robust tests. An effect can be considered statistically significant if the error bar does not overlap with null. Statistical significance is indicated by an asterisk above the effect size, a cross signifies a significant result that did not pass sensitivity analysis. In subgroup analysis an asterisk above the overall effect symbolizes significance of the moderator variable. For the overall effect size Q denotes significant underlying heterogeneity from the original model results.

2.3 Results

2.3.1 Study and data characteristics

The database search yielded 2389 results. From the initial unfiltered reference library, 1122 studies passed the title scan. The following abstract scan reduced the number of studies to 593. This also included additional studies that emerged during full-text scan of literature. After the full-text exam, the total number of accepted studies was 56, 38 of which assessed osmolyte concentrations in tissues, whereas the other studies sampled extracellular fluids (Fig. 2.2). The resulting dataset covered 5 phyla including 43 species. The majority of those were Mollusca ($n = 27$), whereas less than 4 reports were obtained for Annelida ($n = 4$), Cnidaria ($n = 4$), Echinodermata ($n = 1$) and Porifera ($n = 1$) using our research criteria (Fig. 2.3). Reported ionic outcome variables in decreasing abundance

were: sodium, potassium, chloride and magnesium. Organic solutes exist in a wide variety. In total, 60 different substances were identified. The minimum sample size for analysis ($k \geq 3$) was given for 24 of those substances. These substances mainly belonged to the category of free amino acids (FAAs) and derivatives, methylamines and methylsulfonium compounds. All organic solutes and inorganic ions whose concentration was significantly affected by salinity in the following meta-analysis were regarded as osmolytes (*sensu* [332]).

2.3.2 Effect of low salinity stress on osmolyte concentrations

We analysed the overall effect of low salinity stress on a variety of solutes across studies and phyla to identify osmolytes (Fig. 2.4). Significant effect sizes indicate that the osmolyte concentration changes significantly at low salinity compared to high salinity conditions and is thus actively or passively modulated under salinity stress (see Tab. A.6 for statistical outcomes of the meta-analysis). Out of a total of 29 measured organic and inorganic substances, we identified six organic and one inorganic osmolytes. We observed a significant overall effect in 14 outcome variables, however only eight of these passed sensitivity analysis (Tab. A.5). A significant salinity effect was observed for: the total organic osmolyte pool, alanine, betaine, glycine, proline, serine, sodium and taurine. Significant heterogeneity of true effect sizes between studies was observed for all of these compounds.

Few studies measured intracellular ($n = 11$) or tissue ($n = 5$) inorganic ion responses to long-term salinity change. The intracellular potassium concentration was not significantly affected by salinity stress, but residual heterogeneity was observed. Concentrations of chloride and magnesium were not significantly affected by salinity. It has to be noted that magnesium concentrations do not represent intracellular values, but tissue values. Thus, these values include an unquantified extracellular ion component. Intracellular sodium was the only inorganic ion to significantly decrease with decreasing salinity. The meta-analysis re-

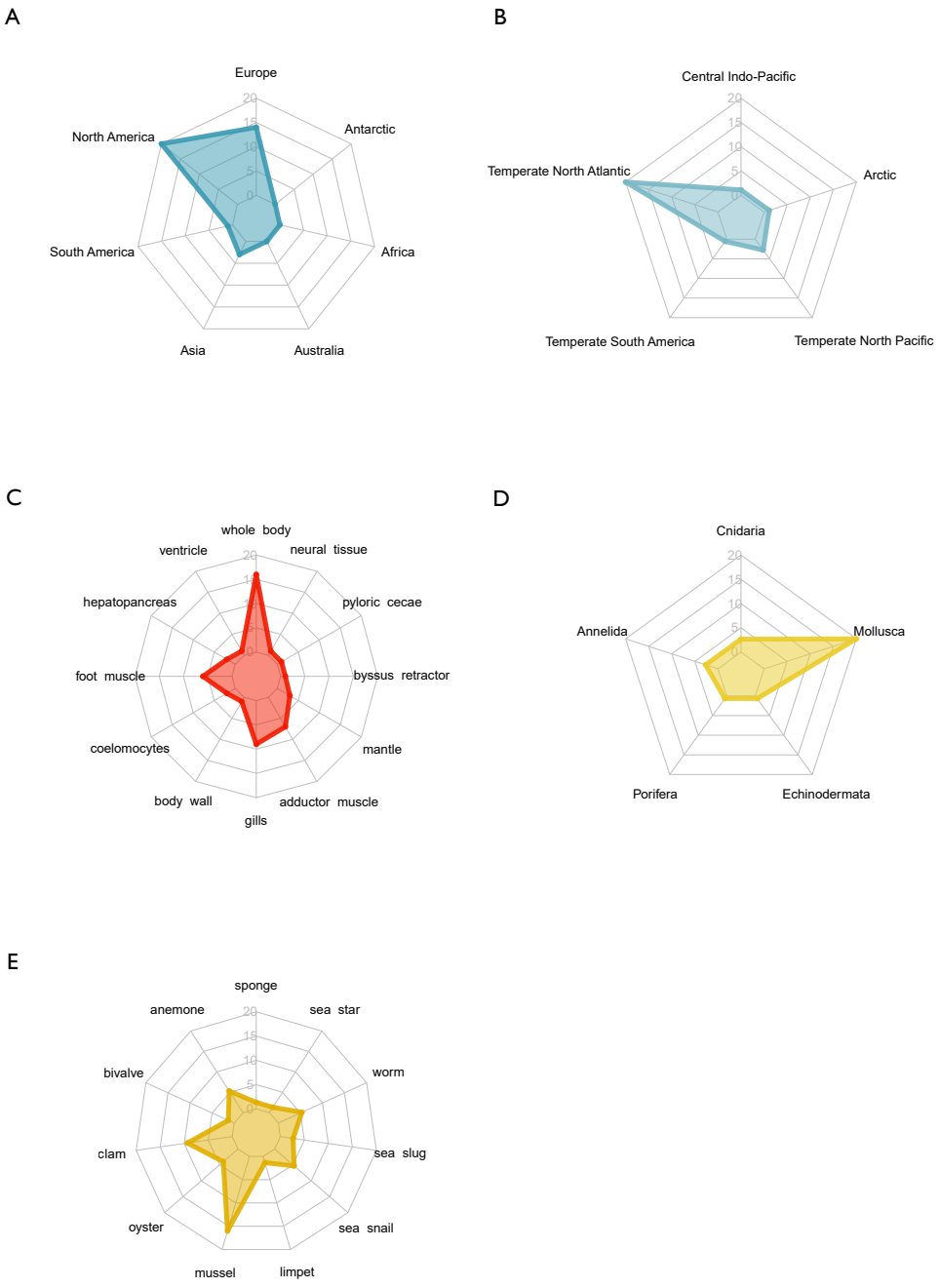

Figure 2.3: Spider plots highlighting the distribution of studies that measured osmolyte concentrations in osmoconformers following long-term acclimation to low salinity regimes. The number of studies increases from center ($n = 0$) to margin ($n = 20$). A) Spread and geographic bias of osmolyte studies. B) Spread and publication bias of osmolyte studies by biogeographic realms. C) Spread and bias of osmolyte studies in different tissues. D) Phylogenetic distribution of study organisms according to taxa. E) Phylogenetic distribution of study organisms according to common name of phylum.

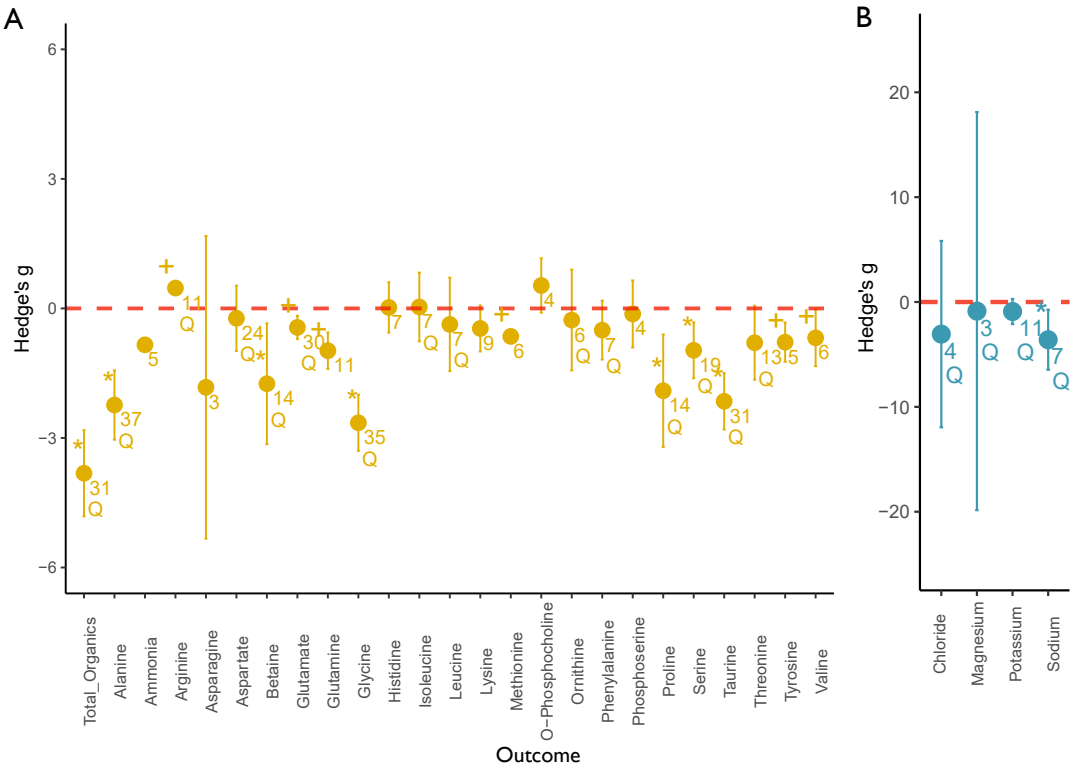

Figure 2.4: Overall meta-analysis of the salinity effect on A) organic and B) inorganic compounds. This figure depicts the overall effect of low salinity on solute concentration of organic compounds (yellow) and inorganic compounds (blue) from the random-effects meta-analysis. Individual outcome variables (compounds) are depicted on the x-axis and the effect sizes expressed as Hedge's g are depicted on the y-axis. The mean effect size for each outcome variable is depicted as a circle with the error bars indicating robust confidence intervals (95%). If the confidence intervals do not overlap with zero (marked in red) an effect size is considered significant. If error bars are not visible, they are smaller than the data point. An asterisk indicates a significant robust difference in effect size that passed sensitivity analysis. Results that were significant, but were flagged during sensitivity analysis were marked with a plus. Q indicates significant residual heterogeneity as tested with the random effects model. Numbers below circles indicate k - the number of studies. All outcome variables can be considered intracellular, except for magnesium. Magnesium concentrations were reported for tissues only.

sults demonstrated a larger variability for inorganic ion concentrations when compared to that of organic solute concentrations. The mean effect sizes for sodium were similar to the effect size of total organics. The effect sizes of individual organic osmolytes were smaller and usually less variable. The random-effects model calculated an initial significant effect for ammonia, glutamate, glutamine, methionine, tyrosine and valine, which we did not consider as robust after sensitivity analysis due to low fail-safe numbers, the effect not being significant after trim and fill procedure, or due to a significant p-curve test (see Tab. A.5 for Sensitivity Analysis). No significant effect, but residual heterogeneity was observed for arginine, aspartate, isoleucine, leucine, ornithine, phenylalanine, threonine. We could not find an effect of salinity on the concentration of asparagine, histidine, lysine, o-phosphocholine, and phosphoserine. Hence, we did not consider them to function as osmolytes. Additionally, there was no significant heterogeneity which would indicate other involved moderator variables. Significant heterogeneity of effect sizes (Q_E) was observed for the majority of solutes analysed ($n = 19$). All substances with underlying heterogeneity were investigated further using the *a priori* defined categories for subgroup analysis if the sample size allowed it.

Phyla

The categorical mixed-effect model analysing the low salinity effect on tissue osmolyte concentrations in different phyla was conducted for 12 organic compounds that exhibited significant residual heterogeneity in the overall effect and provided a large enough sample size in multiple subgroups as well (Fig. 2.5). There was not enough data to include any inorganic osmolyte in this analysis. Supported phyla were: Annelida, Echinodermata and Mollusca. We detected a significant phylogenetic difference for 10 organic compounds. In general, phylum was a significant moderator affecting total organic solute concentrations

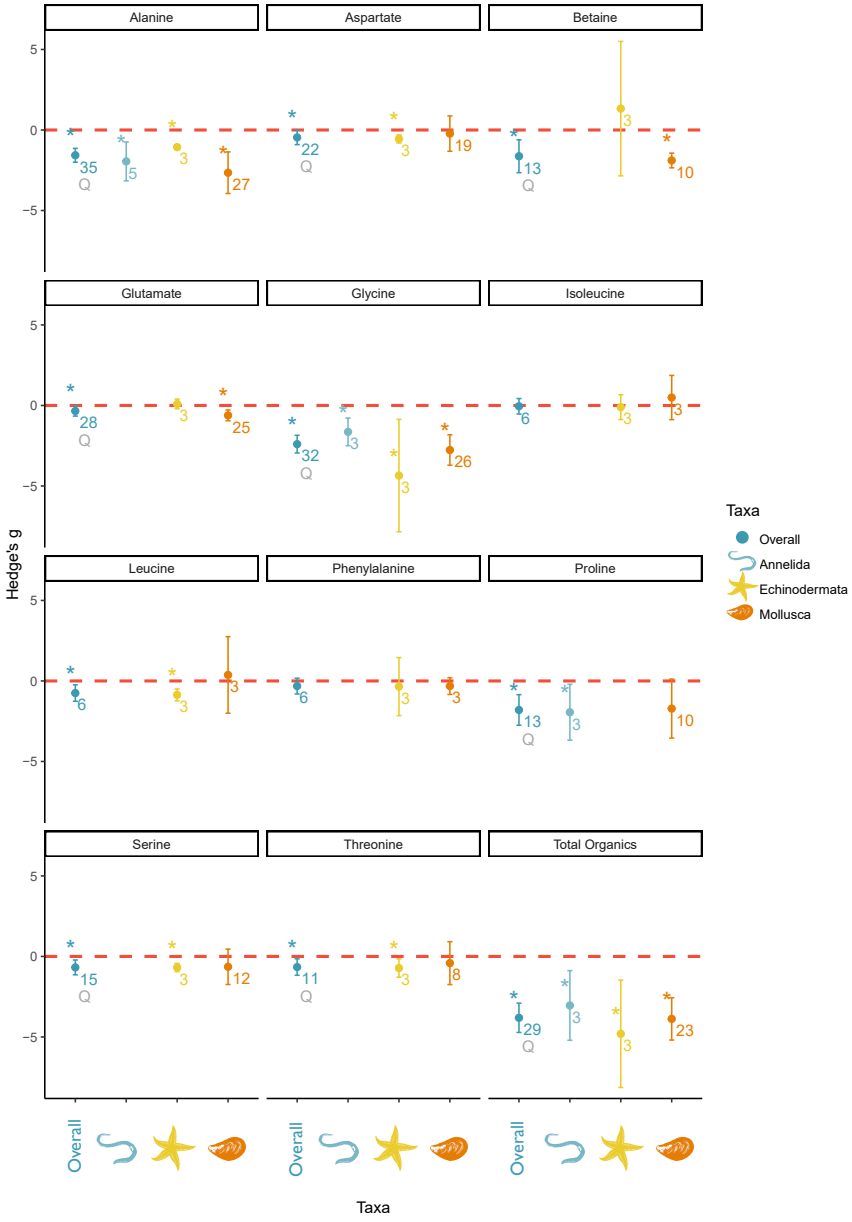

Figure 2.5: Subgroup analysis of salinity effect on organic osmolyte concentrations in tissue by taxa. The panel includes a separate plot for each measured solute. In each plot the overall effect and the subgroups are depicted on the x-axis and the effect sizes as Hedge's g on the y-axis. A positive value indicates a positive salinity effect (i.e. an increase of concentration at decreasing salinities), whereas a negative value indicates a negative salinity effect on solute concentration. The overall mean effect size might differ from Fig. 2.4 due to changed sample size, when subgroups could not be included due to small sample size. Mean effect size for each taxon is depicted as a circle with the error bars indicating robust confidence intervals (95%). An asterisk above the overall effect size indicates taxon affiliation as a significant moderator, asterisks above the individual taxa indicate a significant salinity effect for that specific subgroup. Numbers below circles indicate k - the number of studies. Q indicates significant residual heterogeneity as tested with the mixed effects model.

with respect to salinity stress. Specifically, alanine, aspartate, betaine, glutamate, glycine, leucine, proline, serine and threonine effects sizes were significantly affected by phylum affiliation. Yet, all of these significant effects were accompanied by significant residual heterogeneity, except for leucine. Alanine, betaine, glycine and proline were characterized by the largest effect sizes amongst the organic osmolytes. Alanine and glycine were identified as osmolytes in all taxonomic groups. Betaine and glutamate were identified as osmolytes in Mollusca, but not in Echinodermata. Proline concentration was significantly affected by salinity in Annelida, but not in Mollusca. Concentrations of aspartate, leucine, serine and threonine were only significantly altered by salinity in Echinodermata despite similar effect sizes in Mollusca, because of a high variance in mollusc effect sizes. However, effect sizes of aspartate, leucine, serine and threonine were smaller than those of other osmolytes. To summarize, in Annelida, alanine, glycine and proline were the main osmolytes with an overall smaller effect size for alanine and glycine than in other taxonomic groups. In Echinodermata, glycine was the main organic osmolyte, accompanied by several minor organic osmolytes i.e. alanine, aspartate, leucine, serine and threonine. In Mollusca, the organic osmolyte pool was mostly composed of alanine, betaine and glycine to a similar degree and accompanied by glutamate as minor osmolyte.

Class

For more taxonomic differentiation, we differentiated salinity effects by class for Bivalvia and Gastropoda (see Fig. A.1 Subgroup Analysis Class). Small sample size of phylogenetic groups other than Mollusca and specifically Bivalvia prevented meta-analysis for other phyla and many outcome variables. The effect of class affiliation on effect sizes was tested for total organics and was significantly different between Bivalvia and Gastropoda. Effect sizes were similar, but variance was much larger in Gastropoda. Samples sizes between the

two groups were very different.

Sample type

We analysed further whether there are differences in osmolyte utilization between tissue types. In total, 14 outcome variables fulfilled the prerequisites for subgroup analysis. For those variables, meta-analysis outcomes had a significant residual heterogeneity in their salinity effect in the prior meta-analysis and sufficiently large sample sizes within subgroups. We observed significant differences in effect sizes between sample types in 10 compounds (Fig. 2.6).

There were only two inorganic compounds included in this subgroup analysis. Intracellular sodium concentration was significantly more impacted by salinity stress in muscle tissues than in gills, and variance was sufficiently explained by this moderator. The summary effect of sodium was 1.3-fold higher than the effect of total organic osmolytes and $1.4 - 2.5$-fold larger than the major organic osmolytes. Intracellular potassium concentration was not affected by salinity in gills, whereas a significant salinity effect was observed in muscle tissue. In the case of sodium and potassium the data for muscle tissue originated from one study, which is why no additional robust test was conducted. Total organic osmolyte concentration was significantly impacted by salinity across all sample types. The effect was most pronounced in intestinal and muscle tissue. Glycine and alanine concentrations were significantly affected in all sample types except in mantle tissue and with the largest effect in muscle tissue. Betaine concentration was significantly affected in gill tissue, but not in intestinal tissue. The main osmolytes in gill tissue were: betaine, glycine and taurine. In intestinal tissues the primary osmolyte we observed was glycine. Muscle tissue had a larger number of significantly affected osmolytes than the other tissue types and was the only tissue where sodium concentration was significantly affected by salinity. No osmolytes

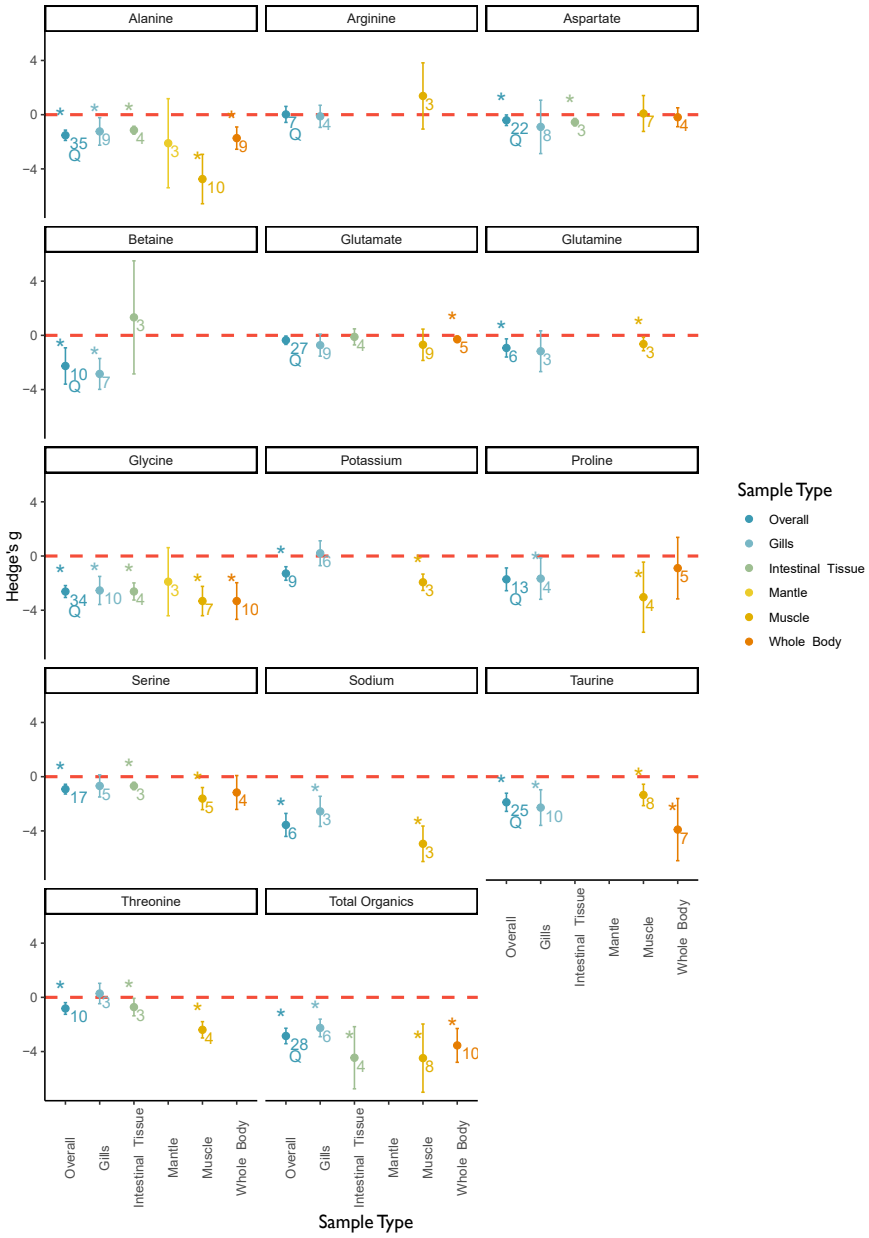

Figure 2.6: Meta-analysis results of the effect of low salinity stress on osmotic compounds by the categorical moderator variable sample type. The panel includes a separate plot for each measured solute. In each plot the overall effect and the subgroups are depicted on the x-axis and the effect sizes as Hedge's g on the y-axis. A positive value indicates a positive salinity effect (i.e. an increase of concentration at decreasing salinities), whereas a negative value indicates a negative salinity effect on solute concentration. The overall mean effect size might differ from Fig. 2.4 due to changed sample size, when subgroups could not be included due to small sample size. Mean effect size for each sample type is depicted as a circle with the error bars indicating robust confidence intervals (95%). An asterisk above the overall effect size indicates sample type as a significant moderator, asterisks above the individual sample types indicate a significant salinity effect for that specific subgroup. Numbers below circles indicate k - the number of studies. Q indicates significant residual heterogeneity as tested with the mixed effects model.

were identified in mantle tissue. We identified sample type as a significant moderator for the following minor osmolytes: aspartate, glutamine, serine and threonine. Among these, the mean effect size of aspartate had a significant residual heterogeneity. Aspartate concentration was only affected by salinity in intestinal tissues. Glutamine concentration was only affected by salinity in muscle tissue. Serine and threonine concentration were significantly impacted by salinity in muscle tissues and less in intestinal tissues. Sample type was a non-significant moderator for arginine, glutamate and proline concentrations and significant residual heterogeneity remained.

2.3.3 Methodological variation in response to low salinity stress

To test for an effect of methodological moderators on the meta-analyses' outcomes, magnitude of salinity stress and experiment duration were tested in separate meta-regression models (see A.7 for statistical output of meta-regression testing of methodological moderators). A significant effect for magnitude of stress (range of salinity reduction $3 - 33$) on mean effect size was observed in 5 of the 29 studied outcome variables, namely alanine, leucine, potassium, proline and valine. Alanine and proline had significant underlying heterogeneity. Experimental duration (range of duration: 1470 days, and *in situ* studies) was significant for 5 of 29 outcome variables, namely ammonia, glutamate, potassium, taurine and valine. When excluding *in situ* studies, there was no significant effect of acclimation duration except for ammonia and potassium.

2.3.4 Impact of salinity stress on osmolyte budgets

Very few studies examined total inorganic ion as well as total organic osmolyte concentration budgets. In total, we identified only three studies that reported the necessary parameters (Fig. 2.7). Two out of the three studies reported tissue and intracellular values, but

for comparative reasons the meta-analysis of budgets was calculated for the tissue data (i.e. non-ECS-corrected) for all studies. Additionally, even though total osmolality was available for one study, we used the sum of the measured total organic and inorganic osmolytes to enable comparable normalization across studies. The unweighted fixed effects meta-analysis revealed no significant effect of salinity on the inorganic-organic osmolyte ratio, for either intracellular or tissue data. The inorganic portion of the total osmolyte pool in tissues and intracellular fluids changes significantly with salinity, however these outcomes were not robust. The same is true for the organic portion of the total pool (see Appendix A.3 for meta-analysis results of budgets).

2.3.5 Sensitivity analysis

Within our data sets, there were some influential studies that were removed (see A.5 for complete list of removed studies). Often the same studies were outliers across multiple outcome variables such as: [140, 277, 308, 316]. The funnel plots together with Egger's test demonstrated significant asymmetry for all major osmolytes (alanine, betaine, glycine, proline, sodium, taurine) and glutamate. The minor osmolytes appeared to have no publication bias. When asymmetry was detected the trim and fill procedure was used to test for robustness of the effect size. The trim and fill procedure revealed that the adjusted effect sizes of alanine, betaine, glycine, proline, taurine and total organics were robust, this was not true for sodium and glutamate. However, p-curve analysis indicates that there was a true effect behind all of those data sets, except for glutamate and glutamine. Outcome variables with significant salinity effect had Rosenberg fail-safe numbers from $5 - 1974$. The majority of effect sizes were robust. A few minor organic osmolytes had low fail-safe numbers, namely valine, methionine, tyrosine, and ammonia. Here, low fail-safe numbers might be due to low sample size in combination with small effect size. In conclusion, there

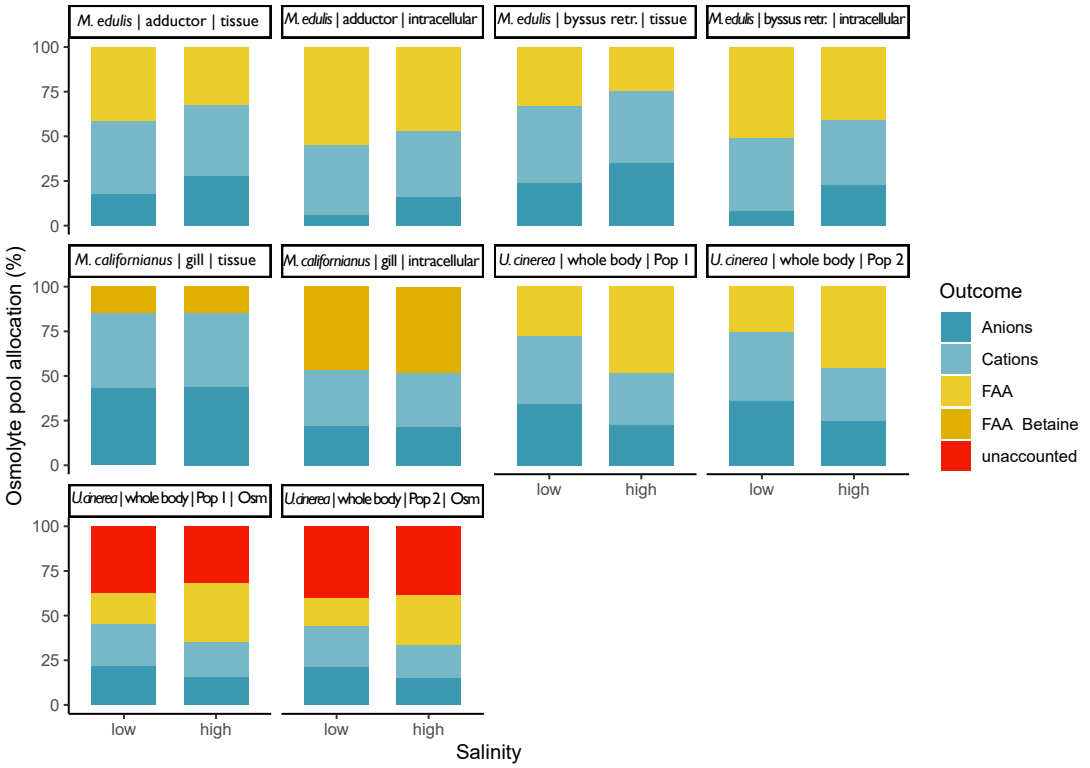

Figure 2.7: This graph shows the composition of the osmolyte pool at low and high salinity for three different bivalve and gastropod mollusc species: *Mytilus californianus* gills [316], *Mytilus edulis* adductor muscle and byssus retractor [267] and *Urosalpinx cinerea* whole body samples from two different populations [358]. The percentage of the total osmolyte pool is shown on the y-axis and salinity on the x-axis. Overall pool size was calculated as sum of measured inorganic and organic pools and also total osmolality for Turgeon [358]. Potts [267] & Silva [316] did not measure total osmolality. Potts [267] & Silva [316] did account for extracellular space and thus have results for whole tissue and intracellular space. Potts [267] and Turgeon [358] did not account for methylamines. Silva [316] did account for betaine, but no other methylamines.

seems to be moderate publication bias for all major osmolytes. Nevertheless, there was a true salinity effect for all major osmolytes and fail-safe numbers were robust. The minor osmolytes ammonia, glutamate, glutamine, methionine, tyrosine and valine results did not pass sensitivity analysis. Further publication bias arises from the unbalanced distribution of studies across continents and biogeographic regions with most studies being conducted in Europe and North America in the North Atlantic temperate region (Fig. 2.3). Also, there is large bias in sampled tissues towards whole-body and muscle tissues (Fig. 2.3). Considering study organisms, mostly molluscs were studied with a specific focus on bivalves (Fig. 2.3).

2.4 Discussion

Up to now, osmolyte data in osmoconformers has never been systematically and statistically analysed using meta-analysis. This holistic approach revealed general patterns of cellular osmolyte composition and concentration changes in osmoconformers. Our review revealed large gaps in knowledge, especially with respect to the role of inorganic ions in osmoregulation. The analysis indicates that to date, not a single study has been able to construct a complete osmolyte budget for intra- and extracellular compartments in any marine invertebrate species.

2.4.1 Inorganic osmolyte pool

Whilst the role of inorganic ions during short-term (minutes-hours) exposure to salinity change has been well documented [317], their utility in long-term salinity acclimation has been disregarded, since significantly altered intracellular concentrations can have strong perturbing effects on cellular protein function [34]. Perturbing effects of mono-

valent ions under low salinity conditions are more likely due to ligand-enzyme interaction rather than due to perturbing effects on protein structures [34, 400]. The perturbing effects of monovalent ions led to the commonly held assumption that all inorganic solutes are strongly conserved or replenished over time following acute osmotic disturbances [309, 324, 330, 398]. Other studies found, however, no effect of low salinity on enzyme activity [12, 302]. Ballantyne Berges [11] suggested that the response to low salinity may involve a reduction of enzyme activity to achieve a homeostasis of metabolic function. It was also found that enzymes from osmoconformers are not inhibited by salt, whereas enzymes from osmoregulators are inhibited [301]. According to Somero & Bowlus [330] a solute must be compatible with protein function as well as protein structure to be considered as overall compatible with macromolecular activity. However, there are differences in the compatibility of inorganic ions with protein functions. Potassium seems to be preferred over sodium as an intracellular cation due its weaker ion-water interaction and thus facilitates a higher solvent capacity of the cell water [331]. Potassium, as a weak protein stabilizer, has a more favourable effect on enzyme activity than sodium, which is a weak protein destabilizer [331]. For example, the enzyme pyruvate kinase is activated by potassium, whereas it is inhibited by sodium [34]. Intracellular chloride concentrations are much lower than potassium concentrations, likely due to the fact that high concentrations of the anion inhibit protein synthesis [331, 373]. There is conflicting evidence regarding the relative importance of inorganic osmolytes in cellular osmolyte acclimation processes [227, 371]. Deaton & Greenberg [66] pointed out already 30 years ago that insights into the role of intracellular ions in cell osmoregulation regulation is precluded by a lack of data. Unfortunately, few studies have added data on intracellular ion concentrations to the scientific record since then. Some authors have postulated that inorganic ions play a similar or even major role during acclimation to altered salinity when compared with the role of

organic osmolytes [61, 227, 250]. This meta-analysis indicates that inorganic ions, specifically sodium, are not exclusively utilized during short-term responses to changed salinity, but play a potentially important role during long-term salinity acclimation. The random effects model computed a significant salinity effect for intracellular sodium concentrations, but no effect was detected for chloride, potassium and magnesium. Lack of a significant decrease in concentration for chloride and magnesium are likely due to the low number of available studies and high variability. Out of the four available chloride studies, three found a significant salinity effect [80, 267, 316]. Intracellular potassium concentration has previously been reported to significantly and non-transiently change with salinity in molluscs [336, 382]. For example, Willmer [382] observed a net loss of both intracellular sodium and potassium ions during acclimation to low salinity that accounted for approximately 40% of the total required reduction in intracellular osmolality. Other studies report an increased contribution of inorganic ions to osmolyte pools during low salinity stress, as concentrations of certain cations (potassium and magnesium) did not decrease permanently, while organic osmolyte concentrations decreased [24]. In general, salinity had a larger effect on sodium than on individual organic osmolytes. This leads us to the hypothesis that intracellular inorganic ions, especially sodium and potentially chloride, are more than a first response strategy to avoid cell damage under acute osmotic stress. Instead, they seem to be as important as organic osmolytes in salinity tolerance of osmoconformers during long-term low salinity acclimation, even though the enzyme activity may be affected by changes in intracellular ion concentration. While it has been shown that inorganic ion concentrations can destabilize protein structure and inhibit protein function, most research focused on hyper-osmotic stress. Protein function and stability at sub-optimal ion concentrations, still allow $>$ 60% enzyme activity within a normal osmolality range [11, 373]. Other studies showed no changes in enzyme activity with reduced salinity [12, 301]. At

the lower salinities experienced and tolerated by euryhaline marine invertebrates, monovalent ion concentrations could still be within the range that allows for sufficient enzyme activity. While there might be species-specific and enzyme-specific variations with regard to the effects of osmolality on protein stability and function, the reduction of inorganic ion concentrations such as chloride and sodium in CVR to adapt to low salinities seems less problematic than acclimation to high osmolalities. Inorganic ions could thus be a crucial component of long-term CVR during low salinity stress.

The categorical meta-analysis revealed that there are significant differences in osmolyte content between tissue types. We considered only studies reporting intracellular ion concentrations, however this reduced the number of available data greatly. For muscle tissues all sodium and potassium concentration data originated from one study, therefore the robust test for random-effects models could not be used. Nevertheless, intracellular sodium concentration differed significantly between gill and muscle tissue ($p < 0.001$). Sodium concentration changed significantly with after long-term acclimation to low salinity in muscle tissue and gills, but the effect size was significantly larger in muscle tissue. Potassium concentration was not significantly impacted by salinity in gills, whereas a significant salinity effect was observed in muscle tissue. This finding is contradictory to the presumption that potassium concentration in tissues is maintained at relatively constant levels due to its important regulatory cell function [238, 404]. Due to the lack of data, a nested design or a more complex meta-regression model was not feasible. Our meta-analysis highlights the lack of intracellular inorganic ion content data in relation to salinity in marine osmoconformers and a strong research bias towards molluscs.

2.4.2 Organic osmolyte pool

Organic osmolytes are key actors during cellular osmolyte regulation. There are many different substances reported as organic osmolytes in the literature, yet reports differ on the role of specific substances or compound classes. Our analysis excluded isolated reports of osmolytes or listings of solutes whose concentrations do not change with salinity thereby generating a more robust candidate list of intracellular osmolytes that are commonly used across taxa. Data of organic osmolyte concentrations discussed in this chapter originated from tissue samples only. However, only 20% of studies corrected tissue concentrations for extracellular volume. This resulted in an under-estimation of intracellular organic osmolyte concentrations, as extracellular volume, which does not contain organic osmolytes, constitutes $10 - 80\%$ of tissues in marine invertebrates (Fig. 2.1) [24, 200]. Over 80% of the identified organic osmolytes were FAAs, which is only one of many compound classes involved in CVR. Our analysis revealed that 85% of the studies did not aim to or detect methylamines or methylsulfonium compounds, which, in the sole case of betaine, were identified as major osmolytes by this meta-analysis. Hence, many of these studies neglected a large part of the organic osmolyte pool. Further, only a few of the studies that included analysis of methylamines met the requirements for meta-analysis. This methodological bias is an issue that has already been raised by other authors who experienced difficulties in estimating the contribution of methylamines due to lack of studies [61, 330]. The contribution of methylamines to the organic osmolyte pool ranged from $3 - 76\%$. Quaternary ammonium compounds (QACs) such as proline-betaine have been identified to constitute the vast majority of the organic osmolyte pool in studies that have measured both FAAs and QACs [254]. Known important methylamines and methylated sulfonium compounds include: glycine-betaine, proline-betaine, DMSP, homarine, sarcosine, or TMAO. Thus,

it is necessary to include the measurement of methylamines when analysing the organic osmolyte pool. Nevertheless, FAAs can be considered a major component of the organic osmolyte pool, and some species (the echinoderm *Asterias rubens*) completely rely on FAAs as their organic osmolytes [308].

When examining the proportions of the organic osmolyte pool in detail it becomes apparent that organic osmolyte pools of osmoconformers consist of a universally distributed set of a few organic compounds which differ in partitioning. Usually major osmolytes account for the large majority of the organic osmolyte pool. Over 70% of the study organisms only employed one or two major substances, which constituted $35 - 70\%$ of the osmolyte pool (Fig. 2.8). The use of more than one primary osmolyte has been suggested as a strategy to guarantee osmoregulatory flexibility through substitution should synthesis of one osmolyte be interrupted or limited [105]. The main osmolyte is often accompanied by $1 - 3$ other minor osmolytes, whose contribution to the total pool is usually $< 20\%$ (Fig. 2.8). Minor osmotic compounds are often intermediate substances to the final major osmolytes [105, 157]. A constraint of our analysis is the large FAA bias in the reviewed literature. Organic osmolyte pool proportions displayed as a heat map (Fig. 2.8) are merely observational. Statistical analysis was not possible due to incomplete datasets or imprecise description thereof.

With our meta-analysis we identified six organic osmolytes across taxa: alanine, betaine, glycine, proline, serine and taurine (Fig. 2.5). Of these alanine, betaine, glycine and taurine were shown to be the main organic osmolytes in benthic marine osmoconformers (Fig. 2.5), which is in agreement with other comparative studies [330]. When all substance classes are considered, betaine is often the main osmolyte accompanied by taurine [232, 316]. The high variation in effect sizes for betaine was likely due to the strong variability of betaine concentration between species (Fig. 2.5). Individual studies reported glu-

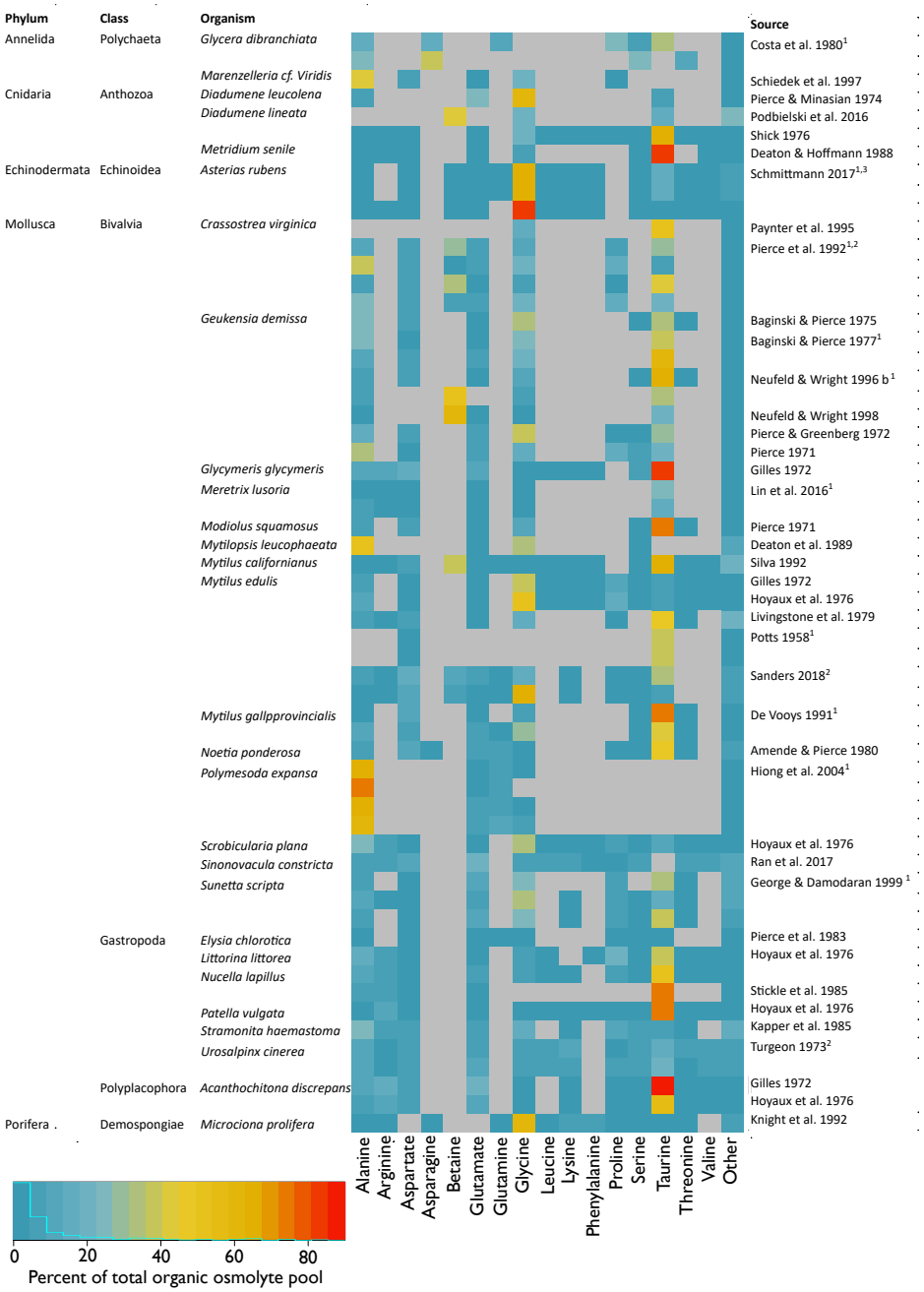

Figure 2.8: Composition of the organic osmolyte pool (% total organic osmolyte pool) at habitat salinity ($11 - 42$). The heatmap indicates percentages of single osmolytes in relation to the total organic pool, where red is a high percentage, blue a low percentage, and grey marks NAs. Only compounds that accounted for $> 5\%$ of the total organic osmolyte pool in a study and were reported in more than 1 study were listed. Compounds $< 5\%$ of the total organic osmolyte pool or with one entry were summed up under *Other*. Multiple entries per study are either different tissue types, separate populations, or independend experiments and are marked with superscripts $1 - 3$, respectively. Studies that passed the full-exam were included. Additionally, studies were included that had to be excluded from meta-analysis due to missing information for calculation of effect sizes, but that contained information that was sufficient to calculate the organic osmolyte pool composition.

tamate or proline to play more important roles than suggested by the compiled dataset in this study [320, 330]. The random effect model revealed significant heterogeneity of variances for most organic osmolytes, indicating further underlying differences in effect sizes possibly due to taxonomic or tissue specific differences.

While this systematic review and meta-analysis focuses on low salinity acclimation of marine osmoconformers, such species can also often tolerate a high level of hyper-osmotic stress. In these cases, CVR is achieved with an initial rapid increase of inorganic ions, which is replaced in a second phase by increases in organic osmolytes [331]. In anthozoa, steep increases in total organic osmolyte concentration were observed under prolonged acclimation to hyper-osmotic conditions [22]. In *Elysia chlorotica* exposed to salinities of 60, initially alanine and subsequently proline-betaine concentrations increased [257], demonstrating the change from a simply structured to a more complex osmolyte.

2.4.3 Taxonomic differences in organic osmolyte concentrations

The categorical subgroup analysis determined significant taxonomic differences in the salinity effects on osmolyte concentration after long-term acclimation to low salinity (Fig. 2.5). All taxa significantly depleted the total organic osmolyte pool during acclimation to low salinity. The main osmolytes alanine, betaine and glycine are used to different extents in different taxa. Glycine is utilized more by Echinodermata and Mollusca than it is by Annelida. Alanine is used most by Mollusca and least by Echinodermata. Minor osmolytes such as aspartate, leucine, serine or threonine, are significantly reduced with decreasing salinity in Echinodermata, but not Mollusca. Some minor osmolytes seem to be phylum-specific: i.e. glutamate in Mollusca, or proline in Annelida. Molluscan organic osmolyte effect sizes have a large variance, which could indicate further underlying heterogeneity within this taxon. Annelida do not utilize a single primary osmolyte and instead the pool

is evenly made up between major and minor, uncommon osmolytes, i.e. asparagine, serine, or proline (Fig. 2.8) [56]. These findings are however contradictory to short-term experiments, where Annelida are reported to use glycine as primary osmolyte accompanied by proline and alanine [134]. Considering alanine, our results are consistent with the literature assessing short-term salinity stress in Annelida [134]. Mollusca were the most represented taxon (30 studies) with Echinodermata the least represented (1 study, 1 species) (Fig. 2.3). In molluscs, taurine has been reported to be ubiquitous [8]. This is in accordance with our findings (Fig. 2.8), but could not be further explored due to lack of data in other groups.

Unfortunately, we did not have a sufficient sample size available for analysis in other taxonomic groups to consider methylamines other than betaine. We did not find a single study on organic osmolyte concentration in tissues of osmoconforming Arthropoda under long-term salinity stress. Cnidaria, Chordata and Porifera could not be included in our subgroup analysis due to low sample size, however, single studies on Cnidaria, Chordata and Porifera reveal that the main osmolytes are the same as in molluscs and annelids [67, 150, 262, 393].

So far, no other study has analysed taxonomic differences in organic osmolyte pools statistically. Some reviews have examined large scale differences in CVR between domains and phyla, or on a small scale between species [24, 332]. But not much information is available for other phylogenetic contrasts. This study demonstrates that phylogeny is a significant underlying factor affecting organic osmolyte adjustments under salinity stress (Fig. 2.5, 2.8). We observed that: 1) Glycine is widely used across taxa, 2) Echinodermata and Porifera rely heavily on glycine and do not utilize taurine. 3) Taurine is mainly used by Mollusca (also present in Cnidaria). Next to taurine, betaine and glycine are major osmolytes in Mollusca. 4) Annelida do not use a single main osmolyte in a large quantity, but use a

variety of osmolytes in smaller amounts. 5) Echinodermata only employ FAAs, whereas methylamines have been reported to play major roles in molluscan and cnidarian CVR. Minor osmolytes are often taxon-specific compounds and could further indicate different taxonomic strategies. There are a few prominent organic osmolytes that are present in many taxa (i.e. alanine, betaine, glycine and taurine). The differences in concentration of such universally employed osmolytes raises the question as to whether different genetically determined physiological strategies are employed, or if the observed differences stem from environmental or nutritional factors. Next to taxonomic differences we find also a large diversity of tissue types that have been sampled for organic osmolytes. Considering the specific physiological roles of different tissues, the question arises whether organic osmolyte pools differ between tissue types.

2.4.4 Cellular strategies of volume regulation differ between tissue types

Salinity had a significantly different effect on inorganic and organic osmolyte concentration between tissues (for discussion of inorganic ions see above chapter). Total organic compounds were significantly affected by salinity in all tissues. Yet, the organic osmolyte pool was less impacted in gill tissues. Also, we observed a more specific composition of osmolytes in gills, whereas muscle tissues employed a wider range of osmolytes. Muscle tissue used more alanine and glycine, whereas taurine was more heavily used in gill tissue. Some minor compounds were more prevalent in certain tissues (such as aspartate in gills, and serine, threonine and aspartate in intestinal tissues and glutamine, serine and threonine in muscle tissue). Differences in osmolyte concentration or their transporters between tissues potentially reflect differences in metabolic pathways between tissues or functional roles of specific osmolytes in different tissues [129, 133]. Overall, the examined tissue types were very diverse, which made generalization for meta-analysis necessary, thereby excluding very

specific measures. Our findings are in accordance with other comparative studies that previously stated differences in osmolyte composition between tissues [90, 157]. Glutamate was unaffected by salinity across individual tissues except for whole-body tissue, which is in contrast to studies that listed glutamate as a major organic osmolyte [34]. Mollusc mantle tissue was seldom examined for organic osmolytes, thus there were only few outcome variables with sufficient data. CVR capacity has been reported to differ between tissues in bivalves. It is reported to be nearly perfect in gills and byssus retractor muscles and incomplete in nervous tissues and adductor muscles [95, 174, 267, 382]. Only few papers compare osmolyte concentrations between tissue types directly and neither discusses possible causes for differences in osmolyte pool composition [56, 61, 122].

2.4.5 Characteristics of major osmolytes

Having determined a pool of prominent osmolytes, despite taxonomic and tissue specific differences in pool composition, the question arises what differentiates the main osmolytes from minor osmolytes or related solutes whose concentration is not modulated during salinity acclimation. Osmolyte utilization is likely governed by compatibility and cytoprotective functions of compounds [332]. According to the preferential hydrations hypothesis certain structure-stabilizing ions and organic osmolytes are preferentially excluded from the immediate space around a protein to facilitate hydration [352]. The preferential exclusion can be directly linked to physiochemical stabilizing effects of these solutes based on the Hofmeister series; osmolytes that have physiochemical similarities with stabilizing ions from the Hofmeister series are regarded as more compatible or fit as an osmolyte [50, 390]. According to the Hofmeister series, destabilizing ions are for example chloride, sodium, calcium and urea, whereas potassium, carboxylate, sulphate, phosphate and methylammonium ions have stabilizing effects (ranked from weak to strong effects, respectively).

Hence, organic osmolytes can be considered to be more compatible compared to inorganic ions. However, not all inorganic ions are protein destabilizers and while they may be less preferred, weak protein destabilizers - such as chloride or sodium - can be tolerated in certain amounts. All primary organic osmolytes identified in this study (alanine, glycine, glutamate and methylamines such as betaine) possess stabilizing effects on protein structures and presumably also membrane systems [332, 394]. The major FAA-osmolytes are mainly non-essential amino acids. Amino acids of the aspartate family (such as threonine and methionine) and the glutamate family (i.e. glutamate, glutamine, proline) were identified amongst the minor osmolytes, which is consistent with the earlier literature (Fig. 2.4, 2.8) [189, 255]. Generally, the predominant organic osmolytes are either uncharged polar molecules or zwitter ions without net charge [332]. Concentrations of essential amino acids such as aromatic amino acids and branched chain amino acids do not appear to be regulated during salinity acclimation. This is due to the differences in protein compatibility of amino acids. Negatively charged osmolytes such as glutamate are only found occasionally (i.e. in bacteria) and in combination with elevated potassium to achieve electroneutrality [72]. Positively charged amino acids such as lysine or arginine can have strong perturbing effects on PEP binding of pyruvate kinase [398]. In contrast, amino acids such as glycine, alanine or taurine did not affect PEP binding at normal cellular concentrations (up to 1 mM). Glycine proved least inhibitory to enzyme reaction compared to other FAAs [34]. Taurine is known for its inert nature [29] and along with betaine, is known to counteract negative impacts of salts on protein structure and function [38, 400], whereas other organic osmolytes seem inferior in structure and function. High proline concentrations, for example, have not been associated with any beneficial effects. Some compounds are preferential to others, as glutamate concentrations for example, are reduced when other osmolytes are available and accumulated [58]. Other osmolytes identified in this meta-

analysis (i.e. arginine, lysine) seem inferior in abilities or even harmful, which might explain their lower concentrations. Additionally, organic osmolytes with a decreased hydrophobic moment (i.e. alanine and glycine) seem to be preferred over solutes with a large hydrophobic moment such as valine and isoleucine that are disruptive of protein structures [332].

Another criterion for osmolyte selection is metabolic availability and energetic cost. The utilization of end products of nitrogen metabolism is a cost-effective way to mobilize organic osmolytes [29]. Alanine is omnipresent in osmolyte pools, likely due to the fact that it is derived from components of key metabolic pathways such as glycolysis and the citric acid cycle. It is thus fast and easy to produce via transamination in all cells. Alanine has also been reported as a first responder in CVR, which is later substituted by structurally more complex organic osmolytes such as taurine [9, 28]. Taurine is also a metabolic end product, produced via degradation of methionine and cysteine [3, 408]. Betaine is derived from catabolism of the common metabolic products, choline and glycine [258]. Glycine has been shown to be universally employed across taxa and tissues. It is the simplest stable amino acid and is characterized by its small size and molecular weight. Despite its ubiquity, the metabolism of glycine and its derivatives (such as betaine) is generally poorly studied in invertebrates [8, 61]. Glycine appears to be generated via active transmethylation from serine [79]. Multiple pathways have been suggested as the main sources for organic osmolytes, such as the uptake via nutrition or directly from seawater and *de novo* synthesis [87, 246, 253, 339, 389]. In corals it has been observed that *de novo* biosynthesis as well as exogenous uptake mechanisms for glycine-betaine are active [233].

2.4.6 What limits the capacity for salinity tolerance?

Up to now, it remains unclear what defines the capacity for salinity tolerance in osmoconforming animals. Species-specific and population-specific differences in osmolyte pool

composition have been suggested as one important mechanism that distinguishes more euryhaline from stenohaline osmoconformers [24, 162]. It has been further hypothesized that the organic osmolyte pool size might be a key factor in CVR capacity [260]. In fact, recent work has shown that organic osmolyte concentrations decrease linearly with decreasing salinity in cnidarians (sea anemones, Fig. 2.9) and echinoderms (sea stars, Fig. 2.9) acclimated for several weeks to different salinities [262, 308]. Podbielski et al. [262] postulated that a critical salinity (S_{crit}) exists that is characterized by a depletion of the organic osmolyte pool and correlated loss of fitness. Using an anemone species as a model, the latter authors could demonstrate that growth and reproduction decreased to zero once low salinities were reached that led to full depletion of organic osmolytes. Similarly, Schmittmann [308] demonstrated that the total organic osmolyte pool in tissues of sea stars decreases to nearly zero with decreasing salinity. Further, the authors speculated that increased costs for ion regulation at and below S_{crit} could cause steep reductions in fitness. However, little is known about the role of intracellular inorganic ions during long-term salinity acclimation. There are studies that report a permanent decrease in intracellular inorganic osmolyte concentrations, as well as studies that report no changes [309, 316, 382, 398]. Thus, the involvement of inorganic osmolytes in salinity acclimation may differ between species and multiple scenarios are conceivable how salinity could affect the intracellular inorganic osmolyte pool: inorganic and organic osmolytes could be equally employed (Fig. 2.9), the inorganic ion pool concentration could remain constant (or be replenished after the initial osmotic shock) while organic osmolyte concentrations are reduced with decreasing salinity (Fig. 2.9), or both osmolyte pools could be utilized to different degrees (Fig. 2.9). Results from our meta-analysis indicate an involvement of both pools in salinity acclimation. However, owing to lack of data, it remains to be established as to how important the relative roles of inorganic vs. organic osmolytes are during long-term acclimation to altered

salinity regimes. It has not yet been sufficiently examined whether the interplay between inorganic and organic osmolyte pools influences the capacity for cellular osmotic regulation and salinity tolerance ranges. Lange [175] defined the difference between eury- and stenohaline organisms via "the range of salinities over which the capacity for cellular volume regulation meets the demands". According to this definition, a critical limit is reached when an organism expresses inability for cellular volume regulation under reduced salinities causing an increase in cell hydration. Oligo- and stenohaline species have been rarely studied in comparison to euryhaline species and thus, we could not retrieve enough data to statistically compare different physiotypes with respect to osmolyte composition and pool size. Deaton [64] studied differences in extracellular fluid ion concentrations between different physiotypes and observed that the most euryhaline species were able to slightly (hyper)regulate ion concentrations in the extracellular fluid at very low salinities. Gainey [95] discovered euryhaline species to maintain a constant ECS volume when exposed to hypoosmotic stress, whereas ECS volume was reduced in stenohaline species. Other hypotheses aiming to explain the ability of euryhaline species to tolerate a broad range of salinities relate to (i) different solute permeability control mechanisms of cells [250, 324] and (ii) an osmolyte pool that consists of QACs and inorganic osmolytes rather than FAAs [250]. Willmer [381] found strongly increased sodium pump (Na^+/K^+-ATPase) activity in nervous tissue of low salinity (salinity of 10) acclimated mussels *Mytilus edulis*. The sodium pump maintains high intracellular potassium and low sodium concentrations and drives many other secondarily active transporters involved in ion- and osmoregulation [102]. Thus, an enhanced activity reflects an enhanced ion regulatory effort to support neuronal functions [349]. In addition, 14-day acclimation to low salinity led to a thickening of the neural lamella, which was hypothesized to counteract the increases in hydrostatic pressure characteristic for low salinity acclimated axons [382]. This points towards inor-

ganic ions as crucial osmolytes as well as the need for additional anatomical modifications to protect cell structure from mechanical damage at very low salinities. A certain degree of hyperosmotic cellular regulation to maintain transmembrane ion gradients under extremely low salinities, at least in neuronal tissue, seems to be key to achieving a very high degree of euryhalinity (e.g. *Mytilus* salinity range: ca. $5 - 35$, [376]. The work by Willmer [381, 382, 380] also illustrates an increase in energetic expenditure for cellular osmoregulation at low salinities to maintain homeostasis and vital cell functions. During embryogenesis in the freshwater bivalve *Dreissena rostiformis* an upregulation of aquaporin (water channel) and v-type H^+ATPase (proton pump) coding genes have been observed, as well as an expansion of the aquaporin gene family [41]. In an euryhaline osmoregulator, the barnacle *Balanus improvisus*, the expression of aquaporin-coding genes was found to be reduced after long-term low salinity acclimation [188]. Thus, modulation of water permeability of cellular membranes via changed aquaporin density and isoform composition might be a potential mechanism during acclimation to very low salinities. However, the role, structure and function of aquaporins in invertebrates remains greatly understudied [42, 53, 155], especially with respect to their role during hypoosmotic stress in osmoconformers [198]. There are a variety of other abiotic and biotic factors that may influence salinity tolerance *in situ* such as temperature, desiccation, anoxia, pollution, nutrition, parasitism or reproduction [135, 141, 244, 313, 372]. In addition to this, there are also other cellular responses of marine invertebrates to hypo-osmotic stress. Some observed physiological responses to low salinity stress are: lower respiration and feeding rates, drop in hear rates, increase in immune response, upregulation of cellular stress response gene and genes involved in catabolism of osmolytes, osmolyte transport and membrane permeability and potentially stability [2, 36, 110, 138, 203, 234, 262, 356].

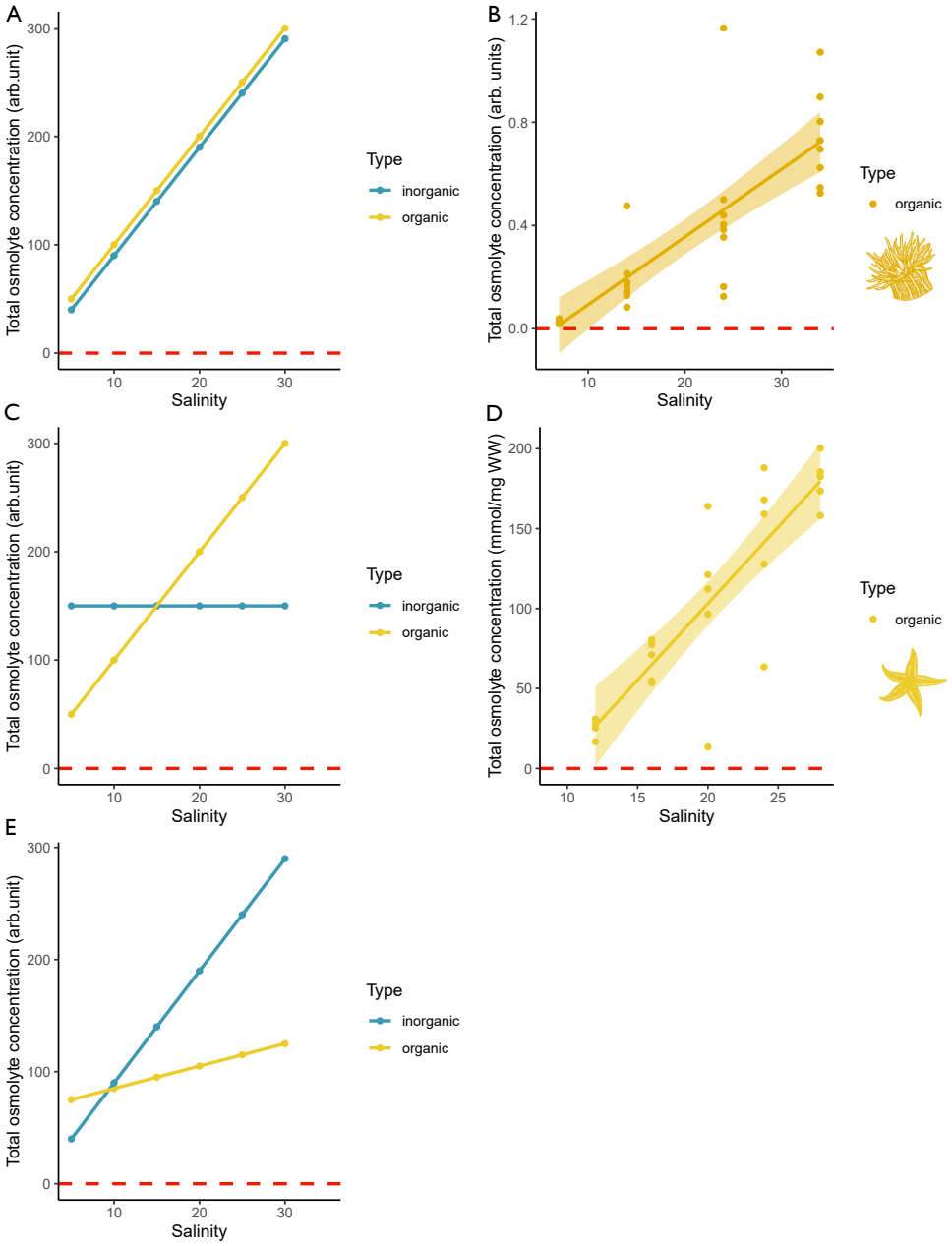

Figure 2.9: Three hypothetical models of how total intracellular inorganic and organic osmolyte concentrations (y-axis) might change in relation to long-term salinity changes (x-axis). A: Inorganic and organic osmolytes are employed equally for cellular volume regulation. B: Only the organic pool is adjusted, the inorganic portion remains constant. C: Inorganic osmolytes change with salinity, organic osmolytes are less affected by salinity stress. D: Example of changes in the organic osmolyte pool of whole-body tissue of the cnidarian *Diadumene lineata* with salinity [262]. E: Example of changes in total organic osmolyte pool of pyloric caecae of the echinoderm *Asterias rubens* with salinity [308].

2.4.7 Estimation of complete intracellular osmolyte budgets

In order to estimate the degree of involvement of inorganic and organic osmolytes in cellular volume regulation (CVR), we examined the osmolyte pool sizes of tissues. There are a number of requirements to estimate a complete tissue osmolyte budget and reliable concentration estimates for intracellular osmolytes. 1) It is necessary to measure both the inorganic and the organic compounds. 2) The estimates of organic and inorganic pool sizes should comprise all relevant compounds including the main inorganic anions and cations, and a variety of organic osmolytes, namely methylamines, FAAs, QACs, methyl-sulfonium compounds, sugars, polyols and urea [124]. 3) The contribution of ECS to tissue solute and water content needs to be accounted for. 4) In addition, intracellular osmolality should be measured in order to determine, whether important osmolytes (and solutes) have not been identified.

Our systematic review revealed that no study fulfilled all of these criteria. Only three studies quantified inorganic (anions and cations) as well as organic osmolytes in tissues following long-term acclimation to different salinity regimes (Fig. 2.7). However, none of these studies constructed a complete budget. Either total osmolality was not measured, ECS was not considered or organic osmolyte analyses were restricted to FAAs. Finally, none of these three studies measured salinity effects on less abundant inorganic ions (such as magnesium, calcium, sulphate, etc.). Both inorganic and organic pools changed similarly during salinity acclimation, thus the ratio of inorganic to organic substances was not altered. This highlights the need to consider contributions of both intracellular inorganic and organic osmolytes during acclimation to different salinity regimes. However, these conclusions have to be considered with caution, because they are based on very low sample sizes of exclusively mollusc species and were not normalized to total osmolality, but to

the sum of the measured inorganic and organic compounds. Only one of the three studies reporting a complete osmolyte budget measured total osmolality. Figure 2.7 shows that a large portion of the total solute pool can remain undetected if total osmolality is not used as control. Since Turgeon [358] only determined FAAs, the unknown portion of the osmolyte pool likely consisted of methylamines or methylsulfonium compounds. ECS was also not considered in the latter study. The reported tissue osmolyte concentrations thus represent a mix of those extracted from intracellular and extracellular compartments. The research of Potts [267] and Silva [316] highlights the importance of considering the ECS in estimation of intracellular osmolyte concentrations. Both studies compared inorganic and organic compounds in the intracellular space and entire tissues in *Mytilus* sp.. Inorganic ion concentrations are higher in tissue samples compared to the corresponding intracellular values, which is due to the high contribution of ECS ions (Fig. 2.7). Yet, despite reporting true intracellular concentrations, these two studies have other drawbacks. Potts [267] disregarded all organic osmolytes other than FAAs whilst Silva [316] determined the concentration of inorganic osmolytes and FAAs plus betaine, but not that of other methylamines or methylsulfonium compounds. It should further be noted that these studies measured the total FAA pool, which also includes solutes that contribute to intracellular osmolality, but do not change in concentration with salinity. The intracellular organic osmolyte pool in these three mollusc studies accounted for ca. 30% of the overall osmolyte pool in tissue and 45% in the intracellular space, previous studies estimated its proportion to account for $\sim$ 50% [61, 90, 131]. Yet, most of the latter estimates are based on data obtained from osmoregulators - often Arthropoda. A short-term experiment on annelid *Arenicola marina* tissues revealed that inorganic ions constitute 60% and organic substances 40% of total osmolality [374]. In conclusion, the overall share of organic osmolytes in CVR has likely been overestimated, while the role of inorganic ions has been underrated. CVR is a prod-

uct of inorganic and organic osmolyte regulation and more studies are needed to quantify the relative importance of organic vs. inorganic osmolytes in long-term salinity acclimation processes across taxa.

2.4.8 Best-practice guidelines

We suggest standard guidelines to study CVR in tissues of marine osmoconforming invertebrates. In the course of this systematic review, we encountered a number of methodological problems in published salinity tolerance research which leads us to make recommendations for best-practices in future research:

(i) Measure intracellular inorganic osmolyte composition

Inorganic osmolytes are estimated to account for half of the intracellular total osmolyte pool, but are often disregarded. We strongly advocate the need to measure both components of the osmolyte pool in tissue samples. To derive intracellular ion concentrations, tissue ion concentrations, extracellular ion concentrations and ECS volume need to be measured (Fig. 2.1). Tissue cation concentrations have been measured in the past via flame photometry [231, 296] and anions via a multitude of specific methods for each individual anion. Chloride was measured via titration methods [316, 358] or a microdiffusion method [267]. Sulphate can be estimated gravimetrically and phosphate colorimetrically [267]. Our group has recently developed a novel protocol using ion chromatography to determine anion concentrations in invertebrate tissues [263].

(ii) Analyse intracellular organic osmolytes using untargeted meth-

ods

Our literature analysis revealed that publication bias towards measuring FAAs likely resulted from the targeted metabolomic HPLC approaches common at the time these studies were published (1958-1992). This is a drawback since methylamines and methylsulfonium compounds remained undetected, despite the fact that these substances account for a large portion ($35 - 75\%$) of the organic osmolyte pool [254, 262, 397]. Recent developments in untargeted metabolomics are able to detect unexpected organic osmolytes. We advocate the need to use such techniques (i.e. HPLC, GC-MS, LC-MS or ^1H-NMR). These techniques are able to capture a large spectrum of solutes and to quantify their concentration. Untargeted methods also provide a solid basis for exploratory analysis of poorly studied species. Another key shortcoming of past osmolyte work is that many studies did not distinguish whether substances were non-existent or not detectable with the chosen methodological approach. Thus, studies should report on the capabilities and constraints of their methods. Finally, compounds not detected (or detected at very low concentrations) should also be reported, even when not significantly impacted by treatments.

(iii) Measure total osmolality of tissues & extracellular fluids

Intracellular osmolality in combination with total inorganic and organic pool size can help to determine whether crucial portions of the osmolyte pool have remained undetected (Fig. 2.7). Such a strategic approach may lead to the identification of other important osmolytes and determine the composition of the entire osmolyte pool [259, 254]. Total osmolality of tissue homogenates is easily measured with an osmometer [358]. To distinguish between extracellular and intracellular osmolality,

osmolality of extracellular fluid samples (blood, coelomic fluid, hemolymph) will have to be measured and the volume of the extracellular space in the tissue in question will have to be determined as well (Fig. 2.1).

(iv) Account for extracellular space

Osmolyte composition between cells and extracellular fluids is very different, but in organic osmolyte studies the ECS is often disregarded to facilitate analysis. The ECS can make up a large portion of tissues ($10 - 80\%$ of tissue mass). In the ECS, inorganic ions are more highly concentrated, whereas organic osmolytes are negligible. Subsequently, ignoring the contribution of the ECS overestimates intracellular inorganic osmolytes when aiming to investigate cellular osmolyte budgets and underestimates intracellular organic osmolyte content (Fig. 2.1). Thus, determination of the ECS is crucial for estimation of reliable organic and inorganic osmolyte data. Determining ECS is a time-consuming process and requires expertise in labelling techniques. Traditionally, the determination of the extracellular volume has been achieved by measuring the dilution of a known volume of radioactively labelled tracers such as ^{3}H-/^{14}C-inulin, ^{3}H-/^{14}C-dextran, ^{13}C-sucrose, ^{131}I-albumin, or ^{14}C-polyethylene glycol injected into the ECS [161, 215, 225, 318, 350, 382]. This requires availability of adequate labs and equipment and also poses an unnecessary safety risk for the investigator. With the advancement in labelling techniques we propose the use of fluorescent tracers such as FITC-inulin instead. There are a few recent studies available that successfully used FITC inulin for ECS determination in mammals [26, 160, 363].

(v) Report major statistical parameters and outcomes and deposit data to public databases

Gaps in documentation of experimental results led to exclusion of many studies in this meta-analysis. This applied largely to older studies, but also recent publications. Absolute osmolyte concentrations and statistical parameters such as sample size, mean and variance, or appropriate terms for other data types should be reported (see [32] for a comprehensive introduction to different data types). A complete record of statistical analysis results is necessary as well for comparative analysis of data sets (i.e. test-statistic, degrees of freedom, p-value). Of course, all data and metadata should ideally be deposited to publicly available databases (e.g. Pangaea) to facilitate future use.

(vi) Use standard units

Units utilized in marine animal CVR research are very diverse. To enable direct comparison between studies, researchers should consent to the same units or provide all necessary information for conversion of units. We recommend the use of $mmol \times L^{-1}$ for extracellular fluids and intracellular water and $mmol \times kg\ dry\ weight^{-1}$ for tissues and whole animal tissue samples.

2.4.9 Future research

This meta-analysis revealed that there are large gaps of knowledge and fragmented data in extracellular and cellular osmolyte dynamics. Our meta-analysis determined that taxonomy is an important factor influencing CVR and osmolyte adjustment in relation to salinity. But there is a large taxonomic bias towards Mollusca and more specifically Bivalvia in the

literature (Fig. 2.3). When choosing study species, a wider taxonomic coverage should be considered. While it is impossible to study a wide range of species, we propose the use of model organisms as representatives for certain taxa. Here it makes sense to focus efforts on models already utilized in other biological sub-disciplines, especially those with broad distribution (e.g. Placozoa: *Trichoplax* sp., Porifera: *Amphimedon queenslandica*, Cnidaria: *Nematostella vectensis*, Ctenophora: *Mnemiopsis leidyi*, Echinodermata: *Strongylocentrotus purpuratus*, Annelida: *Platynereis dumerilii* , Platyhelminthes: *Macrostomum lignano*, Xenacoelomorpha: *Symsagittifera roscoffensis*, Tunicata: *Ciona intestinalis*, Bivalvia: *Mytilus edulis*, Gastropoda: *Aplysia sp.*, Cephalopoda: *Loligo pealei*, Chordata: *Branchiostoma floridae*, etc.). Overall, this study only considered adult stages of osmoconforming species, because data on larvae or juveniles is rudimentary. Nevertheless, future research should consider larval and juvenile ontogenetic stages. The limiting factor for successful acclimation to low salinity might also depend on the physiological capacities of larval or juvenile stages [46]. This study focused on cellular actors in CVR. The metabolic pathways for osmolytes are not fully established and the metabolic cost for cellular osmoregulation remains unclear. The processes involved in CVR, such as compound synthesis, membrane transport, membrane permeability regulation and osmotic sensing are poorly studied and are a promising field for future research [129, 131, 187, 324].

2.5 Conclusion

In conclusion this systematic review and meta-analysis has revealed that:

(i) Osmolyte classes employed by marine osmoconforming invertebrates are: monovalent inorganic ions, methylamines and methylated sulfonium compounds, free amino acids and derivatives.

(ii) Main osmolytes are employed across taxa (i.e. alanine, betaine, glycine and taurine) but in different magnitudes, whereas accompanying minor osmolytes differ between taxa and tissue type. The categorical subgroup analysis and heatmap of the organic osmolyte pool illustrates a diverse osmolyte composition in Annelida. Echinodermata and Mollusca differ in their main osmolyte, but are otherwise characterized by a similar osmolyte composition.

(iii) We hypothesize that methylamines play a similarly important role or even a more important role than FAAs in the organic osmolyte pool, but data is insufficient for meta-analysis.

(iv) The tissue sodium ion pool is modulated during long-term salinity acclimation, but changes in concentration of other inorganic ions are poorly studied due to methodological limitations.

(v) There are significant differences in osmolyte budget composition between tissues. Intestinal tissues and muscles utilize a wider range of osmotic compounds, whereas gill tissue utilizes specific combinations of osmolytes.

Overall, this systematic review reveals large gaps in osmolyte research in osmoconforming marine invertebrates, especially with respect to inorganic ions, methylamines and study organisms. This impairs the explanatory power of meta-analysis, but gives us well-founded indications where more research is needed. We propose a best-practice guideline to improve future research efforts. More work is needed to broaden the scope of osmolyte research and establish a deeper understanding of CVR processes to understand mechanisms that drive changes in coastal biodiversity with ongoing salinity changes.

Acknowledgements

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A

Supplementaries Chapter 2

A.1 Search strings for systematic literature search

1. ISI Web of Science:

   Date of search: 13.04.2018

   Search String:

   (marine OR brackish OR estuar* OR coastal OR sea OR lagoon* OR benth* OR demersal OR shore* OR intertidal OR subtidal OR ocean* OR bay* OR cove* OR

harbo* OR lake* OR pond* OR bog* OR stream* OR river* OR freshwater* OR creek* OR lotic OR lentic OR headwater* OR reservoir* OR brook* OR wetland* OR *pool* OR marsh* OR watershed* OR catchment* OR limnol* OR glacial* OR ”inland waters”) AND (invertebrate OR macrofauna NOT plankton OR sponge OR porifera OR anthozo* OR anemone OR coral OR cnidaria* OR mollus* OR bivalv* OR gastropod* OR clam OR mussel OR snail OR slug OR polyplacophora OR cephalopod* OR polychaete OR worm OR annelid* OR chaetognat*OR entrproct* OR gastrotrich* OR hairyback OR hemichordat* OR kinorynch* OR ”mud dragon” OR arthropod* OR nematod* OR nematomorph* OR nemert* OR phoronid* OR plathelminth* OR flatworm OR priapulid* OR sipuncul* OR xenacoelomorph* OR brachiopod* OR lampshells OR bryozo* OR ”moss animal” OR ”sea mat” OR ectoproct OR chordat* OR tunicat* OR urochordat* OR caphalochordat* OR lancelet* NOT teleost NOT mammal* NOT amphib* NOT reptile NOT vertebrat* OR insect* OR echinoderm* OR ”sea star” OR ”sea urchin” OR ”sea cucumber” OR ”feather star” OR crinoid* OR comatulid* OR ”sand dollar” OR ”sea lil*” OR ”brittle star” OR crustac*) AND (salin* OR ”salt stress” OR osmolality OR osmotic OR osmolarity OR isoosmo* OR hypoosmo* OR osmoregulat* OR osmoconform*) AND (”Free amino acid” OR ”amino acid” OR Methylamin* OR ”methylated ammonium” OR Calcium OR Magnesium OR Bicarbonate OR Sodium Or Chloride OR betaine OR taurine OR alanine OR glycine OR Ion* OR Osmolyte* OR Electrolyte* OR ”volume regulation” OR ”cell volume”) AND (extracell* OR blood OR hemolymph* OR haemolymph* OR ”coelomic fluid” OR intracell* OR cell* OR tissue OR whole-body OR muscle OR gill OR mantle OR heart OR nerv*)

2. Google Scholar

   Date of search: 24.04.2018

   Search String:

   (osmoconformer OR ”volume regulation”OR ”cell volume”)AND(salinity OR osmolality OR osmotic) AND (”amino acid” OR Methylamine* OR Ion* OR Osmolyte*) AND (invertebrate OR macrofauna -fish)AND(marine OR brackish OR estuary* OR benthic OR freshwater)

   Further settings:

   - Without Citations and Patents
   - From 1945 – 2018

3. Scopus

   Date of search 30.01.2019

   Search String:

   (marine OR brackish OR estuar* OR coastal OR sea OR lagoon* OR benth* OR demersal OR shore* OR intertidal OR subtidal OR ocean* OR bay* OR cove* OR harbo* OR lake* OR pond* OR bog* OR stream* OR river* OR freshwater* OR creek* OR lotic OR lentic OR headwater* OR reservoir* OR brook* OR wetland* OR *pool* OR marsh* OR watershed* OR catchment* OR limnol* OR glacial* OR ”inland waters”) AND (”Free amino acid” OR ”amino acid” OR methylamin* OR ”methylated ammonium” OR ion* OR osmolyte* OR electrolyte* OR ”volume regulation” OR ”cell volume”) AND (salin* OR ”salt stress” OR osmolality OR osmotic OR osmolarity OR isoosmo* OR hypoosmo* OR osmoregulat* OR osmoconform*) AND (invertebrate OR macrofauna OR sponge OR porifera OR anthozo* OR anemone OR coral OR cnidaria* OR mollus* OR bivalv* OR gastro-

pod* OR clam OR mussel OR snail OR slug OR polyplacophora OR cephalopod* OR polychaete OR worm OR annelid* OR chaetognat*or AND entrproct* OR gastrotrich* OR hairyback OR hemichordat* OR kinorynch* OR ”mud dragon” OR arthropod* OR nematod* OR nematomorph* OR nemert* OR phoronid* OR plathelminth* OR flatworm OR priapulid* OR sipuncul* OR xenacoelomorph* OR brachiopod* OR lampshells OR bryozo* OR ”moss animal” OR ”sea mat” OR ectoproct OR chordat* OR tunicat* OR urochordat* OR caphalochordat* OR lancelet* OR echinoderm* OR ”sea star” OR ”sea urchin” OR ”sea cucumber” OR ”feather star” OR crinoid* OR comatulid* OR ”sand dollar” OR ”sea lil*” OR ”brittle star” OR crustac* AND NOT plankton AND NOT copepod AND NOT human* AND NOT diatom AND NOT teleost AND NOT mammal* AND NOT amphib* AND NOT reptile AND NOT vertebrat* AND NOT insect* AND NOT algae AND NOT bacteria AND NOT archea AND NOT fish AND NOT plant AND NOT foraminifer*) AND (LIMIT-TO (SUBJAREA , ”AGRI”) OR LIMIT-TO (SUBJAREA , ”ENVI”) OR LIMIT-TO (SUBJAREA , ”BIOC”) OR LIMIT-TO (SUBJAREA , ”MEDI”))

Further Settings:

- Search limited to biological research fields: Agricultural and Biological Sciences, Biochemistry, Genetics and Molecular Biology, Environmental Science, Medicine
- All years (oldest 1960)
- advanced search mode used

Table A.1: List of excluded outcome variables. The table lists all potential outcome variables that were reported in the literature. The list shows whether requirements for meta-analysis were met and if not the reason for exclusion. When sample size was $n \leq 3$, or data was derived from ≤ 3 studies, these outcome variables were not analysed further.

ID	Outcome	Experiments	Studies	Requirements for Meta-Analysis met?	Comment
1	2-Phosphoglycerate	3	1	no	n < 3
2	Alanine	34	22	yes	
3	α-Aminoadipic acid	1	1	no	n < 3
4	α-Aminoisobuytric acid	1	1	no	n < 3
5	α-Methylhistidine	1	1	no	n < 3
6	Ammonia	8	4	yes	
7	Ammonium	1	1	no	n < 3
8	AMP	3	1	no	n = 3
9	Anserine	1	1	no	n < 3
10	Arginine	12	10	yes	
11	Asparagine	3	2	yes	
12	Aspartate	20	14	yes	
13	ATP	4	2	no	substance not relevant
14	β-Alanine	2	2	no	n < 3
15	β-Aminoisobuytric acid	1	1	no	n < 3
16	Betaine	9	6	yes	
17	Bicarbonate	3	1	no	n = 3
18	Calcium	19	9	yes	
19	Carnitine	2	2	no	n < 3
20	Chloride	29	17	yes	
21	Citrulline	1	1	no	n < 3
22	Cystathionine	1	1	no	n < 3
23	Cysteic acid	1	1	no	n < 3
24	Cysteine	1	1	no	n < 3
25	Cystine	1	1	no	n < 3
26	Dimethylsulfone	1	1	no	n < 3
27	γ-Aminoisobuytric acid	2	2	no	n < 3
28	γ-Methylhistidine	1	1	no	n < 3
29	Glutamate	27	18	yes	
30	Glutamine	14	8	yes	
31	Glycine	33	22	yes	
32	Guanidinosuccinaate	2	1	no	n < 3
33	Histidine	7	5	yes	
34	Homarine	1	1	no	n < 3
35	Homocysteine	1	1	no	n < 3
36	Homolysine-A	1	1	no	n < 3
37	Hydroxyacetone	3	1	no	n = 3
38	Hydroxyproline	1	1	no	n < 3
39	Isoleucine	7	5	yes	
40	Leucine	7	5	yes	
41	Lysine	10	7	yes	
42	Magnesium	16	10	yes	
43	Methionine	6	4	yes	
44	Methylamine	3	1	no	n = 3
45	O-Phosphocholine	5	2	yes	
46	O-Phosphoserine	6	3	yes	
47	Ornithine	6	5	yes	
48	Phenylalanine	7	5	yes	
49	Phosphatidylserine	1	1	no	n < 3
50	Phosphoserine	2	2	no	n < 3
51	Potassium	36	25	yes	
52	Proline	13	12	yes	
53	Proline-Betaine	1	1	no	n < 3
54	Sarcosine	1	1	no	n < 3
55	Serine	19	15	yes	

Table A.1: List of excluded outcome variables. The table lists all potential outcome variables that were reported in the literature. The list shows whether requirements for meta-analysis were met and if not the reason for exclusion. When sample size was n $\leq$ 3, or data was derived from $\leq$ 3 studies, these outcome variables were not analysed further.

ID	Outcome	Experiments	Studies	Requirements for Meta-Analysis met?	Comment
56	Sodium	35	17	yes	
57	Succinate	2	2	no	n < 3
58	Taurine	31	18	yes	
59	Threonine	14	7	yes	
60	TMAO	3	1	no	n = 3
61	Total Organics	35	19	yes	
62	Total Osmolality	38	21	yes	
63	Tyrosine	6	4	yes	
64	Urea	2	1	no	n < 3
65	Uric Acid	1	1	no	n < 3
66	Valine	7	5	yes	

Table A.2: Filtered reference list includes all references judged acceptable based on title and abstract, but excluded during full-exam because they did not pass the necessary inclusion criteria. Selection criteria for inclusion in meta-analyses. Criteria for exclusion are listed in columns "Irrelevant data" - "Other". An "x" under the Selection Criteria columns indicates that the experiment did not meet the inclusion criteria. Irrelevant indicates that there was no relevant response data in the study. No original data means that no original results were shown. Not aquatic and no osmoconformer refer to exclusion criteria for study organisms. The next columns focus on salinity as the studied stressor. No salinity treatment indicates that there was either none, or only data for one salinity available, but no salinity effect studied. Salinity unknown indicates that the salinity of the treatment was not reported or a unit was used that was not convertable. Salinity not steady indicates that the salinity conditions during the experiment fluctuated. Short-term salinity treatment means an acclimation time less than 14 day and acclimation time not reported indicates that there is no or insufficient information about the acclimation duration to the salinity treatments. No multiple salinity treatments indicates that there were viable results for control or in situ conditions, but no further salinity treatment was reported. The next columns focus on experimental design and data completeness. Missing control indicates that there was no control condition specified, which is necessary for effect size calculation unless it is a regression design. Lack of relevant treatment means that salinity treatment was hyperosmotic or otherwise irrelevant. Variation not reported indicates that error estimates were not reported in the original experiment or the variation type was not specified. No replication means that sample size = 1. Sample size not reported indicates that there was no information on sample size, this is necessary for effect size calculation. Correlation coefficient addresses regression studies that lacked necessary detail of the correlation coefficient to calculate effect size. The selection criteria is listed in random order, if one exclusion factor was found during the full-exam it was noted. Thus, if an experiment did not meet one criteria, it's ability to meet the additional criteria are not listed.

			Selection criteria for inclusion in meta-analyses															
ID	Author/Year	Title/Journal	Irrelevant data	No original data	Not aquatic	No osmoconformer	No salinity treatment	Salinity unknown	Salinity not steady	Short-term treatment	Acclimation unknown	No multiple treatments	Missing control	Lack of relevant treatment	Variation unknown	No replication	Sample size unknown	Correlation coef. unknown	Other
1	Akberali, H. B., Marriott, K. R. M., & Trueman, E. R. (1977).	Calcium utilisation during anaerobiosis induced by osmotic shock in a bivalve mollusc. Nature, 266(5605), 266852a0. https://doi.org/10.1038/266852a0													x				
2	Abbott, W., & Awapara, J. (1960).	Sulfur metabolism in the lugworm, Arenicola cristata Stimpson. The Biological Bulletin, 119(3), 357–370.					x	x											
3	Abe, H. (2002).	Distribution, metabolism and physiological functions of free D-amino acids in aquatic invertebrates. Bulletin of the Japanese Society of Scientific Fisheries (Japan). http://agris.fao.org/agris-search/search.do?recordID=JP2003000648								x									
4	Ackermann, D. (1955).	Über das Vorkommen von Homarin, Taurocyamin, Cholin, Lysin und anderen Aminosäuren sowie Bernsteinsäure in dem Meereswurm Arenicola marina. Hoppe-Seyler s Zeitschrift für physiologische Chemie, 302(1–2), 80–86.					x												
5	Ackermann, D., & Janka, R. (1954a).	Neues Vorkommen von Mytilit bei Avertebraten (Cionia intestinalis). Hoppe-Seyler' s Zeitschrift für physiologische Chemie, 296(1), 283–286.					x												
6	Ackermann, D., & Janka, R. (1954b).	Über das Vorkommen von Homarin, Glykokollbetain, Cholin, Arginin, Mytilit, Taurin und d, l-Milchsäure in der Meeresschnecke Patella sp. Hoppe-Seyler' s Zeitschrift für physiologische Chemie, 298(1), 65–69.					x												
7	Adolph, E. F. (1936).	Differential permeability to water and osmotic exchanges in the marine worm Phascolosoma. Journal of Cellular and Comparative Physiology, 9(1), 117–135. https://doi.org/10.1002/jcp.1030090111								x									

ID	Author/Year	Title/Journal	Selection criteria for inclusion in meta-analyses																
			Irrelevant data	No original data	Not aquatic	No osmoconformer	No salinity treatment	Salinity unknown	Salinity not steady	Short-term treatment	Acclimation unknown	No multiple treatments	Missing control	Lack of relevant treatment	Variation unknown	No replication	Sample size unknown	Correlation coef. unknown	Other
8	Ahearn, H. R. H., Ahearn, G. A., & Gomme, J. (2000).	INTEGUMENTARY L-HISTIDINE TRANSPORT IN A EURYHALINE POLYCHAETE WORM: REGULATORY ROLES OF CALCIUM AND CADMIUM IN THE TRANSPORT EVENT. The Journal of Experimental Biology 203, 2877–2885.								x									
9	Aladin, N. (1983).	On displacement of the critical salinity barrier in the Caspian and Aral Seas, the Branchiopoda and Ostracoda taken as examples. Zool Z, 62, 689–694.																	Language
10	Aladin, N. V., & Potts, W. T. W. (1995).	Osmoregulatory capacity of the Cladocera. Journal of Comparative Physiology B, 164(8), 671–683. https://doi.org/10.1007/BF00389810					x				x								
11	Allen, J. A., & Garrett, M. R. (1971).	Taurine in Marine Invertebrates. In F. S. Russell & M. Yonge (Hrsg.), Advances in Marine Biology (Bd. 9, S. 205–253). Academic Press. https://doi.org/10.1016/S0065-2881(08)60343-0		x															
12	Allen, K. (1961a).	Amino acids in the Mollusca. American Zoologist, 253–261.		x						x									
13	Allen, K. (1961b).	THE EFFECT OF SALINITY ON THE AMINO ACID CONCENTRATION IN RANGIA CUNEATA (ELECYPODA). The Biological Bulletin, 121(3), 419–424.								x									
14	Amado, E. M., Freire, C. A., Grassi, M. T., & Souza, M. M. (2012).	Lead hampers gill cell volume regulation in marine crabs: Stronger effect in a weak osmoregulator than in an osmoconformer. Aquatic Toxicology, 106–107, 95–103. https://doi.org/10.1016/j.aquatox.2011.10.012								x									
15	Amado, E. M., Vidolin, D., Freire, C. A., & Souza, M. M. (2011).	Distinct patterns of water and osmolyte control between intertidal (Bunodosoma caissarum) and subtidal (Anemonia sargassensis) sea anemones. Comparative Biochemistry and Physiology A-Molecular & Integrative Physiology, 158(4), 542–551. https://doi.org/10.1016/j.cbpa.2010.12.019								x									
16	Amende, L. M., & Pierce, S. K. (1980b).	Free amino acid mediated volume regulation of isolated Noetia ponderosa red blood cells: Control by Ca 2+ and ATP. Journal of Comparative Physiology B: Biochemical, Systemic, and Environmental Physiology, 138(4), 291–298.								x									
17	Anderson, J. W., & Bedford, W. B. (1973).	The physiological response of the estuarine clam, Rangia cuneata (Gray), to salinity. II. Uptake of glycine. The Biological Bulletin, 144(2), 229–247.								x									
18	Apte, S., Khoury, F., Roth, W., & Schlichter, D. (1996).	Transport of amino acids into freshly isolated cells from a sea anemone. Endocytobiosis and Cell Research, 11(2–3), 129–146.	x							x									
19	Asuvapongpatana, S., Weerachatyanukul, W., Buranajitpirom, D., Watthammawut, A., Wongtripop, S., & Withyachumnarnkul, B. (2013).	Salinity tolerance of cross-breed shrimp families: Morphological and biochemical approaches. Aquaculture Research, 44(7), 1152–1161. https://doi.org/10.1111/are.12162	x			x													
20	Augusto, A., Pinheiro, A. S., Greene, L. J., Laure, H. J., & McNamara, J. C. (2009).	Evolutionary transition to freshwater by ancestral marine palaemonids: Evidence from osmoregulation in a tide pool shrimp. Aquatic Biology, 7(1–2), 113–122. https://doi.org/10.3354/ab00183								x									
21	Aunaas, T., Denstad, J.-P., & Zachariassen, K. E. (1988).	Ecophysiological importance of the isolation response of hibernating blue mussels (Mytilus edulis). Marine Biology, 98(3), 415–419. https://doi.org/10.1007/BF00391117							x			x							

ID	Author/Year	Title/Journal	Selection criteria for inclusion in meta-analyses															
			Irrelevant data	No original data	Not aquatic	No osmoconformer	No salinity treatment	Salinity unknown	Salinity not steady	Short-term treatment	Acclimation unknown	No multiple treatments	Missing control	Lack of relevant treatment	Variation unknown	No replication	Sample size unknown	Correlation coef. unknown	Other
22	Awapara, J. (1962).	Free amino acids in invertebrates: A comparative study of their distribution and metabolism. Amino acid pools, 158–175.		x			x												
23	Babarro, J. M. F., Reiriz, M. J. F., Labarta, U., & Garrido, J. L. (2011).	Variability of the total free amino acid (TFAA) pool in Mytilus galloprovincialis cultured on a raft system. Effect of body size. Aquaculture Nutrition, 17(2), e448–e458. https://doi.org/10.1111/j.1365-2095.2010.00781.x										x							
24	Babarro, J. M., Reiriz, M. J. F., Garrido, J. L., & Labarta, U. (2006).	Free amino acid composition in juveniles of Mytilus galloprovincialis: Spatial variability after Prestige oil spill. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology, 145(2), 204–213.											x						
25	Bablanian, G. M., & Treistman, S. N. (1985).	The effect of hyperpolarization of cell R15 on the hemolymph composition of intactAplysia. Journal of Comparative Physiology B, 155(3), 297–303. https://doi.org/10.1007/BF00687471								x									
26	Baginski, R. M., & Pierce, S. K. (1977).	The time course of intracellular free amino acid accumulation in tissues of Modiolus demissus during high salinity adaptations. Comparative Biochemistry and Physiology Part A: Physiology, 57(4), 407–412. https://doi.org/10.1016/0300-9629(77)90137-2										x							
27	Baginski, R. M., & Pierce, S. K. (1978).	A comparison of amino acid accumulation during high salinity adaptation with anaerobic metabolism in the ribbed mussel, Modiolus demissus demissus. Journal of Experimental Zoology Part A: Ecological Genetics and Physiology, 203(3), 419–428.										x							
28	Baldwin Ernest, & Needham Dorothy Mary Moyle. (1937).	A contribution to the comparative biochemistry of muscular and electrical tissues. Proceedings of the Royal Society of London. Series B - Biological Sciences, 122(827), 197–219. https://doi.org/10.1098/rspb.1937.0021					x												
29	Baldwin, E., & Yudkin, W. H. (1950).	The annelid phosphagen: With a note on phosphagen in Echinodermata and Protochordata. Proceedings of the Royal Society of London. Series B-Biological Sciences, 136(885), 614–631.					x	x											
30	Ballantyne, J. S., & Moyes, C. D. (1987).	Osmotic effects on fatty acid, pyruvate, and ketone body oxidation in oyster gill mitochondria. Physiological zoology, 60(6), 713–721.	x																
31	Bamford, D. R., & McCrea, R. (1975).	Active absorption of neutral and basic amino acids by the gill of the common cockle, Cerastoderma edule. Comparative Biochemistry and Physiology Part A: Physiology, 50(4), 811–817. https://doi.org/10.1016/0300-9629(75)90149-8	x				x												
32	Barnes, H., & Blackstock, J. (1975).	Studies in the biochemistry of cirripede eggs. IV. The free Amino-acid pool in the eggs of Balanus balanoides (L.) and B. balanus (L.) during development. Journal of Experimental Marine Biology and Ecology, 19(1), 59–79.					x												
33	Bartberger, C. A., & Pierce Jr, S. K. (1976).	Relationship between ammonia excretion rates and hemolymph nitrogenous compounds of a euryhaline bivalve during low salinity acclimation. The Biological Bulletin, 150(1), 1–14.								x									
34	Bassindale, R. (1942).	The Distribution of Amphipods in the Severn Estuary and Bristol Channel. Journal of Animal Ecology, 11(1), 131–144. JSTOR. https://doi.org/10.2307/1305	x																
35	Battaglia, B., & Bryan, G. W. (1964).	Some Aspects of Ionic and Osmotic Regulation in Tisbe [Copepoda, Harpacticoida] in Relation to Polymorphism and Geographical Distribution. Journal of the Marine Biological Association of the United Kingdom, 44(1), 17–31. https://doi.org/10.1017/S0025315400024632								x									

ID	Author/Year	Title/Journal	Selection criteria for inclusion in meta-analyses															
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36	Beadle, L. C. (1931).	The Effect of Salinity Changes on the Water Content and Respiration of Marine Invertebrates. Journal of Experimental Biology, 8(3), 211–227.	x							x									
37	Beadle, L. C. (1934).	Osmotic Regulation in Gunda ulvae. Journal of Experimental Biology, 11(4), 382–396.				x				x									
38	Beadle, L. C. (1937).	Adaptation to Changes of Salinity in the Polychaetes: I. Control of Body Volume and of Body Fluid Concentration in Nereis Diversicolor. Journal of Experimental Biology, 14(1), 56–70.								x									
39	Beadle, L. C., & Cragg, J. B. (1940).	Studies on Adaptation to Salinity in Gammarus Spp: 1. Regulation of Blood and Tissues and the Problem of Adaptation to Fresh Water. Journal of Experimental Biology, 17(2), 153–163.								x									
40	Bedford, J. J. (1971).	Osmoregulation in Melanopsis trifasciata—IV. The possible control of intracellular isosmotic regulation. Comparative Biochemistry and Physiology Part A: Physiology, 40(4), 1015–1027. https://doi.org/10.1016/0300-9629(71)90291-X								x									
41	Bedford, J. J. (1972).	Osmoregulation in Melanopsis trifasciata. II. The osmotic pressure and the principal ions of the hemocoelic fluid. Physiological Zoology, 45(3), 261–269.								x									
42	Bedford, J. J. (1973).	Osmotic Relationships in a Freshwater Mussel, Hyridella Menziesi Gray (Lamellibranchia: Unionidae). Archives Internationales de Physiologie et de Biochimie, 81(5), 819–831. https://doi.org/10.3109/13813457309074486												x					Freshwater species
43	Bellamy, D., & Jones, I. C. (1961).	Studies on Myxine glutinosa—I. The chemical composition of the tissues. Comparative Biochemistry and Physiology, 3(3), 175–183. https://doi.org/10.1016/0010-406X(61)90053-6										x							
44	Bellis, S., Davis, J. P., & Stephens, G. C. (1987).	The lack of an effect of magnesium on the uptake of amino acids by the sea urchin Strongylocentrotus purpuratus. Journal of Experimental Zoology, 244(3), 383–388. https://doi.org/10.1002/jez.1402440305								x									
45	Belyaev, G. M. (1957).	Physiological characteristics of representatives of the same species in waters of different degrees of salinity. Trudy Vsesoyuznogo Gidrobiologischeskogo Obshchestva (Akad. Nauk SSSR), 8, 321–353.								x									
46	Benson, J., & Treherne, J. (1978).	Axonal Adaptations to Osmotic and Ionic Stress in an Invertebrate Osmoconformer (Mercierella Enigmatica Fauvel): III. Adaptations to Hyposmotic Dilution. Journal of Experimental Biology, 76(1), 221–235.	x																
47	Beres, L. S., & Pierce, S. K. (1981).	The effects of salinity stress on the electrophysiological properties of Mya arenaria neurons. Journal of Comparative Physiology, 144(2), 165–173. https://doi.org/10.1007/BF00802754	x																
48	Berger, V. (1989).	On the adaptation of molluscs to increased salinityBerger1989.pdf. Biologiia moria., 2, 30–35.												x					hyperosmotic stress
49	Berger, E. (1930).	Unterschiedliche Wirkungen gleicher Ionen und Ionengemische auf verschiedene Tierarten. Pflüger's Archiv für die gesamte Physiologie des Menschen und der Tiere, 223(1), 1–39. https://doi.org/10.1007/BF01794065	x																
50	Berger, V. J., & Kharazova, A. D. (1997).	Mechanisms of salinity adaptations in marine molluscs. Interactions and Adaptation Strategies of Marine Organisms, 115–126.		x															
51	Berger, V. Y., Naumov, A. D., & Babkov, A. I. (1995).	The relationship of abundance and diversity of marine benthos to environmental salinity. Russian Journal of Marine Biology, 21, 41–46.	x																

ID	Author/Year	Title/Journal	Selection criteria for inclusion in meta-analyses																
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52	Bethe, A., & Berger, E. (1931).	Variationen im Mineralbestand verschiedener Blutarten. Pflüger's Archiv für die gesamte Physiologie des Menschen und der Tiere, 227(1), 571–584. https://doi.org/10.1007/BF01755356					x				x								
53	Bethe, Albrecht. (1929).	Ionendurchlässigkeit der Körperoberfläche von wirbellosen Tieren des Meeres als Ursache der Giftigkeit von Seewasser abnormer Zusammensetzung. Pflüger's Archiv für die gesamte Physiologie des Menschen und der Tiere, 221(1), 344–362. https://doi.org/10.1007/BF01793988				x													
54	Bialaszewicz, K. (1933).	Contribution A L'étude de la Composition Minérale des Liquides Nourriciers Chez Les Animaux Marins. Archives Internationales de Physiologie, 36(1), 41–53. https://doi.org/10.3109/13813453309142367									x								
55	Bialaszewicz, K., & Kupfer, Ch. (1936).	De La Composition Minérale Des Muscles Des Animaux Marins. Archives Internationales de Physiologie, 42(3), 398–404. https://doi.org/10.3109/13813453609146182					x												
56	Binyon, J. (1962).	Ionic regulation and mode of adjustment to reduced salinity of the starfish Asterias rubens L. Journal of the Marine Biological Association of the United Kingdom, 42(1), 49–64.										x							
57	Binyon, J. (1978).	Some observations upon the chemical composition of the starfish asterias rubens l., with particular reference to strontium uptake. Journal of the Marine Biological Association of the United Kingdom, 58(2), 441–449. Scopus. https://doi.org/10.1017/S0025315400028101										x							
58	Bishop, S. H., Greenwalt, D. E., Kapper, M. A., Paynter, K. T., & Ellis, L. L. (1994b).	Metabolic regulation of proline, glycine, and alanine accumulation as intracellular osmolytes in ribbed mussel gill tissue. Journal of Experimental Zoology Part A: Ecological Genetics and Physiology, 268(2), 151–161.										x							
59	Bishop, S. H. (1976).	Nitrogen metabolism and excretion: Regulation of intracellular amino acid concentrations. In Estuarine Processes: Uses, Stresses, and Adaptation to the Estuary (S. 414–431). Elsevier.		x															
60	Bishop, S. H., Greenwalt, D. E., & Burcham, J. M. (1981).	Amino acid cycling in ribbed mussel tissues subjected to hyperosmotic shock. Journal of Experimental Zoology Part A: Ecological Genetics and Physiology, 215(3), 277–287.										x							
61	Blank, M., Bastrop, R., Röhner, M., & Jürss, K. (2004).	Effect of salinity on spatial distribution and cell volume regulation in two sibling species of Marenzelleria (Polychaeta: Spionidae). Marine Ecology Progress Series, 271, 193–205. https://doi.org/10.3354/meps271193	x																
62	Bowlus, R. D., & Somero, G. N. (1979).	Solute compatibility with enzyme function and structure: Rationales for the selection of osmotic agents and end-products of anaerobic metabolism in marine invertebrates. Journal of Experimental Zoology, 208(2), 137–151. https://doi.org/10.1002/jez.1402080202	x																
63	Boyle, P. R. (1967).	Physiological and Behavioural Studies on the Ecology of some New Zealand Chitons [PhD Thesis]. ResearchSpace@ Auckland.								x									
64	Bradley, T. (1987).	Physiology of osmoregulation in mosquitoes. Annual review of entomology, 32(1), 439–462.	x			x													
65	Buckeridge, J. S. (2012).	Opportunism and the resilience of barnacles (Cirripedia: Thoracica) to environmental change. Integrative Zoology, 7(2), 137–146. https://doi.org/10.1111/j.1749-4877.2012.00286.x	x																
66	Bullaro, C. E. (1969).	OSMOTIC RESPONSES OF TWO SPECIES- OF SIPUNCULIDS TO DIFFERENT SALINITIES AND TEMPERATURES.								x									

ID	Author/Year	Title/Journal	Selection criteria for inclusion in meta-analyses															
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67	Burcham, J. M. (1983).	Amino acid catabolism by ribbed mussel (Modiolus demissus) gill tissue: Studies on isolated mitochondria and the L-amino acid oxidase.	x															
68	Burg, M. B., & Ferraris, J. D. (2008).	Intracellular Organic Osmolytes: Function and Regulation. Journal of Biological Chemistry, 283(12), 7309–7313. https://doi.org/10.1074/jbc.R700042200		x															
69	Burger, J. W. (1957).	The general form of excretion in the lobster, homarus. The Biological Bulletin, 113(2), 207–223. https://doi.org/10.2307/1539079				x													
70	Burnett, L., Terwilliger, N., Carroll, A., Jorgensen, D., & Scholnick, D. (2002).	Respiratory and acid-base physiology of the purple sea urchin, Strongylocentrotus purpuratus, during air exposure: Presence and function of a facultative lung. Biological Bulletin, 203(1), 42–50. https://doi.org/10.2307/1543456								x									
71	Bursey, C. R. (1982).	Salinity Tolerance and Osmotic Response in Two Species of Spider Crabs of the Genus Libinia (Decapoda Brachyura, Majidae). Crustaceana, 42(1–3), 194–200. Scopus. https://doi.org/10.1163/156854082X00867								x									
72	Bursey, C. R., & Harmer, J. A. (1979).	Induced changes in the osmotic concentration of the coelenteron fluid of the sea anemone Condylactis gigantea. Comparative Biochemistry and Physiology Part A: Physiology, 64(1), 73–76. https://doi.org/10.1016/0300-9629(79)90432-8								x									
73	Camien, M. N., Sarlet, H., Duchâteau, G., & Florkin, M. (1951).	Non-Protein Amino Acids in Muscle and Blood of Marine and Fresh Water Crustacea. Journal of Biological Chemistry, 193(2), 881–885.					x												
74	Campillo, J. A., Sevilla, A., Albentosa, M., Bernal, C., Lozano, A. B., Cánovas, M., & León, V. M. (2015).	Metabolomic responses in caged clams, Ruditapes decussatus, exposed to agricultural and urban inputs in a Mediterranean coastal lagoon (Mar Menor, SE Spain). Science of The Total Environment, 524–525, 136–147. https://doi.org/10.1016/j.scitotenv.2015.03.136										x							
75	Cantelmo, A. C., Cantelmo, F. R., & Langsam, D. M. (1975).	Osmoregulatory ability of the rock crab, Cancer irroratus, under osmotic stress. Comparative Biochemistry and Physiology Part A: Physiology, 51(3), 537–542. https://doi.org/10.1016/0300-9629(75)90338-2									x								
76	Cao, C., & Wang, W.-X. (2017).	Copper-induced metabolic variation of oysters overwhelmed by salinity effects. Chemosphere, 174, 331–341. https://doi.org/10.1016/j.chemosphere.2017.01.150	x																
77	Carley, W. W. (1975).	Effects of brain removal on integumental water permeability and ion content of the earthworm Lumbricus terrestris L. General and Comparative Endocrinology, 27(4), 509–516. https://doi.org/10.1016/0016-6480(75)90071-4	x																
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170	Fraga, F., & Lopez-Capont, M. (1958).	Oligosaccharides and the protein factor of the mussel (Mytilus edulis). Invest. Pesquera, 11, 39–52.	x				x												
171	Freas, W., & Grollman, S. (1980).	Ionic and osmotic influence on prostaglandin release from the gill tissue of a marine bivalve, Modiolus demissus. Journal of Experimental Biology, 84(1), 169–185.	x																
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173	Freel, R. W. (1982).	Heterogeneity and dynamics of muscle fiber water in marine crustaceans. Journal of Comparative Physiology B: Biochemical, Systemic, and Environmental Physiology, 146(1), 113–121.								x									
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181	Fyhn, H. J. (1976).	A note on the hyperosmotic regulation in the brackish-water clamRangia cuneata. Journal of comparative physiology, 107(2), 159–167.				x													
182	Gainey, Louis F. (1978a).	The Response of the Corbiculidae (Mollusca: Bivalvia) to Osmotic Stress: The Organismal Response. Physiological Zoology, 51(1), 68–78. https://doi.org/10.1086/physzool.51.1.30158666	x																
183	Gainey, L. F. Jr. (1994).	Volume regulation in three species of marine mussels—ScienceDirect. http://www.sciencedirect.com/science/article/pii/0022098194901287																	
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185	Gangal, S. V., & Magar, N. G. (1964).	Free amino acids in crab muscle. Indian Journal of Biochemistry, 1, 59–60.						x											

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186	Garrett, M. A., & Bradley, T. J. (1987).	Extracellular accumulation of proline, serine and trehalose in the haemolymph of osmoconforming brackish-water mosquitoes. Journal of Experimental Biology, 129(1), 231–238.				x													
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188	Generlich, O., & Giere, O. (1996).	Osmoregulation in two aquatic oligochaetes from habitats with different salinity and comparison to other annelids. Hydrobiologia, 334(1–3), 251–261. https://doi.org/10.1007/BF00017375				x													
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194	Gilles, R. (1988).	Comparative aspects of cell osmoregulation and volume control. Kidney and Blood Pressure Research, 11(3–5), 277–288.	x				x												
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197	Gomme, J. (1982).	Epidermal nutrient absorption in marine invertebrates: A comparative analysis. American Zoologist, 22(3), 691–708.	x																
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200	Gordon, M. S. (1965).	Intracellular osmoregulation in skeletal muscle during salinity adaptation in two species of toads. The Biological Bulletin, 128(2), 218–229.								x									

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201	Gray, J. S. (1976).	The effects of salinity, temperature and mercury on mortality of the trocophore larvae of Serpula vermicularis L. (Annelida: Polychaeta). Journal of Experimental Marine Biology and Ecology, 23(2), 127–134. Scopus. https://doi.org/10.1016/0022-0981(76)90136-2	x																
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254	Kasschau, M. R., & Howard, C. L. (1984).	Free amino acid pool of a sea anemone: Exposure and recovery after an oil spill. Bulletin of environmental contamination and toxicology, 33(1), 56–62.										x							in situ; little change in salinity
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262	Khlebovich, V. V. (1969).	Aspects of animal evolution related to critical salinity and internal state. Marine Biology, 2(4), 338–345. https://doi.org/10.1007/BF00355713	x															
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			Irrelevant data	No original data	Not aquatic	No osmoconformer	No salinity treatment	Salinity unknown	Salinity not steady	Short-term treatment	Acclimation unknown	No multiple treatments	Missing control	Lack of relevant treatment	Variation unknown	No replication	Sample size unknown	Correlation coef. unknown	Other
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			Irrelevant data	No original data	Not aquatic	No osmoconformer	No salinity treatment	Salinity unknown	Salinity not steady	Short-term treatment	Acclimation unknown	No multiple treatments	Missing control	Lack of relevant treatment	Variation unknown	No replication	Sample size unknown	Correlation coef. unknown	Other
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556	Willmer, P. G. (1978).	Volume Regulation and Solute Balance in the Nervous Tissue of an Osmoconforming Bivalve (Mytilus Edulis). Journal of Experimental Biology, 77(1), 157–179.		x															
557	Wittmann, A. C., Storch, D., Anger, K., Poertner, H. O., & Sartoris, F. J. (2011).	Temperature-dependent activity in early life stages of the stone crab Paralomis granulosa (Decapoda, Anomura, Lithodidae): A role for ionic and magnesium regulation? JOURNAL OF EXPERIMENTAL MARINE BIOLOGY AND ECOLOGY, 397(1), 27–37. https://doi.org/10.1016/j.jembe.2010.11.005										x							
558	Wittmann, A. C., Held, C., Pörtner, H. O., & Sartoris, F. J. (2010).	Ion regulatory capacity and the biogeography of Crustacea at high southern latitudes. Polar Biology, 33(7), 919–928. https://doi.org/10.1007/s00300-010-0768-1										x							
559	Wright, S. H., & Secomb, T. W. (1986).	Epithelial Amino Acid Transport in Marine Mussels: Role in Net Exchange of Taurine between Gills and Sea Water. Journal of Experimental Biology, 121(1), 251–270.						x											
560	Wright, S. H., Secomb, T. W., & Bradley, T. J. (1987).	Apical membrane permeability of Mytilus gill: Influence of ultrastructure, salinity and competitive inhibitors on amino acid fluxes. Journal of experimental biology, 129(1), 205–230.						x			x								
561	Wright, S. H., & Secomb, T. W. (1984).	Epidermal taurine transport in marine mussels. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology, 247(2), R346–R355.					x												
562	Wu, H., Liu, X., Zhang, X., Ji, C., Zhao, J., & Yu, J. (2013b).	Proteomic and metabolomic responses of clam Ruditapes philippinarum to arsenic exposure under different salinities. Aquatic Toxicology, 136–137, 91–100. https://doi.org/10.1016/j.aquatox.2013.03.020										x							
563	Wu, H., Zhang, X., Wang, Q., Li, L., Ji, C., Liu, X., Zhao, J., & Yin, X. (2013a).	A metabolomic investigation on arsenic-induced toxicological effects in the clam Ruditapes philippinarum under different salinities. Ecotoxicology and Environmental Safety, 90, 1–6. https://doi.org/10.1016/j.ecoenv.2012.02.022										x							
564	Yamada, A., & Matsushima, O. (1992).	The relation of d-alanine and alanine racemase activity in molluscs. Comparative Biochemistry and Physiology Part B: Comparative Biochemistry, 103(3), 617–621. https://doi.org/10.1016/0305-0491(92)90379-6										x							
565	Yancey, P. H. (2005).	Organic osmolytes as compatible, metabolic and counteracting cytoprotectants in high osmolarity and other stresses. Journal of Experimental Biology, 208(15), 2819–2830. https://doi.org/10.1242/jeb.01730		x															
566	Yancey, P. H., Rhea, M. D., Kemp, K., & Bailey, D. M. (2004).	Trimethylamine oxide, betaine and other osmolytes in deep-sea animals: Depth trends and effects on enzymes under hydrostatic pressure. Cellular and Molecular Biology, 50, 371–376.						x											
567	Yancey, Paul H, Blake, W. R., & Conley, J. (2002).	Unusual organic osmolytes in deep-sea animals: Adaptations to hydrostatic pressure and other perturbants. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology, 133(3), 667–676. https://doi.org/10.1016/S1095-6433(02)00182-4		x															
568	Yancey, Paul H. (2001).	Water Stress, Osmolytes and Proteins. American Zoologist, 41(4), 699–709. https://doi.org/10.1093/icb/41.4.699		x															

			Selection criteria for inclusion in meta-analyses																
ID	Author/Year	Title/Journal	Irrelevant data	No original data	Not aquatic	No osmoconformer	No salinity treatment	Salinity unknown	Salinity not steady	Short-term treatment	Acclimation unknown	No multiple treatments	Missing control	Lack of relevant treatment	Variation unknown	No replication	Sample size unknown	Correlation coef. unknown	Other
569	Yancey, Paul H., Heppenstall, M., Ly, S., Andrell, R. M., Gates, R. D., Carter, V. L., & Hagedorn, M. (2010).	Betaines and Dimethylsulfoniopropionate as Major Osmolytes in Cnidaria with Endosymbiotic Dinoflagellates. Physiological and Biochemical Zoology, 83(1), 167–173. https://doi.org/10.1086/644625		x															
570	Yang, S., Zhong, J., Zhao, L., Wu, H., Du, Z., Liu, Q., Zhang, J., Yan, T., & Huang, X. (2018).	The salinity tolerance of the invasive golden apple snail (Pomacea canaliculata). Molluscan Research, 38(2), 90–98. https://doi.org/10.1080/13235818.2017.1386260								x									
571	Yaroslavtseva, L. M., & Sergeeva, E. P. (2009a).	Adaptation capacity of the larvae of marine invertebrates under variation of environmental parameters as a sensitive test of sea water pollution. Russian Journal of Marine Biology, 35(2), 127–131. https://doi.org/10.1134/S1063074009020035	x																
572	Yaroslavtseva, L. M., & Sergeeva, E. P. (2009b).	Adaptation to reduced salinity in larvae of the mussel Crenomytilus grayanus from spring and summer spawnings. Russian Journal of Marine Biology, 35(4), 335–341. https://doi.org/10.1134/S1063074009040099	x																
573	Yin, M., Palmer, H. R., Bedford, J. J., Fyfe-Johnson, A., Santoso, F., Suko, J., & Yancey, P. H. (1999).	Unusual osmolytes in deep-sea vestimentiferans, gastropods, and echinoderms. American Zoologist, 39, 65A-65A.						x											
574	Yin, M., & Yancey, P. H. (2000).	Methylalamine osmolytes dominate in deep-sea polychaetes, pycnogonids, and octopods. American Zoologist, 40, 1269–1269.						x											
575	Yin, M., Palmer, H. R., Fyfe?Johnson, A. L., Bedford, J. J., Smith, R. A. J., & Yancey, P. H. (2000).	Hypotaurine, N?Methyltaurine, Taurine, and Glycine Betaine as Dominant Osmolytes of Vestimentiferan Tubeworms from Hydrothermal Vents and Cold Seeps. Physiological and Biochemical Zoology, 73(5), 629–637. https://doi.org/10.1086/317749						x											
576	Yin, Q., & Wang, W.-X. (2017).	Relating metals with major cations in oyster Crassostrea hongkongensis: A novel approach to calibrate metals against salinity. Science of The Total Environment, 577, 299–307. https://doi.org/10.1016/j.scitotenv.2016.10.185																	not quantitative
577	Yudkin, W. H. (1954).	Transphosphorylation in echinoderms. Journal of Cellular and Comparative Physiology, 44(3), 507–518. https://doi.org/10.1002/jcp.1030440310	x																
578	Zanders, I. P., & Herrera, F. C. (1974).	Ionic distribution and fluxes in holothurian tissues. Comparative Biochemistry and Physiology Part A: Physiology, 47(4), 1153–1170. https://doi.org/10.1016/0300-9629(74)90090-5									x								
579	Zatta, P., & Cervellin, D. (1987).	Hypo-Osmotic Stress in the Bivalve Mollusc Callista Chione (lam.). Monitore Zoologico Italiano - Italian Journal of Zoology, 21(4), 287–292. https://doi.org/10.1080/00269786.1987.10736532				x													
580	Zebe, E., & Schiedek, D. (1996).	The lugworm Arenicola marina: A model of physiological adaptation to life in intertidal sediments. Helgoländer Meeresuntersuchungen, 50(1), 37. https://doi.org/10.1007/BF02367136						x		x	x								
581	Zerbst-Boroffka, I., Grospietsch, T., Mekhanikova, I., & Takhteev, V. (2000).	Osmotic and ionic hemolymph concentrations of bathyal and abyssal amphipods of Lake Baikal (Siberia) in relation to water depth. Journal of Comparative Physiology B, 170(8), 615–625. https://doi.org/10.1007/s003600000142						x											

ID	Author/Year	Title/Journal	Selection criteria for inclusion in meta-analyses																
			Irrelevant data	No original data	Not aquatic	No osmoconformer	No salinity treatment	Salinity unknown	Salinity not steady	Short-term treatment	Acclimation unknown	No multiple treatments	Missing control	Lack of relevant treatment	Variation unknown	No replication	Sample size unknown	Correlation coef. unknown	Other
582	Zerbst-Boroffka, Irene, Kamaltynow, R. M., Harjes, S., Kinne-Saffran, E., & Gross, J. (2005).	TMAO and other organic osmolytes in the muscles of amphipods (Crustacea) from shallow and deep water of Lake Baikal. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology, 142(1), 58–64. https://doi.org/10.1016/j.cbpa.2005.07.008						x											
583	Zurburg, W., Kluytmans, J. H., Pieters, H., & Zandee, D. I. (1979).	The Influence of Seasonal Changes on Energy Metabolism in Mytilus edulis (L). II. Organ Specificity. In E. Naylor & R. G. Hartnoll (Hrsg.), Cyclic Phenomena in Marine Plants and Animals (S. 293–300). Pergamon. https://doi.org/10.1016/B978-0-08-023217-1.50044-8										x							
584	Zurburg, W., & De Zwaan, A. (1981).	The role of amino acids in anaerobiosis and osmoregulation in bivalves. Journal of Experimental Zoology, 215(3), 315–325. https://doi.org/10.1002/jez.1402150309						x											

Table A.3: Filtered reference list includes all studies that met the inclusion criteria after full-exam

ID	Author/Year	Title/Journal
1	Allen, J. A., & Garrett, M. R. (1972).	Studies on taurine in the euryhaline bivalve Mya arenaria. Comparative Biochemistry and Physiology Part A: Physiology, 41(2), 307–317. https://doi.org/10.1016/0300-9629(72)90062-X
2	Amende, L. M., & Pierce, S. K. (1980a).	Cellular volume regulation in salinity stressed molluscs: The response of Noetia ponderosa (Arcidae) red blood cells to osmotic variation. Journal of comparative physiology, 138(4), 283–289.
3	Austin, H. L. (2007).	Effect of hypo-osmotic stress on pedal disk diameter, volume regulation and concentration of Mg (2+) in the sea anemone Metridium senile. Oregon Institute of Marine Biology.
4	Baginski, R. M., & Pierce, S. K. (1975).	Anaerobiosis: A possible source of osmotic solute for high-salinity acclimation in marine molluscs. Journal of Experimental Biology, 62(3), 589–598.
5	Benson-Rodenbough, B., & Ellington, W. R. (1982).	Responses of the euryhaline sea anemone bunodosoma cavernata (bosc) (anthozoa, actinaria, actiniidae) to osmotic stress. Comparative Biochemistry and Physiology Part A: Physiology, 72(4), 731–735. https://doi.org/10.1016/0300-9629(82)90157-8
6	Berger, V. Ya., Khlebovich, V. V., Kovaleva, N. M., & Natochin, Yu. V. (1978).	The changes of ionic composition and cell volume during adaptation of molluscs (Littorina) to lowered salinity. Comparative Biochemistry and Physiology Part A: Physiology, 60(4), 447–452. https://doi.org/10.1016/0300-9629(78)90015-4
7	Bishop, C. D., Lee, K. J., & Watts, S. A. (1994a).	A comparison of osmolality and specific ion concentrations in the fluid compartments of the regular sea urchin Lytechinus variegatus Lamarck (Echinodermata: Echinoidea) in varying salinities. Comparative Biochemistry and Physiology Part A: Physiology, 108(4), 497–502. https://doi.org/10.1016/0300-9629(94)90333-6
8	Bryan, G. W. (1963).	The accumulation of radioactive caesium by marine invertebrates. Journal of the Marine Biological Association of the United Kingdom, 43(2), 519–539.
9	Costa, C. J., & Pierce, S. K. (1983).	Volume regulation in the red coelomocytes ofGlycera dibranchiata: An interaction of amino acid and K+ effluxes. Journal of Comparative Physiology, 151(2), 133–144. https://doi.org/10.1007/BF00689911
10	Costa, C. J., Pierce, S. K., & Warren, M. K. (1980).	The intracellular mechanism of salinity tolerance in polychaetes: Volume regulation by isolated Glycera dibranchiata red coelomocytes. The Biological Bulletin, 159(3), 626–638.
11	De Vooys, C. G. N. (1991).	Anaerobic metabolism in sublittoral living Mytilus galloprovincialis in the mediterranean—IV. Role of amino acids in adaptation to low salinities during anaerobiosis and aerobiosis. Comparative Biochemistry and Physiology Part A: Physiology, 100(2), 423–431. https://doi.org/10.1016/0300-9629(91)90494-W
12	Deaton, Lewis E., & Hoffmann, R. J. (1988).	Hypoosmotic volume regulation in the sea anemone metridium senile. Comparative Biochemistry and Physiology Part C: Comparative Pharmacology, 91(1), 187–191. https://doi.org/10.1016/0742-8413(88)90185-5
13	Deaton, Lewis E. (1981).	Ion Regulation in Freshwater and Brackish Water Bivalve Mollusks. Physiological Zoology, 54(1), 109–121. https://doi.org/10.1086/physzool.54.1.30155809
14	Deaton, Lewis E., Derby, J. G., Subhedar, N., & Greenberg, M. J. (1989).	Osmoregulation and salinity tolerance in two species of bivalve mollusc: Limnoperna fortunei and Mytilopsis leucophaeta. Journal of Experimental Marine Biology and Ecology, 133(1–2), 67–79.
15	Deaton, L. E. (1992).	Osmoregulation and epithelial permeability in two euryhaline bivalve molluscs: Mya arenaria and Geukensia demissa. Journal of Experimental Marine Biology and Ecology, 158(2), 167–177. https://doi.org/10.1016/0022-0981(92)90224-X
16	Emerson, D. N. (1969).	Influence of salinity of ammonia excretion rates and tissue constituents of euryhaline invertebrates. Comparative Biochemistry and Physiology, 29(3), 1115–1133.
17	Gainey, L. F., & Greenberg, M. J. (1977).	Physiological basis of the species abundance-salinity relationship in molluscs: A speculation. Marine Biology, 40(1), 41–49. https://doi.org/10.1007/BF00390626
18	Gainey, Louis F. (1978b).	The Response of the Corbiculidae (Mollusca: Bivalvia) to Osmotic Stress: The Cellular Response. Physiological Zoology, 51(1), 79–91. https://doi.org/10.1086/physzool.51.1.30158667
19	Henry, R. P., Perry, H. M., Trigg, C. B., Handley, H. L., & Krarup, A. (1990).	Physiology of two species of deep-water crabs, Chaceon fenneri and C. quinquedens: Gill morphology, and hemolymph ionic and nitrogen concentrations. Journal of Crustacean Biology, 10(3), 375–381.
20	Hildreth, J. E., & Stickle, W. B. (1980).	The effects of temperature and salinity on the osmotic composition of the southern oyster drill, Thais haemastoma. The Biological Bulletin, 159(1), 148–161.

Table A.3: Filtered reference list includes all studies that met the inclusion criteria after full-exam

ID	Author/Year	Title/Journal
21	Hiong, K. C., Peh, W. Y. X., Loong, A. M., Wong, W. P., Chew, S. F., & Ip, Y. K. (2004).	Exposure to air, but not seawater, increases the glutamine content and the glutamine synthetase activity in the marsh clam Polymesoda expansa. Journal of Experimental Biology, 207(26), 4605–4614. https://doi.org/10.1242/jel.01334
22	Kapper, M., Stickle, W., & Blakeney, E. (1985).	Volume regulation and nitrogen metabolism in the muricid gastropod Thais haemastoma. The Biological bulletin, 169(2), 458–475.
23	Knight, P.-A., Loomis, S. H., & Fell, P. E. (1992).	The use of free amino acids for osmotic compensation by the euryhaline sponge Microciona prolifera (Ellis & Sollander). Journal of Experimental Marine Biology and Ecology, 163(1), 111–123. https://doi.org/10.1016/0022-0981(92)90150-9
24	Kube, S., Sokolowski, A., Jansen, J. M., & Schiedek, D. (2007).	Seasonal variability of free amino acids in two marine bivalves, Macoma balthica and Mytilus spp., in relation to environmental and physiological factors. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology, 147(4), 1015–1027. https://doi.org/10.1016/j.cbpa.2007.03.012
25	Lee, W.-C., & Chen, J.-C. (2003).	Hemolymph ammonia, urea and uric acid levels and nitrogenous excretion of Marsupenaeus japonicus at different salinity levels. Journal of Experimental Marine Biology and Ecology, 288(1), 39–49. https://doi.org/10.1016/S0022-0981(02)00597-X
26	Lin, C.-H., Yeh, P.-L., & Lee, T.-H. (2016).	Ionic and Amino Acid Regulation in Hard Clam (Meretrix lusoria) in Response to Salinity Challenges. Frontiers in Physiology, 7. https://doi.org/10.3389/fphys.2016.00368
27	Livingstone, D. R., Widdows, J., & Fieth, P. (1979).	Aspects of nitrogen metabolism of the common mussel Mytilus edulis: Adaptation to abrupt and fluctuating changes in salinity. Marine Biology, 53(1), 41–55.
28	Lucu, C., & Devescovi, M. (1999).	Osmoregulation and branchial Na+,K+-ATPase in the lobster Homarus gammarus acclimated to dilute seawater. Journal of Experimental Marine Biology and Ecology, 234(2), 291–304. https://doi.org/10.1016/S0022-0981(98)00152-X
29	Lucu, ?., Devescovi, M., Skaramuca, B., & Kožul, V. (2000).	Gill Na,K-ATPase in the spiny lobster Palinurus elephas and other marine osmoconformers: Adaptiveness of enzymes from osmoconformity to hyperregulation. Journal of Experimental Marine Biology and Ecology, 246(2), 163–178. https://doi.org/10.1016/S0022-0981(99)00179-3
30	Lynch, M. P., & Wood, L. (1966).	Effects of environmental salinity of free amino acids of Crassostrea virginica gmelin. Comparative Biochemistry and Physiology, 19(4), 783–790. https://doi.org/10.1016/0010-406X(66)90434-8
31	Natochin, Yu. V., Berger, V. Y., Khlebovich, V. V., Lavrova, E. A., & Michailova, O. Yu. (1978).	The participation of electrolytes in adaptation mechanisms of intertidal molluscs' cells to altered salinity—ScienceDirect. Comp. Biochem. Physiol., 63A, 115–119.
32	Neufeld, D. S., & Wright, S. H. (1996a).	Response of cell volume in Mytilus gill to acute salinity change. Journal of Experimental Biology, 199(2), 473–484.
33	Neufeld, D. S., & Wright, S. H. (1996b).	Salinity change and cell volume: The response of tissues from the estuarine mussel Geukensia demissa. Journal of Experimental Biology, 199(7), 1619–1630.
34	Neufeld, D., & Wright, S. (1998).	Effect of cyclical salinity changes on cell volume and function in geukensia demissa gills. Journal of Experimental Biology, 201(9), 1421–1431.
35	Oglesby, Larry C. (1968).	Some osmotic responses of the sipunculid worm Themiste dyscritum. Comparative Biochemistry and Physiology, 26(1), 155–177. https://doi.org/10.1016/0010-406X(68)90323-X
36	Paynter, K. T., Pierce, S. K., & Burreson, E. M. (1995).	Levels of intracellular free amino acids used for salinity tolerance by oysters (Crassostrea virginica) are altered by protozoan (Perkinsus marinus) parasitism. Marine Biology, 122(1), 67–72. https://doi.org/10.1007/BF00349278
37	Pierce, Sidney K. (1970).	The water balance of Modiolus (Mollusca: Bivalvia: Mytilidae): Osmotic concentrations in changing salinities. Comparative Biochemistry and Physiology, 36(3), 521–533. https://doi.org/10.1016/0010-406X(70)91028-5
38	Pierce Jr., S. K. (1971a).	A source of solute for volume regulation in marine mussels. Comparative Biochemistry and Physiology Part A: Physiology, 38(3), 619–635. https://doi.org/10.1016/0300-9629(71)90129-0
39	Pierce, Sidney K., Warren, M. K., & West, H. H. (1983).	Non-Amino Acid Mediated Volume Regulation in an Extreme Osmoconformer. Physiological Zoology, 56(3), 445–454. https://doi.org/10.1086/physzool.56.3.30152610

Table A.3: Filtered reference list includes all studies that met the inclusion criteria after full-exam

ID	Author/Year	Title/Journal
40	Pierce, Sidney K., Edwards, S. C., Mazzocchi, P. H., Klingler, L. J., & Warren, M. K. (1984).	Proline betaine: A unique osmolyte in an extremely euryhaline osmoconformer. The Biological Bulletin, 167(2), 495–500. https://doi.org/10.2307/1541294
41	Pierce, S. K., Rowland-Faux, L. M., & O'Brien, S. M. (1992).	Different salinity tolerance mechanisms in Atlantic and Chesapeake Bay conspecific oysters: Glycine betaine and amino acid pool variations. Marine Biology, 113(1), 107–115. https://doi.org/10.1007/BF00367644
42	Podbielski, I., Bock, C., Lenz, M., & Melzner, F. (2016).	Using the critical salinity (S-crit) concept to predict invasion potential of the anemone Diadumene lineata in the Baltic Sea. Marine Biology, 163(11), 227. https://doi.org/10.1007/s00227-016-2989-5
43	Potts, W. T. W. (1958).	The Inorganic and Amino Acid Composition of Some Lamellibranch Muscles. Journal of Experimental Biology, 35(4), 749–764.
44	Ran, Z., Li, S., Zhang, R., Xu, J., Liao, K., Yu, X., Zhong, Y., Ye, M., Yu, S., Ran, Y., Huang, W., & Yan, X. (2017).	Proximate, amino acid and lipid compositions in Sinonovacula constricta (Lamarck) reared at different salinities. Journal of the Science of Food and Agriculture, 97(13), 4476–4483. https://doi.org/10.1002/jsfa.8311
45	Rola, R. C., Souza, M. M., & Sandrini, J. Z. (2017).	Hypoosmotic stress in the mussel Perna perna (Linnaeus, 1758): Is ecological history a determinant for organismal responses? Estuarine, Coastal and Shelf Science, 189, 216–223. https://doi.org/10.1016/j.ecss.2017.03.020
46	Rowland, L. M., & Pierce, S. K. (1985).	The utilization and fate of quaternary ammonium compounds during low salinity adaptation in Elysia chlorotica. Physiological zoology, 58(1), 149–157.
47	Sanders, T. (2018)	Bioenergetik der Kalzifizierung in Mytiliden Muscheln entlang des Salinitätsgradienten der Ostsee (Doctoral dissertation, Christian-Albrechts-Universität Kiel).
48	Schiedek, D. (1997).	Marenzelleria cf. viridis (Polychaeta: Spionidae) – ecophysiological adaptations to a life in the coastal waters of the Baltic Sea. Aquatic Ecology, 31(2), 199–210. https://doi.org/10.1023/A:1009907606161
49	Schiedek, D. (1998).	Ecophysiological capability of Marenzelleria populations inhabiting North Sea estuaries: An overview. Helgoländer Meeresuntersuchungen, 52(3), 373. https://doi.org/10.1007/BF02908911
50	Schmittmann, L. (2017).	Local adaptation of the common sea star Asterias rubens to different salinities (Master thesis, Christian-Albrechts-Universität Kiel).
51	Shumway, S. E. (1977a).	Effect of salinity fluctuation on the osmotic pressure and Na<Superscript>+</Superscript>, Ca<Superscript>2+</Superscript> and Mg<Superscript>2+</Superscript> ion concentrations in the hemolymph of bivalve molluscs. Marine Biology, 41(2), 153–177. https://doi.org/10.1007/BF00394023
52	Silva, A. L. (1992).	Effect of salinity on integumental transport in marine bivalves.
53	Stickle, W. B., Kapper, M. A., Blakeney, E., & Bayne, B. L. (1985).	Effects of salinity on the nitrogen metabolism of the muricid gastropod, Thais (Nucella) lapillus (L.) (Mollusca: Prosobranchia). Journal of Experimental Marine Biology and Ecology, 91(1), 1–16. https://doi.org/10.1016/0022-0981(85)90217-5
54	Stucchi-Zucchi, A., & C. Salomão, L. (1998).	The ionic basis of membrane potentials and adaptation to hyposmotic stress in Perna perna, an osmoconforming mollusc. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology, 121(2), 143–148. https://doi.org/10.1016/S1095-6433(98)10115-0
55	Willmer, P. G. (1978a).	Volume Regulation and Solute Balance in the Nervous Tissue of an Osmoconforming Bivalve (Mytilus Edulis). Journal of Experimental Biology, 77(1), 157–179.
56	Witteveen, J., Verhoef, H. A., & Letschert, J. P. W. (1987).	Osmotic and ionic regulation in marine littoral Collembola. Journal of Insect Physiology, 33(1), 59–66. https://doi.org/10.1016/0022-1910(87)90105-3

Table A.4: Study details on salinity acclimation

Title	Adjustment to experimental salinity	Experimental Duration (days)	S low	S high	Taxa	Species
Allen & Garret 1972	unknown	27	12	34	Mollusca	Mya arenaria
Amende & Pierce 1980	acute	56	11.9	35.9	Mollusca	Noetia ponderosa
Austin 2007	acute	21	16.5	33	Cnidaria	Metridium senile
Baginski & Pierce 1975	unknown	21	12	36	Mollusca	Geukensia demissa
Benson-Rodenbough & Ellington 1982	acute	21			Cnidaria	Bunodosoma cavernata
Berger et al. 1978	unknown	14	16	26.8	Mollusca	Littorina littorea
Bishop et al. 1994a	gradual	32	20	30	Echinodermata	Lytechinus variegatus
Bishop et al. 1994a	gradual (5ppt/day)	32	20	30	Echinodermata	Lytechinus variegatus
Bryan 1963	unknown	28	24	26.9	Cnidaria	Actinia equina
Costa & Pierce 1983	acute	14	16	32	Annelida	Glycera dibranchiata
Costa et al. 1980	gradual (2.6-9.6/48hrs)	14	15	32	Annelida	Glycera dibranchiata
Costa et al. 1980	gradual (2.6-9.6/48hrs)	14	12	32	Annelida	Glycera dibranchiata
De Vooys 1991	acute	14	19.0	38.0	Mollusca	Mytilus gallpprovincialis
Deaton & Hoffmann 1988	acute	14	17	31	Cnidaria	Metridium senile
Deaton 1981	unknown	28	2.20	23.2	Mollusca	Ostrea palmula
Deaton 1981	unknown	28	0.0	35.0	Mollusca	Polymesoda caroliniana
Deaton 1981	unknown	28	2.2	28.8	Mollusca	Polymesoda maritima
Deaton 1992	unknown	35	0.3	28.8	Mollusca	Geukensia demissa
Deaton 1992	unknown	35	1.4	28.8	Mollusca	Mya arenaria
Deaton et al. 1989	acute	21	0.2	17.2	Mollusca	Mytilopsis leucophaeta
Deaton et al. 1989	acute	22	0	19	Mollusca	Mytilopsis leucophaeata
Emerson 1969	acute	16	17.5	35.0	Mollusca	Macoma inconspicua
Gainey & Greenberg 1977	acute	14	0.0	39.0	Mollusca	Polymesoda caroliniana
Gainey & Greenberg 1977	acute	14	2.0	45.0	Mollusca	Pseudocyrena floridana
Gainey 1978b	acute	14	0	10	Mollusca	Polymesoda caroliniana
Henry et al. 1990	gradual (2ppt/day)	21	20.0	35.0	Arthropoda	Chaceon fenneri
Henry et al. 1990	gradual (2ppt/day)	21	20.0	35.0	Arthropoda	Chaceon quinquedens
Henry et al. 1990	gradual (2ppt/day)	21	15.0	35.0	Arthropoda	Chaceon quinquedens
Hildreth & Stickle 1980	gradual (2psu/day)	35	7.5	30.0	Mollusca	Stramonita haemastoma
Hiong et al. 2004	acute	17	10	30	Mollusca	Polymesoda expansa
Kapper et al. 1985	gradual (2ppt/day)	14	5	30	Mollusca	Stramonita haemastoma
Knight et al. 1992	gradual (5ppt/3days)	14	10	30	Porifera	Microciona prolifera
Kube et al. 2007	NA	in situ	3.7	33.6	Mollusca	Macoma balthica
Kube et al. 2007	NA	in situ	6.1	38	Mollusca	Mytilus edulis
Lee & Chen 2003	gradual (2-3 ppt/day)	28	18	34	Arthropoda	Marsupenaeus japonicus
Lin et al. 2016	unknown	28	10.0	20.0	Mollusca	Meretrix lusoria
Livingstone et al. 1979	acute	14	15.0	30.0	Mollusca	Mytilus edulis
Livingstone et al. 1979	acute	21	15.0	30.0	Mollusca	Mytilus edulis
Lucu & Devescovi 1999	acute	15	20.0	38.0	Arthropoda	Homarus gammarus
Lucu et al. 2000	gradual (2ppt/2days)	16	20.0	38.0	Arthropoda	Palinurus elephas
Lynch & Wood 1966	NA	in situ	3.4	26.7	Mollusca	Crassostrea virginica
Natochin et al. 1978	acute	14	10.0	26.8	Mollusca	Mytilus edulis
Neufeld & Wright 1996a	unknown	28	20	33	Mollusca	Mytilus californianus
Neufeld & Wright 1996b	unknown	21	20	33	Mollusca	Geukensia demissa
Neufeld & Wright 1998	unknown	21	20	33	Mollusca	Geukensia demissa
Oglesby 1968	unkown	17	13.1	34.6	Sipuncula	Themiste dycritum
Paynter et al. 1995	NA	70			Mollusca	Crassostrea virginica
Pierce 1970	acute	14	8.0	40.0	Mollusca	Geukensia demissus demissus
Pierce 1970	acute	14	3.0	44.0	Mollusca	Geukensia demissus granosissimus
Pierce 1971	unknown	21	3.0	48.0	Mollusca	Geukensia demissus granosissimus
Pierce 1971	unknown	21	22.0	41.0	Mollusca	Modiolus squamosus
Pierce et al. 1983	gradual (0.2-20 psu/2 weeks)	14	3.1	37.4	Mollusca	Elysia chlorotica
Pierce et al. 1984	unknown	14	3	30	Mollusca	Elysia chlorotica
Pierce$_{et al.}$1992	acute	14	11.0	30.0	Mollusca	Crassostrea$_{v}$*irginica*
Podbielski et al. 2016	gradual (2 psu/day)	28	7	34	Cnidaria	Diadumene lineata
Potts 1958	unknown	14	15.0	30.0	Mollusca	Mytilus edulis
Ran et al. 2017	gradual (3-4 psu/8hrs)	25	13	23	Mollusca	Sinonovacula constricta
Rola et al. 2017	gradual (3 psu/45min)	14	20.0	35.0	Mollusca	Perna perna
Rowland & Pierce 1985	unknown	14	5	30	Mollusca	Elysia chlorotica
Sanders 2018	gradual (2.2psu/day)	28	4.5	16.0	Mollusca	Mytilus sp.
Sanders 2018	gradual (2.2psu/day)	28	4.5	16.0	Mollusca	Mytilus edulis
Schiedek et al. 1997	NA	in situ	1	8	Annelida	Marenzelleria cf. Viridis
Schiedek et al. 1997	gradual (8psu/week)	14	1	24	Annelida	Marenzelleria cf. Viridis
Schiedek et al. 1998	NA	in situ	6	20	Annelida	Marenzelleria cf. Wireni
Schiedek et al. 1998	gradual (8psu/week)	in situ	1	22	Annelida	Marenzelleria cf. Wireni
Schmittmann 2017	gradual (1psu/2-3 days)	31	16	32	Echinodermata	Asterias rubens
Schmittmann 2017	gradual (1psu/2-3 days)	41	16	32	Echinodermata	Asterias rubens
Shumway 1977a	unknown	28	6.4	32.0	Mollusca	Mya arenaria
Silva 1992	gradual (3psu/2days)	14	19	32	Mollusca	Mytilus californianus
Stickle et al. 1985	gradual (2psu/day)	14	15	35	Mollusca	Nucella lapillus
Stucchi-Zucchi & Salomao 1998	unknown	15	15	35	Mollusca	Perna perna
Willmer 1978a	unknown	14	7.0	37.7	Mollusca	Mytilus edulis
Witteveen et al. 1987	acute	21	5.1	40.9	Arthropoda	Anurida maritima

Table A.5: Results of sensitivity analysis per outcome variable. Influential studies were identified and excluded. If the mean effect size (MES) was significant as indicated by an asterisk, further sensitivity analyses were conducted to test whether the results are robust. k denotes the number of studies. The fail-safe number was calculated after Rosenberg. If its robustness is higher than specified in column H it can be considered sufficiently robust. Publication bias was tested via funnel plot asymmetry in the plot and additionally tested via Egger's test. If asymmetry was detected the effect size (ES) was tested for its robustness via the Trim & Fill method. True effect vs. p-hacking was tested for with the p-curve test.

ID	Outcome	Influential studies	Name of removed studies	MES	k	Fail-safe no. (n)	Robustness of fail-safe no. ($n > 5 \times k + 10$	Funnel plot asymmetry	Egger's test	ES robust to trim & fill?	p-curve (true effect present?)
1	Alanine	1	Ran et al. 2017	*	37	1271	195	yes	sig	yes	yes
2	Arginine	1	Ran et al. 2017-2	NA	11	NA	NA	NA	NA	NA	NA
3	Ammonia	1	Silva 1992-2	*	5	5	35	no	ns	NA	error
4	Asparagine	NA	NA	NA	3	NA	NA	no	ns	NA	NA
5	Aspartate	NA	NA	NA	24	NA	NA	yes	ns	NA	NA
6	Betaine	NA	NA	*	14	85	80	yes	sig	yes	yes
8	Chloride	1	Potts 1958	NA	4	NA	NA	NA	NA	NA	NA
9	Glutamate	1	Schiedek et al. 1997-6	*	30	36	160	no	sig	no	no
10	Glutamine	1	Schmittmann 2017-17	*	11	47	65	no	ns	NA	no
11	Glycine	2	Kapper et al. 1985-5 Ran et al. 2017-5	*	34	1893	180	yes	sig	yes	yes
12	Histidine	NA	NA	NA	7	NA	NA	NA	NA	NA	NA
13	Isoleucine	NA	NA	NA	7	NA	NA	NA	NA	NA	NA
14	Leucine	NA	NA	NA	7	NA	NA	NA	NA	NA	NA
15	Lysine	1	Schmittmann 2017-35	NA	9	NA	NA	NA	NA	NA	NA
16	Magnesium	2	Austin - 2007 Berger et al. 1978-2	NA	3	NA	NA	NA	NA	NA	NA
17	Methionine	NA	NA	*	6	11	40	no	ns	NA	low k
18	O-Phosphocholine	1	Schmittmann 2017-44	NA	4	NA	NA	NA	NA	NA	NA
19	Ornithine	NA	NA	NA	6	NA	NA	NA	NA	NA	NA
20	Phenylalanine	NA	NA	NA	7	NA	NA	NA	NA	NA	NA
21	(O-)Phosphoserine	1	Kapper et al. 1985-7	NA	4	NA	NA	NA	NA	NA	NA
22	Potassium	NA	NA	NA	15	NA	NA	NA	NA	NA	NA
23	Proline	NA	NA	*	13	169	75	yes	sig	yes	yes
24	Serine	NA	NA	*	19	180	105	yes	ns	NA	yes
25	Sodium	1	Natochin et al. 1978-2	*	9	154	55	yes	sig	no	yes
26	Taurine	2	Lin et al. 2016-13 Schmittmann 2017-56	*	25	670	135	yes	sig	yes	yes
27	Threonine	NA	NA	NA	13	NA	NA	NA	NA	NA	NA
28	Total Organics	1	Kapper et al. 1985-12	*	31	1974	165	yes	sig	yes	yes
30	Tyrosine	1	Silva 1992-21	*	5	14	35	no	ns	NA	low k
31	Valine	1	Ran et al.2017-17	*	6	8	40	no	ns	NA	low k

A.2 Formulas for calculating effect sizes in meta-analysis

Standardized mean difference (d) (Eq. 1):

1. $d = \frac{\bar{x}_1 - \bar{x}_2}{S_{within}}$

   Where $\bar{x}_1$ and $\bar{x}_2$ are the sample means of the two groups, the high salinity and the low salinity, respectively and the denominator (S_{within}) is the within groups standard deviation, pooled across groups (Eq. 2). Hedge's g corrects for a bias overestimating standardized mean difference for small sample sizes using the correction factor J ([117]).

2. $S_{within} = \sqrt{\frac{(n_1-1)\times S_1^2 + (n_2-1)\times S_2^2}{n_1+n_2-2}}$

   S_1 and S_2 are the Standard Deviation of the two groups and n_1 and n_2 the sample size. Variation parameters were converted to standard deviation if necessary and the standardize mean difference (d) and its variance (V_d) computed.

   The variance of d (Eq. 3) for each study was calculated as:

3. $V_d = \frac{n_1+n_2}{n_1\times n_2} + \frac{d^2}{2\times(n_1+n_2)}$

   and converted to variance of g (V_g) as follows (Eq. 4 & 5):

4. $V_g = J^2 \times V_d$

5. $J = 1 - \frac{3}{4\times df - 1}$

   Meta-analyses weigh the individual effect sizes by the inverse of the effect size variance to account for the precision of each study (Eq. 6).

6. $w = \frac{1}{V_g}$

   Calculation of effect size and variance (Eq. 7 - 9) from correlation coefficient (r) was only necessary for a couple of datapoints:

7. $d = \frac{2r}{\sqrt{1-r^2}}$

8. $V_r = \frac{(1-r^2)^2}{n-1}$

9. $V_d = \frac{4V_r}{(1-r^2)^3}$

Table A.6: Statistical results of robust test for random effects and mixed-effect categorical meta-analysis

Outcome	Overall t-value	df*	p-value	Taxa Q_M	df1	df2	p-value	Class Q_M	df1	df2	p-value	Sample Type Q_M	df1	df2	p-value
Alanine	-5.7241	26	<0.0001	98.5939	3	22	<0.0001					24.9312	5	22	<0.0001
Ammonia	-131.4645	1	0.0048												
Arginine	-8.6303	2	0.0132												
Asparagine	-2.2422	2	0.1542												
Aspartate	-0.6387	17	0.5315	10.9937	2	14	0.0013					5.1903	4	13	0.0101
Betaine	-2.8773	8	0.0206	51.779	2	6	0.0002					18.9747	2	6	0.0025
Chloride	-1.4832	2	0.2763												
Glutamate	-3.3918	30	0.0029	7.7093	2	17	0.0041					3.8606	4	16	0.0222
Glutamine	-5.4873	7	0.0009					11.4652	2	3	0.0394				
Glycine	-8.4089	24	<0.0001	19.9759	3	19	<0.0001					30.8141	5	19	<0.0001
Histidine	0.0948	6	0.9275												
Isoleucine	0.1024	6	0.9218	0.5694	2	4	0.6059								
Leucine	-0.8389	6	0.4337	21.4929	2	4	0.0072								
Lysine	-2.0102	8	0.0793												
Magnesium	-0.5317	2	0.6889												
Methionine	-11.5151	5	<0.0001												
O-Phosphocholine	2.6808	3	0.075												
Ornithine	-0.5919	5	0.5797												
Phenylalanine	-1.8033	6	0.1214	1.6026	2	4	0.3082								
(O-)Phosphoserine	-0.5273	3	0.6345												
Potassium	-1.7538	11	0.1176									39.7932	2	7	<0.0001
Proline	-3.3108	9	0.0091	6.0649	2	7	0.0296					2.9489	3	6	0.1203
Serine	-3.1673	17	0.0056	18.0732	2	12	0.0002					14.3575	4	12	0.0002
Sodium	-3.4867	7	0.0252									75.347	2	4	<0.0001
Taurine	-6.4261	31	<0.0001									13.0932	3	16	0.0001
Threonine	-2.0387	11	0.0662	4.4748	2	8	0.0496					35.3665	3	6	0.0003
Total Organics	-7.8171	25	<0.0001	16.1703	3	23	<0.0001	21.2902	2	16	<0.0001	29.3042	4	21	<0.0001
Tyrosine	-4.7657	4	0.0089												
Valine	-2.6744	5	0.0441												

* df for overall model = no. of cluster - no. of coefficients (here = 1)

Fig. A1 Subgroup analysis – Class: Total Organics

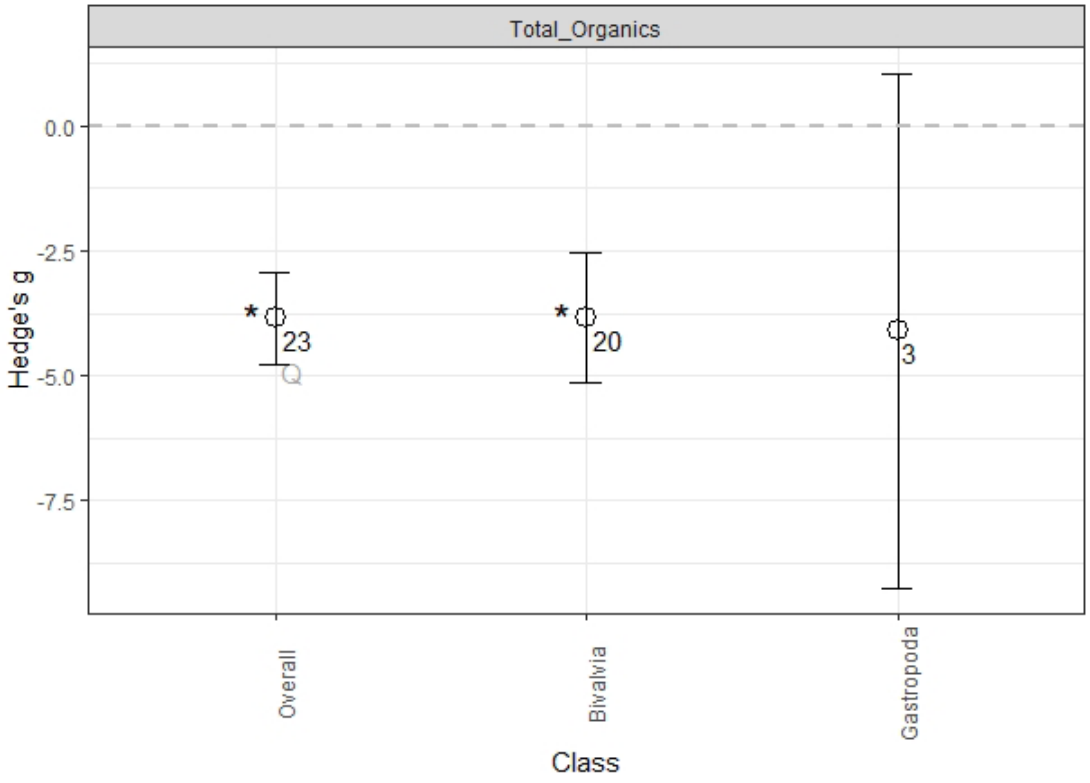

Figure A.1: Subgroup analysis - Effect of low salinity on total organic osmolyte concentration between molluscan classes Bivalvia and Gastropoda. Mean effect size is depicted as a circle with the error bars indicating robust confidence intervals (95%). If the confidence intervals do not overlap with zero an effect size is considered significant. An asterisk indicates a significant robust difference in effect size. Q indicates significant residual heterogeneity as tested with the random effects model. Numbers below circles indicate the number of studies.

Table A.7: Outcome of the meta-regression results for the moderator variables. Moderator variables were magnitude of salinity stress and expermental duration (including and excluding *in situ* studies). The p-value denotes whether an effect significantly influenced effect size (*), or whether it was non significant (ns). Q denotes whether the methodological moderator explained all variance in effect sizes (ns) or whether residual heterogeneity remains (Q). Methodological moderators were only tested if an overall significant effect size was found (overall effect is shown with results from Fig. 2.4 and Tab. A.6), or for non-significant effect sizes if significant residual heterogeneity of effect sizes was found. Analysis was not conducted if no significant effect and no significant residual heterogeneity of effect sizes was found. This is symbolized by NA.

		Overall		Magnitude		Duration (with *in situ*)		Duration (without *in situ*)	
ID	Outcome	p	Q	p	Q	p	Q	p	Q
1	Alanine	*	Q	*	Q	ns	Q	ns	Q
2	Arginine	ns	Q	ns	Q	ns	Q	ns	Q
3	Ammonia	*	ns	ns	ns	ns	ns	*	ns
4	Asparagine	ns	ns	NA	NA	NA	NA	NA	NA
5	Aspartate	ns	Q	ns	Q	ns	Q	ns	Q
6	Betaine	*	Q	ns	Q	NA	NA	ns	Q
7	Chloride	ns	Q	ns	Q	NA	NA	ns	Q
8	Glutamate	*	Q	ns	Q	*	Q	ns	Q
9	Glutamine	*	ns	ns	ns	NA	NA	ns	ns
10	Glycine	*	Q	ns	Q	ns	Q	ns	Q
11	Histidine	ns	ns	NA	NA	NA	NA	NA	NA
12	Isoleucine	ns	Q	ns	Q	ns	Q	ns	Q
13	Leucine	ns	Q	*	ns	ns	Q	ns	Q
14	Lysine	ns	ns	ns	ns	ns	ns	ns	ns
15	Magnesium	ns	Q	ns	Q	NA	NA	ns	Q
16	Methionine	*	ns	ns	ns	ns	ns	ns	ns
17	O-Phosphocholine	ns	ns	ns	ns	NA	NA	ns	ns
18	Ornithine	ns	Q	ns	Q	ns	Q	ns	Q
19	Phenylalanine	ns	Q	ns	Q	ns	Q	ns	Q
20	(O-)Phosphoserine	ns	ns	ns	ns	NA	NA	ns	ns
21	Potassium	ns	Q	*	ns	NA	NA	*	ns
22	Proline	*	Q	*	Q	ns	Q	ns	Q
23	Serine	*	Q	ns	Q	ns	Q	ns	Q
24	Sodium	*	Q	ns	Q	NA	NA	ns	Q
25	Taurine	*	Q	ns	Q	*	Q	ns	Q
26	Threonine	ns	Q	ns	Q	ns	Q	ns	Q
27	Total Organics	*	Q	ns	Q	ns	Q	ns	Q
28	Tyrosine	*	ns	ns	ns	ns	ns	ns	ns
29	Valine	*	ns	*	ns	*	ns	ns	ns

A.3 Meta-analysis outcome for inorganic and organic osmolyte ratios I

Meta-analysis

The raw data from the systematic review was used to calculate the overall effect size, after filtering out influential studies. Standardized mean differences were used to calculate effect size. No variance data was available and could not be re-calculated, hence an unweighted fixed-effects meta-analysis was be conducted.

This document shows the output from the meta-analysis for the total inorganic pool vs. total organic pool. Three ratios were analysed:

- inorganic vs. organic pool
- inorganic vs. total osmolyte pool
- organic vs. total osmolyte pool

Analysis, where possible, was run for intracelluar data (which accounted for extracellular space (ECS)) and whole tissue data (including ECS).

Meta-analysis outcome inorganic vs. organic osmolyte pool

Intracellular values: We found no significant salinity effect on the inorganic/organic osmolyte pool ratio for intracellular values ($g = -0.2542 \pm 0.6867$, df = 2, p-value = 0.4682).

Whole tissue data: We found no significant salinity effect on the inorganic/organic osmolyte pool ratio for tissues ($g = 0.2014 \pm 0.2940$, df = 2, p-value = 0.1795).

Meta-analysis outcome inorganic osmolyte pool vs. total osmolyte pool

Intracellular values: We found an initial significant salinity effect on the inorganic/total osmolyte pool ratio for intracellular space, which was however not robust. To account for

dependent data clusters and adjust for small sample size a robust test was used on the fixed-effects model ($g = -0.1064 \pm 1.0948$, df = 2, p-value = 0.4333).

Whole tissue data: We found an initial significant salinity effect on the inorganic/total osmolyte pool ratio for tissues, which was however not robust. To account for dependent data clusters and adjust for small sample size a robust test was used on the fixed-effects model ($g = 0.0799 \pm 0.4010$, df = 2, p-value = 0.5712).

Meta-analysis outcome organic vs. total osmolyte pool

Intracellular values: We found an initial significant salinity effect on the organic/total osmolyte pool ratio for intracellular space, which was however not robust. To account for dependent data clusters and adjust for small sample size a robust test was used on the fixed-effects model ($g = 0.1166 \pm 1.2022$, df = 2, p-value = 0.4340).

Whole tissue data: We found an initial significant salinity effect on the organic/total osmolyte pool ratio for tissues, which was however not robust. To account for dependent data clusters and adjust for small sample size a robust test was used on the fixed-effects model ($g = -0.1216 \pm 0.7426$, df = 2, p-value = 0.6383).

Subgroup Analyses

No subgroup analyses were conducted since the number data points per subgroup (from independent clusters) was insufficient.

Testing for influential studies and outliers

1. Inorganic vs. organic osmolyte pool

   Intracellular: The analysis identified no outliers.

   Whole tissue: The analysis identified 4 of the 5 results as influential studies. Since this would exclude almost all studies, no studies were removed.

2. Inorganic vs. total osmolyte pool

   Intracellular: The analysis identified 3 of the 3 results as influential studies. Since this would exclude all studies, no studies were removed.

   Whole tissue: The analysis identified 5 of the 5 results as influential studies. Since this would exclude all studies, no studies were removed.

3. Organic vs. total osmolyte pool

   Intracellular: The analysis identified 3 of the 3 results as influential studies. Since this would exclude all all studies, no studies were removed.

   Whole tissue: The analysis identified 5 of the 5 results as influential studies. Since this would exclude all studies, no studies were removed.

Sensitivity Analysis Funnel plots showed no publication bias for the 1) inorganic/organic osmolyte pool ratio. High asymmetry was found for the 2) inorganic/total osmolyte pool ratio and the 3) organic/total osmolyte pool ratio for both tissue and intracellular data.

3

Capacity for cellular osmoregulation defines critical salinity of marine invertebrates at low salinity

Imke Podbielski, Claas Hiebenthal, Mithra - Christin Hajati, Christian Bock, Markus Bleich, Frank Melzner

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Capacity for cellular osmoregulation defines critical salinity of marine invertebrates at low salinity

Imke Podbielski[1], Claas Hiebenthal[2], Mithra - Christin Hajati[3], Christian Bock[4], Markus Bleich[5], Frank Melzner[1]

Affiliations

[1]Experimental Ecology - Benthic Ecology, GEOMAR-Helmholtz Centre for Ocean Research Kiel, Hohenbergstraße 2, 24105 Kiel, Germany

[2]KIMOCC, GEOMAR-Helmholtz Centre for Ocean Research Kiel, Düsternbrooker Weg 20, 24105 Kiel, Germany

[3]Referat 2.5 Hydrogeologische Grundlagen, Landesamt für Bergbau, Energie und Geologie, Stilleweg 2, 30655 Hannover, Germany

[4]Integrative Ecophysiology, Alfred Wegener Institute Helmholtz Centre for Polar and Marine Research, Am Handelshafen 12, 27515 Bremerhaven, Germany

[5]Physiological Institute, Christian-Albrechts-University Kiel, Olshausenstraße 40, 24098 Kiel, Germany

Significance statement

Climate change models predict a salinity decrease ('desalination') for many coastal regions, likely leading to range shifts of marine species. This makes it urgent to understand the cellular basis for marine animal salinity tolerance. We quantified cellular osmolyte systems and related them to growth and mortality of six invertebrate model species acclimated to low salinity. Our results confirm an important role of organic osmolytes, but also stress a previously largely neglected role of inorganic ions in facilitating acclimation to per-

sistent low salinity. However, we also demonstrate that osmolyte pool size cannot predict low salinity thresholds that lead to severe growth reduction and mortality. Thus, we suggest to determine a comprehensive set of parameters to predict animal responses to future desalination.

Abstract

Low salinity can severely affect the fitness of marine organisms. As desalination has been predicted for many coastal areas with ongoing climate change, it is crucial to gain more insight in mechanisms that constrain salinity acclimation ability. Low-salinity induced depletion of the organic osmolyte pool has been suggested to set a critical boundary in osmoconforming marine invertebrates. Whether inorganic ions also play a persistent role during low-salinity acclimation processes is currently inconclusive.

We investigated the salinity tolerance of six marine invertebrate species following a four-week acclimation period around their low-salinity tolerance threshold. To obtain complete osmolyte budgets, we quantified organic and inorganic osmolytes and determined fitness proxies.

Our experiments corroborated the importance of the organic osmolyte pool during low-salinity acclimation. Methylamines constituted a large portion of the organic osmolyte pool in molluscs, whereas echinoderms exclusively utilized free amino acids. Inorganic osmolytes were involved in long-term cellular osmoregulation in most species, thus are not just modulated with acute salinity stress. The organic osmolyte pool was not depleted at low salinities, whilst fitness was severely impacted. Instead, organic and inorganic osmolytes often stabilized at low salinity.

These findings suggest that low salinity acclimation capacity cannot be simply predicted

from organic osmolyte pool size. Rather, multiple parameters (i.e. osmolyte pools, net growth, water content and survival) are necessary to establish critical salinity ranges. However, a quantitative knowledge of cellular osmolyte systems is key to understand the evolution of euryhalinity and to characterize targets of selection during rapid adaptation to ongoing desalination.

Keywords

Salinity stress | Invertebrates | Osmolytes | Cellular osmoregulation | Climate Change

3.1 Introduction

Salinity is one of the most important abiotic factors shaping species composition in marine environments. Low salinity (hypoosmotic) stress as experienced in many estuaries and coastal regions can put severe physiological stress on marine animals, thereby affecting survival, immune responses, metabolism, protein function and various cellular processes [34, 36, 175]. Under moderate hypoosmotic stress, increased costs for osmoregulation are met by an increased routine metabolic rate, or by re-allocation of energy towards osmoregulatory processes. This, however, reduces the scope for growth and reproduction and, ultimately, fitness [240, 262]. Under extreme salinity stress, many marine metazoans are restricted to maintaining basic functions to conserve energetic reserves until environmental conditions improve. Such a state is not sustainable. Avoidance is the only option, if a species' capacity for salinity tolerance via acclimation or rapid adaptation is not sufficient. Persistent salinity changes can lead to geographic range shifts, vertical distribution shifts and, if relocation is not possible, (local) extinction [353]. Benthic marine invertebrates are especially affected by environmental salinity changes as they often have limited mobility

and larval stages with reduced tolerance to low salinity [46, 226].

With ongoing climate change, salinity is projected to decrease in many coastal areas due to an increase in freshwater inflow caused by increased precipitation and glacier melting [1]. The Antarctic shelf, shallow coral reefs in Australia, or the Baltic Sea are prominent regions where expected desalination or prolonged low salinity periods could fundamentally change benthic invertebrate communities [113, 221, 367]. To predict desalination effects on distribution and species fitness, it is crucial to understand the physiological mechanisms, capacity and limits that determine salinity tolerance.

Aquatic organisms can generally be divided into osmoregulators and osmoconformers. While osmoregulators regulate the osmotic pressure of their body fluids independently to that of ambient seawater, osmoconformers are characterized by extracellular fluids (i.e. haemolymph, coelomic fluid, interstitial fluid) that are isosmotic with respect to seawater. Osmoconformers, consisting chiefly of invertebrates, use cellular volume regulation (CVR) to react to osmotic changes. In CVR, small molecules are used to adjust cellular osmotic pressure (i.e. osmolality). To acclimate to reduced seawater salinity, cells release or catabolize some of these osmotic compounds to avoid water inflow and harmful swelling. Only when the capacity for CVR reaches its limit, cellular water content will increase, ultimately leading to cellular damage and cell death [175].

Osmolytes can be of inorganic and organic nature and are defined as compounds whose concentration is regulated in relation to salinity (*sensu* [332]). Organic osmolytes are also termed compatible osmolytes, because they are generally considered non-perturbing with protein function and structure [332]. Many of the major organic osmolytes have even beneficial attributes such as protein and membrane stabilizing abilities. Often, the organic osmolyte pool is composed of one or two major osmolytes accompanied by minor osmolytes [264]. Invertebrates are known to mainly employ free amino acids (FAAs) and methy-

lamines [332]. However, there is a strong methodological bias towards FAAs in the osmolyte literature, disregarding methylamines and methylsulfonium compounds [264]. Yet, these substances can make up the majority of the organic osmolyte pool [163, 254, 262]. The use of non-targeted analysis methods, such as HPLC, GC-mass spectrometry, or NMR spectroscopy is, hence, crucial.

The importance of organic osmolytes for long-term salinity acclimation has been demonstrated for many species and taxa [262, 308]. Nevertheless, approximately half of the intracellular osmolyte pool consists of inorganic ions and this part of the osmolyte pool is poorly characterized [61, 264]. A better understanding of how inorganic osmolyte concentrations are modulated during low salinity acclimation is important, as inorganic ion concentrations also influence many intracellular processes. Inorganic osmolytes were often considered to be utilized exclusively during short-term responses to salinity stress [275]. However, several studies suggested that inorganic osmolytes play an important, yet understudied, role in long-term acclimation as well [267, 296, 382]. Overall, both, the inorganic and organic shares of the total osmolyte pool have to be quantified.

While some mechanisms of CVR are relatively well established, it is unclear, whether unifying mechanisms of salinity tolerance exist in marine invertebrates. Osmolyte pool size, as well as pool composition have been suggested to be key factors in determining species salinity acclimation limits, but have not been formally tested across a broader taxonomic range [24, 162, 249]. Podbielski et al. [262] introduced the concept of critical salinity (S_{crit}), suggesting that fitness (reproductive ability, long-term survival) becomes zero when the organic osmolyte pool is fully depleted, with the implicit assumption that the cellular inorganic osmolyte pool is not modulated strongly. This concept has been successfully applied to a cnidarian and an echinoderm species [262, 308]. However, tissue inorganic ions have not been determined in these studies.

Here we tested, whether the S_{crit} concept is applicable for species from a broad taxonomic range and whether inorganic osmolytes play a substantial role during acclimation to similar experimental salinity regimes. We focused on long-term low salinity acclimation of benthic osmoconformers of the Baltic Sea. We chose six study species from three phyla that fulfill important ecological roles, exhibit a wide geographic distribution and differ in their tolerance of low salinity. These species were: a bivalve and a gastropod (Molluscs: *Mytilus edulis*-like, *Littorina littorea*), a sea anemone (Cnidaria: *Diadumene lineata*) as well as a sea star and two sea urchin species (Echinoderms: *Asterias rubens*, *Psammechinus milliaris*, *Strongylocentrotus droebachiensis*) to determine differences in salinity tolerance mechanisms. They will hereafter be referred to as mussels, snails, sea anemones, sea stars, shore sea urchins and green sea urchins, respectively. We investigated effects of low salinity acclimation on fitness proxies and tissue osmolytes. Specifically, we hypothesized that:

i Tissue organic osmolyte concentrations decrease during acclimation to low salinity.

ii Tissue inorganic osmolyte concentrations decrease during acclimation to low salinity. More specifically, the concentrations of the main monovalent inorganic osmolytes (i.e. sodium and chloride) decrease in tissues, while potassium and calcium concentrations remain constant.

iii Organic osmolyte composition differs between taxa.

iv The critical salinity concept is applicable for all investigated species.

This is the first study to investigate the complete osmolyte pool in a variety of marine invertebrates following long-term acclimation to low salinity using comparable approaches.

3.2 Results

3.2.1 Survival

Following a four-week acclimation interval to seven different salinity treatments, we observed a significant decrease of survival of sea stars, snails, mussels and green sea urchins (Tab. B.1). In mussels, the decrease in survival occurred gradually between salinities 10 to 5. Sea star survival fell abruptly from 100% to 0% between salinities 12 and 10. Animals at salinity 11 had a low body turgor and appeared moribund. The survival of snails steeply declined from salinity 8 to 6. Green sea urchin survival decreased to 67% at salinity 17, whereas no shore sea urchins died during the experimental time period. Survival of sea anemones was not impacted by low salinity, either. However, asexual reproduction rate decreased to zero at salinities of 10 (Fig. 3.1, 3.2).

3.2.2 Growth

Despite being fed *ad libitum* in all treatments, growth decreased significantly with a reduction in salinity (Tab. B.3). Thresholds of zero weight gain and consecutive loss of biomass were located at salinities of 5.6, 9.3, 12.7, 14.6, 14.7 and 20.9 for mussels, sea anemones, sea stars, snails, shore sea urchins and green sea urchins, respectively (Fig. 3.1, 3.2). A significant increase of total wet weight (WW) was observed for all species at higher salinity treatments with an attenuation of growth observed for mussels, sea anemones, sea stars and shore sea urchins.

3.2.3 Water Content

Reduction of salinity significantly increased tissue water content of all species, except for sea stars (Tab. B.2). In molluscs, water content was constant at high and medium salini-

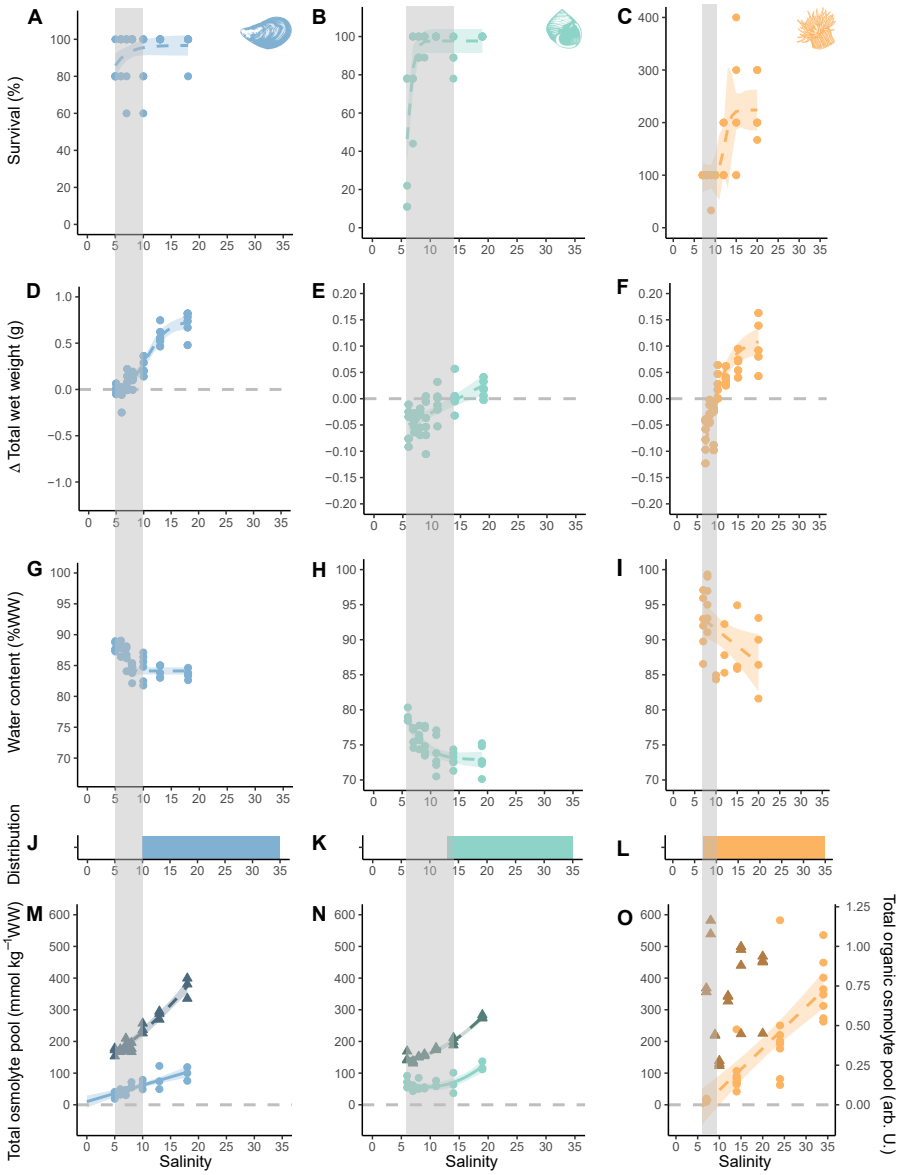

Figure 3.1: Effect of long-term low salinity acclimation on survival, growth, water content, distribution and osmolyte pool size on mussels, snails and sea anemones. Salinity is depicted on the x-axis. On the y-axis survival is presented in %, change in total wet weight in g, water content in % wet weight and osmolyte pools in mmol kg^{-1} wet weight. Osmolyte pools were corrected for relative weight change due to increase in water content with decreasing salinity. The inorganic pool is depicted by triangles and the organic pool by circles. No organic osmolytes were measured for sea anemones, instead osmolyte data from a previous study is shown [262]. Colored bars represent the species' known salinity range in the Baltic Sea. Models were fitted to raw data points for seven treatment levels. If no model is shown, there was no significant model. Models were chosen according to best fit. Model outcomes are listed in Tab. B.1 - B.4. Shaded areas represent a 95% confidence interval. Four treatment levels were clustered around the lower salinity threshold of each species, two levels represented intermediate salinity and the highest one was close to control conditions. Species are marked by color code and symbol: blue - mussels (*Mytilus* sp.), turquoise - snails (*Littorina littorea*), orange - sea anemones (*Diadumene lineata*). Grey vertical bars represent the critical salinity range derived from the salinity thresholds of the complementing measurements of physiological and biochemical biomarkers.

ties, but increased at a salinity threshold of 8 for mussels and 11 for snails. Water content increase was continuous for both sea urchin species and sea anemones. In sea stars, water content was constant above salinity 11 but decreased abruptly below that threshold.

3.2.4 Total osmolality and total osmolyte pool sizes

Tissue osmolality significantly declined in tissues of mussels, snails, sea anemones and sea stars with decreasing salinity (Fig. B.1). Total osmolality in mussels and snails declined linearly, whereas in sea stars it stabilized at very low salinity. Sea anemone tissue osmolality was constant across a wide salinity range but dropped at a salinity threshold of 10. No significant change in osmolality was detectable in the two sea urchin species.

Overall, the concentration of inorganic osmolytes was higher than that of the organic osmolyte pool in all investigated species, besides shore sea urchins. Total inorganic osmolyte concentration decreased linearly with salinity in the tissues of sea stars, whereas a stabilization occurred for mussels and snails at lower salinity (Fig. 3.1, 3.2, Tab. B.4). The concentration of organic osmolytes declined significantly with decreasing salinity in mussels, snails, sea anemones, sea stars and green sea urchins (Tab. B.4). The S_{crit} of mussels, sea stars and green sea urchins was <0, 10 and 14, respectively, as could be derived from extrapolating the linear model until the organic osmolyte concentration reached zero. The S_{crit} of sea anemones from a previous study was located at salinity 6.5 (Fig. 3.1) [262]. S_{crit} could not be estimated in of the other species as the relation was not linear (i.e. snails), not significant (i.e. shore sea urchins), or extrapolation results were negative (i.e. mussels).

3.2.5 Organic osmolytes: pool composition and major osmolytes

In total, 60 compounds were identified (Fig. B.2, Tab. B.6, Tab.B.7, Tab.B.8) by metabolic profiling. The concentration of 39 solutes declined significantly with decreasing salinity

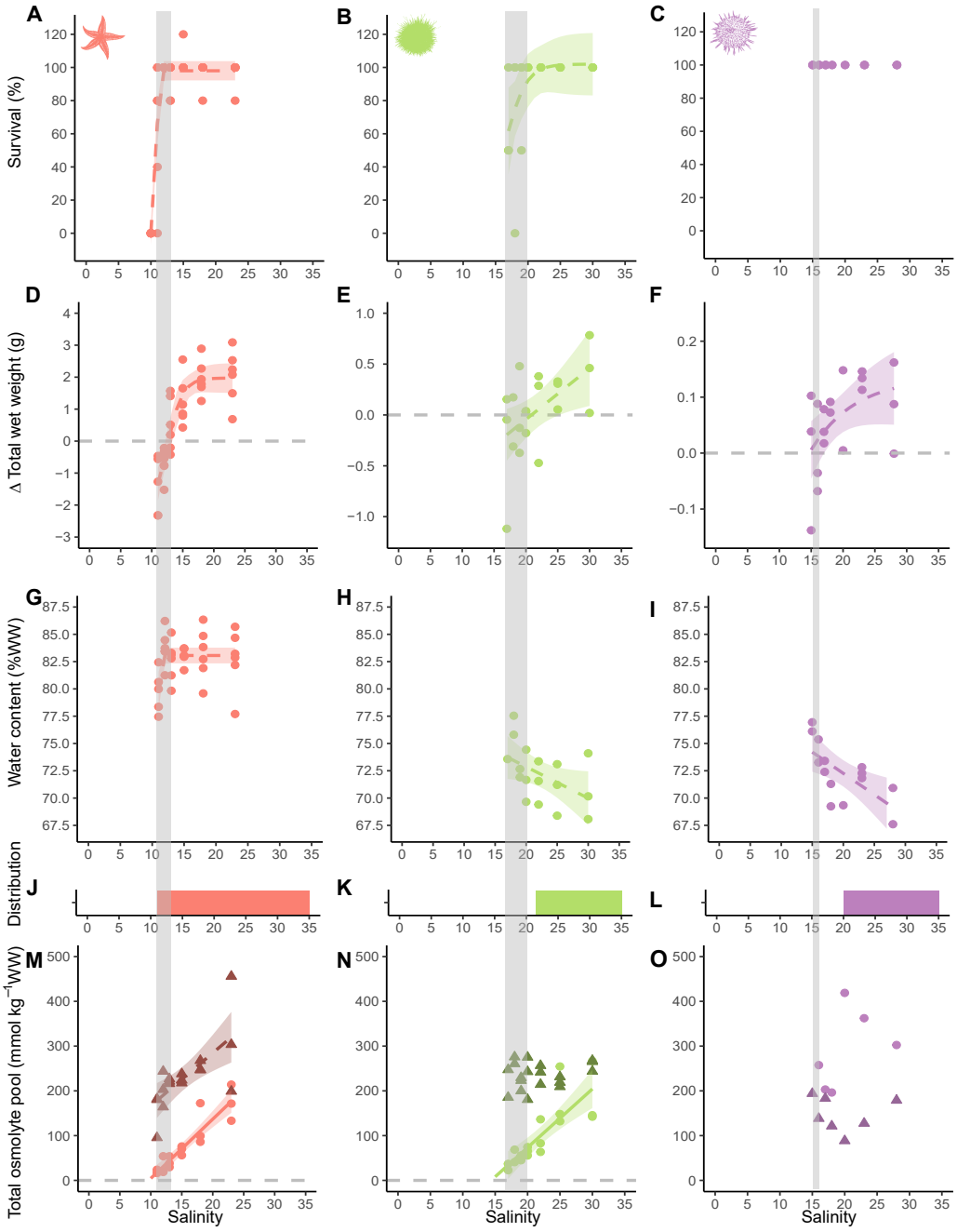

Figure 3.2: Effect of long-term low salinity acclimation on survival, growth, water content, distribution and osmolyte pool size on sea stars, green sea urchins and shore sea urchins. Salinity is depicted on the x-axis. On the y-axis survival is presented in %, change in total wet weight in g, water content in % wet weight and osmolyte pools in mmol kg^{-1} wet weight. Osmolyte pools were corrected for relative weight change due to increases in water content with decreasing salinity. The legend and further graph descriptions are addressed in Fig. 3.1. Species are marked by color code and symbol: red - sea stars (*Asterias rubens*), green - green sea urchins (*Strongylocentrotus droebachiensis*) and purple - shore sea urchins (*Psammechinus milliaris*). Grey vertical bars represent the critical salinity range derived from the salinity thresholds of the measurements of physiological and biochemical markers.

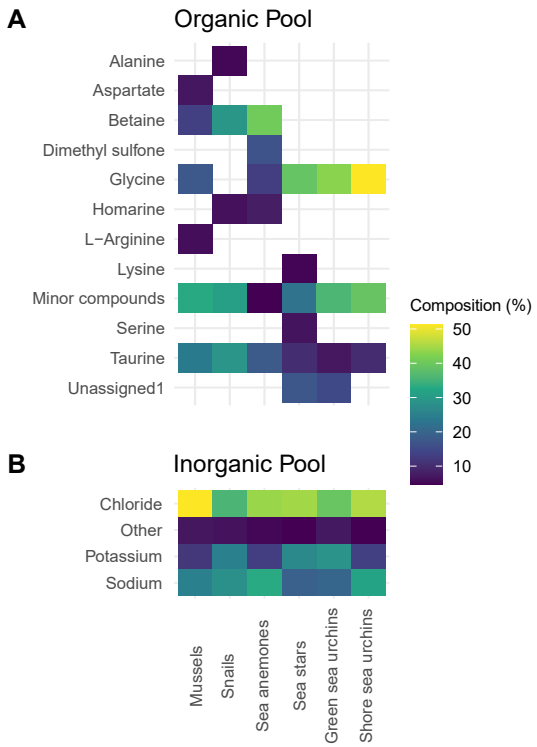

Figure 3.3: Composition of the total organic and inorganic compound pool in tissues. Depicted are the major compounds (>5%) in tissue extracts of the study organisms at the high salinity treatment: snails (*L. littorea*), mussels (*Mytilus* sp.), sea anemones (*D. lineata*), shore sea urchins (*P. milliaris*), green sea urchins (*S. droebachiensis*) and sea stars (*A. rubens*). Organic osmolyte data was retrieved from a previous study [262]. Color of field represent the percentage of the compound in the total pool of measured compounds. If no data is shown for certain species that means that the percentage of this substance is <5%.

and were thus considered osmolytes (*sensu* [332]). The major osmolytes that had a substantial contribution to the total organic osmolyte pool were glycine (echinoderms), betaine (sea anemones and mussels) and betaine and taurine (snails) (Fig. 3.3). The concentration of most of the identified osmolytes was minor (<5% of the total organic pool; Fig. B.2).

Salinity effects on individual osmolytes were not always consistent between species (Fig. 3.4, Tab. B.9). Glycine was one of the few osmolytes to be detected in all species and was the major osmolyte in echinoderms. We found methylamines such as betaine or homarine to be mollusc-specific osmolytes and to reach a constant level at low salinity. Taurine

was a major osmolyte in sea stars and molluscs. Intermediate osmolytes were often species-specific, such as glutamate in green sea urchins, aspartate in mussels, or lysine in sea stars. Serine was specific to echinoderms. Alanine was an intermediate organic osmolyte in echinoderms and snails. Overall, the organic osmolyte pool consisted of 25%, 43% and >60% methylamines in snails, mussels and sea anemones, respectively [262]. On the contrary, methylamines were negligible and FAAs constituted the main portion of the organic osmolytes in echinoderms (78-90%).

3.2.6 Inorganic osmolytes: pool composition and major osmolytes

The main inorganic osmolytes we identified were chloride, potassium and sodium (Fig. 3.3, Fig. B.3, Tab. B.5). The total inorganic ion pool in tissues was chiefly accounted for by sodium and chloride (60-80%) in all species. We observed a significant effect of salinity on tissue chloride concentration in all species except in shore sea urchins (Fig. 3.4). The relationship was linear in sea stars and values were constant at low salinities in mussels, snails and green sea urchins. Similar patterns could be observed for sodium and potassium. Tissue sodium and potassium concentrations changed significantly with salinity in snails, sea stars and mussels - but not in sea urchins. Sodium concentrations were constant at low salinity in mussels, snails and sea stars. Tissue potassium concentration was less strongly impacted by salinity in mussels, snails and sea stars when compared to chloride or sodium. In sea anemones, chloride, sodium and potassium concentrations decreased initially with decreasing salinity until an inflection point at salinity of 10 was reached after which concentrations increased. Concentrations were corrected for relative weight changes resulting from tissue hydration (Fig. 3.1).

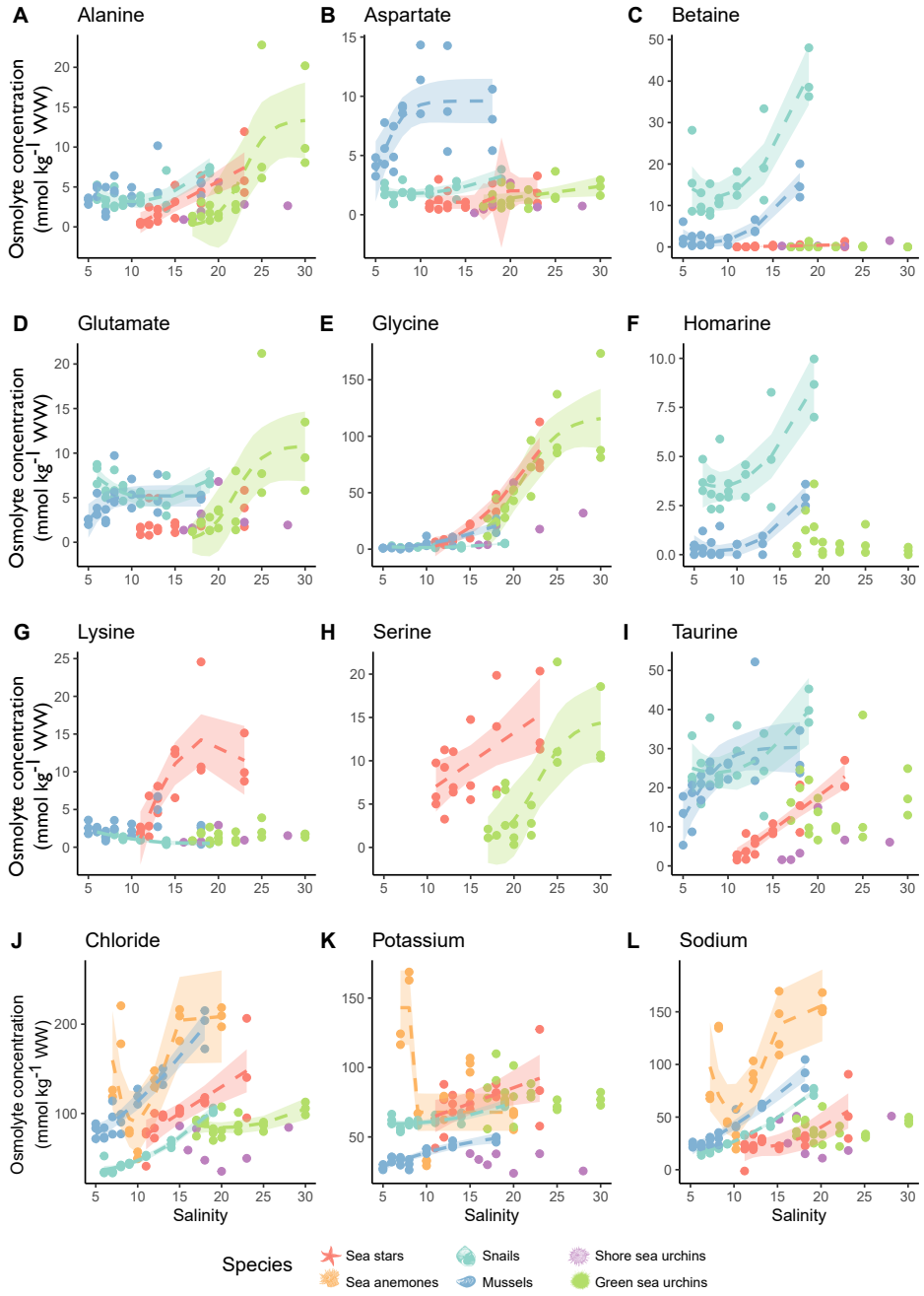

Figure 3.4: Salinity effect of the major inorganic and organic osmolytes. Depicted are the main osmolytes in tissues of the six study organisms: mussels (blue, *Mytilus* sp., snails (turquoise, *L. littorea*), sea anemones (orange, *D. lineata*), shore sea urchins (purple, *P. milliaris*), green sea urchins (green, *S. droebachiensis*) and sea stars (red, *A. rubens*). Osmolyte concentrations were corrected for relative weight changes due to an increase in water content with decrease in salinity. Concentration of the respective osmolyte is shown on the y-axis (mmol kg^{-1} WW) and salinity is depicted on the x-axis. Models were chosen according to best fit and their predicted values are depicted by lines. Only significant models were shown. If no data is shown for certain species that means that this substance was not detected. If only data points are shown without a model, that mean that there was no significant model. Confidence intervals (95%) are shown as shaded area. No organic osmolytes were measured for sea anemones. For model results see Tab. B.9.

3.3 Discussion

3.3.1 Fitness proxies and volume regulation capacity

The applied salinity stress impacted the tested invertebrate species on different levels. The first physiological sign of low salinity stress was a reduction of net growth rates, with negative values at very low salinities. Cessation of growth at low salinities indicates severe salinity stress and an unsustainable energetic state where costs for osmoregulation are too high to allow for allocation of surplus energy into anabolic processes [171]. Below such growth-abolishing salinity levels, energy reserves and tissue have to be catabolized to meet energetic demands. This threshold towards negative growth was located close to salinities (within $\pm 0 - 1.5$) that caused mortality and a change in tissue water content in all species examined here. Changes in cellular water content have previously been hypothesized to indicate that an organism has reached its volume regulation capacity limit [175]. At this point, species cannot further acclimate to low salinity by continued depletion of the osmolyte pool and cellular water content increases. Tissue water content increased in all tested species at low salinities, indicating an impaired volume regulatory ability.

Growth rates were similar or higher compared to literature data in all investigated species [171, 210, 220, 237, 262, 308].

Water content was similar to previously reported values in mussels, snails, sea anemones, green sea urchins and shore sea urchins [37, 130, 173, 313, 382]. We did not observe an increase in water content with decreasing salinity in sea stars, similar to the other species. Instead, the tissue water contents for sea stars were observed to decrease at a salinity of 11 in this experiment, which coincided with a loss of body turgor. Yet, unpublished results showed a steep increase in water content of sea stars at salinities of 11 after a shorter acclimation period of two weeks (Fig. B.4). This indicated that sea star volume regulation

capacity operates over a wide range of salinities until a limit is reached. This limit cannot be tolerated for prolonged periods and thus was only visible under shorter acclimation times. The general response of water content in relation to salinity was thus similar for all investigated species. In sea urchins the relation between water content and salinity was linear. A potential increase in tissue hydration around a threshold salinity of 20 for the green sea urchin and 18 for the shore sea urchin could not be resolved due to a high variance and low level of replication.

Survival rates in this study corresponded to salinities observed at geographic distribution boundaries and/or were similar to published mortality rates for sea stars, green sea urchins, sea anemones and mussels [173, 308, 313, 337]. Although none of our sea anemones died under the applied salinity treatments, the observed cessation of asexual reproduction already at salinity 10 was slightly higher than found previously (salinity 7) [262]. Snails, however, were more tolerant to low salinity during our experiments (salinity 8) than it was found in older studies (salinity limit of 10-12.5) [148]. While we observed no mortality of shore sea urchins even at the lowest applied salinities of 15, in the field, the distribution limit of shore sea urchins has been reported between salinities of 17 and 20 [98].

3.3.2 Cellular osmoregulation

Organic osmolyte concentrations in tissues decreased following long-term low salinity acclimation in all species, except in shore sea urchins, confirming hypothesis 1. This decrease was linear in sea anemones, green sea urchins, sea stars and mussels, whereas it stabilized at low salinity in snails (Fig. 3.1, 3.2, [262]).

Our second hypothesis, namely that tissue inorganic osmolyte concentrations decrease after long-term low salinity acclimation, can be accepted for mussels, snails and sea stars and has to be partially rejected for green sea urchins. Tissue ion concentrations in mussels

decreased with declining salinity, but stabilized at salinity $\leq$ 8, indicating hyperosmotic regulation at low salinity. Similarly, inorganic ion concentration in snail tissues revealed stabilized concentrations at low salinity, indicating hyperregulation. Hyperosmotic intracellular regulation was proposed to occur in mussels acclimated to low salinity [382]. In order to measure this, fine-scale monitoring is needed, but the rare inorganic osmolyte studies were not designed to detect this pattern [267, 316]. This exposes a huge research gap for fine-scale monitoring of inorganic osmolyte profiles of osmoconforming invertebrates.

In green sea urchins, inorganic osmolyte concentrations were stable across salinity treatments. Inorganic osmolytes do not seem to play a role in green sea urchin CVR. In general, tissue inorganic ion concentrations are higher than intracellular values due to the influence of the extracellular fluids. However, consideration of the inorganic ion contribution of the extracellular space does not change the overall results substantially. To do so, we calculated the maximal ECS contribution, compared this to literature values and conservatively estimated a likely ECS fraction (Appendix B.1: Estimation of the intracellular inorganic pool by calculating the likely ECS fraction). Even when factoring in maximal ECS fractions, the inorganic osmolyte concentration was still significantly affected by salinity in mussels, snails and sea stars and inorganic osmolyte pool size remained larger than the organic share (Fig. B.5).

Organic and the inorganic osmolyte pool played important roles in cellular osmoregulation in molluscs and sea stars, whereas in green sea urchins, only the organic pool was involved. Furthermore, the higher inorganic osmolyte concentration (compared to the organic pool) implied an even higher importance of the inorganic pool for the CVR process in molluscs and some echinoderms. Only three other studies are available, (exclusively assessing molluscs) that measured inorganic as well as organic compounds after long-term low salinity acclimation. Similar to our results, these studies found significant effects of

salinity on both osmolyte pools and a larger inorganic pool size [267, 316, 358].

3.3.3 Organic osmolyte concentration

Overall, we observed three recurrent patterns of individual organic osmolyte concentration changes with salinity. First, for some osmolytes (e.g. intermediate osmolytes in sea stars) we detected a steady decrease of osmolyte concentration with decreasing salinity, which agrees well with previous studies ([308], Fig. 3.4). A second pattern we observed was a decline of organic osmolyte concentrations with declining salinity that eventually stabilized - indicating hyperregulation - at low salinity. This pattern was very prominent for the major organic osmolytes betaine (molluscs) and glycine (sea stars; Fig. 3.4) and is also consistent with literature results [65, 296]. Third, the major osmolytes glycine in green sea urchins and taurine and mussels reached maximum concentrations already at intermediate to high salinity. A similar saturation pattern for taurine concentrations was found in bivalves [163]. At lower salinities the third pattern can either show a linear decrease as described in the first pattern (e.g. taurine or aspartate in mussels), or concentrations can stabilize as described in pattern 2 (e.g. aspartate and glutamate in green sea urchins).

Furthermore, a selective utilization of intermediate organic osmolytes below a certain salinity threshold was observed. This indicates that under severe salinity stress a wider variety of organic osmolytes is modulated, whereas under moderate stress only major osmolytes are utilized. Under moderate salinity stress, intermediate osmolytes occurred at low constant concentrations and were not accumulated further at higher salinity. The reason for this could be that many minor osmolytes are intermediates in metabolic pathways that generate major osmolytes - and are thus converted instead of being accumulated [79, 258, 370]. We propose that the examined Baltic Sea invertebrates prioritize a reduction of major osmolytes during low salinity acclimation and only resort to intermediate osmolytes when

salinity stress progresses.

The taxon-specific differences in osmolyte composition (for example methylamines) between echinoderms and molluscs confirmed our third hypothesis. This is consistent with a recent meta-analysis about taxonomic variation of the salinity effect on osmolyte concentrations in tissues of osmoconformers [264]. Our study highlights methylamines as an important part of the organic osmolyte pool in marine invertebrates. However, a large deficit of studies utilizing untargeted metabolic profiling leads to a severe under-representation of methylamines in the osmolyte literature [264].

Within the echinoderms studied, both sea urchin species had an almost identical organic osmolyte composition. The organic osmolyte pool of the sea star was only slightly different in its composition to that of the two sea urchin species. All three species utilized glycine as main osmolyte and serine as accompanying intermediate osmolyte. Previous studies detected similar concentrations for glycine, but smaller values for serine and other minor osmolytes [308]. Sea stars additionally employed taurine and lysine as intermediate osmolytes, the latter of which we only found in sea stars. Sea urchins were fed with algae, while sea stars were fed with mussels that contain a high taurine concentration themselves, which might explain small differences in osmolyte composition. While osmolytes are generated *de novo* [62], diet can also affect osmolyte composition. Amino acids have been shown to be taken up via ingestion of food in green sea urchins, and tissue lipid content was affected by diet in shore sea urchins [44, 178].

Between the two tested molluscs, we found larger differences in osmolyte composition when compared to the variation among echinoderms. The largest difference was observed for betaine. Snails utilized betaite osmolyte. In contrast, glycine concentrations were higher in musne as a major osmolyte, whereas in mussels, betaine was an intermediasels compared to snails, thus substituting betaine. This is consistent with some previous studies on

molluscs [130, 296]. However, utilization of high concentrations of betaine has been observed in a closely related mussel species (*Mytilus californianus*) [316]. Moreover, glycine is a product of betaine catabolism [370]. Osmolyte pathways are linked and changes in these metabolic processes could also be responsible for these observed differences [370]. Also betaine, has been shown to have other cellular functions: it is involved in regulation of enzymes involved in energy metabolism, lipogenesis or beta-oxidation or regulation of transcription factors [88]. Thus, differences in betaine osmolyte concentrations between species could indicate differences in these metabolic pathways.

Why taxonomic group-specific osmolytes (such as methylamines in molluscs) have evolved is yet unclear. Our results demonstrate the presence of betaine in high concentrations in molluscs. While we cannot infer the source from that data, betaine biosynthesis has been demonstrated in *Mytilus galloprovincialis* [62]. In contrast, echinoderms, do not seem to employ betaine in large concentrations albeit the availability of a genomic blueprint for betaine synthesis and precursor uptake via transporters [233]. Choline is a precursor of betaine. Genes coding choline transporters, responsible for choline uptake, as well as enzymes for betaine synthesis (choline dehydroxygenase, betaine aldehyde dehydrogenase, choline oxidase, glycine-sarcosine methyltransferase and sarcosine dimethyl transferase) are present in echinoderms [104, 233].

Generally, methylamines are considered stronger protein stabilizers than non-methylated osmolytes such as glycine [394]. Thus, the use of methylamines in euryhaline mussels and snails could be an adaptation to the low saline conditions of their environment, whereas habitat osmolality is usually higher for the typically more stenohaline echinoderms. The sea star examined in this study is one of the few euryhaline echinoderms. The only notable difference between sea urchins and sea stars were the larger concentrations of intermediate osmolytes (such as alanine, aspartate, lysine, ornithine, serine, etc.) at low to medium

salinities and the use of taurine as an osmolyte in sea stars.

3.3.4 Inorganic osmolyte concentration

Our second hypothesis, stating that tissue chloride and sodium concentrations decrease with decreasing salinity, can be accepted for mussels, snails, sea stars and sea anemones. In green sea urchins, only tissue chloride concentrations were significantly decreased following acclimation to low salinity. Our hypothesis that constant tissue calcium and potassium concentrations are maintained across salinity treatments, can only be accepted for green sea urchins.

In our study, the monovalent ions chloride and sodium were the main inorganic osmolytes involved in long-term low salinity acclimation, which is in line with other studies using mollusc model organisms [24, 267, 296, 316]. However, not all studies detected a salinity effect on tissue monovalent inorganic osmolyte concentrations. In the mussel *Perna perna*, no changes in cellular chloride or sodium concentration were detected [336]. The effect of salinity on potassium concentrations is however controversial. Our results for mussels, snails and sea stars revealed a significant change in tissue potassium concentration acclimated to different salinities, which is corroborated by other bivalve studies [267, 336, 382]. Yet, usually potassium is assumed to be highly regulated in the intracellular space [238], which is also supported by other mollusc studies [24, 296, 316].

Significant changes of other, lower concentrated, tissue inorganic ions (Tab. **??**) may also be of relevance. Ions like calcium and bromide affect organisms by limiting many biological processes, ranging from calcification to cytoprotective functions [33, 297]. Overall, however, our results highlight, the importance of the monovalent ions chloride and sodium as inorganic osmolytes in long-term salinity acclimation processes.

3.3.5 Critical salinity concept

We previously defined a critical salinity (S_{crit}) as the lower salinity threshold at which the organic cellular osmolyte pool and fitness (proxies: mortality, growth) are reduced to zero and postulated (hypothesis 4) that S_{crit} can be identified in all experimental species (5, Fig. 3.5). We could not confirm this hypothesis for any of the investigated species. In contrast, we discovered that organic osmolyte pools in most animals were not yet fully depleted when severe loss of fitness occurred (Fig. 3.5). The previously reported S_{crit} concept for sea anemones [262] and sea stars [308] is thus not transferable across all invertebrate phyla. In green sea urchins, sea anemones and sea stars, the determined S_{crit} was lower than the threshold we would have expected from fitness proxies and volume regulation capacity. In mussels, the negative S_{crit} values are biologically nonsensical and thus the concept was not applicable. In snails, or shore sea urchins, the concept was not applicable to organic osmolyte data.

In snails, the original S_{crit} concept was not applicable as organic osmolyte concentrations stabilized below a lower salinity threshold, indicating hyperosmotic regulation. Similar patterns could be seen for individual osmolytes in the other invertebrates when fitness was reduced (i.e. betaine and homarine in mussels; alanine, glutamate and serine in green sea urchins; glycine in sea stars). Hyperregulation is an energetically cost-intensive, time-limited tolerance process [290]. In mussels, modeling S_{crit} from osmolyte concentrations was not meaningful as a S_{crit} <0 would be calculated, a phenomenon that was also observed in a previous mussel study [296].

A shortcoming of the original concept was its focus on organic osmolytes (Fig. 3.5). This study has, indeed, demonstrated the significant involvement of the inorganic osmolyte pool in active CVR. An altered concept therefore has to include inorganic osmolytes in

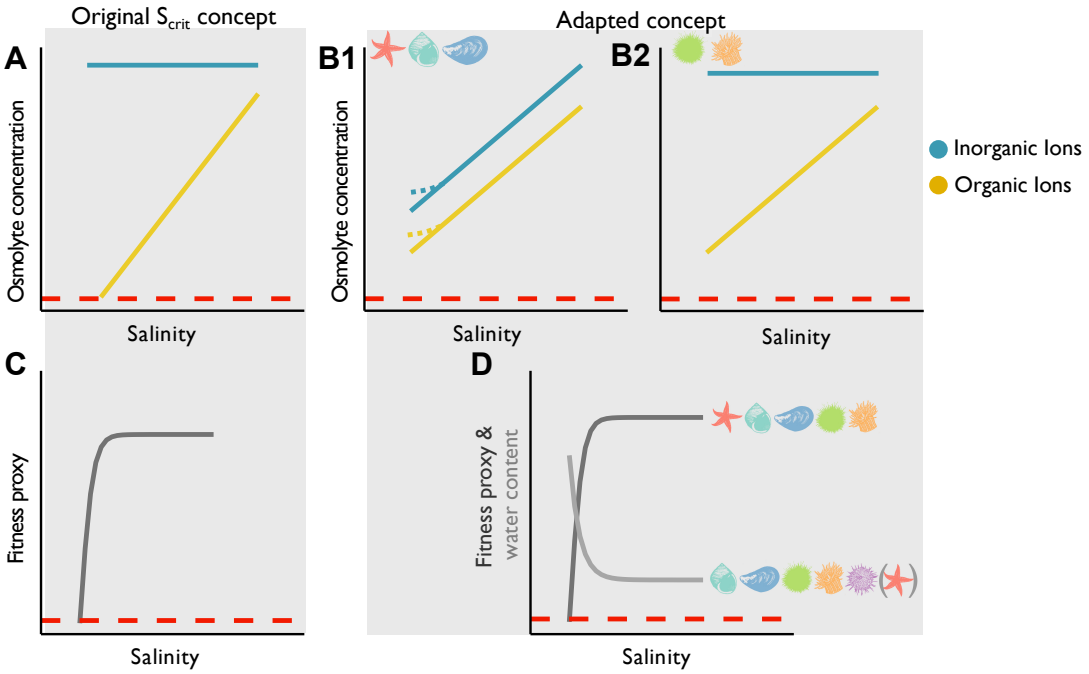

Figure 3.5: Depicted are hypotheses on how salinity affects the osmolyte pool in combination with idealized fitness parameters and capacity for cellular volume regulation. A) the original S_{crit} concept for the total organic osmolyte pool. A linear decrease of organic osmolytes (yellow) is shown until the organic osmolyte pool is fully depleted (red line) while inorganic ion concentration (blue) is constant. S_{crit} is defined as the point when the organic osmolyte pool is depleted. Figure B) shows an altered concept of two scenarios that show a S_{crit} range which incorporates the inorganic (blue) and organic osmolyte pool (yellow). In scenario B1 both pools decrease with salinity (in some species these concentrations stabilize at low salinities), but concentrations do not reach zero (red line). Scenario B2 shows a decrease with salinity in the organic pool, but concentrations do not reach zero (red line), while the inorganic pool remains constant. C) Depicted is a fitness proxy (e.g. survival; dark grey) in relation to salinity, which according to the S_{crit} concept reaches zero when the organic osmolyte pool is depleted. D) Here we see a fitness proxy that drops rapidly after a low salinity threshold is reached (i.e. survival), meanwhile tissue water content (light grey) increases below this limit. The species symbols in figure 5B and 5D symbolize if the suggested concept applies to the investigated species in this study.

long-term acclimation processes to low salinity (Fig. 3.5). There does not seem to be a universal concept based on osmolyte concentrations that defines the lower salinity threshold of species. Rather, the combination of multiple parameters characterizes the mechanistic basis of the lower salinity threshold of study organisms.

For snails the salinity threshold derived from reduced fitness and inorganic and organic osmolyte hyperregulation matched perfectly. A clear tipping point was apparent in snails with a salinity of 14, where biomass is catabolized, volume regulation capacity was impaired, survival was impacted and inorganic and organic osmolyte pools stabilized. While snails can endure lower salinities of 6-13 for some time, living under such conditions will not be sustainable. These findings roughly match known species distribution limits at a salinity of 10-12.5 [148].

In mussels, we saw a clear tipping point at salinity 10 where tissue water content sharply increased and survival declined and the major organic osmolytes betaine and taurine had their inflection points. At salinity $\leq$ 7, this increase stagnated and water content as well as inorganic and organic osmolyte concentrations stabilized. Biomass was not catabolized until salinity dropped below 6. Another study (with individuals of similar size) found an increase of mortality only at salinities $\leq$ 5 [296]. Altogether, a critical salinity range of 7-10 can be suggested, in which a majority of mussels can still survive, yet with energetic trade-offs due to costs for cellular osmoregulation that affect growth, which was demonstrated by a 64% reduction in growth. While they can, hence, tolerate lower salinities under laboratory conditions, *M. edulis*-like genotypes (as used in our study) are replaced in the field by *M. trossolus*-like genotypes already at salinities <10 [152, 337]. The distribution limit of *M. edulis*-like mussels in the Baltic Sea is therefore defined by *M. edulis*' physiological limits.

The fitness parameters, mean water content, concentrations of inorganic osmolytes chloride and sodium as well as the inflection point in total osmolality of sea anemones matched

perfectly to suggest a salinity limit of these animals at 10 (Fig. 3.1). Previous studies on organic osmolytes in sea anemones, however, showed a S_{crit} at salinity 6.5 [262]. No mortality was observed for sea anemones at the measured salinities, but when net growth was zero, asexual reproduction of sea anemones ceased. Thus, despite short-term survival at lower salinities, the long-term lower salinity threshold that permits growth and reproduction of sea anemones appears to be located at salinities around 10. This also agrees with distribution limits of sea anemones in the field [262].

While our calculated S_{crit} values for sea stars (S_{crit} = 10) agreed with literature data [308], S_{crit} was lower than could be expected from fitness proxies. Catabolism of biomass was associated with decrease in survival of sea stars at salinities $\leq$ 13. Furthermore, while tissue total osmolality stabilized at salinities $\leq$ 13 (Fig. B.1), this pattern was not apparent in the inorganic or organic osmolyte pool (Fig. 3.2). However, a closer look at the main organic osmolyte glycine demonstrated stabilizing concentrations at salinities $\leq$ 13 (Fig. 3.4). We can, hence, corroborate a low salinity threshold between salinities of 12 and 13, which was published before [46, 308].

As in sea stars, the S_{crit} = 14.4 calculated for green sea urchins was lower than expected from net growth and survival, which both decreased at salinity 20. No threshold was visible in water content, total organic osmolyte pool and the major osmolyte glycine, while the inorganic ion pool remained constant. Yet, at a salinity of 20, a stabilization of some minor organic osmolytes was observed, agreeing well with the performance fitness parameters. Thus, in green sea urchins no clear salinity threshold was indicated by osmolyte data, whereas fitness parameters proved more useful. The fitness threshold at salinity 20 corresponds well with the distribution limit reported for North Sea populations that occur down to a salinity of 21.5 [173].

Responses in for physiological parameters in shore sea urchins appeared to be simi-

larly associated (even though a low number of replicates hindered the statistical analysis of osmolytes): zero net growth and increasing tissue water content indicated a low salinity threshold at 15, even though survival was not impacted yet. As seen for sea stars, once a tipping point is reached, the survival of marine animals suffering from desalination can decline very rapidly. We hence expect the threshold for mortality to be located close to the lowest salinity treatment (salinity = 15) tested in our experiment. In the field, the distribution limit of shore sea urchins has been reported between salinities 17 and 20 which is higher than could be expected from our data [98].

3.4 Conclusion

Overall, we found that low salinity acclimation had severe effects on the physiological performance and osmotic system of six Baltic Sea invertebrate species.

i We were able to demonstrate that the total organic osmolyte pool was dynamically modulated by changes in salinity in mussels, snails, sea stars and green sea urchins.

ii We detected significant changes in tissue inorganic osmolyte pools in mussels, snails, sea stars and sea anemones. These were mainly driven by modulation of monovalent ion concentrations (sodium, chloride) and less so potassium and calcium.

iii Taxon specific differences in osmolyte composition between studied Echinodermata and Mollusca were mainly manifested in the utilization of methylamines by molluscs (main osmolytes betaine taurine) and the restriction to FAAs in echinoderms (main osmolyte glycine).

iv Lastly, we found the S_{crit} concept *sensu* Podbielski et al. [262] not applicable as osmolyte pools, albeit being reduced with salinity, were not fully depleted at low salin-

ity. Organic osmolyte content alone cannot predict a species' lower tolerance limit. Fitness parameters and osmolyte profiles were found to be linked. However, the relationships are not simple and mechanisms are not uniform between species.

While we found that all species can endure reduced salinity over a certain range, we determined clear physiological limits. Our results nicely show that the low salinity threshold marks a critical salinity range (rather than one specific point). Here, fine-scale monitoring around the anticipated lower salinity threshold is needed to detect the stabilization of osmolyte levels as well as an increase in water content reliably. With respect to future changes in salinity we thus hypothesize that all species studied here will experience a shift in species distribution with the projected salinity changes. Further, fitness of local populations in high salinity environments may be reduced in response to the lowered salinity conditions in the future. As the salinity effects found in this study were species-specific the use of a universal concept is not supported. We propose an alternate comprehensive approach that includes fitness parameters, water content and osmolytes (Fig. 3.5), to understand how distribution limits are determined and identify the mechanisms that control salinity tolerance.

A recent meta-analysis highlighted large gaps of knowledge with respect to intracellular osmolyte concentrations across invertebrate taxa [264]. Research in the field of salinity tolerance, especially cellular osmoregulation, is essential, and certainly needed, to establish reliable physiological limits of species in order to estimate consequences of future salinity changes with ongoing climate change. It is important to assess the salinity tolerance capacity in euryhaline species to obtain a better understanding of the basic mechanisms that are utilized in a wide range of species. It is further crucial to establish cellular inorganic and organic osmolyte profiles that build a foundation for applied cellular physiological research,

for example for designing suitable buffers for *in vitro* assays as these buffers need to incorporate complex organic and inorganic osmolyte changes. Knowledge about cellular and whole-organism biochemistry and physiology is absolutely crucial for characterizing the functions of genes that are under selection by climate change stressors [211]. The Baltic Sea, with its natural salinity gradient and locally adapted populations, can be used to simulate future selection gradients to identify which cellular mechanisms and genes are modulated under persistent salinity reductions. This fundamental knowledge is necessary in order to assess whether there is potential for acclimation and rapid adaptation in changing oceans.

3.5 Material & Methods

3.5.1 Collection & Maintenance

Study organisms were collected in the western Baltic Sea from spring to autumn 2018. All organisms were transported within 1-5 hours (except for green sea urchins from the Kattegat: 2 days) to a climate chamber at GEOMAR, Kiel where they were cultured (Fig. B.6, Tab. B.10, Tab. B.11).

3.5.2 Experimental Set-up

Independent experiments were conducted for each species. After gradual salinity adjustment for 1-3 units day^{-1}, species were exposed to seven salinity treatments for four weeks (Fig. B.7). A minimum of 14 days are necessary to examine stable osmolyte pools [9]. Each tank was considered an experimental unit, with replicate numbers $n = 2 - 6$ (Fig. B.7, Fig. B.8). The number of individuals per tank was adjusted according to size and availability of the animals. In total, we maintained 168 tanks for a minimum duration of six

weeks. Physiochemical water parameters were recorded every 1 - 7 days to ensure high water quality (Tab. B.13). Water was changed depending on accumulation of nitrogenous waste products measured in randomly chosen tanks of the lowest and highest salinity treatment (Tab. B.12). Feeding was stopped a day prior to sampling. At the end of the experiment, two shares of samples were taken from individuals. The first set was used for dry weight measurement, the second one was processed for osmolyte analysis (Fig. B.8).

3.5.3 Fitness proxies & Tissue water content

We investigated fitness proxies (survival, growth) and volume regulation capacity to understand when low salinity stress became severe for our model organisms. Survival rate was utilized as a proxy to estimate critical salinity. Mortality was recorded on a daily basis. Dead individuals were removed from tanks and water was fully exchanged. At the end of the experiment, average survival per tank was compiled. As no mortality of sea anemones was detected in our experiment, instead of survival, asexual reproduction rate was used as an indicator for salinity stress of these animals. Asexually reproducing sea anemones that increased in numbers were expressed as percentages >100%.

Growth was measured as change in total wet weight (WW). All animals were weighed prior to the experiment. At the end of the experiment total WW was recorded. The difference between initial and final total weight (g) was used as measure for net growth. For dry weight (DW) determination, one set of samples was dried for 48-72 hours at 80°C and DW weighed (Fig. B.8). Molluscs tissues were removed from shells and weighed subsequently. Water content was determined using WW and DW values from the growth measurements at the end of the experiment expressed as: $\%WW = \frac{(WW-DW)}{WW \times 100}$

3.5.4 Inorganic Osmolytes & Total Osmolality

Next, we measured the concentrations of nine inorganic ions, as well as the total osmolality in body fluids and tissues. After non-invasive parameters were recorded, body fluids were retrieved from the second set of samples (Fig. B.8, Tab. B.11). Body fluids were centrifuged at 1000 rpm for 1 min. The supernatant was extracted and frozen in liquid nitrogen. Body fluid samples of the same individuals were used for total osmolality, cation and anions analysis. Tissues were shortly immersed in iso-osmotic sucrose solution and flipped over once to remove adherent seawater, blotted dry and snap-frozen in liquid nitrogen. The same tissue material was used for anion, cation, total osmolality and metabolic profiling.

Commonly, concentrations for each anion are titrated separately [267, 316]. We, however, established a novel protocol for ion extraction from tissue samples - involving a sufficient cleaning of proteins to measure anions in tissue extracts via ion chromatography. Our method omits chemical precipitation of proteins which generates a noise that overlaps with the targeted anion signals. If tissue extracts are measured without filters, proteins clog the IC-column eventually leading to increased maintenance costs. As a novel technique, we instead used a mechanical filtering step applying mini dialyzers which are usually used for protein/DNA purification. These filters concentrate the protein fraction of a sample extract via vertical membranes of a specific pore size (here 3K). Instead of keeping the protein fraction, we processed the filtrated (protein-free) fluid sample. We used reference standards (IAPSO Standard Seawater and Merck IC-multi element standard V) to verify that the anion concentration in a fluid sample does not change via this technique and that no additional signal was added. This is the first protocol to sustainably measure anions in filtered tissue extracts via ion chromatography. This method replaces the need for separately conducted measurements of anions via titration. It further allows for a shorter throughput

time and could also be used via auto-analyzer.

For anion, cation and total osmolality analysis, 500 mg powdered tissue samples were used. Material of different individuals from the same tank was pooled if necessary. MilliQ water was added in the smallest dilution possible (usually a 1:1 weight to volume ratio) to retrieve a final sample volume of 400 - 500 μL. The sample was then homogenized in a batch disperser (Ultra-Turrax, IKA, USA) attached to a VWR VDI 12 Homogenisator (IKA, USA) for 60 s and extracts freeze-thawed three times. Afterwards, samples were centrifuged (1500 g, 4°C, 5 min) and supernatants transferred to 3K dialyzers (Amicon Ultra Centrifugal Filter Devices, Merck, Germany). Samples were centrifuged (14000 g, 4°C, 20 min). The eluent was retrieved and used for subsequent analysis. For ion chromatography (Dionex ICS 2100, Thermo Fisher, Germany), fluid and tissue samples were diluted to a final volume ratio of 1:25 for salinity treatments <15 and 1:50 for salinity >15. A conductivity cell and a self-regenerating suppressor were used to reduce background conductivity. An IonPac AS11-HC column was used with potassium hydroxide (KOH) as eluent. We achieved separation with an isocratic flow rate of 0.6 mL min-1 15 mM KOH at 40°C for seawater and body fluid samples. For tissue samples, a ramp protocol was applied with 8 mM KOH increasing to 15 mM after 5.5 min with a flow rate of 0.5 ml min^{-1}. Anion concentrations were computed in mM relative to an IC-multi element standard V (Merck, Germany) containing the main anions fluoride, chloride, bromide, nitrate, sulfate and phosphate.

Aliquots for cation and total osmolality measurements in tissue extracts and fluid samples were used in concentrated form. Total osmolality (osmol kg^{-1}) was determined with a freeze-depression osmometer (Osmomat 030, Gonotec, Germany). Cations were determined via flame-photometry (EFOX 5053 Eppendorf, Germany) using urine standards. Samples were measured in duplicates.

3.5.5 Organic Osmolytes

Metabolic profiling was conducted on the basis of Podbielski et al. [262]. Briefly, metabolites were extracted from 200 mg of tissue (FW) by methanol extraction. The sample suspension was concentrated at 30°C overnight and pellets subsequently re-suspended 1:1 in deuterium oxide (D_2O) containing 0.05% trimethylsilyl propioniate (TSP) (Sigma Aldrich, St. Louis, USA) as internal standard. Untargeted metabolic profiling was conducted at the Alfred-Wegener-Institute (Bremerhaven, Germany). One-dimensional ^{1}H-NMR spectroscopy was performed on a wide-bore 400 MHz spectrometer (9.4 T WB with Advance III HD electronics, Bruker-BioSpin GmbH, Germany) using a triple-tunable (^{1}H, ^{13}C, ^{15}N) probe for 1.7 mm NMR tubes. A sample volume of 50 μL were used for NMR-spectroscopy at room temperature. Each sample was measured four times using a standard one-dimensional Carr–Purcell–Meiboom–Gill (cpmg) sequence with water suppression from the Bruker pulse program library (cpmgpr1). Parameters were as described in [307] using a 5 μs pulse for 90°. 32 scans were usually acquired resulting in a total acquisition time of 4 min 35s. 128 number of scans were used in one sample from *Psammechinus milliaris* in order to increase the signal to noise ratio. Spectra were processed with a Line broadening factor of 0.3, followed by baseline-, shim-, phase-corrections and TSP calibration using the software Chenomx NMR suite 8.1 (Chenomx Inc., Canada). Afterwards metabolites were identified by their chemical shifts using the Chenomx data base. Identification was confirmed by additional 2D ^{1}H-^{13}C HSQC NMR recordings of individual samples from the tissues of the respective species similar to [278]. After assignment, NMR peak integrals were fitted manually to a specific compound and metabolite quantification was based on the TSP standard concentration of 3.2 mM. Across species, we identified and quantified 60 organic substances. All solutes that changed concentration in relation

to salinity were regarded as osmolytes. We categorized them into minor, intermediate and major osmolytes according to their contribution to the overall organic solute pool by <1%, 1-10% and >10%, respectively (Fig. **??**).

3.5.6 Statistics

Statistical analysis was conducted with R (version 4.1.1). All parameters were correlated to salinity and a model was fitted to the data. A range of models was tested for best fit using AIC or R^2 and degrees of freedom. Amongst all significant models, we selected all models with the lowest AIC+5. Amongst those models we always chose the model with the fewest number of parameters. If the selection of significant models only included linear models, we chose the model based on R^2. If the final model was linear, assumptions were tested. If assumptions were flagged, the data set was examined manually. No model was fitted if no salinity effect was found or the number of data points was too low.

Acknowledgements

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B

Supplementaries Chapter 3

Fig. B.1 Total Osmolality

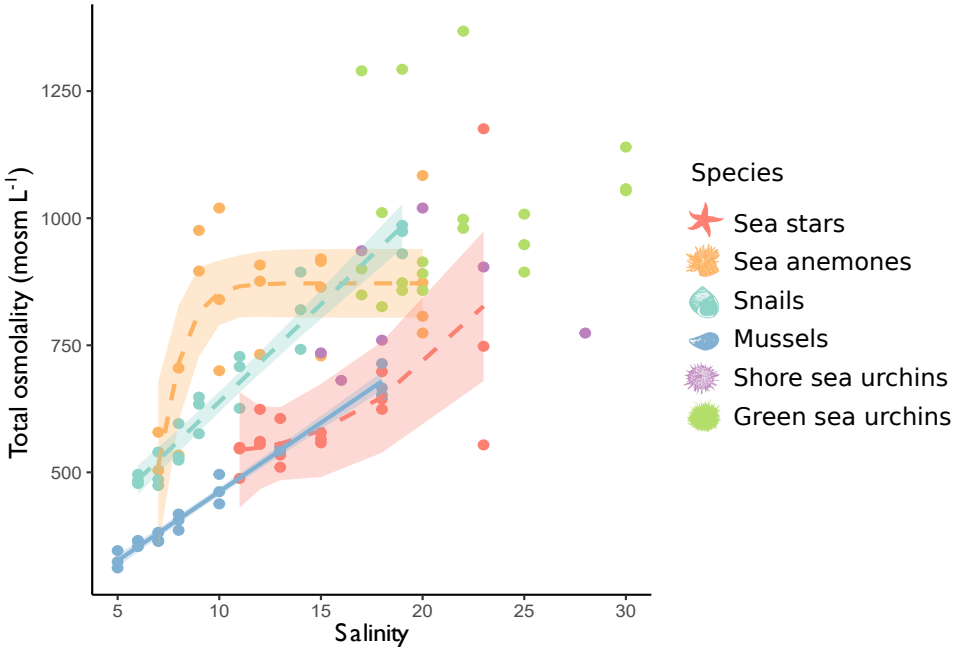
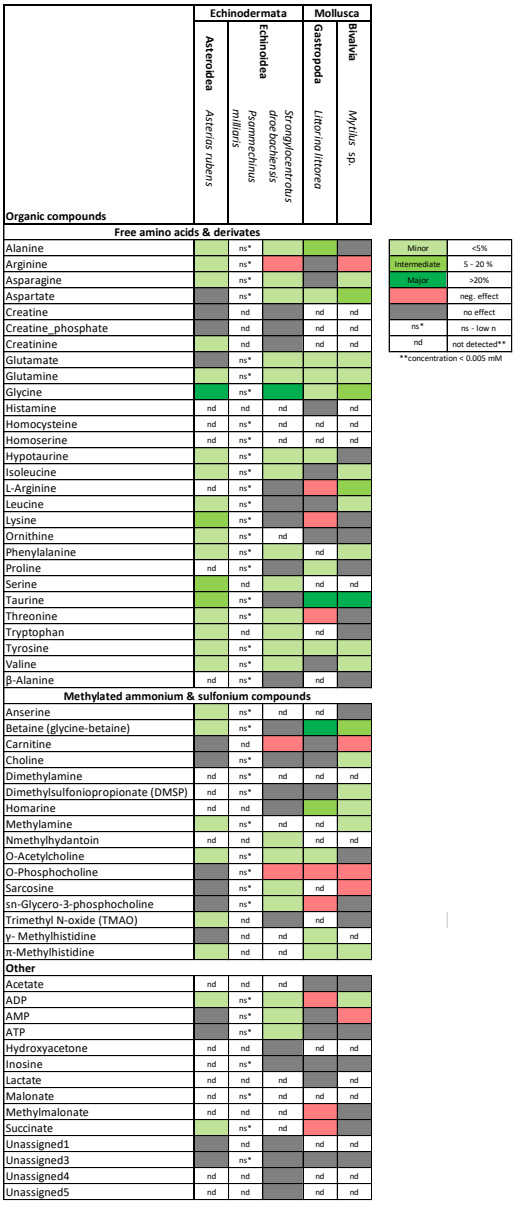


Figure B.1: Effect of salinity on total osmolality of tissues. Depicted is total osmolality in tissue extracts of the six study organisms: mussels (blue, *Mytilus edulis*), snails (turquoise, *Littorina littorea*), green sea urchins (green, *Strongylocentrotus droebachiensis*), sea stars (red, *Asterias rubens*), sea anemones (orange, *Diadumene lineata*) and shore sea urchins (purple, *Psammechinus milliaris*). Total osmolality (mosm L^{-1}) is shown of the y-axis and salinity is depicted on the x-axis. Models were chosen according to best fit and their predicted values are depicted by lines. *A. rubens*: Quadratic model (R^2 = 0.4486, df = 2, 15, F-value = 6.1, p-value = 0.0115), *D. lineata*: 3-parametric logistic model (Residual S.E. = 115.01, DF = 17, b = -1.16, d = 871.90, e = 6.69), *L. lineata*: Linear model (R^2 = 0.9411, df = 1, 19, F-value = 303.7, p-value < 0.001), *M. edulis*: Linear model (R^2 = 0.9793, df = 1, 19, F-value = 896.9, p-value < 0.001). No model is shown for shore and green sea urchins, because there was no significant relationship. Confidence intervals (95%) are shown as shaded area. Abbreviations: b = y-intercept, d = upper limit, e = steepness of increase as x.

Fig. B.2 All identified inorganic & organic compounds, their salinity effect and percentage of the organic osmolyte pool I



Organic compounds	Echinodermata			Mollusca	
	Asteroidea	Echinoidea		Gastropoda	Bivalvia
	Asterias rubens	*Psammechinus millaris*	*Strongylocentrotus droebachiensis*	*Littorina littorea*	*Mytilus sp.*
Free amino acids & derivates					
Alanine		ns*			
Arginine		ns*			
Asparagine		ns*			
Aspartate		ns*			
Creatine		nd		nd	nd
Creatine_phosphate		nd		nd	nd
Creatinine		nd		nd	nd
Glutamate		ns*			
Glutamine		ns*			
Glycine		ns*			
Histamine	nd	nd	nd		nd
Homocysteine	nd	ns*	nd	nd	nd
Homoserine	nd	ns*	nd	nd	nd
Hypotaurine		ns*			
Isoleucine		ns*			
L-Arginine	nd	ns*			
Leucine		ns*			
Lysine		ns*			
Ornithine		ns*	nd		
Phenylalanine		ns*		nd	
Proline	nd	ns*			
Serine		nd		nd	nd
Taurine		ns*			
Threonine		ns*			
Tryptophan		nd		nd	
Tyrosine		ns*			
Valine		ns*			
β-Alanine	nd	ns*		nd	
Methylated ammonium & sulfonium compounds					
Anserine		ns*	nd	nd	
Betaine (glycine-betaine)		ns*			
Carnitine		nd			
Choline		ns*			
Dimethylamine	nd	ns*	nd	nd	nd
Dimethylsulfoniopropionate (DMSP)	nd	ns*			
Homarine	nd	nd			
Methylamine		ns*	nd	nd	
Nmethylhydantoin	nd	nd		nd	nd
O-Acetylcholine		ns*			
O-Phosphocholine		ns*			
Sarcosine		ns*		nd	
sn-Glycero-3-phosphocholine		ns*			
Trimethyl N-oxide (TMAO)		nd		nd	
γ- Methylhistidine		nd	nd		nd
π-Methylhistidine		nd	nd		
Other					
Acetate	nd	nd	nd		
ADP		ns*			
AMP		ns*			
ATP		ns*			
Hydroxyacetone	nd	nd		nd	nd
Inosine	nd	ns*			
Lactate	nd	nd	nd		nd
Malonate	nd	ns*	nd	nd	nd
Methylmalonate	nd	nd	nd		
Succinate		ns*	nd		
Unassigned1		nd		nd	nd
Unassigned3		ns*			
Unassigned4	nd	nd		nd	nd
Unassigned5	nd	nd		nd	nd

Minor	<5%
Intermediate	5 - 20 %
Major	>20%
	neg. effect
	no effect
ns*	ns - low n
nd	not detected**

**concentration < 0.005 mM

Figure B.2: Map of all identified organic compounds, their salinity effect and percentage of the organic osmolyte pool

Fig. B.3 All identified inorganic & organic compounds, their salinity effect and percentage of the organic osmolyte pool II

Inorganic compounds	Echinodermata			Mollusca		Cnidaria
	Asteroidea *Asterias rubens*	**Echinoidea** *Psammechinus miliaris*	**Echinoidea** *Strongylocentrotus droebachiensis*	**Bivalvia** *Mytilus* sp.	**Gastropoda** *Littorina littorea*	**Anthozoa** *Diadumene lineata*
Bromide		ns*			nd	
Calcium		ns*				
Chloride		ns*				
Fluoride		ns*				
Nitrate		ns*				
Phosphate		ns*				
Potassium		ns*				
Sodium		ns*				
Sulfate		ns*				

	sig. effect
	neg. effect
	no effect
ns*	ns - low n
nd	not detected**

**concentration < 0.05 mM

Figure B.3: Map of all measured inorganic compounds, their salinity effect and percentage of the total inorganic compound pool

Fig. B.4 Tissue water content of sea star *Asterias rubens* acclimated to different salinity treatments for two and four weeks

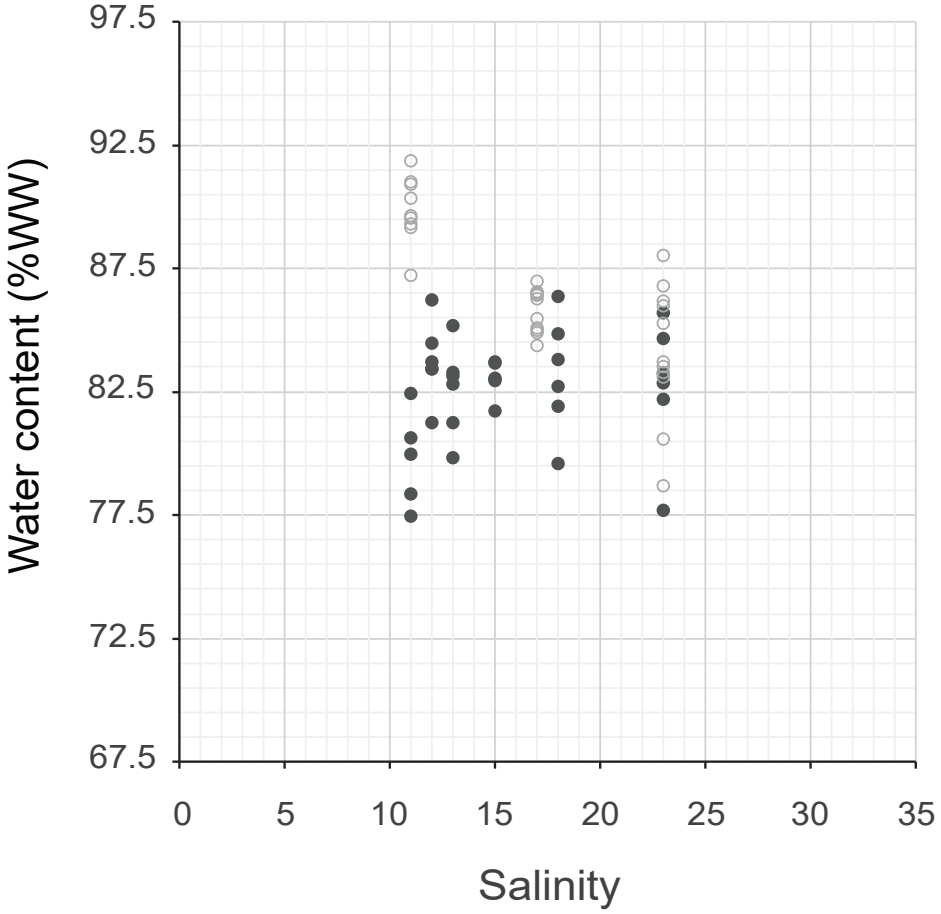


Figure B.4: Tissue water content of sea star *Asterias rubens* acclimated to different salinity treatments. Comparission of a four week acclimation period (solid circles, n = 6) to two week acclimation period (open circles, n = 11). Two-week acclimated animals were exposed to salinities of 11, 17 and 23. Four-week acclimated animals were exposed to seven different salinity treatments (10, 11, 12, 13, 15, 18 and 23). Acclimation rate was 1 S day^{-1}. After two weeks of acclimation to low salinity (11) an increase in water content was observed, whereas after four weeks water content decreased. All animals in the four-week acclimation at a salinity of 10 died. At a salinity of 11 animals lost body turgor. In the two-week acclimation two individuals died at salinity 11, these animals also had a collapsed body turgor. Reference: F. Melzner, F. Bauer, I. Podbielski (unpublished).

Estimation of the intracellular inorganic pool by calculating the likely ECS fraction I

Tissue inorganic ion concentrations are slightly higher than intracellular values due to the influence of the extracellular fluids. The ionic composition of the extracellular space (ECS) in osmoconformers is nearly identical to that of seawater as opposed to the intracellular space, that additionally contains organic osmolytes. Hence, tissue ion concentrations are slightly higher than intracellular values due to the influence of the extracellular fluids. The extracellular space can vary widely depending on species and tissue type (from 8-81% total water content) and the number of studies is unfortunately low. It was, hence, not feasible to apply ECS literature values to calculate intracellular concentrations. Furthermore, obtaining precise ECS estimates for our experimental species was beyond the scope of this study. We therefore estimated likely ECS fractions for mussels, sea stars, green sea urchins and snails. The most likely ECS fractions were determined by calculating the maximally possible ECS fraction (before measured osmolyte tissue concentrations became negative). In our study, the individuals that were used for ion determination were simultaneously sampled for their body fluids (hemolymph or coelomic fluid), except for sea anemones which were to small to extract coelenteron fluid. Echinoderms were drained of coelomic fluid first and tissue sampled afterwards. Thus, mainly intersittial fluids remain in the measured tissues, reducing the ECS bias. For molluscs the minimum amount of hemolymph extracted was added to the calculated ECS fraction. The formula for calculating ECS adapted from Freel [93] is documented below (Eq.1). Estimation of intracellular inorganic pool sizes with the most likely ECS-coefficients resulted in a maximum ECS volume of 50-60%, 10-20%, 20-30% and 45-55%in mussels, sea stars, green sea urchins and snails, respectively.

Eq.(1) $[C_i] = \frac{[C_t]-[C_{bf}]\times ECS}{(1-ECS)}$

$[C_i]$ = intracellular solute concentration in (mmol kg^{-1} cell water), $[C_t]$ = tissue solute concentration (mmol kg^{-1} tissue water), $[C_{bf}]$ = body fluid solute concentration (mmol L^{-1} body fluid) and ECS = the extracellular space of the tissue (kg kg^{-1} tissue water).

Fig. B.5 Estimation of the intracellular inorganic pool by calculating the likely ECS fraction II

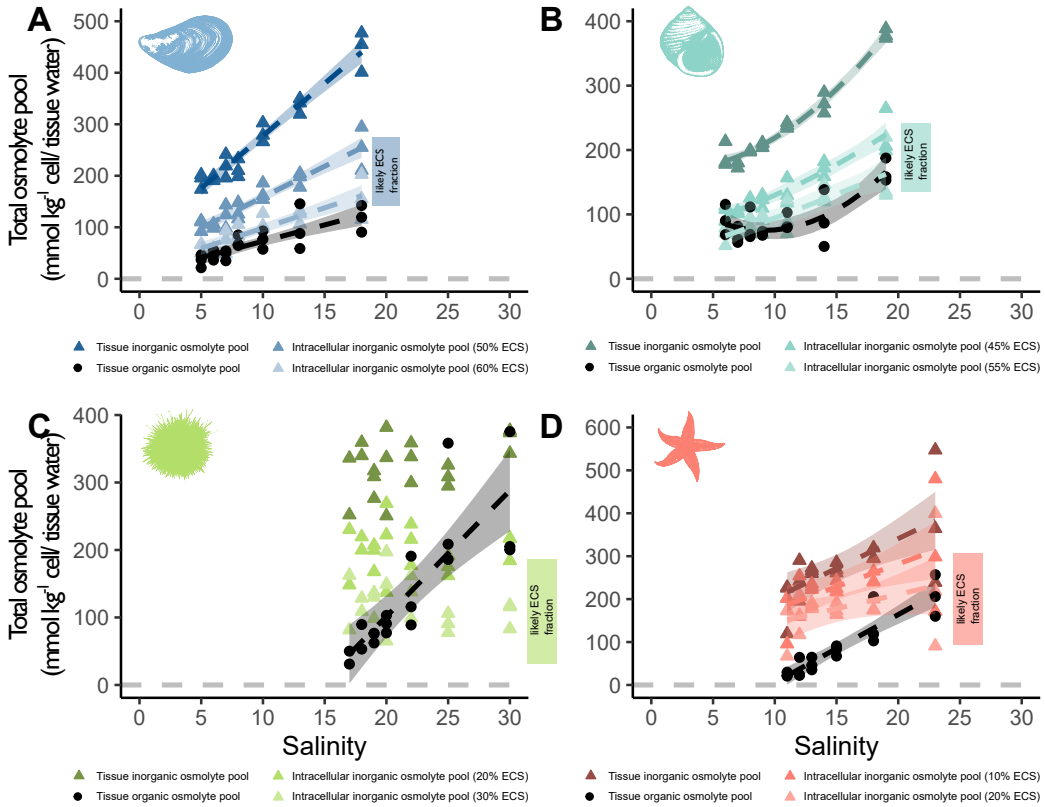


Figure B.5: Depicted is the total inorganic compound pool and the total organic osmolyte pool for A) mussels (blue, *M. edulis*, B) snails (turquoise, *L. lineata*), C) green sea urchins (green, *S. droebachiensis*) and D) sea stars (red, *A. rubens*). Concentration of the osmolyte pool is shown on the y-axis (mmol L^{-1} cell or tissue water) and salinity is depicted on the x-axis. Models correspond to the models depicted in Fig. 3.1 & Fig. 3.2. Their predicted values are depicted by dashed lines. Confidence intervals (95%) are shown as shaded area. If no model is shown, there was no significant model. The osmolyte concentrations were corrected for relative weight change due to increased tissue water content with decreasing salinity. The inorganic pool is marked with triangles, the organic pool with circles. The calculation of the intracellular inorganic osmolyte pool with the most likely fractions of extracellular space (ECS) is shown with a gradual color scale for each species. The darker the colour, the lower the ECS volume. The darkest color shows the original inorganic tissue data. Total organic osmolyte concentation of tissues in shown in black. The most likely ECS fractions were determined by calculating the maximally possible ECS fraction (before measured osmolyte tissue concentrations became negative).

Fig. B.6 Experimental set-up & sampling procedure I

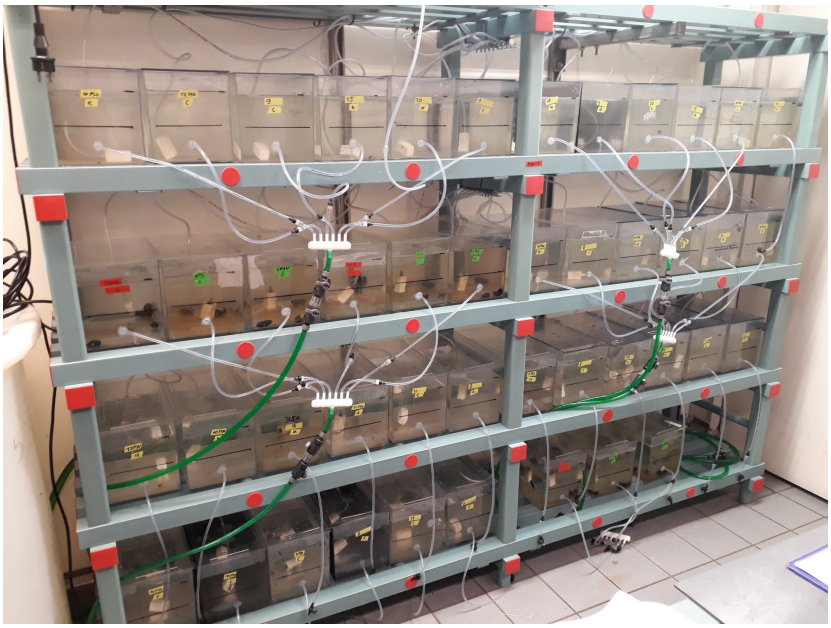


Figure B.6: This figure depicts a part of the climate chamber set up. Here tanks (10 L aquaria, filtered Balitc sea water 0.2 μm) of sea anemones, snails and mussels are shown. Air saturation in experimental tanks was maintained above 80% with air diffusor stones that were placed in each experimental unit. Salinity treamtments were positioned randomly. Prior to experiments animals were acclimated to climate chamber conditions.

Fig. B.7 Experimental set-up & sampling procedure II

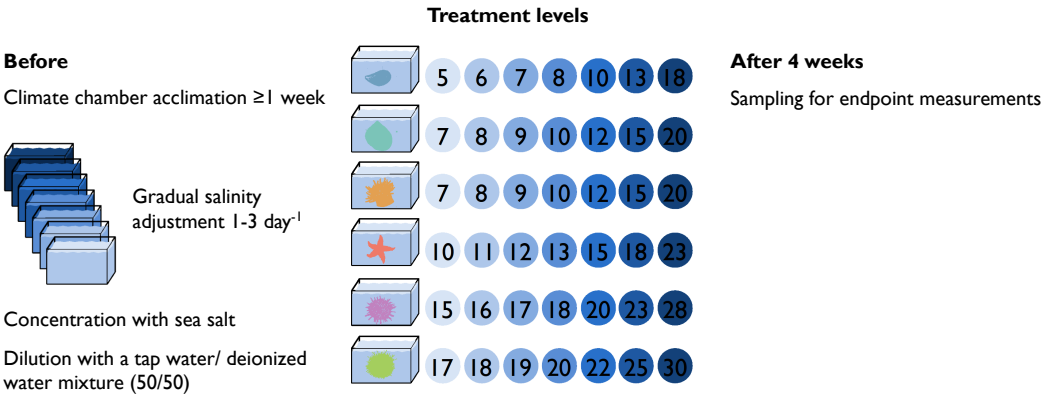


Figure B.7: In total, six different species were studied and each species acclimated to seven salinity treatments, of those four treamtents were clustered around the species lower salinity limit. Salinity was gradually adjusted. Experimental exposure lasted four weeks. In total 168 tanks were mainted for a minimum of 6 weeks.

Fig. B.8 Experimental set-up & sampling procedure III

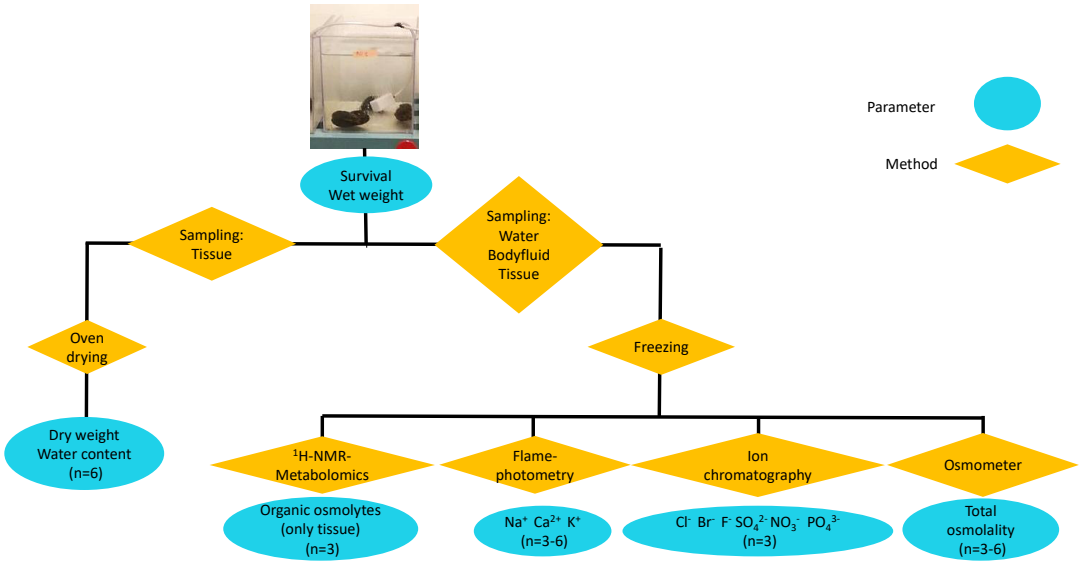


Figure B.8: Flow chart of sampling procedure and division of sampling material for the different measurement with mussels as example.

Table B.1: Model Outcomes Figure 3.1 & 3.2 I

				Survival					
Species	**Model**	**Residual S.E.**	**DF**	**Coefficient**	**Estimate**	**S.E.**	**t-value**	**p-value**	
A. rubens	Logistic	15.7029	39	b	-6.2055	8.5761	-0.724	0.474	
				d	98.0083	2.8738	34.104	<0.001	***
				e	10.8782	0.1745	62.323	<0.001	***
D. lineata	Logistic	52.4035	38	b	-1.3626	2.2193	-0.614	0.543	
				c	95.4403	14.0599	6.788	<0.001	***
				d	223.9324	19.1994	11.664	<0.001	***
				e	12.2445	0.7987	15.331	<0.001	***
L. littorea	Logistic	15.5605	39	b	-1.8485	0.5850	-3.160	0.003	**
				d	97.6991	3.1103	31.412	<0.001	***
				e	6.0552	0.1381	43.835	<0.001	***
Mytilus sp.	Asymptotic	11.0189	40	d	96.6959	2.7599	35.035	<0.001	***
				e	2.2845	0.4563	5.007	<0.001	***
S. droebachiensis	Logistic	24.1626	18	b	-0.6028	0.4143	-1.455	0.163	
				d	101.9907	9.0475	11.273	<0.001	***
				e	16.2945	1.1631	14.009	<0.001	***

Abbreviations: b = y-intercept, c = lower limit, d = upper limit, e = steepness of increase as x

Table B.2: Model Outcomes Figure 3.1 & 3.2 II

Tissue water content										
Species	**Model**	**Residual S.E.**	**R^2**	**DF**	**Coefficient**	**Estimate**	**S.E.**	**t-value**	**F-value**	**p-value**	
A. rubens	Logistic	1.9324		32	b	-5.2272	6.3357	-0.825		0.416	
					d	83.0595	0.3538	234.748		<0.001	***
					e	10.3893	0.7417	14.008		<0.001	***
D. lineata	Linear		0.2091	22					5.818	0.025	*
L. littorea	Asymptotic	1.6424		35	c	122.1071	40.8192	2.991		0.005	**
					d	72.8413	0.6212	117.253		<0.001	***
					e	2.8115	1.0482	2.682		0.011	*
Mytilus sp.	Logistic	1.2598		38	b	3.7080	4.2202	0.879		0.385	
					c	84.1215	0.2925	287.620		<0.001	***
					d	87.9517	0.4122	213.368		<0.001	***
					e	7.1374	0.1949	36.626		<0.001	***
P. milliaris	Linear		0.4369	12					9.309	0.010	*
S. droebachiensis	Linear		0.2355	15					4.621	0.048	*

Abbreviations: b = y-intercept, c = lower limit, d = upper limit, e = steepness of increase as x

Table B.3: Model Outcomes Figure 3.1 & 3.2 III

Total WW											
Species	**Model**	**Residual S.E.**	**R^2**	**DF**	**Coefficient**	**Estimate**	**S.E.**	**t-value**	**F-value**	**p-value**	
A. rubens	Logistic	0.7182		32	b	-0.7730	0.2076	-3.724		<0.001	***
					d	4.4718	0.2291	19.522		<0.001	***
					e	12.3660	0.2763	44.760		<0.001	***
D. lineata	Asymptotic	0.0414		40	d	1.1170	0.0145	76.909		<0.001	***
					e	4.1221	0.2315	17.807		<0.001	***
L. littorea	Linear		0.4660	40					34.910	<0.001	***
Mytilus sp.	Logistic	0.0949		38	b	-0.5287	0.1338	-3.952		<0.001	***
					c	1.4466	0.0598	24.177		<0.001	***
					d	2.2375	0.0479	46.721		<0.001	***
					e	10.6017	0.4913	21.580		<0.001	***
P. milliaris	Asymptotic	0.0697		17	d	1.1354	0.0460	24.683		<0.001	***
					e	6.9117	1.3970	4.948		<0.001	***
S. droebachiensis	Linear		0.2814	18					7.048	0.016	*

Abbreviations: b = y-intercept, c = lower limit, d = upper limit, e = steepness of increase as x

Table B.4: Model Outcomes Figure 1 & 2 III

Inorganic and organic osmolyte pools								
Species	**Pool**	**Model**	**R^2**	**DF**	**F-value**	**p-value**	**Significance**	**Source**
A. rubens	Inorganic	Linear	0.4796	1, 16	14.750	0.0014	**	this study
	Organic	Linear	0.8512	1, 16	91.540	<0.001	***	this study
D. lineata	Inorganic	NA						
	Organic	Linear	0.6837	1, 30	64.850	<0.001	***	Podbielski et al. 2016
L. littorea	Inorganic	Quadratic	0.9639	2, 18	240.400	<0.001	***	this study
	Organic	Quadratic	0.6511	2, 18	16.800	<0.001	***	this study
Mytilus edulis-like	Inorganic	Quadratic	0.9406	2, 18	142.500	<0.001	***	this study
	Organic	Linear	0.6485	1, 19	35.050	<0.001	***	this study
P. milliaris	Inorganic	NA						
	Organic	NA						
S. droebachiensis	Inorganic	NA						this study
	Organic	Linear	0.6680	1, 17	34.210	<0.001	***	this study

Table B.5: Concentration of inorganic solutes (mmol g^{-1}WW) in tissue of the six study organisms

Species	Salinity	Tank	Individual	Medium	Bromide	Chloride	Fluoride	Nitrate	Phosphate	Sulfate	Calcium	Potassium	Sodium
Asterias	11	C	1	Tissue	0.07	40.98	0.36	0.18	0.18	0.82	0.38	42.00	10.32
Asterias	11	D	1	Tissue	0.15	79.51	0.38	0.16	4.14	2.28	0.61	63.36	29.76
Asterias	11	F	1	Tissue	0.15	76.85	0.39	0.19	5.01	2.16	0.70	62.40	34.08
Asterias	12	A	3	Tissue	0.19	83.62	0.93	0.39	8.22	2.53	0.88	76.86	29.30
Asterias	12	B	1	Tissue	0.13	100.24	0.76	0.17	8.12	2.64	0.85	87.89	42.84
Asterias	12	C	2	Tissue	0.09	72.42	0.56	0.22	4.80	2.23	0.76	49.77	33.08
Asterias	13	A	1	Tissue	0.18	96.28	0.69	0.16	7.67	3.66	0.87	80.10	37.20
Asterias	13	E	1	Tissue	0.17	98.73	0.60	0.22	5.84	2.82	0.76	67.80	39.20
Asterias	13	F	1	Tissue	0.20	95.89	0.74	0.29	9.18	4.00	1.06	73.80	36.20
Asterias	15	B	1	Tissue	0.16	105.49	1.12	0.44	6.73	3.47	0.73	73.93	31.71
Asterias	15	C	3	Tissue	0.11	99.99	1.02	0.31	12.03	3.30	0.99	69.89	29.90
Asterias	15	D	2	Tissue	0.11	103.01	1.20	0.22	12.92	5.12	0.85	81.81	32.72
Asterias	18	A	1	Tissue	0.21	113.30	1.23	0.40	11.64	5.44	0.85	81.95	32.66
Asterias	18	C	2	Tissue	0.24	117.98	1.40	0.33	10.77	5.95	0.96	88.19	41.81
Asterias	18	D	2	Tissue	0.21	111.66	1.97	0.32	10.19	3.60	0.98	78.83	38.90
Asterias	23	C	1	Tissue	0.15	95.13	1.52	0.26	6.17	3.90	0.72	57.80	39.20
Asterias	23	D	2	Tissue	0.35	206.51	2.55	0.23	13.07	7.68	1.59	127.50	96.30
Asterias	23	F	1	Tissue	0.26	140.29	1.37	0.33	12.75	4.81	1.40	83.40	58.80
Diadumene	7	AEF	pooled	Tissue	0.37	126.19	6.55	1.04	20.08	12.18	2.94	124.25	74.55
Diadumene	7	BCD	pooled	Tissue	0.27	118.71	7.55	0.53	20.37	11.67	2.81	116.46	77.81
Diadumene	8	ABF	pooled	Tissue	0.36	177.99	8.84	0.46	21.68	16.79	4.84	168.78	139.17
Diadumene	8	CDE	pooled	Tissue	0.46	220.65	11.24	0.58	25.83	17.98	5.13	162.86	137.19
Diadumene	9	ACE	pooled	Tissue	0.19	77.84	3.64	0.23	11.00	6.98	1.93	66.95	52.79
Diadumene	9	BDF	pooled	Tissue	0.19	81.05	3.22	0.23	9.48	7.49	1.94	60.98	54.21
Diadumene	10	AD	pooled	Tissue	0.12	49.28	1.76	0.12	4.31	4.11	1.07	32.48	29.70
Diadumene	10	E	pooled	Tissue	0.13	57.01	1.56	0.12	3.77	4.15	1.16	29.23	41.99
Diadumene	10	F	pooled	Tissue	0.12	49.91	1.58	0.12	4.40	3.77	1.03	33.18	35.83
Diadumene	12	AB	pooled	Tissue	0.29	137.94	4.44	0.21	8.73	11.08	2.95	79.38	96.80
Diadumene	12	DE	pooled	Tissue	0.29	147.85	3.86	0.20	7.34	11.13	2.72	62.44	108.17
Diadumene	12	C	pooled	Tissue	0.28	129.62	4.35	0.22	9.54	11.67	2.23	77.92	90.99
Diadumene	15	A	pooled	Tissue	0.36	181.30	5.86	1.00	8.61	13.47	3.11	103.09	122.96
Diadumene	15	B	pooled	Tissue	0.42	216.44	4.18	0.39	8.89	14.78	4.18	79.49	170.15
Diadumene	15	C	pooled	Tissue	0.19	209.12	4.91	0.20	9.08	15.82	3.75	96.60	150.14
Diadumene	15	F	pooled	Tissue							3.23	106.81	113.44
Diadumene	20	A	pooled	Tissue							3.64	66.80	154.80
Diadumene	20	B	pooled	Tissue	0.41	197.11	4.17	0.37	7.92	13.87	3.39	68.40	154.50
Diadumene	20	C	pooled	Tissue	0.43	218.55	3.13	0.33	4.39	13.84	3.84	55.20	168.90
Diadumene	20	F	pooled	Tissue	0.27	209.46	3.38	0.24	5.27	14.01	3.87	66.00	151.80
Littorina	6	A	pooled	Tissue	0.00	52.41	6.76	3.62	5.00	4.47	1.89	66.41	28.42
Littorina	6	B	pooled	Tissue	0.00	33.43	7.06	3.64	3.79	3.82	2.81	62.19	24.20
Littorina	6	D	pooled	Tissue	0.00	34.41	7.52	3.07	6.36	4.14	2.14	59.38	26.17
Littorina	7	A	pooled	Tissue	0.00	33.37	5.77	3.33	2.24	3.83	2.70	53.63	26.11
Littorina	7	B	pooled	Tissue	0.00	35.05	5.50	3.41	1.49	3.90	3.36	54.57	30.34
Littorina	7	E	pooled	Tissue	0.00	34.72	5.09	3.27	1.39	3.43	3.53	56.68	28.93
Littorina	8	B	pooled	Tissue	0.00	41.05	5.39	3.62	2.44	4.31	3.45	61.34	30.32
Littorina	8	D	pooled	Tissue	0.00	40.13	5.49	3.70	1.15	5.14	3.71	60.16	30.55
Littorina	8	F	pooled	Tissue	0.00	42.32	5.33	3.60	1.21	4.43	4.04	58.52	31.96
Littorina	9	A	pooled	Tissue	0.00	44.36	5.03	3.67	1.00	5.00	3.96	62.13	34.60
Littorina	9	B	pooled	Tissue	0.00	42.87	4.94	3.52	0.97	5.04	4.05	58.27	34.37
Littorina	9	D	pooled	Tissue	0.00	44.34	5.36	3.70	1.04	5.13	4.01	60.54	34.14
Littorina	11	B	pooled	Tissue	0.00	51.99	5.21	3.59	0.86	4.74	4.49	62.52	42.80
Littorina	11	C	pooled	Tissue	0.00	52.04	4.98	3.56	0.83	6.06	3.99	65.88	42.38
Littorina	11	F	pooled	Tissue	0.00	53.29	4.50	3.41	0.71	5.43	3.92	60.21	41.75
Littorina	14	A	pooled	Tissue	0.00	61.60	4.13	3.28	0.70	5.41	4.11	59.28	49.70
Littorina	14	C	pooled	Tissue	0.00	65.51	3.69	3.27	1.01	5.98	4.17	64.47	50.50
Littorina	14	E	pooled	Tissue	0.00	70.10	4.72	3.52	0.61	5.82	4.95	67.32	54.71
Littorina	19	B	pooled	Tissue	0.00	100.84	4.44	5.12	0.52	7.28	4.50	73.00	77.40
Littorina	19	E	pooled	Tissue	0.00	106.24	3.87	4.92	0.53	6.87	4.54	73.20	83.80
Littorina	19	F	pooled	Tissue	0.00	102.43	3.63	4.25	0.61	6.56	4.38	71.80	82.40
Mytilus	5	B	1	Tissue	0.10	88.53	3.00	0.23	17.05	5.34	1.51	26.34	36.11
Mytilus	5	C	2	Tissue	0.21	83.37	2.74	0.45	15.58	4.88	1.42	30.19	34.04
Mytilus	5	D	2	Tissue	0.18	71.69	2.19	0.34	14.13	4.89	1.12	27.53	31.38
Mytilus	6	B	3	Tissue	0.15	73.51	2.49	0.66	21.02	5.03	1.37	35.56	31.64
Mytilus	6	D	3	Tissue	0.12	72.20	2.12	0.43	18.86	5.17	1.20	35.56	31.64
Mytilus	6	F	2	Tissue	0.14	83.97	2.67	0.28	15.73	4.94	1.29	31.92	34.72
Mytilus	7	A	1	Tissue	0.18	76.80	2.43	0.36	13.78	4.55	1.48	29.70	40.45
Mytilus	7	D	2	Tissue	0.26	114.02	2.93	0.38	13.65	5.01	1.51	33.54	38.14
Mytilus	7	E	1	Tissue	0.25	96.23	2.63	0.38	14.67	5.85	1.36	35.33	33.28
Mytilus	8	C	1	Tissue	0.24	99.14	2.13	0.26	13.30	5.11	1.18	30.45	44.52
Mytilus	8	E	2	Tissue	0.26	90.30	1.96	0.35	12.73	4.65	1.26	26.25	39.27
Mytilus	8	F	1	Tissue	0.26	77.09	2.28	0.34	14.45	4.57	0.97	33.81	33.39
Mytilus	10	A	3	Tissue	0.29	110.35	2.43	0.32	14.37	5.35	1.17	41.36	50.38
Mytilus	10	D	1	Tissue	0.31	119.08	1.88	0.38	17.32	5.72	1.21	41.58	49.28
Mytilus	10	E	2	Tissue	0.33	127.44	2.28	0.32	15.98	5.96	1.56	38.06	65.12
Mytilus	13	A	3	Tissue	0.37	132.15	1.93	0.35	15.47	6.57	1.89	44.10	66.57
Mytilus	13	E	3	Tissue	0.40	142.02	3.60	0.39	16.61	7.85	1.64	47.25	68.25
Mytilus	13	F	3	Tissue	0.35	150.49	2.98	0.71	19.13	7.09	1.66	42.63	69.93
Mytilus	18	A	1	Tissue	0.46	172.35	1.99	0.56	16.42	8.40	2.08	49.20	84.00
Mytilus	18	B	1	Tissue	0.48	215.17	1.78	0.56	14.24	8.75	2.68	46.20	109.40

Table B.5: Concentration of inorganic solutes (mmol g^{-1}WW) in tissue of the six study organisms

Species	Salinity	Tank	Individual	Medium	Bromide	Chloride	Fluoride	Nitrate	Phosphate	Sulfate	Calcium	Potassium	Sodium
Mytilus	18	C	1	Tissue	0.49	204.04	2.14	0.58	14.85	8.76	2.18	50.00	97.60
Psammechinus	15	BC	pooled	Tissue	0.15	86.09	0.88	0.46	5.90	4.97	1.34	38.02	56.16
Psammechinus	16	ABC	pooled	Tissue	0.05	58.98	0.59	0.35	4.55	3.80	1.15	33.79	34.94
Psammechinus	17	ABC	pooled	Tissue	0.08	82.97	0.47	0.46	3.31	4.38	2.43	29.99	59.02
Psammechinus	18	ABC	pooled	Tissue	0.05	47.67	0.57	0.41	5.04	2.99	1.18	37.80	25.62
Psammechinus	20	AB	pooled	Tissue	0.03	35.28	0.72	0.43	3.59	1.94	0.85	23.85	21.73
Psammechinus	23	AB	pooled	Tissue	0.04	49.78	0.69	0.53	5.08	3.17	1.64	37.91	28.55
Psammechinus	28	ABC	pooled	Tissue	0.09	84.43	0.56	0.37	2.98	3.86	1.92	25.50	59.10
Strongylocentrotus	17	A	1	Tissue	0.09	91.42	1.20	0.39	13.74	8.20	1.13	85.50	45.49
Strongylocentrotus	17	B	1	Tissue	0.11	92.66	0.86	0.45	8.05	12.92	1.37	77.98	56.77
Strongylocentrotus	17	C	1	Tissue	0.14	74.56	0.99	0.64	3.41	6.89	1.57	55.75	41.38
Strongylocentrotus	18	A	1	Tissue	0.12	88.89	1.04	0.56	6.65	11.82	1.08	109.89	55.62
Strongylocentrotus	18	B	1	Tissue	0.10	93.80	0.99	0.49	8.42	13.00	1.17	90.72	51.84
Strongylocentrotus	19	A	1	Tissue	0.11	83.06	0.92	0.37	8.52	11.70	1.12	77.04	46.55
Strongylocentrotus	19	B	1	Tissue	0.10	69.78	0.81	0.55	6.11	9.70	3.02	74.79	34.99
Strongylocentrotus	19	C	1	Tissue	0.11	78.23	0.94	0.44	8.70	8.16	1.61	82.02	42.89
Strongylocentrotus	20	A	1	Tissue	0.09	80.90	1.97	0.90	6.41	7.10	1.13	101.43	42.84
Strongylocentrotus	20	B	1	Tissue	0.15	73.07	0.90	0.48	8.33	5.90	0.88	57.16	33.67
Strongylocentrotus	20	C	1	Tissue	0.15	107.61	0.84	0.43	9.47	8.45	1.29	79.07	67.73
Strongylocentrotus	22	A	1	Tissue	0.11	87.02	1.04	0.52	10.29	9.74	0.78	88.37	43.88
Strongylocentrotus	22	B	1	Tissue	0.17	101.15	1.14	0.50	12.68	7.63	1.32	81.58	50.26
Strongylocentrotus	22	C	1	Tissue	0.16	80.04	0.99	0.46	11.68	9.38	0.72	78.69	32.14
Strongylocentrotus	25	A	1	Tissue	0.16	90.35	0.93	0.36	12.44	6.29	0.76	76.80	43.20
Strongylocentrotus	25	B	1	Tissue	0.16	86.80	0.72	0.42	12.08	4.46	0.90	71.80	41.60
Strongylocentrotus	25	C	1	Tissue	0.16	79.78	0.94	0.37	10.43	7.06	0.86	69.60	40.00
Strongylocentrotus	30	A	1	Tissue	0.20	98.39	1.03	0.36	9.62	7.92	0.98	72.80	52.40
Strongylocentrotus	30	B	1	Tissue	0.20	105.74	0.84	0.33	9.86	9.85	1.11	82.20	55.20
Strongylocentrotus	30	C	1	Tissue	0.12	112.82	0.93	0.22	10.52	6.93	1.02	77.00	58.20

Table B.6: Concentration of organic solutes (mmol g^{-1}WW) in tissue of the six study organisms I

Species	Salinity	Tank	Individual	Medium	ADP	AMP	ATP	Acetate	Alanine	Anserine	Arginine	Asparagine	Aspartate	Betaine	Carnitine	Choline	Creatine	Creatine phosphate	Creatinine	Dimethylsulfone	Dimethylamine	Glutamate
Asterias	11	C	1	Tissue	0.0489	0.2994	0.1264		0.2998	0.0585	1.3716	0.4692	0.6545	0.0000	0.1228	0.0870	0.0000	0.0537	0.0000			1.5268
Asterias	11	D	1	Tissue	0.0741	0.4086	0.1258		0.5816	0.0633	1.1727	0.1317	0.5305	0.0000	0.2073	0.2985	0.0000	0.0000	0.0000			0.8428
Asterias	11	F	1	Tissue	0.0850	0.4863	0.1231		0.4889	0.1038	1.3078	0.5749	0.9307	0.0000	0.0867	0.5199	0.0000	0.0000	0.0000			1.7049
Asterias	12	A	3	Tissue	0.1046	0.9758	0.2018		2.4788	0.1491	3.2299	0.6528	2.9836	0.0000	0.0650	0.1947	0.0000	0.0000	0.0000			4.9690
Asterias	12	B	1	Tissue	0.0851	0.7244	0.3492		0.4524	0.0637	1.4397	0.1200	1.2707	0.0000	0.2747	0.5499	0.0000	0.0000	0.0000			0.7778
Asterias	12	C	2	Tissue	0.1120	0.3735	0.2001		0.3091	0.1019	0.5291	0.1298	0.4499	0.0442	0.0000	0.3046	0.0418	0.0000	0.0000			1.8950
Asterias	13	A	1	Tissue	0.1444	0.5055	0.1072		1.4687	0.1837	1.9869	0.1624	0.5681	0.0000	0.4190	0.5115	0.0000	0.0000	0.0000			1.2798
Asterias	13	E	1	Tissue	0.0598	0.3021	0.1417		0.6828	0.0930	1.8191	0.6935	0.8433	0.0406	0.0845	0.3792	0.0000	0.0000	0.2446			1.5811
Asterias	13	F	1	Tissue	0.0407	0.4228	0.2608		2.2082	0.2394	3.1966	1.3094	1.9993	0.2655	0.3352	0.3152	0.0769	0.0000	0.0000			4.9128
Asterias	15	B	1	Tissue	0.0710	0.5436	0.3363		1.0999	0.1232	2.0844	0.3331	0.6046	0.1853	0.1126	0.5334	0.1318	0.0000	0.4613			1.0743
Asterias	15	C	3	Tissue	0.0569	0.4295	0.1205		5.2359	0.2832	5.8094	1.0163	1.0659	0.4712	0.3193	0.5145	0.0512	0.0672	0.0000			2.1988
Asterias	15	D	2	Tissue	0.0859	0.6127	0.2017		3.1698	0.2325	3.3875	0.5731	0.7169	0.0833	0.2334	0.4518	0.1583	0.1609	0.8944			1.8435
Asterias	18	A	1	Tissue	0.1204	0.7766	0.2177		6.4278	0.5781	5.9382	3.0634	2.6504	0.1611	0.5233	0.7794	0.0000	0.0000	1.6914			4.9987
Asterias	18	C	2	Tissue	0.0689	0.6827	0.3491		2.9068	0.3041	2.8741	0.4331	0.8143	0.0473	0.2968	0.4248	0.1299	0.1406	1.4313			1.8035
Asterias	18	D	2	Tissue	0.2055	0.5711	0.1249		4.3980	0.0785	2.8353	0.6248	0.6680	0.5730	0.2623	0.2272	0.0000	0.0000	0.0000			2.3184
Asterias	23	C	1	Tissue	0.1675	0.5071	0.1913		4.3047	0.2296	1.4079	0.5859	0.9754	0.2564	0.0000	0.5146	0.3504	0.0000	3.2462			1.7451
Asterias	23	D	2	Tissue	0.1486	0.3227	0.2065		5.7757	0.4527	2.4053	0.9745	1.8145	0.0000	0.6361	0.9401	0.0000	0.0000	7.1063			3.8519
Asterias	23	F	1	Tissue	0.2060	0.5881	0.1347		11.9348	0.7234	1.2394	3.4661	3.2787	1.3524	0.4271	0.1966	0.0000	0.0000	1.9195			5.8273
Littorina	6	A	pooled	Tissue	0.2242	0.6626	0.5013	0.6387	3.4154		0.4854	0.6290	2.1993	8.6094	0.4397	0.2047				0.1271		6.5472
Littorina	6	B	pooled	Tissue	0.5376	0.8144	0.9680	1.1627	5.2488		0.7803	0.1313	1.6676	28.1479	0.2863	0.5431				0.3845		8.2824
Littorina	6	D	pooled	Tissue	0.3798	0.7822	0.4551	0.9748	5.2140		0.6812	0.2097	2.6673	15.3782	0.2531	0.4665				0.2717		8.7307
Littorina	7	A	pooled	Tissue	0.2507	0.2392	0.4688	0.4042	2.6292		0.6128	0.0658	0.9099	8.5357	0.2884	0.1670				0.0197		5.6738
Littorina	7	B	pooled	Tissue	0.3542	0.7200	0.6258	0.7077	3.3295		0.5807	0.1012	1.6329	13.0556	0.2212	0.1187				0.1475		5.7896
Littorina	7	E	pooled	Tissue	0.2922	0.2583	0.6746	0.8555	3.3394		0.7091	0.0969	0.9849	9.6406	0.2995	0.2260				0.1635		5.0102
Littorina	8	B	pooled	Tissue	0.1637	0.3874	0.1459	0.2073	2.4158		0.6240	0.1028	1.8663	8.6114	0.1985	0.2825				0.0357		5.8017
Littorina	8	D	pooled	Tissue	0.3160	0.5586	0.5957	0.5136	2.6409		1.8194	0.3272	1.8838	7.6183	0.1234	0.1708				0.0886		5.0752
Littorina	8	F	pooled	Tissue	0.1900	0.9627	0.6511	1.6996	5.0448		0.7081	0.4604	2.9510	15.2479	0.1835	0.4906				0.5544		8.1142
Littorina	9	A	pooled	Tissue	0.2637	0.5921	0.5456	0.3507	2.6675		0.9829	0.0477	1.6380	10.3733	0.1279	0.1871				0.0440		4.6284
Littorina	9	B	pooled	Tissue	0.1554	0.0952	0.5392	0.3627	2.8280		1.5466	0.0912	1.9181	10.7302	0.2270	0.6056				0.0533		5.9597
Littorina	9	D	pooled	Tissue	0.3473	0.2559	0.8809	0.5865	3.4862		0.7536	0.1252	1.4985	11.7604	1.1994	0.4314				0.3554		6.3957
Littorina	11	B	pooled	Tissue	0.1462	0.1985	0.7522	0.7515	3.8981		0.5143	0.1995	1.8111	18.1982	0.6139	0.8151				0.1079		5.6599
Littorina	11	C	pooled	Tissue	0.2043	0.3652	0.3687	0.6862	2.9943		0.0000	0.1408	2.1748	12.4352	0.2389	0.3569				0.2191		4.3004
Littorina	11	F	pooled	Tissue	0.0952	0.3154	0.8075	0.3417	3.3285		0.6060	0.0619	1.7029	14.5573	0.0760	0.2041				0.0187		5.2476
Littorina	14	A	pooled	Tissue	0.0796	0.1175	0.3922	0.3312	2.3110		0.1742	0.0345	1.2798	11.2799	0.2649	0.1534				0.2259		2.9758
Littorina	14	C	pooled	Tissue	0.0426	0.2004	0.6784	0.3957	2.7261		0.3261	0.1576	2.3480	19.0166	0.5053	0.2381				0.4065		4.1293
Littorina	14	E	pooled	Tissue	0.1717	0.6140	0.4706	0.7452	7.0757		0.4048	0.0583	2.8232	33.3520	0.5033	1.3114				0.1923		7.4811
Littorina	19	B	pooled	Tissue	0.0988	0.3707	0.3470	0.8660	7.4625		0.8376	0.1415	2.8582	48.0377	0.4432	0.5304				0.3497		7.5993
Littorina	19	E	pooled	Tissue	0.2290	0.5551	0.3320	0.5685	6.7142		0.4764	0.0584	3.8286	36.2802	0.8388	0.3906				0.0342		6.8432
Littorina	19	F	pooled	Tissue	0.2347	0.2268	0.6542	0.6652	7.3272		1.1552	0.4585	3.0264	38.5412	0.0000	0.3323				0.3591		6.5427
Mytilus	5	B	1	Tissue	0.1255	0.9260	0.3651		2.8130	0.0884	0.9592	0.4171	4.1089	6.0945	0.4341	0.2578				0.4780		2.5921
Mytilus	5	C	2	Tissue	0.2615	1.1469	0.1646		3.4044	0.1551	2.0890	0.5895	3.2464	0.7977	0.0950	0.1698				0.2365		1.9771
Mytilus	5	D	2	Tissue	0.4894	1.5043	0.2626		3.6378	0.1064	1.9733	0.4063	4.8267	1.8923	0.2269	0.1788				0.5691		2.5459
Mytilus	6	B	3	Tissue	0.5141	1.4350	0.2814		4.8952	0.0701	2.9266	0.4686	4.2629	0.2607	0.2521	0.2691				0.5209		3.8273
Mytilus	6	D	3	Tissue	0.1718	0.8709	0.2597		5.1261	0.0759	0.6598	0.6793	7.7745	2.5861	0.0000	0.6038				0.3814		5.5115
Mytilus	6	F	2	Tissue	0.1827	1.2761	0.6976		3.7944	0.1266	2.4024	0.7074	5.5884	0.5407	0.4171	0.1634				0.8053		3.0948
Mytilus	7	A	1	Tissue	0.2990	1.5520	0.3507		1.3014	0.0532	0.8911	0.7227	4.8707	0.4356	0.7099	0.1833				0.1796		2.1725
Mytilus	7	D	2	Tissue	0.6109	0.7945	0.9623		4.9693	0.3397	0.7237	1.8675	7.4639	0.4503	0.0000	0.4588				0.5757		3.6833
Mytilus	7	E	1	Tissue	0.3395	1.3887	0.5093		1.8531	0.0623	0.7654	1.1071	3.6154	2.8390	0.0000	0.2129				0.8731		2.4782
Mytilus	8	C	1	Tissue	0.2542	1.3586	0.4001		4.5203	0.1646	0.3219	1.1730	9.1851	0.4799	0.0000	0.2145				0.5456		4.4995
Mytilus	8	E	2	Tissue	0.5500	1.2314	0.8216		6.4189	0.1026	0.4478	3.0102	8.5643	2.0197	0.0000	0.5862				1.0933		9.7243
Mytilus	8	F	1	Tissue	0.3567	1.2554	0.5112		4.2330	0.0445	0.0874	0.8950	9.0422	2.1101	0.0000	0.3945				0.4706		5.6246
Mytilus	10	A	3	Tissue	0.2166	1.2499	0.3914		2.9926	0.0428	0.5506	1.0645	11.3828	2.1611	0.0000	0.2266				1.1328		5.3401

Table B.6: Concentration of organic solutes (mmol g^{-1}WW) in tissue of the six study organisms I

Species	Salinity	Tank	Individual	Medium	ADP	AMP	ATP	Acetate	Alanine	Anserine	Arginine	Asparagine	Aspartate	Betaine	Carnitine	Choline	Creatine	Creatine phosphate	Creatinine	Dimethylsulfone	Dimethylamine	Glutamate
Mytilus	10	D	1	Tissue	0.2336	0.3864	0.6513		4.9542	0.0000	0.4180	1.2066	14.3326	0.4577	0.0000	0.7082				0.8935		7.0995
Mytilus	10	E	2	Tissue	0.1195	0.3120	0.2338		3.8295	0.0000	0.2251	0.7422	8.5236	2.0125	0.0000	0.3117				0.7216		3.8293
Mytilus	13	A	3	Tissue	0.0858	0.1634	0.3156		4.3016	0.0000	0.3658	2.3202	8.7108	3.6444	0.0000	0.3600				1.0045		4.6031
Mytilus	13	E	3	Tissue	0.0826	0.1698	0.2913		10.1743	0.0000	0.8548	4.1274	14.2702	6.5937	0.0000	1.2754				2.7139		7.6352
Mytilus	13	F	3	Tissue	0.0849	0.0898	0.3209		4.0990	0.0000	0.3297	0.4921	5.3338	3.8334	0.0000	0.4589				0.6603		3.3372
Mytilus	18	A	1	Tissue	0.0887	0.0876	0.1299		5.5828	0.0000	0.4842	1.4987	8.0562	20.0692	0.0000	1.0463				2.5885		4.8273
Mytilus	18	B	1	Tissue	0.0395	0.0379	0.0598		3.9253	0.0000	0.1583	1.9483	5.4133	12.0153	0.0000	0.5702				3.0788		3.0964
Mytilus	18	C	1	Tissue	0.2994	0.3910	0.5475		5.4659	0.1131	0.8303	1.3540	10.6009	14.5026	0.0000	0.2319				1.9176		5.3002
Psammechinus	15	BC	pooled	Tissue																		
Psammechinus	16	ABC	pooled	Tissue	0.0393	0.2268	0.0636		0.9193	0.0609	0.3873	0.0535	0.1567	0.2260		0.0465				0.6606	0.1408	1.3556
Psammechinus	17	ABC	pooled	Tissue	0.0491	0.1818	0.0696		1.0011	0.0633	0.2563	0.2406	0.4602	0.0877		0.0752				0.5135	0.1856	1.6038
Psammechinus	18	ABC	pooled	Tissue	0.0221	0.2571	0.0673		1.9323	0.1372	0.3154	0.3654	0.8563	0.0062		0.1382				0.7326	0.4392	3.1876
Psammechinus	20	AB	pooled	Tissue	0.1213	0.7259	0.1221		5.5845	0.4003	0.5156	2.1518	2.6801	0.2489		0.4041				2.6525	1.0481	6.8133
Psammechinus	23	AB	pooled	Tissue	0.0907	0.2149	0.0948		2.8261	0.0888	0.4997	0.5834	0.6482	0.1320		0.0778				0.3035	0.4899	2.2526
Psammechinus	28	ABC	pooled	Tissue	0.0258	0.1982	0.0606		2.6499	0.0882	0.1555	0.6362	0.7300	1.5195		0.0365				1.1560	0.2255	1.9331
Strongylocentrotus	17	A	1	Tissue	0.0628	0.0392	0.1465		1.3515		1.2374	0.5052	0.6986	0.0000	0.0000	0.1217	0.2666	0.1291	0.0000	0.6357		1.1885
Strongylocentrotus	17	C	1	Tissue	0.0470	0.0487	0.0792		0.5548		0.7188	0.7903	0.7521	0.0000	0.3030	0.1735	0.0818	0.3634	0.0604	2.1118		1.2295
Strongylocentrotus	18	A	1	Tissue	0.0840	0.1601	0.2513		3.1646		1.5010	1.2533	1.3248	0.0000	0.4338	0.2695	0.3201	0.2245	0.1256	3.4843		2.0907
Strongylocentrotus	18	B	1	Tissue	0.0459	0.1159	0.1151		1.3079		2.4618	1.9331	1.7145	0.0000	0.4371	0.3003	0.1452	0.0000	0.3259	3.9377		2.2516
Strongylocentrotus	19	A	1	Tissue	0.0629	0.0497	0.1318		2.7264		1.3908	0.4828	0.9865	0.0000	0.0000	0.0872	0.0516	0.1808	0.0346	1.5189		1.3564
Strongylocentrotus	19	B	1	Tissue	0.0780	0.1005	0.1083		0.7954		2.2588	0.5966	0.5498	1.3906	0.2855	0.3633	0.4218	0.0000	0.0539	3.0736		1.2917
Strongylocentrotus	19	C	1	Tissue	0.0534	0.1148	0.1901		1.7481		1.5184	2.7552	2.7375	0.4784	0.4277	0.7131	0.5248	0.3972	0.2957	3.4929		2.8057
Strongylocentrotus	20	A	1	Tissue	0.0552	0.1294	0.1392		4.6468		2.1398	1.6891	2.4053	0.0000	0.0000	0.3372	0.2880	0.2601	0.1537	1.5320		3.5081
Strongylocentrotus	20	B	1	Tissue	0.0550	0.0986	0.1041		1.5941		0.6194	0.6792	0.7895	0.0000	0.0000	0.1459	0.1035	0.0733	0.0724	0.6182		3.6543
Strongylocentrotus	20	C	1	Tissue	0.0919	0.0835	0.2171		1.1488		1.0159	1.2593	1.2513	0.0000	0.0000	0.5894	0.2536	0.2075	0.0512	1.4322		1.5846
Strongylocentrotus	22	A	1	Tissue	0.1573	0.0552	0.1493		2.1640		0.5825	0.5505	0.5045	0.2088	0.0000	0.6942	0.1457	0.0846	0.0677	1.3528		1.3836
Strongylocentrotus	22	B	1	Tissue	0.1295	0.4450	0.5325		4.7848		0.3808	2.5698	2.1176	0.0000	0.0000	0.0900	0.2083	0.2137	0.2560	0.6650		8.0007
Strongylocentrotus	22	C	1	Tissue	0.0474	0.1422	0.0743		2.8143		1.4579	1.1029	1.0863	0.0000	0.0000	0.1458	0.1600	0.0507	0.0000	1.1482		2.2983
Strongylocentrotus	25	A	1	Tissue	0.0827	0.2892	0.1632		7.4906		0.3395	4.0251	1.4901	0.2294	0.0962	0.0709	0.2078	0.1108	0.0987	0.3669		5.5675
Strongylocentrotus	25	B	1	Tissue	0.0957	0.2778	0.1539		6.1188		0.7459	1.7923	1.3630	0.0000	0.0000	0.0710	0.0379	0.0000	0.0000	0.7491		7.6992
Strongylocentrotus	25	C	1	Tissue	0.1562	0.1456	0.5554		22.8008		0.4141	5.1108	3.6607	0.0000	0.0000	0.0927	0.0000	0.0000	0.0000	3.9620		21.1862
Strongylocentrotus	30	A	1	Tissue	0.0628	0.2113	0.1692		9.8452		0.1678	4.0745	1.6148	0.0000	0.0000	0.2083	0.0561	0.1230	0.1110	3.3639		9.4906
Strongylocentrotus	30	B	1	Tissue	0.3097	0.4056	0.2740		20.1962		0.7418	5.1151	2.9449	0.0000	0.0000	0.2992	0.0984	0.0000	0.1138	3.1083		13.4825
Strongylocentrotus	30	C	1	Tissue	0.0870	0.4564	0.1452		8.0494		0.7200	5.3323	2.3685	0.0000	0.0000	0.2153	0.2576	0.2120	0.1697	2.5246		5.8026

Table B.7: Concentration of organic solutes (mmol g^{-1}WW) in tissue of the six study organisms II

Species	Salinity	Tank	Individual	Medium	Glutamine	Glycine	Histamine	Homarine	Homo-cysteine	Homo-serine	Hydroxy-acetone	Hypo-taurine	Inosine	Isoleucine	L-Arginine	Lactate	Leucine	Lysine	Malonate	Methyl-amine	N-Methyl-hydantoin	Methyl-malonate
Asterias	11	C	1	Tissue	0.3691	6.4173						0.0006		0.0955			0.2379	1.9658		0.0922		
Asterias	11	D	1	Tissue	0.0912	1.8390						0.0002		0.1808			0.3192	2.6849		0.0529		
Asterias	11	F	1	Tissue	0.4626	2.0179						0.0010		0.1197			0.2755	1.7287		0.2087		
Asterias	12	A	3	Tissue	0.9435	8.6068						0.0024		1.5649			1.5082	6.8013		0.2006		
Asterias	12	B	1	Tissue	0.2805	4.1547						0.0004		0.1812			0.3447	2.7988		0.1345		
Asterias	12	C	2	Tissue	0.1350	5.3186						0.0001		0.0674			0.1440	1.3923		0.0567		
Asterias	13	A	1	Tissue	0.5386	7.9944						0.0002		0.4450			0.5912	4.5498		0.1320		
Asterias	13	E	1	Tissue	0.6623	6.8781						0.0009		0.1802			0.3451	6.4502		0.2058		
Asterias	13	F	1	Tissue	3.5436	8.2490						0.0046		1.8204			2.0285	8.0859		0.1365		
Asterias	15	B	1	Tissue	0.5963	22.3824						0.0009		0.2858			0.6370	6.5492		0.2980		
Asterias	15	C	3	Tissue	2.4532	9.7170						0.0064		1.9940			1.6906	12.3925		0.1898		
Asterias	15	D	2	Tissue	3.0134	21.4042						0.0011		0.9064			0.9423	12.9365		0.3249		
Asterias	18	A	1	Tissue	5.0466	43.8051						0.0196		6.5892			6.0286	24.5629		0.5825		
Asterias	18	C	2	Tissue	2.8964	48.5751						0.0113		0.4818			0.9533	10.2189		0.3666		
Asterias	18	D	2	Tissue	3.0179	33.0528						0.0012		0.7495			0.9445	10.6468		0.2917		
Asterias	23	C	1	Tissue	1.6822	71.8551						0.0014		0.8277			1.1187	8.7547		0.6295		
Asterias	23	D	2	Tissue	3.2645	112.6838						0.0084		2.0734			2.2886	15.1410		1.0645		
Asterias	23	F	1	Tissue	7.9430	76.6134						0.0256		3.6528			4.4491	9.9075		0.5328		
Littorina	6	A	pooled	Tissue	3.5057	1.5476	0.1756	3.2559				0.0032	0.0793	0.3097	10.1104	0.0000	0.6403	2.0328				1.0152
Littorina	6	B	pooled	Tissue	3.8985	1.6860	0.1088	4.8633				0.0045	0.1755	0.3964	13.0215	0.0000	0.8583	2.4935				1.6029
Littorina	6	D	pooled	Tissue	4.7498	3.0792	0.1205	3.6737				0.0032	0.2784	0.3053	8.4786	0.0000	0.7779	2.1687				1.0612
Littorina	7	A	pooled	Tissue	2.5781	0.7716	0.0769	2.3208				0.0018	0.1804	0.1624	6.7408	0.0000	0.4606	1.1223				0.3329
Littorina	7	B	pooled	Tissue	3.4917	1.6258	0.1002	3.8577				0.0043	0.0812	0.2735	8.1959	0.0000	0.7337	1.3399				0.8149
Littorina	7	E	pooled	Tissue	3.3234	1.3034	0.0545	3.0856				0.0026	0.1659	0.2269	4.4125	0.0000	0.5726	1.2936				0.5648
Littorina	8	B	pooled	Tissue	4.1229	1.4165	0.0866	2.3274				0.0042	0.0758	0.2370	7.9381	0.0000	0.4879	1.7508				0.7973
Littorina	8	D	pooled	Tissue	2.3609	1.4725	0.0355	2.9276				0.0047	0.0862	0.2128	3.6971	0.0000	0.5771	1.6289				0.8794
Littorina	8	F	pooled	Tissue	4.2867	2.1279	0.1098	5.8880				0.0060	0.0731	0.3825	8.6779	0.0000	0.8995	2.1630				0.9910
Littorina	9	A	pooled	Tissue	2.1170	1.5428	0.1058	2.9332				0.0043	0.1999	0.1799	5.6365	0.3919	0.4634	0.9774				0.3375
Littorina	9	B	pooled	Tissue	1.2494	2.2585	0.1011	3.2241				0.0038	0.3624	0.2868	4.5479	0.5041	0.6406	1.1055				0.5078
Littorina	9	D	pooled	Tissue	2.3191	1.9089	0.1019	3.6258				0.0052	0.2549	0.2464	5.9225	0.7115	0.6681	1.4051				0.4871
Littorina	11	B	pooled	Tissue	2.2045	1.9718	0.1894	4.0867				0.0070	0.3318	0.2447	6.2821	0.0000	0.6333	1.1027				0.6181
Littorina	11	C	pooled	Tissue	2.0341	1.1551	0.0937	2.9299				0.0040	0.1245	0.2668	0.7236	0.0000	0.6485	0.9474				0.4949
Littorina	11	F	pooled	Tissue	2.2606	1.7843	0.0998	4.5755				0.0054	0.1707	0.2094	5.8673	0.8007	0.5393	0.9389				0.3037
Littorina	14	A	pooled	Tissue	1.2774	0.8999	0.0566	2.4145				0.0045	0.0604	0.1268	1.9392	0.2740	0.3365	0.3430				0.1829
Littorina	14	C	pooled	Tissue	2.0430	1.1450	0.1282	4.8470				0.0070	0.0428	0.1947	2.6934	0.0000	0.4693	0.4925				0.3996
Littorina	14	E	pooled	Tissue	1.5261	3.1474	0.1328	8.2697				0.0096	0.3315	0.3237	4.1552	0.9417	0.7621	0.8818				0.5860
Littorina	19	B	pooled	Tissue	4.8064	5.3457	0.0903	9.9696				0.0187	0.2006	0.3122	4.6831	0.1912	0.7683	0.4876				0.5895
Littorina	19	E	pooled	Tissue	4.3453	5.0934	0.1269	8.6727				0.0138	0.1721	0.3127	3.3984	0.2543	0.6917	0.5382				0.5226
Littorina	19	F	pooled	Tissue	3.8367	4.7517	0.1469	7.0051				0.0214	0.1537	0.3125	2.7353	0.4527	0.7994	0.4544				0.6336
Mytilus	5	B	1	Tissue	0.4230	1.0003		0.3080				0.0041	0.0343	0.2776	5.4507		0.4267	3.5995		0.3555		0.9756
Mytilus	5	C	2	Tissue	0.3469	0.9980		0.0000				0.0009	0.1628	0.2399	3.2733		0.5276	1.7816		0.1801		0.7512
Mytilus	5	D	2	Tissue	0.6385	0.7646		0.4962				0.0132	0.1042	0.2337	4.4403		0.4607	2.5533		0.3255		0.5322
Mytilus	6	B	3	Tissue	0.5235	1.7357		0.0703				0.0078	0.3172	0.3944	7.7969		0.8666	2.0392		0.1571		0.8746
Mytilus	6	D	3	Tissue	1.1102	1.8729		1.2044				0.0067	0.6131	0.4256	6.2730		0.5047	2.6573		0.2758		0.5676
Mytilus	6	F	2	Tissue	0.8473	0.9666		0.3135				0.0140	0.2185	0.2639	5.1793		0.4270	2.3348		0.5846		0.8992
Mytilus	7	A	1	Tissue	0.4639	0.4143		0.0860				0.0023	0.1260	0.1080	2.3075		0.2010	0.8211		0.5979		1.0615
Mytilus	7	D	2	Tissue	0.4639	0.0223		0.0000				0.0183	0.3848	0.2511	8.2053		0.5937	2.1381		0.4093		2.0256
Mytilus	7	E	1	Tissue	0.6871	0.4763		0.6186				0.0014	0.2376	0.1039	6.2067		0.2237	1.4184		0.5857		0.7260
Mytilus	8	C	1	Tissue	1.2441	2.3250		0.0713				0.0027	0.5156	0.3722	7.3499		0.7037	2.4540		0.5013		0.9585
Mytilus	8	E	2	Tissue	1.4966	0.9102		0.0000				0.0353	0.3444	0.6482	12.2346		0.9472	3.5613		1.1894		1.3395
Mytilus	8	F	1	Tissue	1.3056	1.8381		1.4529				0.0421	0.4509	0.3789	9.6712		0.6193	2.4166		0.3369		0.6503
Mytilus	10	A	3	Tissue	1.6833	5.1053		0.5308				0.0089	0.1418	0.3726	7.2104		0.6365	2.1407		0.6091		1.0918

Table B.7: Concentration of organic solutes (mmol g^{-1}WW) in tissue of the six study organisms II

Species	Salinity	Tank	Individual	Medium	Glutamine	Glycine	Histamine	Homarine	Homo-cysteine	Homo-serine	Hydroxy-acetone	Hypo-taurine	Inosine	Isoleucine	L-Arginine	Lactate	Leucine	Lysine	Malonate	Methyl-amine	N-Methyl-hydantoin	Methyl-malonate
Mytilus	10	D	1	Tissue	1.9042	11.6965		0.5269				0.0176	0.4750	0.6142	9.3705		0.9666	3.0844		0.4349		0.5363
Mytilus	10	E	2	Tissue	1.4084	3.0578		0.0000				0.0101	0.1396	0.3166	3.4211		0.5202	1.5245		0.5309		0.8400
Mytilus	13	A	3	Tissue	3.4095	10.7605		0.0000				0.0264	0.3232	0.9078	7.3668		1.0344	2.7166		0.7027		0.4622
Mytilus	13	E	3	Tissue	4.6827	10.5287		0.9484				0.0797	0.6923	0.9563	10.5936		1.4616	4.9834		1.6688		2.0379
Mytilus	13	F	3	Tissue	1.0540	2.9495		0.5511				0.0140	0.1245	0.2099	7.0641		0.3869	6.7250		0.6745		0.5381
Mytilus	18	A	1	Tissue	3.4601	27.0272		3.5923				0.0487	0.3612	0.4737	8.6533		0.7169	2.9074		0.8171		0.5615
Mytilus	18	B	1	Tissue	1.9305	14.5630		2.9009				0.0359	0.3122	0.2680	4.1846		0.4408	2.6125		0.5867		0.3265
Mytilus	18	C	1	Tissue	3.6133	20.4782		2.4897				0.0294	0.1914	0.7991	7.8794		1.1914	0.3810		0.4979		0.5827
Psammechinus	15	BC	pooled	Tissue																		
Psammechinus	16	ABC	pooled	Tissue	0.2359	3.8102			0.0000	0.7304		0.0003	0.1866	0.1894	2.2798		0.7310	0.6554	0.5842	0.0264		
Psammechinus	17	ABC	pooled	Tissue	0.3167	3.9871			0.0000	0.7146		0.0004	0.1455	0.1778	2.1308		0.4716	0.8067	0.5516	0.0450		
Psammechinus	18	ABC	pooled	Tissue	1.1482	21.7394			0.0000	1.5989		0.0006	0.5189	0.3065	5.5751		1.0712	0.7357	1.1352	0.2323		
Psammechinus	20	AB	pooled	Tissue	3.1923	58.8686			0.9180	3.7070		0.0025	0.9112	0.8064	5.5675		2.3524	2.9231	3.6990	0.4359		
Psammechinus	23	AB	pooled	Tissue	0.2947	17.7149			0.0000	0.8870		0.0005	0.4024	0.3262	1.1996		0.7413	0.9330	3.6188	0.1164		
Psammechinus	28	ABC	pooled	Tissue	0.8652	31.9274			0.0000	0.9885		0.0013	0.2132	0.8485	2.4675		1.2696	1.5414	1.1087	0.1489		
Strongylocentrotus	17	A	1	Tissue	0.7062	27.3039		0.0545			0.1747	0.0010	0.0000	0.3316	6.5136		1.0212	0.8763			0.1774	
Strongylocentrotus	17	C	1	Tissue	1.5932	11.4609		0.4347			0.1789	0.0013	0.0000	0.3457	1.8330		1.9155	0.8442			2.8465	
Strongylocentrotus	18	A	1	Tissue	2.6146	44.9840		2.2557			0.2005	0.0086	0.0794	0.4702	12.2626		2.8218	1.9528			1.7291	
Strongylocentrotus	18	B	1	Tissue	2.3434	23.8628		1.2482			0.1797	0.0106	0.0593	0.2805	8.7804		1.7454	1.7901			2.0399	
Strongylocentrotus	19	A	1	Tissue	0.8130	37.1375		1.4211			0.0672	0.0068	0.0009	0.3800	7.3171		1.1960	0.8778			0.9699	
Strongylocentrotus	19	B	1	Tissue	1.9679	27.8630		3.6044			0.7223	0.0024	0.0000	0.2828	7.1298		1.6445	0.4902			1.7723	
Strongylocentrotus	19	C	1	Tissue	1.5560	35.5169		0.6899			0.2532	0.0053	0.0641	0.4390	7.2493		1.6003	2.6658			0.8938	
Strongylocentrotus	20	A	1	Tissue	2.4507	51.4649		0.1657			0.1714	0.0136	0.0287	0.4691	10.2232		1.3057	1.8199			1.2978	
Strongylocentrotus	20	B	1	Tissue	1.7670	52.6663		0.0000			0.2209	0.0009	0.0308	0.2843	6.0394		1.0614	0.7302			0.1660	
Strongylocentrotus	20	C	1	Tissue	1.5979	42.8624		0.6355			0.1931	0.0019	0.0203	0.2506	5.5953		1.4292	1.2324			2.3032	
Strongylocentrotus	22	A	1	Tissue	0.9902	72.6795		0.1293			0.2310	0.0316	0.0185	0.2377	7.8996		0.6692	0.6453			0.5880	
Strongylocentrotus	22	B	1	Tissue	2.9259	96.2870		0.2363			0.7789	0.0014	0.1325	0.9386	6.8995		1.9839	2.0610			0.3653	
Strongylocentrotus	22	C	1	Tissue	2.0785	46.6952		0.5607			0.1536	0.0037	0.0367	0.4914	4.9725		1.4095	0.9551			0.3474	
Strongylocentrotus	25	A	1	Tissue	19.2123	85.1840		0.1113			0.6030	0.0069	0.0464	1.3723	6.8374		2.4185	1.9707			0.1516	
Strongylocentrotus	25	B	1	Tissue	4.5955	89.9204		0.4607			0.3042	0.0109	0.0536	0.9633	9.5110		1.6299	1.3201			0.6657	
Strongylocentrotus	25	C	1	Tissue	8.3586	137.2001		1.5471			0.5234	0.0006	0.0757	3.3976	12.6336		5.8491	3.9014			2.4624	
Strongylocentrotus	30	A	1	Tissue	6.3343	87.6242		0.0000			0.2404	0.0192	0.0577	0.9606	8.7658		1.9239	1.3181			4.1261	
Strongylocentrotus	30	B	1	Tissue	8.5068	173.3421		0.3810			0.2892	0.0223	0.1389	1.4076	9.7909		3.2967	1.7306			4.5847	
Strongylocentrotus	30	C	1	Tissue	8.7891	81.1313		0.2020			0.2756	0.0132	0.0860	1.2564	7.6800		2.5393	1.7604			6.2439	

Table B.8: Concentration of organic solutes (mmol g^{-1}WW) in tissue of the six study organisms III

Species	Salinity	Tank	Individual	Medium	O-Acetylcholine	O-Phosphocholine	Ornithine	Phenylalanine	Proline	Sarcosine	Serine	Succinate	Taurine	Threonine	Trimethylamine	Tryptophan	Tyrosine	Unassigned 1	Unassigned 3	Unassigned 4	Unassigned 5	Valine	sn-Glycero-3-phosphocholine	β-Alanine	π-Methylhistidine	τ-Methylhistidine
Asterias	11	C	1	Tissue	0.0910	0.6139	0.4212	0.0368		0.2283	9.7551	0.0238	1.4924	0.3020	0.0222	0.0444	0.0448	89.3294	0.5004			0.1210	1.5336		0.0231	
Asterias	11	D	1	Tissue	0.2136	1.2374	0.3946	0.1478		0.3468	5.0093	0.0095	2.6466	0.3422	0.0109	0.0528	0.1435	59.1778	0.4435			0.2819	3.6939		0.1241	
Asterias	11	F	1	Tissue	0.2266	1.4284	0.5541	0.0876		0.4319	5.8372	0.0452	2.8807	0.3880	0.0276	0.0000	0.0519	20.6572	1.5451			0.1612	2.8602		0.0000	
Asterias	12	A	3	Tissue	0.3065	1.1035	1.8291	1.1232		0.5286	11.2579	0.0622	8.3120	1.1814	0.0043	0.1676	1.4253	21.8739	0.2308			1.7737	6.1844		0.2714	
Asterias	12	B	1	Tissue	0.3030	2.5314	0.4091	0.1451		0.5405	3.2621	0.0130	3.7149	0.4279	0.0162	0.0000	0.0417	84.6787	2.9578			0.2170	3.3302		0.1577	
Asterias	12	C	2	Tissue	0.1393	1.0456	0.1626	0.0267		0.2158	9.2434	0.0131	1.6587	0.2361	0.0050	0.0000	0.0219	17.0671	0.2251			0.1099	1.4513		0.0620	
Asterias	13	A	1	Tissue	0.2930	1.1894	0.6180	0.4121		0.5206	11.0507	0.0481	5.7541	0.4490	0.0039	0.0326	0.2609	35.8195	2.1736			0.5868	4.0705		0.2715	
Asterias	13	E	1	Tissue	0.2179	0.8315	0.6280	0.0729		0.3722	6.3708	0.0757	2.9566	0.3035	0.0395	0.0349	0.0962	14.4023	1.0326			0.2155	8.4346		0.0571	
Asterias	13	F	1	Tissue	0.2868	0.4963	1.3433	1.2904		0.4810	6.9222	0.0930	6.9147	2.0036	0.0211	0.1098	1.3308	4.1567	0.3036			1.7358	0.7007		0.6118	
Asterias	15	B	1	Tissue	0.3231	1.4938	1.1095	0.2252		0.1941	7.1372	0.0406	10.8266	0.4514	0.0177	0.1062	0.2616	2.7166	0.6390			0.3817	6.8277		0.1500	
Asterias	15	C	3	Tissue	0.8160	1.0048	3.5896	0.9498		0.4517	14.7584	0.1332	8.2212	2.1829	0.0093	0.1650	1.0927	6.2872	0.3108			2.1944	1.6641		0.4149	
Asterias	15	D	2	Tissue	0.2462	0.7138	2.8820	0.7067		0.2258	5.5094	0.0657	9.0273	0.9456	0.0119	0.2200	0.9094	4.2643	0.3878			0.9048	2.7151		0.6251	
Asterias	18	A	1	Tissue	0.2635	1.1217	7.9206	2.9011		0.9676	19.8618	0.2246	20.7161	2.7852	0.0982	0.4137	3.8008	22.2299	0.4994			6.7493	3.4108		2.1286	
Asterias	18	C	2	Tissue	0.1687	0.6016	2.9204	0.3953		0.1849	6.6326	0.0803	15.4393	0.7027	0.0242	0.1125	0.3301	11.1015	0.7292			0.5385	1.5296		0.3066	
Asterias	18	D	2	Tissue	0.2309	1.1093	3.0569	0.3586		0.1898	13.9555	0.0359	8.5797	0.7878	0.0150	0.1465	0.3405	5.9352	0.3395			0.8129	6.0921		0.3525	
Asterias	23	C	1	Tissue	0.4343	0.7568	3.8275	0.1400		0.3681	11.3286	0.0000	20.3181	0.5024	0.0410	0.0256	0.3635	88.0237	1.7122			0.9595	3.6075		0.2447	0.2214
Asterias	23	D	2	Tissue	0.4069	0.8589	4.5255	1.0073		0.5308	20.3507	0.1897	27.0230	2.1859	0.0397	0.1759	1.4248	12.5048	0.9416			2.3968	1.7634		0.8880	0.1942
Asterias	23	F	1	Tissue	0.4687	0.5691	3.0102	1.4896		0.3071	12.0797	0.0900	20.3259	2.6679	0.1603	0.2533	2.6200	14.7841	0.4216			3.5736	1.9801		0.8483	0.0882
Littorina	6	A	pooled	Tissue	0.2779	3.5643	0.2176		2.3737			2.2672	21.7562	1.7230			0.1803		0.0637			0.4757	5.4930		0.5191	0.1449
Littorina	6	B	pooled	Tissue	0.2699	4.8010	0.3142		3.7342			3.0922	33.3090	6.1641			0.1075		0.1083			0.6693	8.6816		0.1978	0.1887
Littorina	6	D	pooled	Tissue	0.3759	2.8206	0.0672		4.4105			1.5388	22.7541	2.1022			0.0934		0.1116			0.5681	4.5668		0.3583	0.1539
Littorina	7	A	pooled	Tissue	0.2207	1.7125	0.0192		3.3549			0.8876	15.8338	1.1079			0.1134		0.0518			0.3296	3.6132		0.0814	0.0877
Littorina	7	B	pooled	Tissue	0.3252	2.0147	0.0000		2.2072			1.3839	26.3165	1.9760			0.1331		0.1451			0.4847	4.2662		0.0761	0.1154
Littorina	7	E	pooled	Tissue	0.1633	1.5658	0.2047		2.8863			1.1726	16.7207	1.5839			0.1489		0.3820			0.4830	3.5303		0.0725	0.0927
Littorina	8	B	pooled	Tissue	0.3756	2.6667	0.1179		1.8447			0.8290	20.8251	1.1617			0.1280		0.0555			0.4494	4.1569		0.0943	0.0761
Littorina	8	D	pooled	Tissue	0.2056	2.2412	0.1144		2.3012			0.9668	25.8109	1.3692			0.0786		0.0416			0.4205	4.5913		0.0385	0.0996
Littorina	8	F	pooled	Tissue	0.1415	2.5398	0.2786		2.8979			1.5223	37.8981	1.9710			0.1226		1.2085			0.6538	8.4342		0.1169	0.2188
Littorina	9	A	pooled	Tissue	0.2098	1.1523	0.1388		1.4808			0.8289	23.0877	0.7885			0.1696		0.0345			0.3754	3.2755		0.0585	0.1773
Littorina	9	B	pooled	Tissue	0.1043	1.8113	0.1157		2.8459			1.0073	22.0111	0.9201			0.1843		0.0497			0.5079	3.5450		0.0903	0.1208
Littorina	9	D	pooled	Tissue	0.1957	2.3536	0.3397		3.1384			1.1876	20.8653	0.6249			0.1930		0.0613			0.5533	4.2522		0.1135	0.2305
Littorina	11	B	pooled	Tissue	0.1176	2.2210	0.3205		1.9407			1.0574	35.9484	1.2128			0.1718		0.0687			0.5267	4.0075		0.1890	0.0972
Littorina	11	C	pooled	Tissue	0.1813	1.8260	0.2481		1.7549			0.9376	29.4302	0.7977			0.1580		0.0858			0.4959	3.2488		0.1222	0.1460
Littorina	11	F	pooled	Tissue	0.2809	1.3864	0.2449		1.5624			0.9239	23.1447	0.6214			0.1722		0.0489			0.4774	2.9140		0.0889	0.2689
Littorina	14	A	pooled	Tissue	0.1548	0.6235	0.1580		0.9280			0.5263	12.7473	0.2963			0.1000		0.1004			0.2696	1.5933		0.0659	0.3141
Littorina	14	C	pooled	Tissue	0.2463	1.2241	0.1233		2.0155			0.3608	24.2889	0.9658			0.1023		0.3194			0.3647	2.5898		0.1295	0.3209
Littorina	14	E	pooled	Tissue	0.3354	1.2663	1.3281		2.5896			2.2209	33.8728	0.9574			0.2035		0.2707			0.7356	3.8340		0.0824	0.4685
Littorina	19	B	pooled	Tissue	0.4712	1.2280	0.4499		4.8261			0.8870	45.2772	0.9780			0.2276		0.3795			0.6742	4.5559		0.1820	
Littorina	19	E	pooled	Tissue	0.3090	1.0859	0.2419		3.5199			0.8655	39.7745	0.8572			0.2655		0.2110			0.6444	3.0370		0.1622	
Littorina	19	F	pooled	Tissue	0.5906	1.1127	1.3878		3.1371			0.9963	36.6970	1.1160			0.3096		0.2176			0.6515	3.2651		0.2829	
Mytilus	5	B	1	Tissue	0.1104	3.5104	2.2332	0.1726	0.7838	1.4738		2.8998	17.7896	0.8970	0.0845	0.0522	0.0420		0.6635			0.4967	1.1677	0.8519	0.0540	
Mytilus	5	C	2	Tissue	0.0648	4.2892	2.3607	0.1896	0.6204	1.1470		4.0366	5.3132	0.7751	0.0213	0.0685	0.1674		0.5562			0.3363	4.0545	0.3533	0.1412	
Mytilus	5	D	2	Tissue	0.1372	3.4200	3.4157	0.0978	0.6712	1.5072		1.1457	13.4680	0.6909	0.0490	0.1169	0.1809		0.5251			0.4299	4.3744	0.9561	0.1018	
Mytilus	6	B	3	Tissue	0.2591	3.5953	3.1602	0.2110	1.0371	1.7006		1.5467	8.7220	1.4925	0.1428	0.0641	0.3297		0.5186			0.7689	2.3652	1.3625	0.1638	
Mytilus	6	D	3	Tissue	0.1121	1.8956	3.0716	0.1410	1.2086	1.6453		1.2071	18.7180	1.0858	0.3175	0.0000	0.0801		0.4238			0.7395	1.8851	1.5642	0.1550	
Mytilus	6	F	2	Tissue	0.2118	7.2552	1.2249	0.0629	1.0809	2.1480		0.5229	20.6780	0.8155	0.1204	0.0000	0.0256		2.5484			0.4134	1.4864	2.1104	0.0280	
Mytilus	7	A	1	Tissue	0.1985	4.8227	0.9898	0.0403	1.4047	1.4332		0.1295	16.8448	0.2752	0.0570	0.0631	0.0495		1.1610			0.1795	1.4577	0.9111	0.1842	
Mytilus	7	D	2	Tissue	0.3433	5.7288	3.0538	0.1147	1.1273	2.9667		1.1407	21.0560	0.9279	0.6031	0.0696	0.0443		1.4934			0.5600	2.2655	1.8271	0.2179	
Mytilus	7	E	1	Tissue	0.0829	3.0666	1.5316	0.0242	0.8968	1.1901		0.2172	23.1680	0.3660	0.1042	0.0000	0.0000		0.6427			0.1535	2.3342	0.8626	0.0700	
Mytilus	8	C	1	Tissue	0.1167	2.8467	2.2890	0.1764	0.8388	1.5578		1.5000	24.3495	0.7993	0.0820	0.0000	0.1578		0.7644			0.7521	1.8823	1.0820	0.1773	
Mytilus	8	E	2	Tissue	0.2973	4.8663	4.9126	0.2343	2.2950	2.1324		10.2771	26.6175	1.1043	0.4240	0.0000	0.2317		1.1545			1.1054	2.1238	4.1357	0.3897	

Table B.8: Concentration of organic solutes (mmol g^{-1}WW) in tissue of the six study organisms III

Species	Salinity	Tank	Individual	Medium	O-Acetylcholine	O-Phosphocholine	Ornithine	Phenylalanine	Proline	Sarcosine	Serine	Succinate	Taurine	Threonine	Trimethylamine	Tryptophan	Tyrosine	Unassigned 1	Unassigned 3	Unassigned 4	Unassigned 5	Valine	sn-Glycero-3-phosphocholine	β-Alanine	π-Methylhistidine	τ-Methylhistidine
Mytilus	8	F	1	Tissue	0.2946	3.3173	3.2024	0.2003	0.7614	1.9037		1.2170	20.3910	1.0775	0.2365	0.0718	0.1973		0.5194			0.7025	2.8173	1.6300	0.2201	
Mytilus	10	A	3	Tissue	0.2294	4.0880	2.0787	0.2583	0.7555	1.0753		0.3654	26.3670	0.8690	0.1262	0.0000	0.1223		0.3854			0.6722	2.8723	0.6885	0.1942	
Mytilus	10	D	1	Tissue	1.9549	1.4039	2.6587	0.2982	1.3346	1.2686		0.5764	25.9710	1.5067	0.2837	0.1060	0.4412		0.3434			1.1937	2.8453	1.8883	0.5094	
Mytilus	10	E	2	Tissue	0.1382	2.7852	1.6877	0.1702	0.8100	1.0360		0.3177	22.1320	0.8484	0.2780	0.0000	0.1459		0.7137			0.5821	3.2123	1.0159	0.1696	
Mytilus	13	A	3	Tissue	0.2186	2.5386	1.5213	0.2021	1.6681	0.8493		0.2199	26.8590	1.4310	0.1390	0.0000	0.3508		0.3796			1.5841	3.9297	0.7967	0.2588	
Mytilus	13	E	3	Tissue	0.3268	4.5232	4.8652	0.3716	1.7631	2.6529		0.4667	52.1850	2.5711	0.0980	0.1802	0.4982		0.7670			1.9241	9.1739	4.3661	0.2431	
Mytilus	13	F	3	Tissue	0.0818	1.2344	0.8964	0.0775	0.5637	0.3971		0.1713	21.8190	0.3884	0.0731	0.0401	0.1332		0.2490			0.3319	2.0110	1.0752	0.1407	
Mytilus	18	A	1	Tissue	0.2124	1.2951	1.2155	0.1680	1.7463	0.4665		0.1876	34.6500	0.9301	0.1846	0.0611	0.3012		0.3940			0.8762	2.5286	2.3879	0.1557	
Mytilus	18	B	1	Tissue	0.1901	0.8147	1.2947	0.1213	1.4150	0.6134		0.0000	23.7900	0.5303	0.5619	0.1014	0.1334		0.2288			0.5205	3.1491	1.3776	0.1649	
Mytilus	18	C	1	Tissue	0.2933	1.5247	0.5655	0.3342	1.1817	0.4096		0.8792	25.7600	1.1382	0.6460	0.1147	0.4287		0.3905			1.8317	2.9234	3.3262	0.4707	
Psammechinus	15	BC	pooled	Tissue																						
Psammechinus	16	ABC	pooled	Tissue	0.0201	1.1898	0.0869	0.1834	0.3091	0.3681		0.0319	1.5826	0.4221			0.1862		0.3178			0.3059	0.4509	0.0613		
Psammechinus	17	ABC	pooled	Tissue	0.0232	0.6992	0.0447	0.1088	0.1416	0.1988		0.0243	1.5939	0.4266			0.1185		0.3063			0.2880	1.2540	0.1207		
Psammechinus	18	ABC	pooled	Tissue	0.0716	0.9934	0.5437	0.2441	1.1885	0.5895		0.0830	3.2617	0.8303			0.2316		0.2522			0.5077	1.0424	0.4613		
Psammechinus	20	AB	pooled	Tissue	0.3579	2.0369	2.1501	0.5576	1.9593	1.0926		0.2099	14.9774	2.0085			0.7891		1.1478			1.2555	3.2275	0.9616		
Psammechinus	23	AB	pooled	Tissue	0.0703	0.4599	0.3718	0.1457	0.0514	1.9981		0.0307	6.6278	0.8325			0.1196		0.1430			0.4798	1.2410	0.3399		
Psammechinus	28	ABC	pooled	Tissue	0.1839	0.4423	0.1774	0.4993	0.1645	0.2266		0.0423	6.0715	0.8719			0.8196		0.1855			1.3568	0.2606	0.2802		
Strongylocentrotus	17	A	1	Tissue	0.1900	5.3346		0.1128	1.4869	0.1489	2.1135		16.2253	0.7602	0.0160	0.0000	0.1276	17.5045	1.1558	0.1585	0.1425	0.4331	0.4331	0.3649		
Strongylocentrotus	17	C	1	Tissue	0.1738	4.5482		0.0901	2.2674	0.1597	1.1024		11.6090	0.8689	0.0403	0.0000	0.0424	37.1290	2.0282	1.0562	0.3762	0.3194	0.2699	0.4207		
Strongylocentrotus	18	A	1	Tissue	0.2028	5.1013		0.3412	3.3007	0.2987	6.1163		24.6189	1.4602	0.0661	0.1146	0.2419	76.7500	2.4679	1.2942	1.0442	0.6681	1.1115	0.6284		
Strongylocentrotus	18	B	1	Tissue	0.1768	4.3335		0.2746	3.1723	0.2149	1.3943		19.9741	0.9090	0.1436	0.1818	0.5559	42.6187	2.0047	1.5559	0.9688	0.4330	0.6021	0.7391		
Strongylocentrotus	19	A	1	Tissue	0.0772	2.3120		0.2092	1.2291	0.2073	7.4242		8.1504	0.8398	0.0592	0.0435	0.2048	16.7983	0.5239	0.2930	0.7474	0.4986	0.7771	0.3349		
Strongylocentrotus	19	B	1	Tissue	0.2872	2.4950		0.0477	2.7283	0.5442	6.6438		9.7587	0.9880	0.0477	0.0139	0.0375	44.9995	1.5346	0.5733	0.6035	0.3708	0.7018	0.5461		
Strongylocentrotus	19	C	1	Tissue	0.2831	3.5336		0.1952	2.1611	0.5162	2.5419		22.0136	0.7774	0.1801	0.0728	0.1837	59.7274	1.2537	1.1590	1.4424	0.5592	0.9207	1.0849		
Strongylocentrotus	20	A	1	Tissue	0.1464	2.2638		0.3004	2.9113	0.4923	2.6794		17.3341	1.1542	0.0694	0.0272	0.2269	30.4643	2.0236	0.5271	0.9119	0.7044	0.3027	0.7075		
Strongylocentrotus	20	B	1	Tissue	0.1320	1.0435		0.1199	2.6751	0.1734	1.1239		6.6193	0.9227	0.0254	0.0347	0.1752	8.5012	0.9127	0.2919	0.4781	0.4047	0.5900	0.6223		
Strongylocentrotus	20	C	1	Tissue	0.1990	3.3338		0.0929	2.6046	0.2621	0.3248		13.8337	0.7879	0.0539	0.0000	0.1473	33.7522	1.2005	0.3814	1.1363	0.3810	0.7889	0.5897		
Strongylocentrotus	22	A	1	Tissue	0.1307	1.4125		0.0633	1.7146	0.1364	1.4018		9.1570	0.6031	0.0155	0.0422	0.1165	23.7694	0.8366	0.1714	0.8457	0.3245	0.5233	0.4009		
Strongylocentrotus	22	B	1	Tissue	0.1021	0.9344		0.6712	2.6463	5.1573	4.8820		10.0725	2.4537	0.0517	0.0000	0.5474	1.9002	0.6318	0.8075	0.0938	1.6506	1.8680	0.4205		
Strongylocentrotus	22	C	1	Tissue	0.1864	1.0710		0.2452	0.8782	0.4968	2.7753		9.6270	1.1992	0.0306	0.0911	0.3174	18.9299	0.9801	0.5302	0.7542	0.6966	0.4041	0.5755		
Strongylocentrotus	25	A	1	Tissue	0.4361	0.4661		1.1257	1.1915	4.0551	11.0262		7.3959	2.6138	0.0042	0.1531	1.0425	3.4792	0.8852	0.2914	0.4494	2.1636	0.5024	0.0970		
Strongylocentrotus	25	B	1	Tissue	0.4616	0.0993		0.6852	1.6780	2.1841	9.8207		9.8722	1.7535	0.0069	0.1022	1.0797	6.6906	0.3536	0.2412	0.1116	1.4550	0.8628	0.4450		
Strongylocentrotus	25	C	1	Tissue	0.6940	0.2400		2.0792	4.5505	6.9744	21.4107		38.6153	5.9048	0.1888	0.0613	2.9148	43.5457	3.7607	2.5323	0.0000	5.0323	4.3340	1.1244		
Strongylocentrotus	30	A	1	Tissue	0.3414	0.1208		0.7801	2.3421	2.8269	10.6913		17.1383	2.4145	0.0461	0.1073	1.0187	32.0952	1.6669	1.9999	0.6060	1.4478	1.4676	0.6182		
Strongylocentrotus	30	B	1	Tissue	0.5334	0.2394		1.1230	2.7329	4.2981	18.5696		24.9005	3.3805	0.0417	0.1378	1.4591	38.3955	1.6538	1.3203	0.6722	2.4727	4.0005	1.0844		
Strongylocentrotus	30	C	1	Tissue	0.2612	0.3796		1.0856	0.0000	4.4610	10.3167		12.9919	2.5213	0.0418	0.2388	1.3579	46.6273	1.9266	1.2445	0.4683	1.7978	0.5356	0.6431		

Table B.9: Statistical outcomes of osmolyte concentrations

Type	Compound	Species	Model	RSE	R^2	DF	Coefficient	Estimate	F-value	p-value			
Organic													
	Alanine	Snails	Quadratic		0.6019	2,18					13.610	<0.001	***
		Sea stars	Linear		0.6592	1,16					30.950	<0.001	***
		Sea urchins	Logistic	4.3059		16	b	-0.6735	0.4521	-1.4900		0.1557	
							d	13.4389	2.4143	5.5663		<0.001	***
							e	22.8421	1.0726	21.2955		<0.001	***
	Aspartate	Snails	Quadratic		0.4666	2,18					7.874	0.0035	**
		Mussels	Logistic	2.5788		18	b	-0.7664	0.3155	-2.4294		0.0258	*
							d	9.6000	0.8946	10.7311		<0.001	***
							e	5.6320	0.6311	8.9247		<0.001	***
		Sea stars	Logistic	0.8958		14	b	-2.3171	15.8731	-0.1460		0.8860	
							c	1.0515	0.2587	4.0649		0.0012	**
							d	2.0230	0.5172	3.9116		0.0017	**
							e	18.2960	2.3742	7.7061		<0.001	***
		Sea urchins	Cubic		0.2361	1,17					5.255	0.0349	*
	Betaine	Snails	Quadratic		0.7551	2,18					27.760	<0.001	***
		Mussels	Quadratic		0.8773	2,18					64.320	<0.001	***
		Sea stars	Linear		0.3042	1,16					6.995	0.0177	***
	Glutamate	Snails	Quadratic		0.3724	2,18					5.341	0.0151	*
		Mussels	Logistic	1.7928		18	b	-0.9770	0.6094	-1.6033		0.1263	
							d	5.2018	0.5722	9.0904		<0.001	***
							e	5.1625	0.7175	7.1955		<0.001	***
		Sea urchins	Logistic	3.8149		16	b	-0.6257	0.3373	-1.8550		0.0821	
							d	10.8249	1.9257	5.6213		<0.001	***
							e	21.9189	1.1248	19.4864		<0.001	***
	Glycine	Snails	Quadratic		0.7758	2,18					31.150	<0.001	***
		Mussels	Quadratic		0.8343	2,18					45.310	<0.001	***
		Sea stars	Quadratic		0.9248	2,15					92.300	<0.001	***
		Sea urchins	Logistic	23.4866		16	b	-0.4256	0.1578	-2.6970		0.0159	*
							d	117.9502	15.2492	7.7349		<0.001	***
							e	20.8121	1.0046	20.7163		<0.001	***
	Homarine	Snails	Quadratic		0.6494	2,18					16.670	<0.001	***
		Mussels	Quadratic		0.7838	2,18					32.640	<0.001	***
	Lysine	Snails	Quadratic		0.7627	2,18					28.920	<0.001	***
		Sea stars	Quadratic		0.6329	2,18					12.930	<0.001	***
	Serine	Sea stars	Linear		0.3571	1,16					8.889	0.0088	**
		Sea urchins	Logistic	4.3059		16	b	-0.6735	0.4521	-1.4900		0.1557	
							d	13.4389	2.4143	5.5663		<0.001	***
							e	22.8421	1.0726	21.2955		<0.001	***
	Taurine	Snails	Quadratic		0.3912	2,18					5.784	0.0115	*
		Mussels	Logistic	7.0450		18	b	-0.5191	0.2651	-1.9586		0.0658	
							d	30.3416	3.1982	9.4872		<0.001	***
							e	5.7416	0.7441	7.7165		<0.001	***
		Sea stars	Linear		0.8596	1.16					97.960	<0.001	***
Inorganic													
	Chloride	Snails	Quadratic		0.9601	2,18					241.400	<0.001	***
		Mussels	Quadratic		0.9147	2,18					96.470	<0.001	***
		Sea anemones	Cubic		0.5467	3,14					7.835	0.0026	**
		Sea stars	Linear		0.5385	1,16					20.830	<0.001	***
		Sea urchins	Quadratic		0.2850	2,17					4.787	0.0224	*
	Potassium	Snails	Quadratic		0.6825	2,18					19.350	<0.001	***
		Mussels	Logistic	3.4013		18	b	-0.1549	0.0609	-2.5424		0.0204	*

Table B.9: Statistical outcomes of osmolyte concentrations

Type	Compound	Species	Model	RSE	R^2	DF	Coefficient	Estimate	F-value	p-value			
							d	54.9503	7.2920	7.5357		<0.001	***
							e	4.3863	1.4046	3.1229		0.0059	**
		Sea anemones	Logistic	25.3201		16	b	18.8807	106.9202	0.1766		0.8620	
							c	67.8068	6.3315	10.7094		<0.001	***
							d	143.0856	12.6605	11.3018		<0.001	***
							e	8.5466	2.8415	3.0078		0.0083	**
		Sea stars	Linear		0.2742	1,16					6.046	0.0257	*
	Sodium	Snails	Quadratic		0.9890	2,18					812.300	<0.001	***
		Mussels	Quadratic		0.9342	2,18					127.800	<0.001	***
		Sea anemones	Cubic		0.6540	3,16					10.080	<0.001	***
		Sea stars	Quadratic		0.5000	2,15					7.499	0.0055	**

Table B.10: Methods I: Maintenance

Species	Collection site	Replicates	Individuals tank^{-1}	Diet	Feeding quantity	Feeding frequency	Water change frequency
Mussels	Kiel Bight, Baltic Sea, Germany	6	5	*Rhodomonas balthica*	10000 cells mL^{-1}	2 x day^{-1}	bi-weekly
Snails	Kiel Bight, Baltic Sea, Germany	6	10	*Fucus vesiculosus*	2 g	weekly	bi-weekly
Sea anemones	Kiel Bight, Baltic Sea, Germany	6	1-4	*Artemia fransiscana*	6000 ind tank^{-1}	3x week^{-1}	weekly/bi-weekly
Green sea urchins	Kattegat, Baltic Sea, Germany	3	2	*Fucus vesiculosus*	1 g	3x week^{-1}	3x week^{-1}
Shore sea urchins	Eckernförder Bight, Baltic Sea, Germany	2-3	1-2	*Fucus vesiculosus*	0.85 g	3x week^{-1}	3x week^{-1}
Sea stars	Kiel Bight, Baltic Sea, Germany	6	5	*Mytilus* sp.	5 ind	weekly	bi-weekly

Table B.11: Methods II: Sampling

Species	Extracted body fluid (mL)	Methods of body fluid extraction	Tissue
Mussels	0.2	via syringe from adductor mussel	entire soft body
Snails	0.02	tissue slashed and drained via centrifuge (Betzer et al. 1974)	entire soft body
Sea anemones	NA*	NA	Whole-body
Green sea urchins	0.2	drawn via syringe through peristomial membrane	entire soft body
Shore sea urchins	1.5	see above	entire soft body
Sea stars		drained from body cavity	pyloric cecae

* sea anemones were too small for fluid extraction

Table B.12: Monitoring of physio-chemical water parameters

Parameters	Monitoring frequency	Mean	SD	Min	Max	Device
Salinity	daily		0.2			WTW Cond 315i
Temperature (C)	weekly	14.5	0.3	13.7	15.8	WTW Cond 315i
pH	weekly	8.23	0.15	7.79	10.1	WTW pH 3110 probe
Toxic end products:	bi-weekly/weekly					JBL quick tests
PO_4^{3+}		0.17	0.51	0	5	
NO_3^-		1.18	2.69	0	15	
NO_2^-		0.17	0.26	0	1	

Table B.13: Monitoring of physio-chemical water parameters

Species	Treatment	Mean	SD	Min	Max
Asterias rubens	10	10.05	0.10	9.9	10.3
	11	11.05	0.11	10.9	11.4
	12	12.07	0.16	11.9	12.7
	13	13.07	0.17	12.9	13.8
	15	15.04	0.10	14.9	15.3
	18	18.07	0.49	17.8	23.1
	23	23.05	0.13	22.8	23.6
Mytilus edulis	5	5.01	0.11	4.8	5.3
	6	6.01	0.13	5.8	6.5
	7	7.00	0.11	6.8	7.3
	8	7.99	0.12	7.8	8.6
	10	9.98	0.11	9.8	10.3
	13	12.96	0.09	12.8	13.4
	18	17.95	0.11	17.6	18.5
Littorina littorea	6	6.07	0.22	5.9	8.1
	7	7.08	0.28	5.9	9.6
	8	8.08	0.22	7.9	10.2
	9	9.07	0.26	8.9	12.1
	11	11.04	0.26	10.9	14.1
	14	14.06	0.17	13.5	15.8
	19	19.02	0.17	17.5	19.9
Strongylocentrotus droebachiensis	17	17.11	0.21	17.00	18.30
	18	18.12	0.22	17.90	19.10
	19	19.13	0.20	19.00	20.20
	20	20.11	0.19	19.90	21.10
	22	22.11	0.19	21.90	23.10
	25	25.11	0.21	24.60	26.10
	30	30.02	0.14	29.00	30.20
Psammechinus milliaris	15	15.12	0.22	15.00	16.20
	16	16.11	0.20	15.90	17.10
	17	17.10	0.19	17.00	18.10
	18	18.12	0.41	17.90	20.20
	20	20.14	0.39	19.80	22.20
	23	23.19	0.72	22.90	28.10
	28	28.00	0.57	23.00	28.30
Diadumene lineata	7	6.95	0.07	6.80	7.10
	8	7.95	0.07	7.70	8.20
	9	8.98	0.08	8.90	9.30
	10	9.97	0.09	9.80	10.30
	12	11.93	0.19	9.90	12.20
	15	14.97	0.08	14.80	15.30
	20	19.95	0.07	19.80	20.20

4

Effect of long-term low salinity stress on gene expression of two locally-adapted *Mytilus* sp. populations from the Baltic Sea hybridzone

Imke Podbielski, Trystan Sanders, Uwe John, Pierre De Wit, Frank Melzner

In preparation

Effect of long-term low salinity stress on gene expression of two locally-adapted *Mytilus* sp. populations from the Baltic Sea hybridzone

Imke Podbielski[1*], Trystan Sanders[1a], Uwe John[2,3], Pierre De Wit[4], Frank Melzner[1]

Affiliations

[1]Experimental Ecology - Benthic Ecology, GEOMAR-Helmholtz Centre for Ocean Research Kiel, Hohenbergstraße 2, 24105 Kiel, Germany

[2] Ecological Chemistry - Biosciences, Alfred Wegener Institute Helmholtz Centre for Polar and Marine Research, Am Handelshafen 12, 27515 Bremerhaven, Germany

[3] Helmholtz Institute for Functional Marine Biodiversity at the University of Oldenburg (HIFMB), Ammerländer Heerstraße 231, 26129 Oldenburg

[4] Department of Marine Sciences, Tjärnö Marine Laboratory, University of Gothenburg, Strömstad, Sweden

[a]currently at: School of Ocean and Earth Science, National Oceanography Centre Southampton, University of Southampton, Southampton, UK

*Corresponding author: e-Mail: ipodbielski@geomar.de, Phone: (+49) 431 600 4275

Abstract

Low salinity has been demonstrated to reduce fitness of organisms, due to energetic expense for cellular osmoregulation and stress response mechanisms. However, little is known about transcriptomic responses enabling cellular acclimation to low salinity. The Baltic blue mussel population is a hybrid-population, characterized by a Western Baltic *Mytilus edulis*-like population living at high salinities and an Eastern Baltic population with higher *Mytilus trossulus* allele-frequencies living at low salinities. In *Mytilus* sp. lar-

vae higher *M. trossulus* allele-frequency is coupled with better low salinity tolerance and suggests a phenotype-genotype association of salinity tolerance. We studied the population-specific and conserved transcriptomic responses in Kiel (native salinity 16) and Usedom (native salinity 7) populations acclimated to five different salinity treatments (4.5, 5, 6, 7 and 16) for four weeks. We found a larger number of differentially expressed genes in Kiel mussels. Additionally, many of the genes upregulated in Kiel population were downregulated in Usedom mussels. The transcriptomic response to low salinity of Kiel mussels was an upregulation of solute transport and amino acid metabolism, which is related to cellular volume decrease. Transcripts involved in stress response, energy storage and lipid metabolism were also upregulated, indicating that individuals were stressed by the low salinity conditions and the costs of osmoregulation were compensated via catabolism of energy stores. Our results demonstrated that gene expression of Usedom mussels was not strongly affected by the low salinity treatments, whereas results indicated a clear stress response and a selection for more tolerant individuals in the Kiel population under low salinity stress. However, Usedom mussel mortality at the highest salinity treatment was equal to that in low salinity, compared to a low mortality in Kiel mussels at their native salinity. Thus, persistent low salinity has a severe effect on the transcriptomic response in the low salinity susceptible Kiel population, whereas the low salinity tolerant Usedom mussels show a significantly smaller number of differentially expressed genes. Furthermore, these responses are not just upregulated under acute osmotic shock, but are persistent after long-term (four weeks) acclimation to low salinity stress in Kiel mussels. In the future, Kiel mussels will likely experience stress under reduced salinities, which will cause an upregulation of cellular volume regulatory processes which in turn will increase the energy demand and lead to energetic trade-offs.

4.1 Introduction

Environmental stress affects biogeography of marine organisms. The physiological capacity towards extreme environmental conditions determines the fundamental niche of organisms [325]. The fundamental niche together with biological interactions then defines species distribution boundaries [329].

Salinity is an important stressor in the marine environment. Unlike other well studied abiotic factors such as temperature or carbon-dioxide, less is known about how salinity impacts the physiological capacity of species [263]. Salinity stress can, however, have severe impacts on physiological functions such as respiration, feeding, heart rate, fertilization and strongly reduce an organisms fitness [111, 262, 331] and in turn lead to geographic range shifts. While small changes in salinity can lead to an increased metabolic rate to cover rising energetic costs of osmoregulation and ensure cellular homeostasis [120], prolonged severe osmotic stress can cause fundamental changes in metabolic processes and immune responses that ultimately lead to cell death [111, 306].

Brackish species already living at their tolerance limits could be severely impacted by changing salinity. Thus, the ability to acclimate and adapt to osmotic changes in their environment will be crucial for marine organisms in the future.

While the negative effects of salinity stress on marine organisms are universally acknowledged and more climate change scenarios emerge that predict salinity changes in aquatic regions, little is known about the cell physiological mechanisms that shape the resilience to low salinity conditions. There are two universal techniques of aquatic organisms to adapt to their ambient osmotic conditions.

Osmoregulators, such as fish and mammals, can keep the osmolality of their body fluids constant, independent of the environmental conditions. Osmoconformers on the other

hand, have body fluids that are nearly isoosmotic to the ambient salinity [395]. Osmoconformers are thus strongly affected by salinity changes [23]. To avoid cell damage from passive swelling or shrinking, osmoconformers undertake active cellular volume regulation to maintain a relative constant cell volume utilizing inorganic or organic solutes, so called osmolytes [250, 263, 264, 300, 360]. Under hypoosmotic stress, cells release or catabolize osmotically active solutes [354]. These substances are taken-up again or re-synthesized when salinity increases [398]. The first response to hypoosmotic shock usually involves an immediate change in intracellular ion concentration. This is followed by a more gradual modification of organic osmolyte concentrations during longer-term acclimation to low salinity [324, 365].

In the course of climate change, salinity stress is projected to increase around the world, which will have severe impacts on marine organisms. Overall precipitation has been shown to increase in the northern hemisphere since 1951 [1, 207]. Generally, regions with low salinity will very likely become less saline, whereas regions with high salinity will become more saline. Higher precipitation, extreme rain events, melt water and increased surface run-off in certain regions will lead to a decrease in salinity of marine habitats and increase environmental stress on local species [218, 219, 327, 357]. Decreases in ambient salinity have been observed in tropical coral reefs as well as in polar regions. These occurrences are predicted to increase in magnitude or frequency in the future [27, 228, 369], impacting the structure, health and recruitment of benthic communities [57, 113, 221, 310]. The Baltic Sea, characterized by a strong salinity gradient that defines the aquatic fauna, is another example for a region that will likely experience desalination in the future [107, 147, 209, 283].

Thus, if intense long-term habitat salinity changes occur that bring species to their tolerance limits, these will be forced to either acclimate, adapt or migrate (and suffer from local

extinction). However, phenotypic plasticity may not only enable a species to tolerate extreme conditions, but also induce fitness costs that hinder evolution of plasticity [81]. It is assumed that, once a critical cost is reached plasticity has no further positive impact on range extension [81].

Mytilid mussels are important foundation species in many temperate benthic ecosystems and play ecologically and economically important roles. The Mytilid species (*Mytilus edulis*, *Mytilus trossulus* and *Mytilus galloprovincialis*) are known to readily hybridize when occurring sympatrically, thus creating the so-called *Mytilus* species complex [361]. The Baltic *Mytilus* sp. population is located in such a hybrid zone (Fig.4.1). Extraordinarily, this population is exclusively comprised of hybrid populations. Along the salinity gradient we find a high frequency of *M. edulis*-like alleles, whereas at low salinities there is an increase in *M. trossolus*-like alleles [286] (Fig.4.1). In the Baltic Sea, the euryhaline *M. edulis x trossulus* forms large mussel reefs and is estimated to account for a 90% dry weight of animal biomass in some regions of the Baltic Sea [142].

The American *M. trossolus* is less tolerant to low salinity, but inhabits more wave exposed niches compared to *M. edulis*. Additionally, the *M. edulis x trossulus* hybrid zone on the American Atlantic coast is quite different in patterns of gene introgression and niches occupied [285]. Pure *M. edulis* and *M. trossolus* individuals are abundant along the American coast [285]. On the other hand, there has been complete asymmetric introgression of the female mtDNA of *M. edulis* into the Baltic Sea population so that no pure *M. trossulus* individuals can be found.

Future salinity changes will likely impact Baltic Sea blue mussel populations, yet the consequences and potential for acclimation and rapid adaptation remain unclear. With the projected changes in salinity of the Baltic Sea, *Mytilus* sp. could lose >100 km^2 of habitat leading to a massive shift in species distribution and community composition [264, 296,

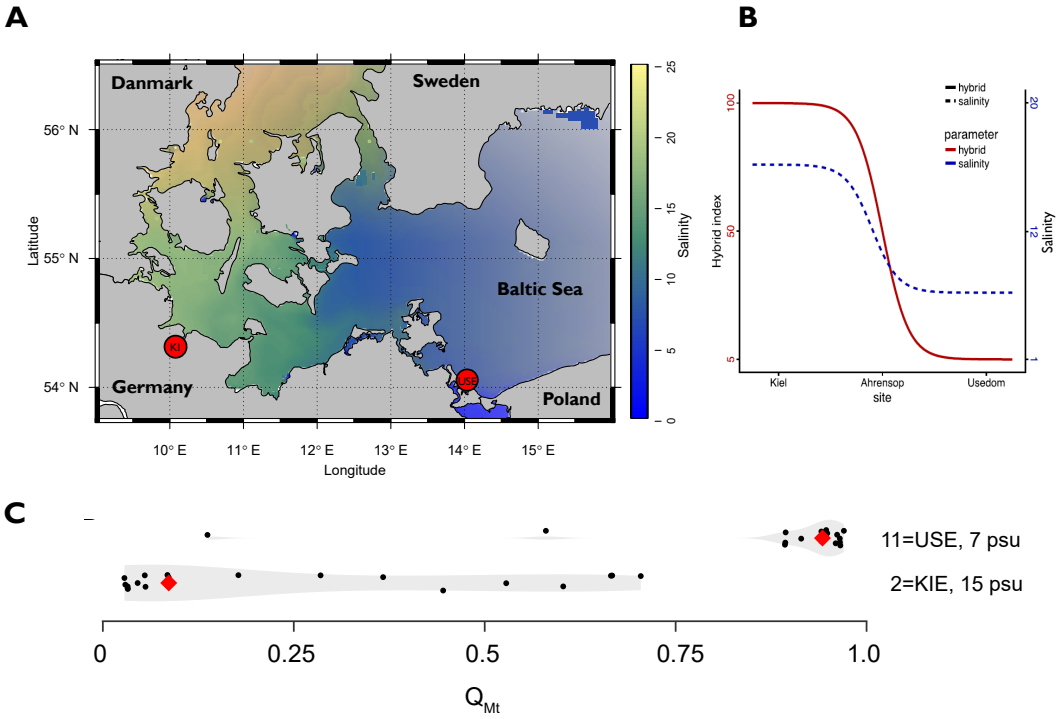


Figure 4.1: Salinity gradient in the Baltic Sea, distribution of Baltic *Mytilus* sp. allele frequencies and sampling localities. A: Sampling localities for the population comparison experiment (Kiel, KIE; Usedom, USE) and the salinity gradient (Graph modified from Knöbel et al. [152]). B: Graph showing the hybrid index and salinity gradient along the geographical distribution of the sample locations (from Sanders [296]). C: Distribution of hybrid classes expressed as QMT values, i.e., assignment of individuals to genetic clusters based on Bayesian analyses using STRUCTURE software for the two populations used for RNAseq analysis (Kiel & Usedom) [85]. Median QMT values are indicated by red diamonds. Salinity is given for each locality according to hydrodynamic mode data obtained from Stuckas et al. [337]) (Graph modified from Knöbel et al. [152]).

367]. Baltic blue mussels can colonize habitats with far lower salinities compared to other *Mytilus* congeners.

Salinity has been shown to act as a selective pressure on larvae driving local adaption to low salinity in Baltic mussel populations [152]. Translocation experiments of Baltic and North Sea *Mytilus* mussels found differences in physiology and genetic composition between the two populations even after one year acclimation that indicate differential selection [136, 345]. *Mytilus*-hybrid zones are therefore most suitable to study basic mechanisms of interspecific hybridization, as well as the adaption potential towards changing environmental conditions [151, 337].

This study investigated the transcriptomic response of *Mytilus* sp. populations from the Baltic Sea to low salinity stress. We compared the transcriptomic response of two locally adapted populations in order to assess population-specific responses to hypoosmotic stress and gain a better understanding of the active cellular mechanisms involved in osmoregulation. We sequenced mantle tissue transcriptomes to test for differential gene expression in *M. edulis*-like mussels from Kiel (Salinity 16) and *M. trossulus*-like mussels from Usedom (Salinity 7) acclimated to salinities of 4.5, 5, 6, 7 and 16 for four weeks.

Our comparative transcriptomic approach is a companion study to an osmolyte analysis measuring organic osmolytes, cations and survival of these two populations in response to salinity [296]. The companion study discovered that the organic osmolyte pool was overall smaller in Usedom mussels than in Kiel mussels, whereas the cation pool was larger. Usedom mussels were more tolerant to low salinities. In fact, the mortality rate in Usedom mussels was unchanged by salinity, but overall at $\sim$ 20%. In Kiel mussels, mortality increased with salinity (Fig. 4.2).

This study addresses uncertainties regarding the genetic regulatory mechanisms of cellular volume regulation under current and future hypoosmotic stress. Adding transcriptomic

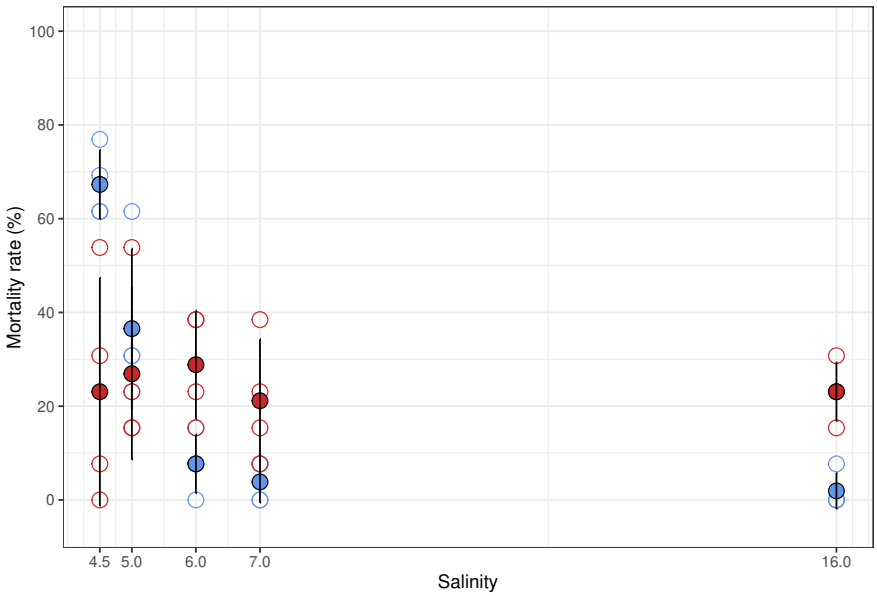


Figure 4.2: Total mortality rates per tank after the termination of the experiment expressed as a percentage of the initial number of animals per tank. The Kiel populations is depicted in blue, the Usedom population in red. All replicates (n = 4) are shown in circles, solid points represent the mean. Error bars represent standard deviation. Data from Sanders [296].

data to existing physiological data is a powerful approach, because it allows us to determine whether the observed phenotypic differences are mirrored by the gene expression data. We hypothesize that, in accordance with their phenotypic differences in salinity tolerance, derived from mortality and osmolyte data of the companion study, *Mytilus* sp. populations will exhibit divergent transcriptomic responses under low salinity:

i At low salinity mussels will upregulate processes involved in cellular volume decrease (i.e. reduction of osmolyte concentrations via release or degradation).

ii Stress response in Kiel population will be enriched at low salinities.

iii Usedom mussels will have less changes in gene regulation than Kiel mussels at low salinities.

iv Severely stressed organisms will show an enrichment in gene expression of stress re-

sponse genes (i.e. heat-shock proteins) and pathways involved in apoptosis.

4.2 Material and Methods

4.2.1 Study Organisms and Experimental Design

Adult *M. edulis x trossulus* mussels (shell length 31 ± 3mm) were collected for common garden experiments from Kiel Fjord (54° 19' 48.846" N, 10° 8' 59.6436" E) and Usedom island (54° 3' 20.5668" N, 14° 0' 40.0572" E) in September 2016 (Fig.4.1). The populations are characterized by their differences in habitat salinity with an average habitat salinity of 16 and 7, for Kiel and Usedom mussels, respectively. Kiel mytilids are *Mytilus*-hybrids with high *M. edulis*-like allele frequencies, where as in Usedom mytilids high *M. trossolus*-like allele frequencies predominate [337] (Fig.4.1). Mussels were transported to the climate chambers of GEOMAR as quickly as possible in temperature controlled, aerated cooling boxes. Mussels were allowed to acclimatize to climate chamber conditions (10°C) for 2 weeks. Organisms were kept in 20 L aerated aquaria with filtered natural Baltic Sea water (collected from Kiel Bight) at their native salinities. Water changes were made every 2 days. Physiochemical water parameters (temperature, salinity, pH) were monitored frequently. Mussels were fed with *Rhodomonas balthica* (15000 cells mL^{-1}) twice a day. Mortality was monitored daily. The experimental design consisted of two mussel populations acclimated to 5 salinity levels with four replicates per treatment level. Treatment levels were 4.5, 5, 6, 7 and 16, representing the natural salinity gradient from the Western Baltic Sea to their physiological tolerance limit. Each experimental unit was gradually acclimatized to their final salinity at a rate of 2-3 day^{-1}. Experimental animals were acclimated to the different salinity treatments for four weeks and mortality monitored with subsequent osmolyte analysis (published in companion paper [296]. A total of 13 mussels were cultivated

per 10 L tank. Inner mantle samples (the central portion of the mantle closest to the inner shell surface) from one mussel per tank were dissected and quickly rinsed in isoosmotic solution to remove seawater and afterwards blotted dry. Tissue samples were weighed and frozen until further analysis.

4.2.2 RNA extraction and sequencing

Frozen mantle tissues of one animal per replicate *M. edulis x trossolus* were ground in liquid nitrogen. Total RNA was extracted from mantle tissues using the Qiagen RNA extraction kit (Qiagen, Germany). RNA yield and purity were assessed determining A260/A230 and A260/A280 ratios with a NanoDrop spectrophotometer (NanoDrop 2000, Thermo Scientific). A bioanalyzer was used to observe the integrity of extracted RNA (Experion, Bio-Rad). The mRNA was purified and cDNA synthesized using the TruSeq stranded mRNA HT sample preparation kit (Illumina, USA) according to manufacturer's instructions. The quality and quantity of the resulting cDNA libraries were validated with a bioanalyzer (LabChip GX, Perkin Elmer, USA). Equal molar pooled libraries were sequenced on an Illumina NextSeq500 sequencer to generate 75 bp single end reads. Illumina BCL files were converted to fastq files and de-multiplexed using bcl2fastq (v2.17; Illumina) using default settings.

4.2.3 Data processing

All bioinformatics analyses were carried out using default parameters, unless otherwise specified. After completing sequencing, data were retrieved and checked for quality. Sequence quality was checked using FastQC v.0.11.9 (Babraham Bioinformatics). Data were trimmed using cutadapt v.3.3 [201]. This includes the removal of sequencing adapters and the removal of low-quality bases by setting the phred-score to 30. Results were then

controlled by running a second quality control. We use a previously published, filtered transcriptome of adult mantle tissue of a Baltic Sea *M. edulis*-like population from Kiel Fjord for mapping reads [276] (PRJNA494236), collected from the same geographic coordinates as the Kiel population animals in this study. The cleaned reads were mapped to the Baltic *M. edulis*-like transcriptome using Bowtie2 v2.4.2 [176]. Because there was a large number of duplicates, data were deduplicated using Picard tools v.2.25.3 (`http://broadinstitute.github.io/picard/`). All duplicate reads were removed. The transcript abundance was quantified using RSEM (RNA-Seq by Expectation-Maximization) v.1.3.3 [185], using the transcript-to-gene-map option, obtaining read counts at the gene level. Mapping statistics were acquired with samtools v.1.12 [186]. Genes with raw count values < 10 were excluded from downstream analyses.

Contigs from the mantle transcriptome were annotated with Trinotate v.3.2.1 (`https://trinotate.github.io/`). TransDecoder v.5.5.0 was used to identify protein-coding sequences within the transcriptome. Sequence similarity searches of the transcript sequences were performed using Basic Local Alignment Search Tool (BLAST) v2.10.1+ [4] with an E-value cut-off of 1e−5 against the SwissProt database and using hmmer [89] to search the Pfam-A database [271] (downloaded 07.2021). hmmer v.3.3.2, signalp v.5.0b [247] and tmmhmm v.2.0c [159] were used to identify protein families, proteins containing signal peptides and transmembrane domains.

4.2.4 Data analysis

Differential gene expression analysis was conducted in R v.4.1.1 (R Core Team, 2021) using the `DEseq2` package v.1.32.0 [192]. We tested for differential gene expression using the model factors "salinity" (levels: 4.5, 5, 6, 7 with 16 as reference level), "population" (levels = Usedom with Kiel as reference) and an interaction factor, resulting in 13 contrasts

(model design = ~ population + salinity + population:salinity). DEseq2 uses the so-called Benjamini-Hochberg (BH) adjustment with an alpha = 0.01 to correct for multiple testing, this method calculates for each gene an adjusted p-value. We assessed overall patterns of the expression data by plotting a two-dimensional principal component analysis (PCA) using the plotPCA function in deseq2, which analyses 500 genes with the highest variance across samples. Count data for PCA were transformed with variance stabilizing transformation with blind dispersion set to false. Transcripts that increased or decreased in response to low salinity were identified using the contrast of low salinity treatment vs. 16 for the more tolerant (Usedom) and less tolerant (Kiel) population. Salinity tolerance was derived from the mortality results of the companion study [296] (Fig. 4.2). Secondly, the interaction effects identified transcripts that differed in abundance in response to salinity by population. Transcripts with an adjusted p-value of 0.05 were considered significantly differentially expressed. Additionally, normalized expression data of genes of interest were visualized. Data were visualized using `pheatmap` v.1.0.12, `ggvenn` v.0.1.9 and `ggplot2` v.3.3.5 [378] packages in R for expression pattern heatmaps, Venn diagrams and all other plots respectively. We performed a rank-based gene ontology (GO) analysis with ermineJ v.3.1.2 [13] and GO_MWU (`https://github.com/z0on/GO_MWU`) as described in [388] on the DEseq contrasts to identify GO categories that were significantly enriched by up- or downregulated genes under low salinity stress.

4.3 Results

4.3.1 RNA sequencing/ Transcriptomic data

The RNA sequencing resulted in an average of raw reads $12,219,495 \pm 4,418,337$ (all values are given as mean $\pm$ standard deviation). Phred scores were above 30. After

quality filtering and deduplication $6,372,187 \pm 2,120,728$ reads remained (Tab. C.1). The reads matched $25,631 \pm 792$ contigs (Reference transcriptome: 29,177 contigs, [402], PRJNA494236). Mapping rate was $26.05\% \pm 3.49$ when mapped to a reference transcriptome from mantle tissue of *M. edulis*-like Kiel population. We found no significant difference between mapping rate for Kiel ($24.89\% \pm 3.72$) and Usedom ($27.01\% \pm 3.05$) mussels (t-value = -1.77, df = 36.57, p-value = 0.08; assumptions of normal distribution and homogeneity of variances were met). Annotation was conducted for the *M. edulis*-like reference mantle transcriptome. 46.86% of all contigs were annotated using Blastx, 40.86% were annotated by hmmer with an overlap of 37.91% (Tab. C.2).

4.3.2 General patterns of transcriptomic change

Gene expression data were first assessed using PCA to determine variation among samples (Fig. 4.3). The PCA plot shows two separate clusters for the Kiel and Usedom mussels and indicates that population was the dominant source of variation (PC1: 32% variance), which is consistent with the known differences in allele-frequencies. Salinity explained another large part of transcriptomic change (PC2: 21% variance) (Fig. 4.3). In the Kiel mussels, higher salinities 7 and 16 were more closely clustered compared to the low salinity treatments 4.5, 5 and 6. In Usedom mussels, all salinity treatments were more dispersed and overlapped, indicating larger variation in the individual gene expression response to low salinity. Only the lowest salinity 4.5 treatment was located slightly apart from the others.

4.3.3 Differential gene expression

We used the R package `DEseq2` to analyze differential gene expression by conducting pairwise comparisons between low salinity treatments and control group for each population. These significant changes were compiled for each contrast and separated for up- and down-

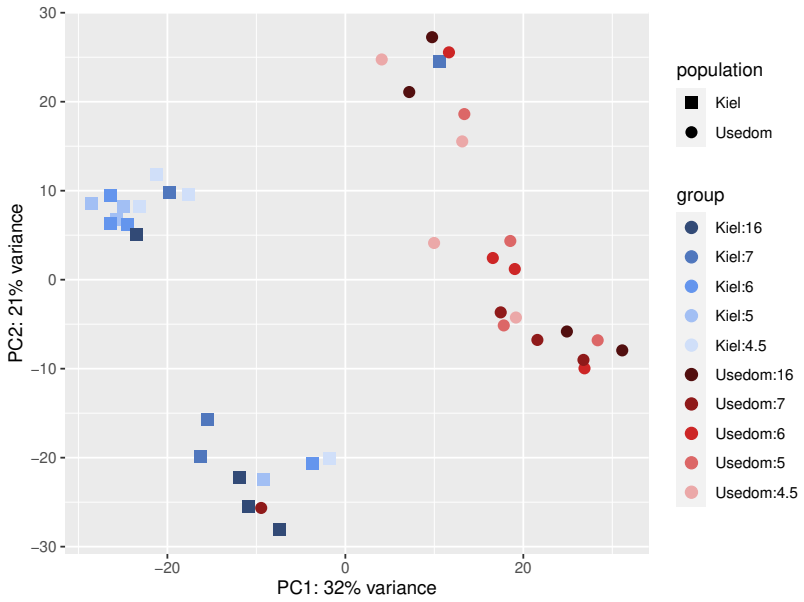


Figure 4.3: Principle components analysis for the RNA-seq data. Blue and red colours represent the Kiel and Usedom populations, respectively. The color gradient depicts the salinity treatments, with lighter shades representing low salinity and darker shades high salinity treatments (4.5, 5, 6, 7 and 16).

regulation (Tab. 4.1). In total, we found 4091 genes differentially expressed between the two mussel populations without considering salinity effects. Overall, 3491 transcripts (12% of all contigs) underwent significant changes in abundance in response to low salinity in both populations. At moderate salinity stress (salinity 7 vs. 16), 326 genes were differentially expressed in the Kiel population. This number increased under low salinity stress (salinity 4.5 vs 16), where we identified 1398 differentially expressed genes in Kiel mussels (Fig. 4.4). There was a clear trend of an increase in abundance of transcripts with decrease of salinity for both up- and downregulated genes, however, the abundance of upregulated genes was almost two-fold higher (Fig. 4.4). Of the 1398 transcripts differentially regulated at extreme salinity stress (salinity 4.5 vs 16) in Kiel mussels, 844 were upregulated and 554 downregulated. Overall, 101 transcripts changed significantly in abundance under low salinity stress in all treatments in the Kiel population (Fig. 4.5), of these 70 were annotated.

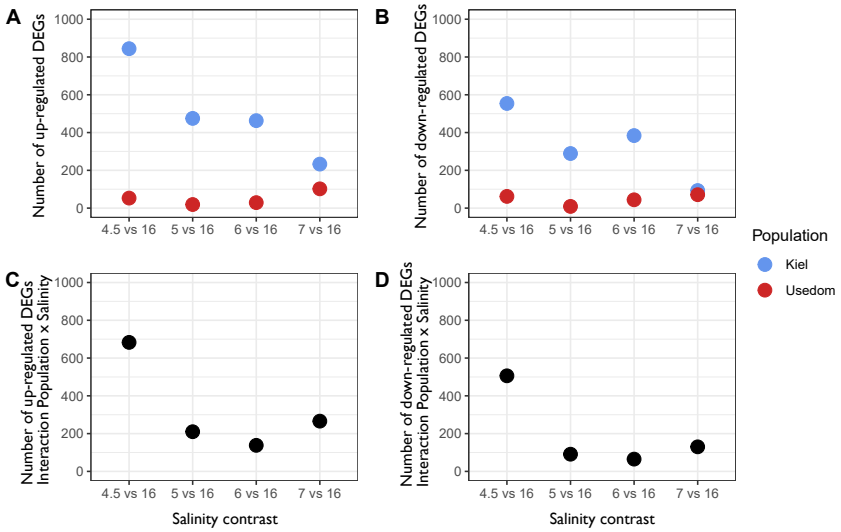


Figure 4.4: Total number of differentially expressed genes depicted for each contrast. Blue and red colours represent the Kiel and the Usedom populations, respectively. Black represents the interaction of population x salinity. Depicted are the A) upregulated genes per population, B) the downregulated genes per population, C) the significantly upregulated genes for the interaction and D) the significantly downregulated genes for the interaction.

In the tolerant Usedom mussels, the total number of differentially expressed genes was smaller than in Kiel mussels (Fig. 4.4). The number of DEGs was highest at moderate salinity stress (salinity 7) with 173 genes, and 115 under extreme stress (salinity 4.5). There was no overall increasing trend in number of DEGs (Fig. 4.4). The trend was similar for up- and downregulated genes. Of the transcripts that were differentially regulated at extremely low salinity, 53 were upregulated in Usedom mussels and 62 downregulated. Only 5 genes were differentially expressed across all low salinity treatments in Usedom mussels, none of these genes was annotated (Fig. 4.5).

Table 4.1: Number of significantly differentially expressed genes by contrasts

	Total			Annotated		
Comparison	Total	Up	Down	Annotated	Up	Down
Kiel vs. Usedom Population	4091	1671	2420	1445	874	571

Table 4.1: Number of significantly differentially expressed genes by contrasts

	Total			Annotated		
Comparison	Total	Up	Down	Annotated	Up	Down
Kiel - Salinity treatment						
4.5 vs. 16	1398	844	554	814	536	278
5 vs. 16	764	475	289	502	360	142
6 vs. 16	847	463	384	579	350	229
7 vs. 16	326	233	93	238	163	75
Usedom - Salinity treatment						
4.5 vs. 16	115	53	62	49	12	37
5 vs. 16	28	19	9	8	2	6
6 vs. 16	73	29	44	25	1	24
7 vs. 16	173	102	71	48	23	25
Interaction Population x Salinity						
4.5 vs. 16 Use 4.5	1189	683	506	717	340	337
5 vs. 16 Use 5	301	210	91	158	118	40
6 vs. 16 Use 6	203	138	65	88	40	48
7 vs. 16 Use 7	396	266	130	132	83	49

Shared responses to low salinity stress

We plotted the log-fold change (LFC) of common genes that were differentially expressed in both populations for the most extreme contrast (4.5 vs. 16, n = 49) and the onset of salinity stress (7 vs. 16, n = 39) (Fig. 4.6 & Fig. 4.7). Moreover, the outlier transcripts with very high or low LFC were not annotated. The salinity response at low and moderate stress was regulated in the same direction, but the slope for the most extreme contrast was nearly on the 1:1 line, whereas the under moderate salinity stress the slope is smaller, indicating that transcripts of Usedom mussels are more highly regulated compared to Kiel mussels (Fig. 4.7). All common genes correlated negatively between Kiel and Usedom mussels (i.e. upregulated genes in Kiel population were down regulated in the Usedom population and *vice versa*). Hence, the shared differentially expressed genes (n = 49) in Kiel and Usedom mussels did not indicate a conserved response to extremely low salinity (4.5) across popula-

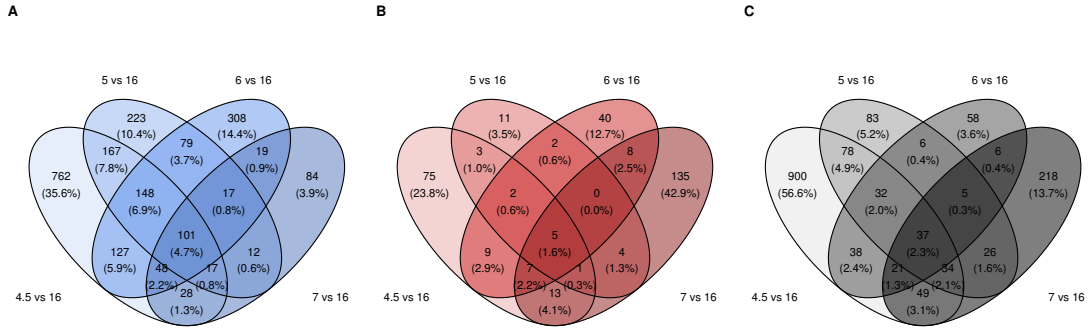


Figure 4.5: Venn diagrams showing the number of differentially expressed genes within contrasts and shared between contrasts as determined by the deseq2 analyses for each population and their interaction separately. Blue, red and grey colours represent the Kiel and Usedom population and their interaction, respectively. Lighter shades represent the low salinity contrasts, while darker shades represent the high salinity contrasts (contrast 4.5 vs. 16, 5 vs. 16, 6 vs. 16 and 7 vs. 16).

tions, since commonly significant DEGs were regulated in opposite directions (Fig.4.5).

20 of the commonly differentially expressed transcripts (at salinity contrast 4.5 vs. 16) were annotated. The upregulated genes in Kiel mussels (downregulated -Usedom) (n = 13) included: negative regulation of apoptosis and stress response (Hypoxia up-regulated protein 1 (*HYOU1*), DNA-(apurinic or apyrimidinic site) endonuclease (*APEX1*)), genes involved in lipid metabolism and homeostasis (3-keto-steroid reductase/17-beta-hydroxysteroid dehydrogenase 7 (*HSD17B7*), Coatomer subunit gamma-1 (*COPG1*), Polypeptide N-acetylgalactosaminyltransferase 2 (*GALNT2*)) and genes involved in transcription and cell cycle (Serine/arginine-rich splicing factor 4 (*SRSF4*), PR domain zinc finger protein 5 (*PRDM5*), Spindle assembly abnormal protein 6 homolog (*SASS6*)). Amongst the down-regulated genes in Kiel mussels (upregulated - Usedom) (n = 7) were: immune response genes (Protein unc-79 homologs (*UNC93A*, *UNC79*)), a DNA stress response gene (Replication stress response regulator (*SDE2*)), a gene regulating energy expenditure and body mass (Arrestin domain-containing protein 3 (*ARRDC3*), a glucose transporter (Solute carrier family 2, facilitated glucose transporter member 3 (*SLC2A3*)) and a phospholipid-

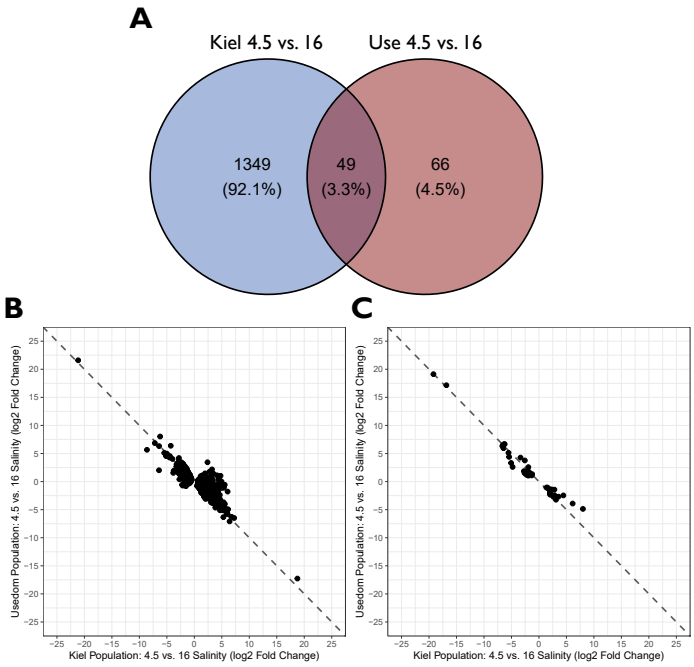


Figure 4.6: Differentially expressed genes between low and high salinity (4.5 vs. 16) in Kiel and Usedom population. A) Venn Diagram showing the number of differentially expressed genes detected during analysis based on within-location salinity response. Blue and red colors represent the Kiel and Usedom population, respectively. B) Scatterplot of the log2 fold changes in gene expression in response to salinity stress in the Kiel vs. Usedom mussels for the 1349 genes that were unique to the Kiel low salinity vs. control comparison (4.5 vs. 16). C) Scatterplot of the log2 fold changes in gene expression in response to salinity stress in the Kiel vs. Usedom mussels for the 49 genes that were commonly differentially expressed in both populations in the low salinity vs. control comparison (4.5 vs. 16). Each circle represents an individual contig. The line represents one-to-one log2fold change. Dots above the line had a larger magnitude log2fold change in the more tolerant Usedom population compared to the susceptible Kiel population under extreme salinity stress. The negative correlation indicates that upregulated genes in the Kiel population are downregulated in the Usedom population and *vice versa*.

transporting ATPase (*ABCA1*).

Only 11 of the 39 differentially expressed genes common in both populations at contrast of salinity 7 vs. 16 were annotated (Fig. 4.7). Again, the relationship between Kiel and Usedom gene expression was negatively correlated. Amongst the upregulated genes in Kiel mussels were: calmodulin mediating control of ion channels and other enzymes (Calmodulin (*CALM*)), genes indicating stress response and involved in apoptosis (Galectin-8 (*LGALS8*), Embryonic polarity protein dorsal (*dl*)), genes involved in diverse metabolic pathways such as protein catabolism (Ubiquitin-protein ligase E3A (*UBE3A*)), glycogen metabolism (Serine/threonine-protein phosphatase PP1-beta catalytic subunit (*PPP1CB*)) and lipid synthesis (Acyl-CoA 6-desaturase (*FADS2*)). Only two genes were significantly downregulated in Kiel mussels (i.e. upregulated in Usedom mussels). First, the arrestin domain-containing protein 3 (*ARRDC3*) which was also differentially expressed under extreme salinity stress but at 35% higher LFC rate. Secondly, a gene responsive to oxidative stress that acts as cell death activator (*CHRB*).

Given indications that gene expression was stimulated in susceptible Kiel mussels during low salinity exposure, we sought to identify unique transcripts increasing in abundance as a result of greater transcription and translation (n = 1349, annotated = 749) and compare the log-fold changes in Kiel mussels vs. more salinity tolerant Usedom mussels (Fig. 4.6). To do so, we isolated 30 transcripts undergoing the lowest and highest fold change increase (range = -21.13 — -2.06-fold, 3.34 — 5.35-fold) during low salinity exposure (Tab. 4.2& Tab. 4.3). We presumed that the transcripts with the largest increases in abundance in the Kiel mussels under extreme salinity stress yield key indicators of the cellular stress response. Amongst the upregulated response to low salinity were genes involved in amine metabolism (hydroxylases (*TBH1*) and aminopeptidases (*LPQL*)), stress response (several heat shock proteins: *HSP68*, *HSP70B2*, *HSPA4*, Mitochondrial uncoupling protein

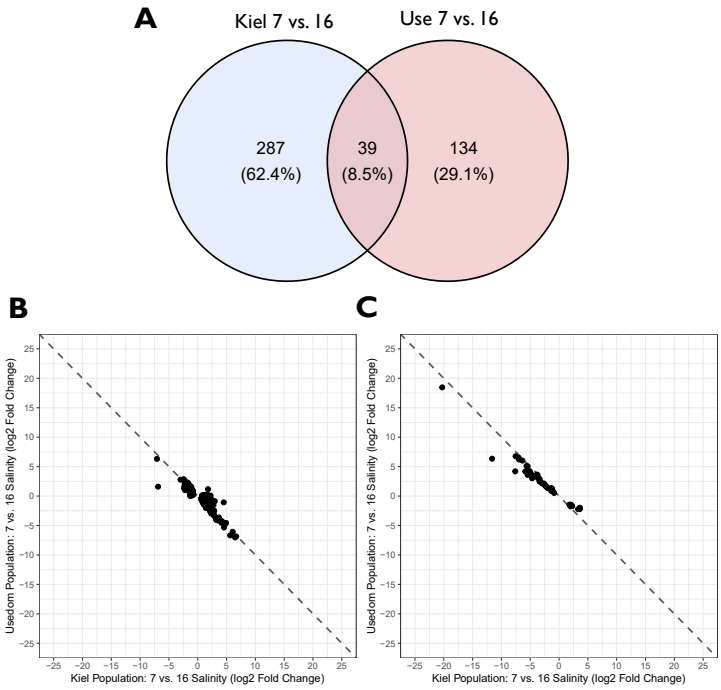


Figure 4.7: Differentially expressed genes between moderate low and high salinity (7 vs. 16) in Kiel and Usedom population. A) Venn Diagramm showing the number of differentially expressed genes detected during analysis based on within-location salinity response. Blue and red colors represent the Kiel and Usedom population, respectively. B) Scatterplot of the log2 fold changes in gene expression in response to salinity stress in the Kiel vs. Usedom mussels for the 287 genes that were unique to the Kiel moderate low salinity vs. control comparison (7 vs. 16). C) Scatterplot of the log2 fold changes in gene expression in response to salinity stress in the Kiel vs. Usedom mussels for the 39 genes that were commonly differentially expressed in both populations in the moderate low salinity vs. control comparison (7 vs. 16). Each circle represents an individual contig. The line represents one-to-one log2fold change. Dots above the line had a larger magnitude log2fold change in the more tolerant Usedom population compared to the susceptible Kiel population under extreme salinity stress. The negative correlation indicates that upregulated genes in the Kiel population are downregulated in the Usedom population and *vice versa*.

2 (*SLC25A8*), WAP four-disulfide core domain protein 18 (*WFDC18*)), ion transport (Sodium-independent sulfate anion transporter (*SLC26A11*)), organic solute transport (organic anion transporter (*SLCO4A1*), Sodium- and chloride-dependent glycine transporter (*SLC6A5*)), water transmembrane transporter (*SLC5A1*), Phospholipid-transporting ATPase (*ABCA1*), septate junction assembly (*CNTN*), calmodulin mediated control of ion channels and other enzymes (*CALM*), biomineralization (*TEMPT*), ion homeostasis & myelination (*HEXB*) (Tab. 4.2). Notable downregulated genes were involved in solute transport for inorganic ions, organic ions or energy substrates (*SLC6A5*, Organic cation transporter (*ORCT*), glucose transporter (*SLC2A2*), Proton myo-inositol cotransporter (*SLC2A13*), ligand-gated ion channels (ligand-gated sodium channel (*ASIC2*), glutamate-gated calcium ion channel (*GRIN2B*)), calmodulin (*CALM*), metabolism of carbohydrates (*B3GAT1*, *B3GAT3*), metabolism of amino acids (*XPNPEP1*, *SULT1B1*), acid-base balance (Carbonic anhydrase-related protein (*CA8*)), immune response & apoptosis (*MAP3K14*, *HERC5*, *ADAMTS7*, *DEPTOR*, *CHRB*), protein stability (*ADGRV1*). Others were involved in RNA regulation, signaling pathways and processing (Tab. 4.3).

Table 4.2: Top 30 up-regulated genes uniquely differentially expressed in the Kiel population at low salinity (4.5) and the significant changes in log-fold change compared to control conditions (16) for Kiel and Usedom mussels

	Gene name	Gene symbol	e-value	GO id	Kiel LFC	Use LFC
1	Temptin	TEMPT	3.44E-26	GO:0005576	5.35	-1.10
2	Tyramine beta-hydroxylase	TBH1	6.31E-15	GO:0005615	4.96	0.01
3	Putative tyrosinase-like protein tyr-3	TYR3	1.35E-57	GO:0046872	4.69	-3.14
4	Mitochondrial uncoupling protein 2	UCP2	3.00E-97	GO:0016021	4.63	-2.38
5	Laccase-4	LAC4	2.06E-62	GO:0005576	4.54	-3.73
6	Tyramine beta-hydroxylase	TBH1	2.32E-17	GO:0005615	4.46	0.29
7	Tyramine beta-hydroxylase	TBH1	1.97E-40	GO:0005615	4.43	0.32
8	Temptin	TEMPT	4.14E-27	GO:0005576	4.43	0.49
9	Contactin	CONT	7.06E-147	GO:0031225	4.41	-0.35
10	Calmodulin	CALM	2.54E-31	GO:0005509	4.27	-4.04
11	Patched domain-containing protein 3	PTHD3	2.80E-114	GO:0016021	4.24	-3.27
12	MAM and LDL-receptor class A domain-containing protein 2	MLRP2	7.87E-61	GO:0005576	4.21	-1.03

Table 4.2: Top 30 up-regulated genes uniquely differentially expressed in the Kiel population at low salinity (4.5) and the significant changes in log-fold change compared to control conditions (16) for Kiel and Usedom mussels

	Gene name	Gene symbol	e-value	GO id	Kiel LFC	Use LFC
13	Probable lipoprotein aminopeptidase LpqL	LPQL	4.34E-07	GO:0005576	4.14	-2.44
14	Solute carrier organic anion transporter family member 4A1	SO4A1	1.37E-90	GO:0005887	4.10	-1.17
15	Beta-hexosaminidase subunit beta	HEXB	4.57E-52	GO:0001669	4.02	-2.80
16	Ras-related C3 botulinum toxin substrate 1	RAC1	1.02E-34	GO:0005884	3.83	-2.51
17	WAP four-disulfide core domain protein 18	WFD18	3.73E-11	GO:0005615	3.80	-1.63
18	Retinal-binding protein	RALB	5.48E-75	GO:0050896	3.66	-0.61
19	Zinc finger protein 862	ZN862	2.46E-11	GO:0005634 GO:0046872	3.64	-3.26
20	Low-density lipoprotein receptor-related protein 3	LRP3	3.51E-14	GO:0005905	3.61	-1.01
21	Putative ariadne-like RING finger protein R811	YR811	1.75E-06	GO:0046872	3.55	-2.86
22	Heat shock 70 kDa protein IV	HSP74	7.51E-93	GO:0005524	3.46	-3.23
23	Sodium-independent sulfate anion transporter	S2611	1.17E-138	GO:0005783	3.37	0.14
24	Sodium- and chloride-dependent glycine transporter 2	SC6A5	1.28E-141	GO:0031045	3.35	-0.34
25	Heat shock protein 68	HSP68	7.75E-103	GO:0005737 GO:0005829 GO:0005634 GO:0005886	3.34	-3.00
26	Heat shock protein 70 B2	HSP74	3.67E-103	GO:0005524	3.32	-3.07
27	Sarcoplasmic calcium-binding protein	SCP	3.17E-06	GO:0005509	3.31	-1.88
28	Transmembrane protein 45B	TM45B	4.14E-44	GO:0016021	3.29	-2.94
29	Phospholipid-transporting ATPase ABCA1	ABCA1	4.14E-35	GO:0030139	3.26	-2.08
30	Sodium/glucose cotransporter 1	SC5A1	5.59E-131	GO:0016324	3.26	-1.46

Table 4.3: Top 30 down-regulated genes uniquely differentially expressed in the Kiel population at low salinity (4.5) and the significant changes in log-fold change compared to control conditions (16) for Kiel and Usedom mussels

	Gene name	Gene symbol	e-value	GO id	Kiel LFC	Use LFC
1	Mitogen-activated protein kinase kinase kinase 14	M3K14	4.01E-07	GO:0005829 GO:0001650	-21.13	21.60
2	Proton-coupled folate transporter	PCFT	7.16E-20	GO:0016021	-6.42	2.01
3	Probable glycosyltransferase STELLO1	STL1	4.96E-30	GO:0005768 GO:0005794	-5.46	5.10

Table 4.3: Top 30 down-regulated genes uniquely differentially expressed in the Kiel population at low salinity (4.5) and the significant changes in log-fold change compared to control conditions (16) for Kiel and Usedom mussels

	Gene name	Gene symbol	e-value	GO id	Kiel LFC	Use LFC
4	DEP domain-containing mTOR-interacting protein	DPTOR	8.44E-71	GO:0035556	-4.29	6.38
5	Ras-related protein Rab-30	RAB30	1.99E-22	GO:0005801	-3.82	1.55
6	Organic cation transporter protein	ORCT	8.26E-66	GO:0016021	-3.27	2.64
7	Galactosylgalactosylxylosylprotein 3-beta-glucuronosyltransferase 1	B3GA1	3.58E-64	GO:0005789	-3.18	2.35
8	Ultraviolet-B receptor UVR8	UVR8	2.74E-17	GO:0000785 GO:0005829 GO:0005634 GO:0009536 GO:0003682	-2.70	2.03
9	Xaa-Pro aminopeptidase 1	XPP1	2.16E-112	GO:0005737 GO:0005829 GO:0070062	-2.67	2.57
10	Glutamate receptor ionotropic, NMDA 2B	NMDE2	9.76E-96	GO:0005856 GO:0005887	-2.63	2.79
11	Carbonic anhydrase-related protein	CAH8	2.33E-89	GO:0005737 GO:0004089	-2.57	1.41
12	Calmodulin	CALM	7.18E-90	GO:0005509	-2.35	2.61
13	Sarcoplasmic calcium-binding protein	SCP	3.44E-66	GO:0005509	-2.33	2.07
14	Sulfotransferase family cytosolic 1B member 1	ST1B1	8.96E-44	GO:0005737 GO:0004062	-2.33	1.55
15	Spondin-1	SPON1	4.52E-16	GO:0031012	-2.26	2.10
16	Helicase POLQ-like	HELQ		GO:0005634 GO:0017117	-2.24	1.28
17	Helicase POLQ-like	HELQ	1.47E-71	GO:0005634 GO:0005524	-2.24	1.70
18	Adhesion G-protein coupled receptor V1	AGRV1	6.78E-81	GO:0016021	-2.22	1.28
19	Solute carrier family 2, facilitated glucose transporter member 2	GTR2	6.30E-10	GO:0016021	-2.20	1.82
20	E3 ISG15–protein ligase HERC5	HERC5	1.12E-17	GO:0005737 GO:0005829 GO:0048471	-2.15	1.83
21	A disintegrin and metalloproteinase with thrombospondin motifs 7	ATS7	2.18E-10	GO:0009986	-2.09	-0.72
22	Proton myo-inositol cotransporter	MYCT	1.04E-46	GO:0097450	-2.09	1.13
23	Protein charybde	CHRB	3.17E-19	GO:0005737 GO:0006915	-2.07	1.62
24	Galactosylgalactosylxylosylprotein 3-beta-glucuronosyltransferase 3	B3GA3	2.13E-52	GO:0005801	-2.06	1.68
25	Sulfotransferase family cytosolic 1B member 1	ST1B1	7.76E-44	GO:0005737 GO:0004062	-2.06	2.30

Table 4.3: Top 30 down-regulated genes uniquely differentially expressed in the Kiel population at low salinity (4.5) and the significant changes in log-fold change compared to control conditions (16) for Kiel and Usedom mussels

	Gene name	Gene symbol	e-value	GO id	Kiel LFC	Use LFC
26	Sodium- and chloride-dependent glycine transporter 2	SC6A5	2.93E-148	GO:0031045	-2.05	2.11
27	Tryptase	TRYB1	2.76E-42	GO:0005615	-2.03	3.14
28	Degenerin-like protein asic-2	ASIC2	3.80E-33	GO:0005887	-2.02	0.89
29	Beta-1,3-galactosyltransferase 1	B3GT1	1.56E-32	GO:0000139	-2.00	1.65
30	Ephrin type-B receptor 4b	EPB4B	7.34E-06	GO:0005887	-1.92	1.91

Population-specific responses to low salinity

Within the Kiel population we found an overlap of 101 genes that were differentially regulated across all salinity treatments (annotated n = 70) (Fig. 4.5, Tab. 4.4). These were mostly upregulated (n = 63). Amongst those genes were amino acid transporters (amino acid transporter (*SLC7A7*, *SLC7A9*), Sodium-coupled monocarboxylate transporter (*SLC5A12*), Sodium- and chloride-dependent glycine transporter (*SLC6A5*), and other solute carriers (*SLC12A2*, *SLC13A5*) and ion transporters (Sodium/glucose cotransporter (*SLC5A1*, *SLC5A9*), Sodium-dependent phosphate transporters (*SLC34A1*, *SLC34A2*), sulfate anion transporter (*SLC26A11*). Upregulated were further, genes involved in amino acid metabolism (Glycine, proline and pyruvate dehydrogenase (*GLDC*, *PRODH*, *PDK2*), Carnosine synthase (*CARNS1*), Choline-phosphate cytidylyltransferase (*PCYT1B*), Serine/threonine-protein phosphatase (*PPP1CB*), Cysteine sulfinic acid decarboxylase (*CSAD*), Ornithine aminotransferase (*OAT*), peptidases (*NAALAD2*), genes associated with gap/septate junctions (*UNC9*, *CNTN*) and lipid metabolism (*NVD*, *ADIPOR*, *CPT1A*). In concert with upregulated stress response (Hypoxia up-regulated protein (*HYOU1*), Hypoxia-inducible factor (*HIF1A*) and immune response genes (*YES1*)), pathways relating to apoptosis (*GHITM*, *ADA10*, *TAX1BP1*, *PDIA6*) and proteolysis (*BIP*) were significantly up-

regulated. Notable downregulated genes were involved in protein glycosylation (*B3GALT6*) and response to oxidative stress (*CHRB*). Simultaneous up- and down regulation of amino acid transporter (*SLC6A5*) suggests that the glycine transport pathways was being balanced, but that cellular response was not uniform.

Table 4.4: List of commonly differentially expressed genes in the Kiel population across low salinity treatments compared to control salinity

Gene name	Gene symbol	e-value	GO id
Up-regulated			
Carnosine synthase 1	CRNS1	1.94E-10	GO:0005524
Roquin-1	RC3H1	0	GO:0005737
			GO:0010494
Y+L amino acid transporter 1	YLAT1	9.38E-151	GO:0016323
Pyruvate dehydrogenase (acetyl-transferring) kinase isozyme 2, mitochondrial	PDK2	1.12E-149	GO:0005829
			GO:0005759
Choline-phosphate cytidylyltransferase B	PCY1B	8.91E-100	GO:0005737
			GO:0005783
Innexin unc-9	UNC9	2.91E-68	GO:0005911
Proline dehydrogenase 1	PROD	2.15E-152	GO:0005759
Hippocampus abundant transcript 1 protein	MF14A	0	GO:0016021
Serine/threonine-protein phosphatase PP1-beta catalytic subunit	PP1B	5.77E-153	GO:0005737
			GO:0005634
			GO:0072357
Contactin	CONT	7.06E-147	GO:0031225
Endoplasmic reticulum chaperone BiP	BIP	0	GO:0005788
Ras-related protein Rab-4B	RAB4B	4.12E-122	GO:0005768
			GO:0032593
Cholesterol 7-desaturase nvd	NVD	2.63E-111	GO:0016021
Adiponectin receptor protein	ADRL	4.63E-141	GO:0016021
Eukaryotic translation initiation factor 4 gamma 3	IF4G3	4.71E-116	GO:0005829
			GO:0016281
Ras-related protein Rab6	RAB6	1.08E-122	GO:0005776
			GO:0031410
Sodium-dependent phosphate transport protein 2B	NPT2B	4.10E-59	GO:0016324
Sodium-dependent phosphate transport protein 2A	NPT2A	4.80E-88	GO:0016324
b(0,+)-type amino acid transporter 1	BAT1	1.15E-129	GO:0016324
Cathepsin L	CATL	7.06E-125	GO:0005764
			GO:0004197
Cysteine sulfinic acid decarboxylase	CSAD	0	GO:0005737
			GO:0004068
Acid phosphatase type 7	ACP7	1.66E-163	GO:0005576
Tyrosine-protein kinase yes	YES	1.32E-160	GO:0005829
			GO:0031234

Table 4.4: List of commonly differentially expressed genes in the Kiel population across low salinity treatments compared to control salinity

Gene name	Gene symbol	e-value	GO id
Coatomer subunit gamma-2	COPG2	0	GO:0030126
Sodium-coupled monocarboxylate transporter 2	SC5AC	1.08E-147	GO:0016324
Alpha-N-acetylgalactosaminidase	NAGAB	7.41E-126	GO:0005737 GO:0005764 GO:0004557
Receptor-transporting protein 3	RTP3	3.89E-10	GO:0005737 GO:0016021
Growth hormone-inducible transmembrane protein	GHITM	1.25E-89	GO:0070062
Pregnancy zone protein	PZP	2.64E-127	GO:0072562
Coatomer subunit alpha	COPA	0	GO:0030126
AFG3-like protein 2	AFG32	0	GO:0005745
Sodium-independent sulfate anion transporter	S2611	1.17E-138	GO:0005783
Glycine dehydrogenase (decarboxylating), mitochondrial	GCSP	0	GO:0005739 GO:0004375
Sodium/glucose cotransporter 1	SC5A1	5.59E-131	GO:0016324
Sodium/glucose cotransporter 4	SC5A9	5.69E-68	GO:0016021
Protein misato homolog 1	MSTO1	1.89E-113	GO:0005741
Sodium-dependent phosphate transport protein 2B	NPT2B	2.07E-148	GO:0016021
Collagen alpha-2(IV) chain	CO4A2	2.10E-13	GO:0005604
Collagen alpha-2(IV) chain	CO4A2	1.32E-08	GO:0005604
Neprilysin-4	NEP4	2.61E-10	GO:0005615
Neprilysin-1	NEP	0	GO:0016021
Sodium- and chloride-dependent glycine transporter 2	SC6A5	1.28E-141	GO:0031045
Carnosine synthase 1	CRNS1	6.25E-174	GO:0005829 GO:0005524
Solute carrier family 13 member 5	S13A5	1.01E-79	GO:0005829 GO:0016021
Solute carrier family 12 member 2	S12A2	0	GO:0016324
Ornithine aminotransferase	OAT	0	GO:0005737 GO:0005829, GO:0005759
Protein sel-1 homolog 1	SE1L1	7.76E-172	GO:0036513
N-acetylated-alpha-linked acidic dipeptidase 2	NALD2	0	GO:0016021
Protein RFT1 homolog	RFT1	2.95E-113	GO:0005789
Nuclear export mediator factor Nemf	NEMF	0	GO:0005634, GO:1990112
Hypoxia-inducible factor 1-alpha	HIF1A	1.59E-109	GO:1904115
Lysine-specific demethylase 3B	KDM3B	0	GO:0000785 GO:0000118
Protein strawberry notch homolog 1	SBNO1	0	GO:0005634 GO:0031490
Disintegrin and metalloproteinase domain-containing protein 10	ADA10	8.80E-163	GO:0005912

Table 4.4: List of commonly differentially expressed genes in the Kiel population across low salinity treatments compared to control salinity

Gene name	Gene symbol	e-value	GO id
Tax1-binding protein 1	TAXB1	1.72E-51	GO:0005829
			GO:0070062
Collagen alpha-1(IV) chain	CO4A1	4.81E-38	GO:0005604
Protein transport protein Sec24C	SC24C	0	GO:0030127
Hypoxia up-regulated protein 1	HYOU1	1.28E-177	GO:0034663
Carnitine O-palmitoyltransferase 1, liver isoform	CPT1A	1.13E-59	GO:0016021
Protein disulfide-isomerase A6 homolog	PDIA6	2.22E-98	GO:0009986
Apolipophorins	APLP	6.17E-61	GO:0005576
Legumain	LGMN	6.92E-113	GO:0045177
Carnosine synthase 1	CRNS1	6.43E-16	GO:0005829
			GO:0005524
Down-regulated			
Beta-1,3-galactosyltransferase 6	B3GT6	7.10E-87	GO:0005794
Spondin-1	SPON1	4.52E-16	GO:0031012
Protein charybde	CHRB	3.17E-19	GO:0005737
			GO:0006915
Transcription intermediary factor 1-beta	TIF1B	3.38E-11	GO:0000785
			GO:0000791
			GO:0000792
			GO:0005654
			GO:0005634
			GO:0032991
Pescadillo homolog	PESC	2.24E-07	GO:0005730
			GO:0005654
			GO:0070545
Sodium- and chloride-dependent glycine transporter 2	SC6A5	2.93E-148	GO:0031045
Integrator complex subunit 6-B	INT6B	1.34E-31	GO:0005634

The total number of DEGs that was differently affected by an interaction of the two factors salinity x population was highest at the most extreme salinity 4.5 (Fig. 4.4, Fig. 4.5). The trend was similar for up- and downregulated genes. In total, 37 transcripts (annotated n = 19) exhibited a significant interaction of salinity and population, i.e. a population-specific response to low salinity across all low salinity treatments (salinity 4.5 -7) (Fig. 4.5). The annotated "interaction" genes were filtered for genes that were upregulated in Kiel mussels and downregulated in Usedom mussels (n = 16). These included genes that were previously reported from the conserved response to low salinity in Kiel mussels, namely

genes involved in amino acid metabolism (*CARNS1*, *GLDC*, *OAT*), amino acid other solute transporters (*SLC7A9*, *SLC5A12*, *SLC6A5*, *SLC22A3*), ion transporter (*SLC26A11*) and septate junction assembly (*CNTN*).

4.3.4 Identification of pathways involved in cellular osmoregulation

The primary objective of this study was to identify candidates of proteins, pathways and functions potentially involved in osmoregulation and differentiate between conserved responses and population-specific responses of a highly salinity tolerant and a moderately salinity tolerant mussel population. Functional enrichment of the GO-terms was tested with a rank-based Mann-Whitney-U test for each contrast to determine transcripts amplified or inhibited as part of the response to low salinity. However, enriched GO terms were few in number and categories broad.

In response to moderate low salinity stress (salinity 7), seven GO-terms were significantly enriched in Kiel mussels, three of those were upregulated. These included protein binding, receptor binding, and peptidases. Four GO terms were downregulated, namely, transferases, fatty acids synthases and nucleotide binding. At more severe salinity stress (salinity 4.5) more GO categories were enriched. Protein binding, nucleotide binding and peptidases were further enriched. Upregulated were further hydrolases, hexosaminidase and calmodulin. Additional downregulated GO categories included rRNA binding and structural molecules.

In response to moderate salinity stress, Five GO-terms were significantly enriched in Usedom mussels. Three of those were upregulated (transferases, fatty acid synthases and serine-type endopeptidase), 2 were down-regulated (protein binding and phosphotransferases). With decreasing salinity, number of enriched GO-categories increased and more peptidases were upregulated. Upregulated categories under moderate salinity stress were

further enriched. Phosphotransferases were more downregulated under more severe salinity stress. Additional GO terms downregulated under severe salinity stress were involved in DNA/RNA binding.

GO categories that were significantly enriched in the interaction of population and salinity included mainly downregulation of transferases, kinases, GTP binding and RNA/DNA binding. Upregulated GO categories included fatty acid synthase, peptidases, hydrolases, oxidoreductases, amino acid dehydrogenases, hexosaminidases and calcium-ion binding.

4.4 Discussion

4.4.1 Population-specific responses to low salinity

The most remarkable results were the different transcriptomic responses to low salinity observed in Kiel vs. Usedom mussels. Since *M. trossulus*-like populations are known to be more tolerant to low salinities, we expected no significant response at moderate salinity stress (salinity 7) which corresponds to their habitat salinity at Usedom. However, under severe salinity stress (salinity 4.5), which poses a physiological limit for both species, we would have expected to see a similar response in gene expression in the Usedom population compared to the Kiel population. Instead we found a consistent negative correlation of differentially expressed genes across salinity treatments between Kiel and Usedom mussels. Similar patterns of differential gene expression were observed between *M. trossulus* and *M. galloprovincialis* under acute salinity stress (0.17% of all genes). However, these populations exhibited a much larger parallel response (1.7% of all genes assayed, [191]).

This raises the question: do the two populations employ different strategies of salinity acclimation? The companion study illustrated a significant increase in mortality of Kiel mussels from $2\% \pm 4$ at salinity 16 to $67\% \pm 7$ at salinity 4.5 with decreasing salinity [296]

(Fig. 4.2). It is notable that these results are similar to other studies, where survival of *M. edulis*-like mussels was maintained until a critical salinity threshold was reached at a salinity $\leq$ 10 [263]. Mortality rate in Usedom mussels was constant at $\sim$ 20% across salinity treatments, which corresponds to the low number of differentially expressed genes that were unaffected by salinity. The mean salinity at Usedom lies at 7 ± 0.6, but fluctuates between 2.8 to 8.6 [297]. Our findings point to a higher tolerance for low salinity in Usedom mussels. Our results are consistent with the notion that Usedom mussels are locally adapted to low salinity conditions and do not have to significantly change gene expression to successfully acclimate to very low salinity. Another option could be that gene expression in Usedom mussels was generally elevated compared to Kiel mussel, as was shown for two coral populations with different heat stress responses [18].

A different explanation could be that instead of a change in gene expression, Usedom mussels used post-translational modifications to cope with salinity stress. It has been shown that the initial response to salinity stress is mediated via cell signaling and post-translational changes of pre-existing proteins [164]. For example, short-term hypoosmotic stress in *M. galloprovincialis* and *M. trossulus* caused changes in the phosphorylation states of proteins involved in diverse cellular regulatory processes mediated by protein kinases, such as suppression of apoptosis [84]. A higher level of phosphorylated proteins could indicate a higher ability to regulate adaptive processes of cellular osmoregulation via MAPKs during low salinity stress [166].

Since the transcriptomic data was mapped to a *M. edulis*-like reference transcriptome, another explanation for the low number of differentially expressed genes in Usedom mussels compared to Kiel mussels could be suboptimal mapping rates. However, compared to Kiel mussels there were no significant differences in mapping rates. The mapping rates in Usedom mussels were even slightly higher (SI1). The generally lower mapping rate can

be attributed to the fact that our reference transcriptome was heavily pruned [276, 402]. Specifically, amino acid metabolic pathways and solute transporters were differentially expressed between the two mussel populations under salinity stress. In Kiel mussels, these processes were upregulated while they were downregulated in Usedom mussels. These results point to an active cellular volume regulation in Kiel mussels. Under hypo-osmotic stress cells release osmolytes, passively via channels or actively via transporters, to avoid an increase of water and cell damage through swelling, the so-called regulatory volume decrease [172, 306]. Free amino acids are common organic osmolytes used for cellular volume regulation [264, 395]. Another mechanism to reduce osmolyte content is the catabolism of organic osmolytes [49]. Kiel mussels were generally characterized by a higher organic osmolyte content compared to Usedom mussels [296]. This could explain why metabolic pathways involving organic osmolytes are enriched in the Kiel population, but downregulated in the Usedom population. We identified genes that are involved in both mechanisms: release and degradation of osmolytes. Genes coding enzymes involved in the degradation of organic osmolytes were ornithine aminotransferase (*OAT*, conversion of ornithine to glutamate) and a glycine dehydrogenase (*GLDC*, degradation of glycine). These amino acids were also detected during metabolic profiling in both mussel populations [296]. Glutamate content was significantly higher in Kiel mussels compared to Usedom mussels. Glycine was one of the main organic osmolytes in *Mytilus* sp. and showed the largest magnitude of change with decreasing salinity. *GLDC* has been previously found to be upregulated in Arctic *M. edulis* [17]. Several solute transporters were differentially expressed between populations in relation to salinity, that are likely engaged in reduction of intracellular osmolyte content. The upregulation of the glycine transporter (*SLC6A5*) fits to the previously mentioned role of glycine in osmoregulation in Kiel mussels. Whereas *SLC6A5* was found to be downregulated under acute low salinity stress in *M. trossulus* and *M. gallo-*

provincialis [191]. *SLC22A3* was another osmolyte transporter that was upregulated in Kiel mussels (downregulated in Usedom mussels). *SLC22A3* codes a symporter that is responsible for co-transport of sodium and quaternary ammonium compounds (such as betaine). Betaine was one of the main osmolyte in Kiel mussels, but its concentration was low in Usedom mussels. This could explain the significant interaction of a quaternary ammonium compound transporter between salinity and population.

Surprisingly, we did not find significantly differentially expressed genes coding ion channels for the interaction of population and salinity. This might be explained by the fact that both populations showed a common phenotypic response of cation content decrease with salinity [296]. However, there was no common change in gene expression data between the two populations, either. Instead, we found the sodium-independent sulfate anion transporter gene (*SLC26A11*) to be differentially expressed. An upregulation of sulfate transport in Kiel mussels is plausible, as sulfate has been shown to significantly decrease in tissues of Kiel mussels [263]. Foshtomi et al. [91] further identified sulfate as inorganic osmolyte involved in cellular volume regulation in Ctenophores. However, we have no information on sulfate content in tissues of Usedom mussels.

Significantly different gene expression results further included genes involved in septate junction assembly (*CNTN*). This could be related to the observed differences in tissue water content between the two populations, where a higher water content was found in tissues of Usedom mussels. When salinity is reduced and organisms reach capacity for volume regulation the cellular water content increases [175]. An upregulation of *CNTN* could change septate junction organization and paracellular barrier functions and serve as a way to impede diffusion under hypoosmotic stress in Kiel mussels. This could further be an indicator, that Kiel mussels have i) reached CVR capacity and ii) have to actively adapt extracellular space structuring to counteract structural damages that might otherwise be cause

by an increase in water content, while the low salinity-adapted Usedom mussels are already equipped with a higher tissue water content. An increase of genes involved in cell adhesion was also detected in arctic *M. edulis* under low salinity stress [17].

4.4.2 Common response to low salinity in Kiel mussels

We found a strong common transcriptomic response across all low salinity treatments in Kiel mussels, as well as uniquely differentially expressed genes in this population. This suggests that Kiel mussels were severely stressed under low salinity conditions in this experiment and there was a common response at all low salinity treatments. The identified genes mainly comprised the physiological functions: osmoregulation, metabolism and cell cycle.

Surprisingly, we did not find many ion channels or any aquaporins (which were annotated in the reference transcriptome) differentially expressed in response to salinity stress, which we would expect with regard to other transcriptomic studies of *Mytilus* sp. to reduced salinities [17, 191, 212]. This could be due to the difference of long-term acclimation in this study (four weeks) vs. relatively short acclimation (4 hrs - 6 days) in the other studies. Ion channels and aquaporins might rather be upregulated as an immediate response to hypo-osmotic stress and are downregulated during long-term acclimation if enough protein has been synthesized or to protect against water currents [194, 212]. Contradicting this, is a previous long-term study on snails that found a variety of ion channels upregulated at low salinity [123].

We found many genes involved in solute transport to be upregulated in Kiel mussels, indicating an active cellular osmoregulation of both inorganic and organic osmolytes. Surprisingly, we did not find an upregulation of taurine transporters, which were upregulated in other salinity bivalve studies [187, 292]. Taurine was one of the major osmolytes in *Mytilus* sp. and heavily regulated with salinity [296]. However, we found an upreg-

ulation of amino acid transporters for glycine (*SLC6A5*), neutral or dibasic amino acids (*SLC7A9*, *SLC7A7*) and transporters for quaternary ammonium compounds (i.e. betaine) (*SLC22A3*) and other organic solute (*SLC5A12*, *SLC5A1*, *SLC5A9*, *SLCO4A1*, *SLC13A5*) under low salinity stress. Literature results corroborate our findings in terms of active organic osmolyte transporters [123]. We further found an upregulation of ion transporter genes for sodium, chloride, potassium (*SLC12A2*), phosphate (*SLC34A1*, *SLC34A2*) and sulfate (*SLC26A11*) at low salinity. Positive regulation of ion transport was also detected in transcriptomes of *M. galloprovincialis* after long-term low salinity acclimation [292]. However, we also detected simultaneous downregulation of some organic osmolyte transporters which suggests that the cellular response is not uniform. Additionally, many of the solute-transporters are sodium or chloride dependent. This indicates that the two osmolyte pools did not operate separately, but were intertwined. For example, sodium is needed for co-transport with amino acids and a certain concentration is required for maintenance of membrane potential, downregulation of ligand-gated ion channels might thus prevent uncontrolled sodium efflux or disruption of homeostasis. We further identified enriched transcripts that are connected to ion homeostasis (*HEXB*), that support this assumption.

In addition to osmolyte transporters, many amino acid metabolic enzymes were upregulated. This suggests a degradation and transformation of organic osmolytes. Enriched were hydroxylases, aminopeptidases, dehydrogenases and phosphatases (*CARNS1*, *CSAD*, *GLDC*, *LPQL*, *NAALAD2*, *OAT*, *PRODH*) that are involved in metabolic pathways of amino acids and their precursors such as aspartate, beta-alanine, carnosine, choline, cysteine, glutamate, glycine, histidine, hypotaurine, ornithine, proline, taurine and tryptophan. This corroborates findings of other bivalve hypo-osmotic stress studies [212]. The main osmolytes in Kiel mussels were aspartate, betaine, glycine and taurine, which we find represented in these pathways [296].

An active osmoregulatory response is further highlighted by an upregulation of genes involved in myelination (*HEXB*, *AFG3L2*) and the sodium/glucose transporter (*SLC5A1*). Sodium/glucose transporters are known to be involved in pumping water or facilitating passive water transmembrane transport in humans [82, 304, 387]. This suggests that Sodium/glucose transporters might adopt the role of aquaporins under low salinity stress to contribute to regulatory volume decrease via water transport out of the cells. Myelination is a process that was previously observed in salinity-stressed *M. edulis*, that increased the thickness of the myelin sheath of nerve cells, likely in order to support maintenance of membrane potential [382]. Upregulation of water transport likely counteracts cell swelling after increases in water content at low salinity. A steep increase in water content has been documented for *M. edulis*-like mussels at salinities $\leq$ 8 [263]. An upregulation of water transport has also been observed in the salinity stressed mussel *Xenostrobus securis* [292].

The second physiological function that was heavily expressed in salinity stressed Kiel mussels was energy metabolism. Growth rate of *M. edulis*-like mussels has been shown to deteriorate until biomass is catabolized under severe stress, likely due to depletion of energy reserves [263]. We found an upregulation of genes related to metabolic pathways of glycogen, glucose and fatty acid metabolism and lipid storage (*ADIPOR*, *CPT1A*, *NAGA*, *PDK2*, *PPP1CB*). This suggests an energetic cost of salinity acclimation. This is further supported by upregulation of cholesterol metabolism (*NVD*) involved in body growth throughout development in invertebrates. We did find upregulated as well as down regulated glucose transporters, that indicate that energy metabolism is enhanced but not uniform across cells. Upregulation in sugar and lipid metabolic pathways was observed in a Spanish *in situ* study in the invasive and less tolerant *X. securis*, whereas in the native more tolerant *M. galloprovincialis* only the regulation of glucose metabolic process was also observed under prolonged low salinity stress [292].

Other changes in lipid metabolism and transport could indicate changes in membrane composition (*ABCA1*, *APOLP2*, *PCYT1B*, *SEL1L*). Salinity has been previously shown to affect membrane composition in *M. edulis* [230], as well as phospholipid transport via ATPase [17]. Changes in the lipid composition of membranes affect the fluidity, permeability or activate signal transduction pathways [103, 110]. Furthermore, changes in membrane lipid bilayer composition and fluidity in mud crabs was correlated to modifications in ATPase activity which in turn is involved in regulation of intracellular ion content [25]. Willmer [381] discovered an increase of $\sim$ 70% in ATPase activity in low salinity stressed *M. edulis*. An increase in phosphorylation of lipids via calmodulin pathways in molluscs was linked to an increase in membrane permeability that allows efflux of taurine and water [251]. We also found up- as well as downregulation of calmodulin (*CALM*) in Kiel mussels, which suggests that this pathway is operated by activation and deactivation of *CALM*. The upregulation of genes (*HEXB*, *AFG3L2*) involved in calcium homeostasis supports this. Calmodulin is a calcium-binding protein that is activated by calcium ions and involved in signaling cascades involved in many biological processes such as muscle contraction, cell proliferation, or apoptosis. Downregulation of calmodulin was also observed in salinity stressed oysters [409].

In concert with a differential expression of genes involved in apoptosis, stress response genes were uniquely upregulated in Kiel mussels. Heat-shock proteins perform chaperone functions to stabilize protein structures and are known to be upregulated under stressful conditions. They have been first described to be upregulated under thermal stress, but that are also upregulated under osmotic stress [71, 213, 234, 265]. This corroborates our findings of upregulation of HSPs (*HSP68*, *HSP70B2*, *HSPA4*, *BIP*). We further identified stress response genes that are usually linked to hypoxia (*HYOU1*, *HIF1A*). This could indicate that i) mussels were simply stressed by lack of oxygen due to valve closure under

low salinity stress, or ii) that hypoxic stress response genes react to multiple stressors, such as HSPs, and are also upregulated under osmotic stress. In support of the first explanation, Kiel mussels were observed to be closed more often and have reduced clearance rates, while no such effect was found for Usedom mussels (pers. communication T. Sanders). Other stress response genes that were enhanced in Kiel mussels under low salinity were genes that reduced production of reactive oxygen species and are also otherwise active in cellular stress response to cold, hypoxia and starvation (*SLC25A8*). We found indicators for severe salinity stress uniquely in Kiel mussels. Severe salinity stress can cause cell damage and death. Upregulated apoptotic pathways are indicators of this process (*ADAM10*). On the contrary, several genes were upregulated that potentially interfere with apoptotic process to promote cell survival (*GHITM*, *PDK2*, *SLC25A8*, *TAX1BP1*) [236]. We further found genes involved in the regulation of immune response, cell survival and apoptosis (*WFDC18*, *YES1*). A study on two mussels subjected to low salinities also found a significant enrichment of GO terms related to immune response including apoptotic processes [292]. Downregulated genes, were related to enhancing of signaling cascades mediated by tumor necrosis factor involved in immune response and apoptosis (*CHRB*, *HERC5*, *MAP3K14*). MAPKs are a family of proteins that has previously been shown to be an important part of salinity adaptation in mussels and fish and are further important regulators of the cell cycle [166, 165, 191]. MAPKs were upregulated in response to hypo-osmotic stress [165, 191], yet our results showed an extreme downregulation of *MAP3K14*.

With respect to the identified genes involved in osmoregulation, metabolism and stress response we would have expected significant and meaningful results from the GO enrichment analysis, however this was not the case. Insignificant GO analysis results, were also experienced by other researchers examining salinity effects between species [191]. They attributed this result, to the relatively small response of hypo-osmotic stress versus other

stressors, but also pointed out that it could be incomplete description of molecular functions and biological processes of genes. We think the latter most likely, since we studied a non-model organism. Most of the annotations refer to model-organisms such as human and mice, and their genetic functions. In invertebrates these gene functions could be very different. Furthermore, cellular volume regulation involves an array of intertwined cellular functions. Organic osmolytes, intracellular inorganic ions, ion channels and solute transporters are all involved in a variety of other metabolic and regulatory processes. Thus, GO categories of involved genes could be widespread. Another explanation linked to the latter is, that, instead of a group of genes belonging to a specific GO category, potentially only few individual genes (i.e. channels and key enzymes) are involved in adaptive processes of CVR. A third explanation can be the difficulty to assess physiological states due to their time-depend responses [199]. Time-dependent processes such as CVR, which are enriched at the beginning of osmotic stress, but can be downregulated during acclimation and thus not detected in molecular probes from long-term experiments.

4.4.3 *Mytilus* sp. in changing salinities of the Baltic Sea

With ongoing climate change the salinity of the Baltic Sea is predicted to decrease by 1.5 – 2 until 2100, shifting the salinity gradient [107, 209]. The physiological threshold of *Mytilus* sp., derived from its current distribution [288] and mortality data from this study (Fig. 4.2), was found to lie at a salinity of 4.5-6. According to the most severe desalination model the salinity isoline of 5 would shift hundreds of kilometers westwards into the Baltic Proper [209]. We assume that desalination will result in a southward range shift of the entire species complex, as was previously proposed by [367]. This would cause the mussels to lose huge areas of potential habitat. *Mytilus* sp., as a reef-building species, is one of the main Baltic ecosystem engineers [142]. Thus, any ecological displacement would also affect

benthic communities severely [367].

With the observed differences in the transcriptomic responses to low salinity between Kiel and Usedom mussels it can be suspected that the two population may fare differently under future desalination. The projected future reduction of the ambient salinities at Usedom to $\sim$ 5 will likely be tolerated by *M. trossulus*-like individuals as no increase mortality or gene expression was observed at salinities <7 in this study. Yet salinities <5 constitute a physiological threshold for *M. trossulus*-like individuals [288], which would likely cause a westward range-shifts of populations from the Bothnian Sea and the Gulf of Finland. Further, salinity fluctuations at Usedom currently range from 2.8 to 8.6 [297]. But with a reduced mean salinity, fluctuations below a salinity of 5 could become more common. Such fluctuations would likely reduce the fitness of Usedom mussels.

The Kiel population (current native salinity 16) will be subjected to lower salinities of approx. 10-13 according to desalination scenarios [107, 209]. The high number of differentially expressed genes observed in Kiel mussel demonstrated that Kiel mussels are severely stressed at salinities $\leq$ 7. It is thus likely that already moderate salinity reductions (10-13) will cause an increased transcriptomic response of osmoregulatory and metabolic pathways and affect fitness of adult mussels. Also larvae mortality has been shown to increase with decreasing salinity in Kiel mussels [152]. Furthermore, salinity fluctuations, common to the western Baltic Sea [297], will expose mussels to salinities similar to the here applied treatments which will cause a more severe stress response.

Moreover, the predicted future habitat salinity of Kiel mussels (10-13) would lie directly in the genetic transition zone of *Mytilus* hybrids, in which an ongoing selection for low salinity adapted genotypes takes place in *Mytilus* larvae [152, 337]. It is therefore likely that Kiel mussels will undergo low salinity selection in the future which would change their current *M. edulis*-like allele frequencies to a more *M. trossulus*-like genome. The high mortal-

ity of Kiel mussel at low salinities can already be an indication of such natural selection process towards low salinity tolerant individuals (4.2). However, a change in allele-frequencies towards the more tolerant *M. trossulus* genotype, would not guarantee better performance under higher salinity conditions. A reduced fitness of Usedom mussels was visible in the relatively high mortality rates at the high salinity for adult mussels in this study (4.2), as well as for larvae in a previous experiment [152]. An overall shift in the Baltic Sea hybrid gradient would have implications for ecosystem structure, since Usedom mussels and Kiel mussel differ in size and shell thickness [143, 297]. Such morphological changes of mussel reefs would likely have consequences for predator-prey interactions [156].

With the projected salinity changes, lower habitat salinities will likely affect both populations. An increase in cellular osmoregulation processes and subsequent energetic trade-offs were observed in salinity stressed Kiel mussels. Such a consequence will likely also be observed in Usedom mussels under prolonged low salinity stress. *M. trossulus*-like mussels were found to have a higher phenotypic plasticity than *M. edulis*-like mussels, established by the low number of differentially expressed genes and low mortality. While this tolerance capacity may permit survival, the increasing costs for osmoregulation (i.e. plasticity) can potentially hinder evolution of plasticity [81]. As the Kiel population was found more affected by salinity stress in terms of mortality rates and gene expression response, Kiel mussels would likely show a higher osmoregulatory response and thus suffer larger trade-offs in their energy budget. This lower plasticity in Kiel mussels could also facilitate rapid adaptation [74].

Our transcriptomic data suggests that adaptive change would likely be located in the genetic markers for osmolyte transport and organic osmolyte degradation and potentially cell-cell adhesion and myelination, since this was heavily upregulated in Kiel mussels, but was downregulated or showed no response in Usedom mussels. Changes in energetic

metabolism and immune response, seen in Kiel mussels, are likely an energetic trade-off due to insufficient acclimation. Based on the companion paper results [296], it can also be assumed that the osmolyte pool would undergo changes in the course of low salinity acclimation towards a higher percentage of inorganic cations, as well as shifts in organic osmolyte composition. The salinity-driven selection of F1 generation of experimental mussels originating from the transition zone, has shown that there is capacity for adaptation [152]. We thus think that the Baltic hybrid population has the potential for adaptation, albeit only within their current salinity tolerance range down to 4.5 [288, 376].

Yet, also other environmental factors have to be considered for estimation of future mussel performance under low salinity. With climate change, not only salinity is predicted to decrease, but also increase sea surface temperatures and decrease pH are predicted [1, 107, 112, 283]. Temperature has been shown to function as a synergistic factor with salinity stress and thus higher future temperatures would decrease capacity for salinity tolerance in *Mytilus* sp. [17, 191, 121]. Ocean acidification might further increase energetic costs for calcification [297], coupled with increased costs for osmoregulation this could further reduce mussel fitness in a future Baltic Sea.

4.5 Conclusions

This study demonstrated a distinctive gene expression response to low salinity in the two mussel populations. This points towards a potential local adaption of Kiel and Usedom mussels adapted to their habitats. Future desalination will likely shift species current range limits southwards. Kiel populations reach their tolerance capacity at a salinity <6, while the most eastern distribution of *Mytilus* sp. has been described for salinities of 4.5. Or results suggest that the Kiel population is more susceptible to low salinity stress,

compared to the more tolerant Usedom mussels. This was illustrated by the overall larger transcriptomic response of Kiel mussel at low salinity, while Usedom mussels had a much lower number of differentially expressed genes. This is shown specifically in the upregulation of genes responsible for osmoregulation processes in Kiel mussels including ion, amino acids and quaternary ammonium compound transport and amino acid degradation, water transport, consumption of energy reserves as well as active stress response to stabilize protein structures and suppress apoptosis. In this regard, interesting field for future research are the mechanisms of water transport and myelination, which could be additional factors in CVR, next to osmolytes, that influence CVR capacity. Due to the susceptibility of Kiel mussels to low salinity stress we expect a change in allele-frequency towards low salinity adapted genotypes. As can be seen by the increased lipid metabolism of Kiel mussels, an increased osmoregulatory response has energetic trade-off which will likely affect scope for growth, evolution of plasticity and fitness of the entire future Baltic hybrid population. Such extensive range shifts of an ecosystem engineer will likely affect the entire benthic community and cause massive ecological changes in the future.

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C

Supplementaries Chapter 4

Table C.1: Mapping output *M. edulis x trossolus*

	Total		Kiel		Usedom	
	Means	**SD**	**Means**	**SD**	**Means**	**SD**
Total number of raw read	12,219,495	4,418,337	12,240,357	4,230,744	12,198,634	4,708,582
Total number of quality controlled reads	6,372,187	2,120,728	6,913,339	2,135,390	6,033,059	2,104,679
Number of contigs matched	25,632	792	26,176	674	25,149	585
Number unaligned reads	4,566,798	1,697,230	5,029,397	1,737,649	4,259,027	1,641,325
Number aligned reads	1,805,389	468,307	1,883,942	471,439	1,774,032	475,226
Number multiple aligned reads	193,569	47,943	199,221	46,206	192,646	50,807
Number secondary alignments	13,169,495	4,595,137	13,310,834	4,421,840	13,428,252	4,862,949
Number single aligned reads	1,611,820	421,693	1,684,722	426,746	1,581,386	425,378
Number qualitative singles	1,608,615	420,877	1,681,501	425,969	1,578,117	424,488
Mapping rate (%)	26.05	3.49	24.89	3.72	27.01	3.05

Table C.2: Overview of annotation rates

Information	Value
Number of unique transcripts	29177
Number of annotated transcripts (Blastx and/or PfamA)	14533
Number of annotated transcripts (Blastx)	13673
Number of annotated transcripts (PfamA)	11922
Number of overlapping annotated transcripts (Blastx & PfamA)	11062
Annotation rate (Blastx)	46.86
Annotation rate (Hmmer)	40.86
Annotation rate overlap (Blastx & Hmmer)	37.91

5

Discussion

In my thesis I studied the effects of low salinity stress on marine benthic osmoconformers on multiple levels ranging from organismal to a biochemical and a transcriptomic level. This was done using the example of the Baltic Sea, which is characterized by a pronounced horizontal salinity gradient and thus is an ideal model to investigate future salinity changes and selection of locally adapted populations. An integrative approach was applied to understand the mechanisms of cellular volume regulation (CVR), capacity for salinity tolerance and the potential for acclimation and adaptation of salinity tolerance in osmo-

conforming model species.

This thesis broadened the scope of osmolyte research and increased our understanding of the mechanisms of CVR. It is an important contribution to climate change research as it elucidates mechanisms that drive changes in coastal biodiversity with regards to salinity.

Study 1 reports the results of the first systematic literature review with subsequent meta-analysis of osmolyte concentrations. The specific aim was to assess the state of the art and highlight overall similarities of CVR response to low salinity between phyla. A precise filtering procedure and thorough scanning of the included reference library assured a reduction of subjective bias towards evidence-based outcomes. This approach identified inorganic ions, free amino acids (FAA) as well as methylamines as osmotic effectors. Additionally, taxonomic differences in organic osmolyte composition were discovered. This review further revealed large biases in the current osmolyte literature. This suggests that it is crucial to investigate the complete palette of osmolytes to understand the CVR system and its potential for acclimation and adaptation to persistent low salinities. It is further crucial to implement this for a broad taxonomic range to identify potentially distinct mechanisms specific for each taxon. The meta-analysis established a useful baseline for active osmolyte systems (study 1). This knowledge was then implemented in study 2.

Study 2 reports an integrative experimental approach to measure tissue inorganic ion and organic osmolyte concentrations in parallel with proxies for fitness after four weeks of low salinity stress. The aim was to compile comprehensive osmolyte budgets in addition to physiological biomarkers for multiple species from different phyla and to examine them for their capacity and similarities. A series of long-term low salinity acclimation experiments were conducted using six model invertebrate species (two molluscs, three echinoderms, one cnidarian). A decline in fitness (i.e. survival and net growth) and an increase in tissue water content (as measure for cellular volume regulation capacity) occurred simultaneously

at low salinities. These organismal responses were associated with a reduction in organic and inorganic osmolyte pools. Yet, the S_{crit} hypothesis could not be validated for any of the studied species. In this study, there was no universal concept defining species' tolerance limits, instead multiple factors were needed to outline a critical salinity range. While this study investigated the cellular osmolyte aspect of salinity tolerance, determinants for CVR capacity could also be related to other cellular processes. Therefore, the next study assessed transcriptomic responses to acclimation to low salinity to better constrain the complex suite of cellular processes impacted by osmotic stress.

Study 3 investigated the transcriptomic response to long-term low salinity stress in two locally adapted mussel populations of the *Mytilus edulis x trossulus* hybrid zone in the Baltic Sea. *M. edulis*-like mussels from Kiel live in the high salinity environment of the Western Baltic Sea, whereas the more tolerant *M. trossulus*-like mussels from Usedom predominate in the low salinity environment of the Eastern Baltic Sea. The aim of this study was to compare two locally adapted populations that differ in habitat salinity and identify differences in their transcriptomic response to salinity acclimation. After four weeks of acclimation to five salinity levels, the number of differentially expressed genes was highly different between the two populations. While there was a clear stress response and upregulation of CVR mechanisms in Kiel mussels an opposite response was observed in Usedom mussels. This suggests that Usedom mussels are not severely affected by low salinity stress. On the contrary, the transcriptomic response in salinity stressed Kiel mussels involved an upregulation of osmoregulatory transport pathways, enzymes involved in catabolism of organic osmolytes, proteins connected to changes in cellular structures that increase stability and limit water flow and upregulation of protein stabilizers, as well as proteins inhibiting apoptotic pathways. The upregulation of pathways involving energy metabolism further indicate an energetic trade-off towards these osmoregulatory processes in Kiel mussels. This

indicates that with decreasing salinity Usedom mussels will be better equipped to cope with low salinity environments and thus *M. trossulus* allele frequencies will become more frequent in the Western Baltic Sea.

5.1 Integrative Approach

While all three studies had the aim to understand how salinity tolerance works in marine invertebrates and what defines the capacity for salinity tolerance, this was investigated on different levels and with different approaches.

The main mechanism assessed in study 1 and 2 was CVR. The analysis of osmolyte pools in both studies therefore made it possible to compare meta-analysis results (study 1) to experimental results (study 2). The systematic review illustrated the state of the art and recommended increased research efforts towards filling specific research gaps. These suggestions were thus implemented in both subsequent experiments (study 2 and 3).

Specifically, the systematic review revealed three important biases in the osmolyte literature. The first bias is the *organic osmolyte bias*: Inorganic osmolytes were often not measured leading to a bias towards organic osmolytes. Even fewer studies from the reference library ($n = 3$) investigate both pools simultaneously. Consequently, it is difficult to evaluate the relative importance of inorganic vs. organic osmolyte pools during salinity acclimation. Thus, in study 2, I investigated all potentially relevant substance classes. The second bias, the *FAA bias*, was generated by targeted osmolyte analysis in many older studies that neglected detection of methylamines and methylated sulfonium compounds leading to a bias towards FAAs in the current literature. Thus, in study 2, organic osmolyte pools were assessed via untargeted metabolomic profiling that allows for detection of FAAs as well as methylamines. The third bias, the *mollusc bias*, consists of a strong focus on mollusc study

organisms in the literature, with emphasis on bivalve species (study1: Fig.2.3). In study 1, the analysis of two entire phyla (i.e. Cnidaria, Porifera) was impaired by the low number of studies available. Therefore, the use of model organisms as reference for a wider variety of phyla was advocated. Following this suggestion study 2 thus investigated six different species of three different phyla (Molluscs: one bivalve, one snail; Echinoderms: one sea star, two sea urchins; Cnidarians: one sea anemone).

Study 2 examined the phenotypic plasticity of osmolyte systems and physiological biomarkers. It was then suggested to investigate the plastic response of salinity acclimation on a transcriptomic level. The analysis of gene expression can give insight into the molecular basis of salinity tolerance [191, 63]. It can further indicate candidate osmoregulatory genes and specific pathways that are upregulated during salinity acclimation with potential for selection for low salinity tolerance [78, 63].

Lastly, all three studies focused on long-term salinity acclimation to study the effects of persistent low salinity predicted by future desalination scenarios. While the systematic review used an inclusion criterion of more than two weeks of acclimation time to altered salinity regimes, which is sufficient for most invertebrates [260] and ensured a large enough number of studies for meta-analysis, other researchers recommend longer acclimation periods. According to [145] a period of up to four weeks can be required for full acclimation in some species. This period is determined by the duration of modulation of intracellular osmolyte pools in response to changes in salinity. The experimental duration in study 2 and 3 were thus chosen to last four weeks. This is already a major asset of this study, distinguishing it from most published salinity tolerance experiments.

5.2 Osmolyte pools

Osmoconformers adjust to changes in seawater salinity via cellular volume regulation [101]. This process is achieved by modulating concentrations of osmotic solutes in the cell (i.e. osmolytes) [300, 126, 375]. The main actors in CVR are inorganic and organic solutes [250, 101, 132, 47]. The time-dependent changes in intracellular osmolyte concentration usually involve an immediate reduction in inorganic ion concentration under hypo- osmotic stress [324]. Organic osmolyte concentrations are reduced minutes to hours later [324, 365]. Full acclimation, however, takes 1-4 weeks [145, 260]. Meanwhile the organic osmolyte composition is still modulated for 2-4 weeks, within which readily available, simpler structured organic osmolytes are replaced by more complex compounds [9]. While most studies agree on the role of inorganic ions as an immediate response [315, 173, 55, 300, 324], most studies do not consider inorganic solutes in long-term acclimation. Instead, it is presumed that inorganic ion pools are being replenished after the immediate response [55, 330, 398, 39].

Yet, it has been postulated by a few studies, that the organic osmolyte pool size alone is not solely responsible for long-term salinity acclimation [250, 23]. The meta-analysis demonstrated that inorganic ions as well as organic osmolytes are utilized in cellular volume regulation after long-term acclimation to low salinity (study 1). Yet, there were only 3 references that reported complete osmolyte budgets including both inorganic and organic osmolytes [316, 267, 358]. Additionally, the systematic review revealed that the number of references investigating organic osmolytes vs. inorganic compounds in tissues was nearly thrice as high and there is a lack of research investigating intracellular inorganic ion concentrations after long-term acclimation to low salinity (study 1). This lack of data on inorganic ion concentrations was already stated 30 years ago [66]. Unfortunately, the situation has

not improved much. There is not a single invertebrate species for which detailed information of intracellular ionic parameters exists (i.e. osmolytes, acid-base and carbonate chemistry) [211]. The overall proportion of the intracellular inorganic vs. the organic osmolyte pool did not change with salinity in study 1, which indicates that both pools are reduced simultaneously with salinity. The overall pool proportion of approximately 30% organic osmolytes vs. 70% inorganic osmolytes (study 2) agrees with literature values [250, 269].

However, the meta-analysis of osmolyte pool size is based solely on mollusc studies. Thus, there is a huge need for experimental studies investigating complete osmolyte budgets in a variety of phyla subjected to long-term low salinity stress. This was implemented in the second study. Salinity acclimation experiments demonstrated that the overall inorganic osmolyte pool is reduced along with salinity in mussels, snails and sea stars, but not in sea urchins (study 2). The organic osmolyte pool was found to change significantly with salinity in mussels, snails, sea stars, green sea urchins and sea anemones. The utilization of inorganic and organic osmolytes is further corroborated by enriched gene expression of pathways linked to both compound classes acclimated to low salinities was then shown in study 3. Thus, this thesis demonstrated, by measuring both osmolyte systems in parallel, that inorganic and organic osmolytes play a crucial role in long-term acclimation to low salinity in molluscs and an echinoderm species.

5.3 Inorganic osmolytes

5.3.1 Lack of studies & methodological challenges

After having established that inorganic osmolyte pools are persistently reduced under hypo-osmotic conditions in most taxa (study 1 and 2), the goal was to gain more information on the specific inorganic ions that are being modulated. Yet, clear results of the

literature review were precluded by a general lack of data. While [66] criticizes the lack of data on inorganic ion concentrations in general, the meta-analysis expands this statement onto specific substances. The reference library established via the systematic review comprised data on 7 inorganic ions. However, only 4 inorganic compounds had a large enough sample size to permit a meta-analysis. Most studies assess sodium, potassium and to a lesser degree chloride. Yet, other potentially important ions are neglected, such as e.g. calcium, magnesium, sulfate, phosphate and bromide.

The reason for this lack of data, specifically with respect to anions, is the time-consuming methodology. Up to now, anion measurements are rare and concentrations were usually titrated separately for each ion [267, 316]. The majority of studies in the reference library analysed cations, because they are more easily measured via flame-photometry [24, 296, 227]. Additionally, more studies in the reference library reported body fluid concentrations than tissue concentrations, presumably due to extraction issues or to bypass the complexity of estimation of interstitial fluids in tissues. Thus, osmolyte analysis in this study attempted another, more comprehensive approach by measuring cation and anion concentrations in parallel. Cation concentrations were measured via flame-photometry. To assess inorganic anions in tissues a novel protocol was established that allowed for the measurement of several ions in tissue extracts via ion-chromatography.

An additional obstacle that likely hampered intracellular ion determination in previous osmolyte studies, is the complex procedure of accounting for the contribution inorganic ion concentrations in extracellular fluids within tissues. Currently, extracellular volume is estimated via radioactively labelled tracers [215, 350, 382]. Due to time constraints and lack of adequate lab space such experiments were not conducted as part of the salinity acclimation experiments presented in study 2, instead, tissue extracts were used. To estimate to which degree changes in inorganic ion concentrations of tissues are overlain by extracel-

lular fluid concentrations, a likely extracellular space (ECS) fraction was estimated using equations from [93] (study 2). The maximal possible ECS fraction was calculated using measured ion concentrations in tissue and body fluid data (study 2: Supplementary text and Eq1). The maximal possible ECS fraction represents the largest value that can be inserted in the calculation for the ECS parameter before intracellular osmolyte concentrations become negative. Together with available literature data, a likely ECS fraction was then estimated (study 2: Figure S4). This bears some uncertainty with regard to absolute intracellular concentrations (which are lower than tissue concentrations). Yet this approach is sufficient to conclude whether the effects seen in tissue extracts represent intracellular signals, or whether they are an artefact of extracellular fluid ion concentration.

5.3.2 Specific inorganic osmolytes

The results of the meta-analysis indicated a significant salinity effect for intracellular sodium concentrations, while intracellular chloride, intracellular potassium and tissue magnesium concentrations did not change with salinity. The effect found for sodium concentration in the meta-analysis was confirmed by the osmolyte analysis in study 2. Here, a significant effect of salinity on tissue sodium concentration was detected in mussels, snails, sea stars and sea anemones, but not in sea urchins. Yet, results presented in study 2 indicated that not only sodium, but also chloride and potassium concentrations were significantly reduced with salinity. Chloride and sodium were the main inorganic ions with respect to their concentration. Other studies have also highlighted the role of sodium, chloride and potassium as inorganic osmolytes [101, 324, 23, 68].

The differences between the meta-analysis and the experimental ion analysis could be due to the relatively low sample size (n=4) for chloride in the meta-analysis. Only one of four studies found no decrease in intracellular chloride concentration, two found a signif-

icant effect, and another had a high within-study variation and therefore no significant effect. In fact, the original random-effects model showed a significant effect size for chloride, but the robust test that was used because of low sample size generated more conservative confidence intervals. Only then did the mean effect size become insignificant. It is thus likely, that a larger sample size would generate a significant effect for intracellular chloride concentration at low salinity. Furthermore, all of these studies investigated bivalves, thus the results may not be entirely transferable to the results for snails, sea stars, sea anemones and green sea urchins investigated in study 2. This is, thus, an example, where results of the meta-analysis were impaired by the low sample size for inorganic ion concentrations in tissues, as well as by the mollusc bias in the literature. It is thus necessary to gain more knowledge of ion concentrations across a wide range of phyla.

While this may explain the different results of meta-analysis and osmolyte analysis for chloride, this was not the case for intracellular potassium effect size, where variation around the mean effect size in the meta-analysis was low. Usually, potassium is considered to be tightly regulated around constant concentrations intracellularly [238, 404], and thus was not expected to change with salinity. However, there are studies within the reference library that report a decrease of intracellular potassium concentrations with decreasing salinity, which corroborate the osmolyte analysis results [382, 267, 336]. This suggests that different species may utilize specific inorganic ions differently to either reduce osmotic pressure or maintain resting potential in neuronal tissues [336, 382].

While the meta-analysis could not offer insight into the salinity effect of minor intracellular inorganic ions, ion chromatography and flame-photometry together allowed for analysis of nine inorganic compounds: sodium, potassium, chloride, calcium, fluoride, bromide, sulfate, nitrate and phosphate. The analysis revealed that most of the minor ions are also modulated in relation to salinity in tissues in at least one of the investigated species (study

2, SI03). While there were no overall patterns of minor inorganic ion utilization between species, bromide, calcium and sulfate were modified with changing salinities in most species (namely: calcium and sulfate in sea stars, mussels, snails and sea anemones, but not sea urchins; bromide in sea stars, mussels and green sea urchins). Few studies on minor inorganic osmolytes were found during the systematic literature review. A previous blue mussel study found no salinity effect on intracellular calcium concentration [296]. Whereas sulfate has been detected to decrease with salinity in body fluids of ctenophores [91]. Minor ions are rarely studied, but can affect organisms by limiting many biological processes, ranging from calcification (i.e. calcium) to cytoprotective functions (i.e. bromide) [297, 33].

The transcriptomic responses, presented in study 3, to low salinity stress in mussels validate the findings regarding major inorganic osmolytes and some minor ions. They illustrated an up-regulation in sodium and chloride, sulfate, potassium and phosphate transmembrane ion exchange proteins as well as sodium/chloride-dependent solute co-transporters. The transmembrane transport of inorganic ions via transporters and channels has previously been demonstrated to be stimulated by acute low salinity stress [101, 68, 191, 16, 69, 205]. These results provide evidence, that despite species-specific differences, most taxa utilize inorganic ions as an osmolyte system during long-term acclimation to low salinity.

5.4 Specific organic osmolytes

Alanine, betaine, glycine and taurine were identified as the main osmolytes in the meta-analysis. The metabolic profiling confirms this for betaine, glycine and taurine, whereas alanine comprised only minor to moderate concentrations (study 2: supplementary information SI 03). These results agree with the literature, where it is stated that

the most abundant organic osmolytes in marine animals are non-essential amino acids (i.e. taurine, glycine, alanine, proline, hypotaurine, aspartate, serine and glutamate) and methylamines (i.e. trimethylamine-N-oxide (TMAO), glycine-betaine, sarcosine, dimethylsulfoniopropionate (DSMP)) [39, 101, 331].

Study 1 revealed that methylamines, specifically betaine, are an important component of the organic osmolyte pool. Osmolyte analysis in study 2 supported this, but also detected other methylamine compounds (such as DSMP, homarine, methylamine, sarcosine, etc.). While the main organic osmolytes are reported in most studies, there are also many individual reports of potentially meaningful, but largely unknown, osmolytes. Out of the 60 organic compounds that were mentioned in the literature, only 24 had a large enough sample size to permit meta-analysis. This excluded methylamines to a large degree (such as proline-betaine, homarine or DSMP) but also rarer FAAs their derivatives (such as beta-alanine, study 1: Tab. A.1). Albeit the scarcity of methylamine data, these substances can comprise the majority portions of the osmolyte pool [254, 262, 125, 314, 393].

Most of the earlier osmolyte studies indicated that FAAs are the main solute source modified under long-term salinity stress [250, 252, 259]. However, these results were often generated using targeted analysis methods, which do not detect methylamines [62]. By now it has been sufficiently established that methylamines are at least equally important osmolytes [39, 101, 331, 259, 371, 62]. Untargeted methods such as ^{1}H-nuclear magnetic resonance have the ability to measure methylamines. Yet, there are still recent studies that do not incorporate them in their osmolyte profiling [162, 122, 187, 277]. Untargeted metabolic profiling is crucial to reveal the true composition of osmolyte pools and the specific influence of all major osmolytes.

5.5 Differences of osmolyte pools between taxa

5.5.1 Taxonomic differences of inorganic osmolytes

Throughout the meta-analysis, the number of studies on inorganic ion concentration was so small, that no subgroup analysis on taxonomic differences was possible. In study 2, differences in the overall utilization of specific inorganic ions between phyla were detected. Molluscs, cnidarians and some echinoderms (i.e. sea stars) used a large variety of inorganic ions as osmolytes (i.e. chloride, sodium, potassium, calcium and sulfate tissue concentrations changed significantly with). An exception were sea urchins, which only reduced very few inorganic ions (i.e. chloride, phosphate and bromides) in tissues after long-term acclimation to low salinity.

Since differences in inorganic ion utilization in body fluids are known to exist in bivalves with different salinity tolerances, it is thus likely that such differences can also be found for tissues [64]. According to the systematic review, up to now no study exists that compared inorganic ion concentrations of tissues between phyla after prolonged low salinity stress.

5.5.2 Taxonomic differences of organic osmolytes

Considering organic osmolytes, taxonomic differences were clearly carved out by the meta-analysis, as well as by the osmolyte analysis. In study 1, taxonomic affiliation had as a significant influence on osmolyte concentrations in tissues. However, out of the five phyla that were mentioned in the literature, only molluscs, echinoderms and annelids had a large enough sample size to permit meta-analysis, thus excluding jellyfish and sponges. Interestingly, differences were found between all three analysed phyla, but the number of studies targeting annelids or echinoderms was low compared to molluscs. Therefore, study 2 incorporated multiple study species from non-molluscan phyla (i.e. cnidaria: sea anemones;

echinoderms: sea stars, shore sea urchins and green sea urchins).

The meta-analysis showed, that all major osmolytes were present in molluscs, echinoderms and annelids. However, the proportion of the major organic osmolytes differed between phyla. More specifically, the primary organic osmolyte was different for each taxon. Echinoderms used primarily glycine, while molluscs used mainly betaine and taurine (study 1: Fig. 2.8). A statistical comparison for taurine between phyla could not be conducted due to lack of data on taurine concentration in other phyla, yet in molluscs taurine always comprised a major portion of the osmolyte pool (study 1: Fig. 2.8). Annelids did not have a primary osmolyte, instead they used a variety of FAAs in moderate concentrations (i.e. alanine, glycine, or proline). Other reports measured high levels of asparagine, serine or taurine in annelids that could not be statistically tested in the meta-analysis due to low study numbers [56, 305]. Study 2 could confirm these results for molluscs, echinoderms and cnidaria and expand them in terms of variety of osmolytes. Glycine was also found to be the primary osmolyte in echinoderms (sea stars, green sea urchins and shore sea urchins). In molluscs, taurine could be identified as major osmolyte alongside betaine for snails and mussels. Sea anemones were found to utilize betaine as well as taurine as main osmolytes.

In study 2, all main osmolytes were found to be present in each taxon and species. Not every organic solute changed significantly with salinity, thus identifying some osmolytes as species- or even taxon-specific. Differences were also found for minor organic osmolyte compositions. The echinoderm-specific use of leucine, serine and threonine found in study 1, could be confirmed via osmolyte analysis in sea stars, green sea urchins and shore sea urchins in study 2. On the contrary, results for aspartate and glutamate differed between the two studies (study 1: Fig. 2.5; study 2: Fig. 3.4 SI08). Reasons for the differences between study 1 and 2 could be the following: considering glutamate, the low sample size of echinoderm studies in the meta-analysis, which included exclusively sea stars and not sea

urchins, could have affected the non-significant results. Additionally, tissue type and experimental duration was found to have a significant effect on glutamate effect size and could thus explain the significant heterogeneity. Considering aspartate, the effect size for molluscs had a large within-study variation in the meta-analysis which could have affected the results (study 1).

Other studies on organic osmolyte content of multiple species are rare - only seven studies were found via the systematic review. Although studying different species, all studies were exclusively conducted for one taxon, i.e. molluscs. [100, 130] found, similar to the findings in study 2, that there is no uniform pattern of FAA utilization amongst mollusc species. For example, alanine was exclusively utilized by mussels, while glycine and taurine were reduced by snails and mussels alike [100]. Also, the degree to which the main osmolytes alanine, glycine and taurine are used differs between species [260]. It was further found that the overall FAA pool size can vary strongly between mollusc species [65, 100, 175].

Another interesting finding is the taxon-specific utilization of methylamines in study 2. Pierce [250] and Burg & Ferraris [39] suggested that the basic CVR mechanisms utilized by all cells are likely very similar and that the major organic osmolyte systems include the same compounds (i.e. polyols, FAAs, urea and methylamines). The osmolyte pool were proposed to only differ in relative contribution of inorganic and organic osmolytes rather than the osmolyte type [250]. These statements could not be confirmed by the results of the osmolyte analysis in study 2. While the previous statement was supported for FAAs, because most phyla seemed to include the same FAAs (study 1: Fig. 2.8, study 2: SI03), the major contribution of methylamines to the organic osmolyte pools of molluscs vs. the negligible proportion of methylamines in echinoderms stands out. While mussels and snails utilize betaine, homarine and other methylamines, echinoderms (i.e. sea stars, green sea urchins

and shore sea urchins) did not employ methylamines or only in negligible amounts (study 2, Fig. 2.4). This is corroborated by meta-analysis results for betaine (study 1, Fig. 2.5). Yet, other studies mention a large contribution of methylamines to the osmolyte pool in corals, sea anemones, sponges, gastropods and polychaetes [254, 262, 125, 314, 393]. However, there are no studies that directly compare multiple species or phyla after prolonged low salinity acclimation that utilized untargeted techniques to quantify FAA as well as methylamines.

5.6 Fitness parameters and tissue hydration

In this work, survival, growth and water content were monitored in parallel with osmolyte systems. Survival is the ultimate parameter of defining a tolerance limit and was used here as a fitness proxy. Mortality increased with decreasing salinity in mussels, snails, sea stars and green sea urchins (study 2). The results agree with distribution limits of said species [308, 173, 367, 337]. Study 3 demonstrated differences in salinity tolerance between locally adapted blue mussel populations. The results for the Kiel population agree with results from experiments in study 2. Overall, the results support the previously established distribution limits of the *Mytilus* species complex [152, 337, 376, 287]. Here, mortality of the more tolerant Usedom mussels was not impacted by changes in salinity but was overall high ($\sim$ 20%) across all salinity treatments. This indicates that Usedom mussels are locally adapted to low salinity conditions, but have a lower fitness at high salinities. On the other hand, mortality of the less tolerant Kiel population was significantly impacted by salinity, which could indicate a potential selection towards low salinity tolerant individuals. Survival, as fitness proxy, has been previously used to discuss potential patterns of selective differentiation in bivalves with different salinity tolerances [78, 152]. However, survival

alone does not offer much understanding of the non-lethal effects under sub-optimal salinity conditions, which is why other fitness parameters are necessary.

Growth rates were impacted by salinity in all studied species (study 2). Previous studies have reported reduced growth for Baltic Sea blue mussels under salinity stress, as well as for sea anemones and sea stars [171, 346, 308, 262]. This study also documented that biomass was catabolized under severe stress. This was supported by gene expression results that showed an upregulation of lipid metabolism (consumption of energy stores) in salinity stressed mussels (study 3). This indicated a re-allocation of energy towards osmoregulatory processes and supports previous studies that postulated that salinity tolerance strategies come at a considerable metabolic cost[116]. Loss of biomass has been well established in other studies, e.g. for sea stars, sea anemones, or mussels under low salinity stress [308, 262, 303]. Modulation of energy metabolism is a central aspect of the cellular stress response [164]and low salinity stress has been shown to result in energetic trade-offs and catabolism of energy stores [138, 123, 292, 202, 347]. It can therefore be concluded that such an energetic trade-off will likely affect the organism's scope for growth and overall fitness of Baltic Sea populations.

Other studies refer to changes in the water content of tissues or cells when discussing CVR capacity, i.e. an organisms' ability to keep or reinstate its cell volume after osmotic challenges [175, 95, 94, 45]. A measure of change in water content is thus often applied as an indicator for completeness of CVR [299, 95, 92]. Osmoconformers have been found to have an overall larger capacity for water regulation than osmoregulators [92]. The water content measurement demonstrated an increase of water content for all investigated species (study 2). This has previously been described for many marine invertebrates, but only rarely in combination with osmolyte analysis [140, 256, 313]. Also, gene expression analysis corroborated these results. An upregulation of pathways involved in transmem-

brane water transport or structural changes in the extracellular space that control diffusion was found at low salinity. However, no upregulation of aquaporins was observed. Other studies also detected an upregulation of pathways involved in water content and permeation [17, 123, 409, 212]. However, most of these studies highlighted aquaporins and utilized short-term acclimation times. This indicates that synthesis of aquaporin proteins is upregulated as an immediate response to osmotic stress and cellular volume decrease. It is likely that this is a time-dependent response, similar to Na^+ K^+ ATPase activity observed in echinoderms [199], and that no elevated gene expression of this component can be detected after long-term acclimation. On the other hand, the pathways observed in this study seem to constitute a long-term response to low salinity. They presumably lead to expression of structural proteins that counteract persistent increases in water content (seen in study 2) by stabilizing cell structures against increased water pressure and induce structural changes to inhibit water inflow into cells.

5.7 S_{crit} CONCEPT

One of the main goals of this project was to better understand what defines the capacity for salinity tolerance in marine invertebrates. Euryhalinity in osmoconformers is assumed to be directly proportional to CVR capacity [250, 92, 299]. Theories have linked the capacity for salinity tolerance to organic osmolyte pool size, FAA pool size and organic osmolyte composition [262, 256, 260, 328, 379]. Thus, one of the first objectives was to test these hypotheses, primarily the S_{crit}-concept. The S_{crit}-concept states that the critical salinity of an organism is reached when the organic osmolyte pool is depleted, which further coincides with a loss of fitness [262]. Study 2 applied the S_{crit}-concept to the organic osmolyte pool of the study organisms. While the S_{crit}-concept was previously found appli-

cable for sea stars and sea anemones [308, 262], study 2 could not find a total depletion of the organic osmolyte pool at the perceived salinity threshold for any species. A linear reduction in organic osmolyte concentration with salinity was found in mussels, sea stars, sea anemones and green sea urchins (but not in snails and shore sea urchins), yet the extrapolated S_{crit}-values were below the critical salinity threshold of fitness proxies, or it was not possible to fit a linear relationship that included [organic osmolytes] $= 0$. No further study is currently known that tested the S_{crit}-concept. The integrative approach of study 2 revealed that fitness proxies, water content and osmolyte profiles were linked (Fig. 5.1). Study 3 validated this, as it demonstrated an upregulation of pathways involved in osmoregulation, as well as energy metabolism, apoptosis, or water transport. Similar findings have been reported for lipid and energy metabolism pathways as well as anti-apoptotic pathways in comparable studies using mollusc model species [410, 409, 138].

Throughout the analysis of multiple parameters across species, no uniform pattern of salinity acclimation could be detected. This agrees with studies that have previously reported species-specific cellular osmoregulation strategies [101, 331, 260, 96, 130]. However, no studies investigated the entirety of osmolyte systems between species or phyla, which is a huge drawback since only looking at the complete osmolyte system allows to understand the underlying mechanisms of salinity tolerance and predict species distribution in a changing world.

While the S_{crit}-concept defines a specific point a as low salinity threshold, the results from study 2 point towards a critical salinity range during which inorganic and organic osmolyte systems as well as fitness parameters and tissue hydration indicate severe salinity stress (Fig. 5.1). Within this range, survival can be achieved by more tolerant individuals, albeit with major trade-offs for example in net growth, while less tolerant individuals do not survive (indicated by the onset of mortality).

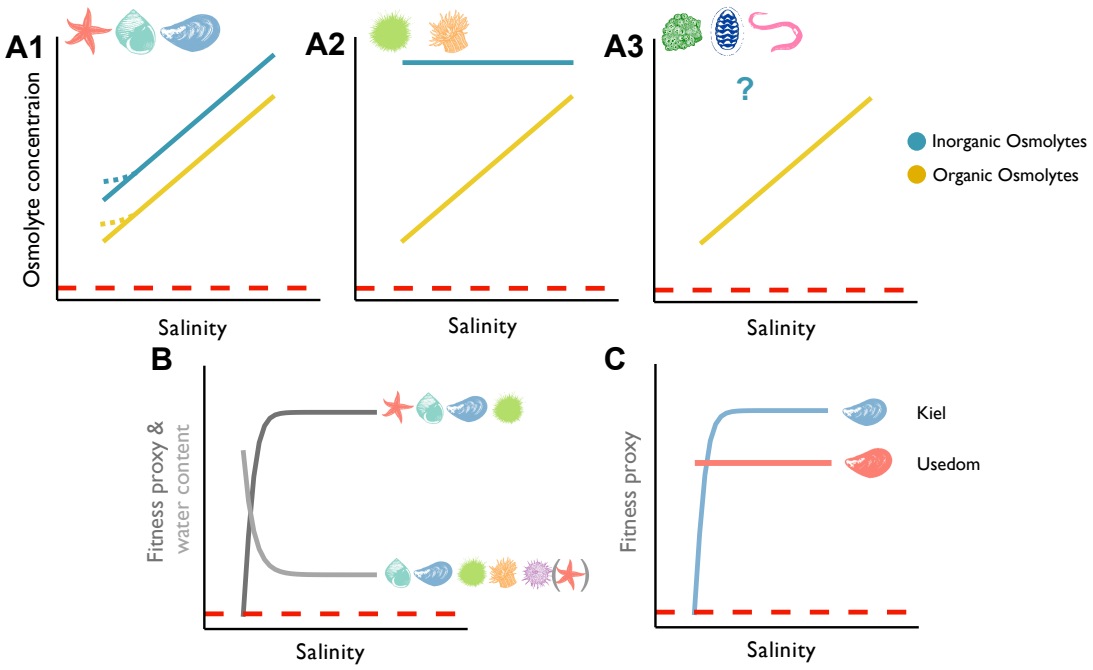

Figure 5.1: Depicted are hypotheses on how salinity affects the osmolyte pool in combination with idealized fitness parameters and capacity for cellular volume regulation. S_{crit} is defined as the point when the organic osmolyte pool is depleted. Figure A) shows an altered concept of a S_{crit} range which incorporates the inorganic (blue) and organic osmolyte pool (yellow). In Scenario A1: the organic and the inorganic pool are depleted with decreasing salinity, but concentrations do not reach zero (red line) In some cases concentrations stabilize at low salinities indicating hyperregulation (blue and yellow dashed lines). Study 2 showed that this was applicable for mussels, snails and sea stars. Scenario A2: shows a linear decrease of organic osmolytes, while inorganic osmolyte concentration is constant. This concept was found applicable for green sea urchins and sea anemones in the scope of study 2. Scenario A3: Additionally, study 1 revealed a depletion of organic osmolytes for polyplacophora, sponges, and annelids. However, no data exists on inorganic osmolyte concentrations after long-term acclimation for these taxa. B) Depicted is a fitness proxy (e.g. survival) (dark grey) that drops rapidly after a low salinity threshold is reached, meanwhile tissue water content (light grey) increases below this limit. Results from study 2. C) Depicted is a fitness proxy (e.g. survival) (dark grey) for two locally adapted mussel populations with different salinity tolerances. The Kiel population (blue line) is adapted to high salinity environments and showed a significant decrease in fitness with decreasing salinity, whereas Usedom mussels (red line) are more tolerant to low salinities, but have a lower fitness at high salinities.

In combination with the reduction of osmolyte systems water content increased and fitness decreased (net growth and/or survival) under hypoosmotic stress (Fig. 5.1). An overall outcome, resulting in an adapted concept, was that (i) either the organic osmolyte pool, (ii) or both the inorganic and organic osmolyte pools, were reduced with salinity (Fig. 5.1). In some species this was linear (i.e. mussels, sea stars, green sea urchins), in other species osmolyte concentrations stabilized at low salinities (i.e. snails), indicating hyperregulation of cellular osmolality. Hyperregulation can stabilize cellular homeostasis and guarantee cellular functioning and a working membrane potential, but is also costly osmoregulatory process that affects the energy budgets of organisms and thus likely can only be maintained in a certain salinity range and for a limited time period. Furthermore, species differ in their ability to hyperregulate.

Other osmolyte studies have previously shown that fine-scale monitoring can help to elucidate changes in pool composition at different salinity treatments [259, 195, 140]. The approach of study 2 further illustrates the usefulness of a fine-scale monitoring to detect potential hyperregulation of osmolyte systems, detect the onset of water content increase (i.e. limit of CVR capacity) and anticipate the low salinity threshold.

5.8 Differential gene expression in mussels with diverging salinity tolerance

After having established the phenotypic plasticity of osmolyte systems and physiological biomarkers in study 2, the investigation of molecular basis of salinity tolerance can give further insights into this process. Investigation of the transcriptomic response to persistently low salinity can give further indication for candidate osmoregulatory genes and specific enriched pathways.

The Baltic Sea salinity gradient is a useful tool to investigate selection for tolerance to low salinity environments. In such conditions the Baltic Sea offers a unique setting to examine salinity stress in locally adapted populations. Thus, this was utilized in the third study. The differences of transcriptomic response of a highly salinity tolerant and a more susceptible locally adapted mussel population from two divergent salinity regimes were studied.

Changes in the transcriptome drive the ability of organisms to react to environmental stress [266]. The transcriptomic response of Kiel and Usedom *Mytilus* sp. populations to low salinity indicated local adaptation to their native habitat salinity, as number of low salinity significantly increased differential gene expression and mortality in Kiel mussels, whereas Usedom mussel mortality and the number of differentially expressed genes was unaffected by salinity. A previous study on mussel larvae reached similar conclusions [152]. Transplantation experiments of Baltic Sea *Mytilus* further demonstrated phenotypic differences of *Mytilus* populations, which are likely based on the underlying genetic adaption to local salinity conditions [345, 143, 136]. However, epigenetic differences cannot be ruled out at this stage and multigenerational common garden experiments are needed to estimate the influence of epigenetic factors [152, 239, 298].

It was, nevertheless, clearly demonstrated that the Usedom population is more tolerant and the Kiel population is more susceptible to low salinity stress. These results are corroborated by the previous salinity acclimation experiments (study 2). Here, the critical salinity range of mussels was located at salinities <10. Other references determined a similar salinity threshold [152, 156, 289, 288]. The overall transcriptomic response was much stronger in Kiel mussels, compared to Usedom mussels. This indicates that Kiel mussels were more heavily stressed at low salinities. The specific pathways that were enriched under persistent low salinity conditions in Kiel mussels (but not in Usedom mussels) further support this conclusion. CVR pathways were differentially expressed alongside pathways that are

related to energy metabolism, growth, water permeability and cell survival.

The annotations of the enriched genes revealed differential regulation of various solute transporters that are responsible for transport of amino acids, other organic anions (e.g. methylamines) and inorganic ions. Also, genes that code enzymes involved in the degradation of amino acids were upregulated. These findings indicate that different mechanisms (e.g. release and degradation) are utilized to reduce intracellular osmotic pressure. And further, that multiple osmotic effectors are modulated. This was supported by the results from meta-analysis and osmolyte analysis, where the reduction of FAAs, methylamines and inorganic ion concentration in response to low salinity acclimation was demonstrated.

Another group of pathways that was upregulated under low salinity conditions in Kiel mussels were lipid and energy metabolism, as well as factors involved in organism development and growth. As shown in study 2, growth was affected in all species under low salinity stress. This is mirrored in the transcriptomic response that indicates an energetic trade-off due to the increased energy demand of osmoregulatory processes. The catabolism of energy stores in mussels at low salinity, retrieved from gene expression and net growth results, suggests that salinity conditions <8 are not sustainable for Kiel mussels.

Furthermore, the transcriptomic response of salinity-stressed Kiel mussels included the upregulation of stress response genes (i.e. heat-shock proteins, oxidative stress response) and the inhibition of apoptotic pathways. This cellular stress response is supported by organismic responses that showed a decrease of fitness and capacity limit for volume regulation at low salinity.

Overall, the transcriptomic response to low salinity acclimation is similar to that obtained for other bivalve species [392, 123, 410, 411, 191, 212]. My study, however, is the only study that illustrates differences between *Mytilus edulis x trossulus* hybrid populations that are locally adapted to different salinity regimes. This study highlights the differences

in salinity tolerance of the *Mytilus* species complex and presents further evidence for local adaption of Baltic Sea *Mytilus* populations.

Subsequently, the question arises whether gene expression patterns in response to low salinity are similar in other species. However, little is known about transcriptomic responses for non-bivalve invertebrate species. Literature research revealed almost no transcriptomic studies on salinity tolerance of the study organisms investigated in study 2. The exception is a single study on green sea urchins, were low salinity acclimation resulted in an enrichment of heat-shock proteins [223].

Despite this lack of knowledge, it has been estimated that, similar to blue mussels, many populations of dominant marine species inhabiting the Baltic Sea are locally adapted, have lost genetic variation and are relatively isolated in terms of gene flow [137]. It is postulated that if such locally adapted populations were to go locally extinct, their key roles in their local ecosystems may not be easy to replace by migration of individuals from North Sea populations, because Baltic Sea populations are evolutionary tailored to the particular local conditions [137]. It is thus crucial to gain more knowledge on phenotypic plasticity and local adaption in Baltic Sea key species to estimate the consequences of future desalination on marine invertebrate populations.

5.9 Future projections

With ongoing climate change, salinity is predicted to decrease in many oceanic coastal regions worldwide [214, 335, 27, 107, 113, 75]. It is thus necessary to determine if species and populations in the affected regions are able to cope with changing conditions. This includes researching whether high phenotypic plasticity or rapid adaptations are the underlying mechanisms for survival. Hence, it is essential to understand how salinity tol-

erance functions and determine current tolerance ranges of species in order to estimate the potential for acclimation and rapid adaptation to persistently low salinity environments.

Based on the critical salinity ranges for Baltic study organisms that were established in study 2, range shifts along the Baltic Sea salinity gradient of these species due to future desalination are likely. This is further emphasized for the Baltic *Mytilus* species complex presented in study 3. Future range shifts of foundation species and affiliated organisms will likely affect entire benthic communities and cause ecological changes [367]. Expected range shifts due to climate change, specifically salinity changes, have been postulated for many oceanic and coastal regions world-wide from tropical mangrove forests to polar regions [367, 127, 14, 221, 57, 75, 335, 341]. Empirical data on observed range shifts due to ocean warming is mounting [183, 139, 40].

In the course of desalination, a shift in allele frequencies is predicted for the Baltic *Mytilus* species complex (study 3). This was previously suggested by [152, 337]. A shift in the Western mussel populations from the *M. edulis*-like genotype towards the more salinity tolerant *M. trossulus*-like genotypes (study 3) could possibly result in an extended low-salinity tolerance of Western Baltic mussel populations.

The loss of fitness in populations under hypo-osmotic stress has been established in this study. The other side of the coin is the potentially reduced performance of low salinity adapted populations that are faced with higher salinities. This was demonstrated by the relatively high mortality of Usedom mussels at the highest salinity treatment. Selection towards low salinity genotypes does not guarantee a good performance under moderate or high salinity conditions. Transplantation experiments of locally adapted Baltic Sea mussels in to North Sea waters demonstrated a reduced performance (i.e. survival, shell size, weight, growth rate, clearance rate) despite long acclimation times (one year) [143, 136, 345, 297]. The Western Baltic Sea experiences larger and more frequent fluctuations in salinity than

the Central Baltic [297]. Thus, if Baltic Sea populations were to shift westwards, the fitness of low-salinity adapted species may be reduced by high salinity inflow events.

To make future projections on how Baltic Sea species will cope with future changes in salinity it is necessary to assess species' potential for acclimation through phenotypic plasticity vs. rapid adaptation. Investigating the effects of low salinity on cellular osmoregulation as the foundation for salinity tolerance together with other phenotypic parameters can give insights on the present phenotypic plasticity, whereas the investigation of gene expression under low salinity can give insights on both phenotypic plasticity of cellular pathways as well as potential osmoregulatory candidate genes for rapid adaptation. For this, however, genome scans would be necessary to identify targets of selection and quantify underlying genetic variation of assumed adaptive genetic markers [30]. In copepods an invasion into freshwater environments was achieved by the evolution of physiological tolerance and plasticity [179]. This freshwater invasion was accompanied by an evolutionary shift to regulate body fluids and ion transporter activity and expression [181, 179]. It was found that balancing selection in the native range, which is presumably facilitated by environmental fluctuations, can promote freshwater invasions and enable rapid adaptation [333].

The potential for acclimation through phenotypic plasticity can be a key factor in defining species capacity to tolerate salinity changes [384]. Diverse phenotypic plasticity was previously found in Hong Kong oysters living in extreme conditions across a natural salinity gradient [391]. High phenotypic plasticity has been suggested to facilitate the survival of Baltic barnacle and isopod populations in future climate change scenarios, whereas no indication for local adaptation was found in these studies [384, 386]. Indicators for phenotypic plasticity was tested in this study by way of the S_{crit}-concept and osmolyte concentrations. The salinity tolerance of species assessed in study 2 gives evidence on the phenotypic plasticity that may enable individuals to buffer effects of salinity changes without

genetic selection for low salinity adapted genotypes. The salinity acclimation experiments revealed the different degrees of phenotypic plasticity for each study organism. Here, mussels and snails showed a large plasticity, whereas an intermediate tolerance was seen in sea anemones and sea stars and least capacity for salinity tolerance was observed for both sea urchin species. Generally, it was found that euryhaline animals utilize the inorganic osmolyte pool, whereas the stenohaline species did not. Furthermore, only euryhaline species were found to employ the more complex methylamines in their organic osmolyte pool. Methylated osmolytes are known to be better protein stabilizers than non-methylated compounds [394] and could be an adaptation of euryhaline species to lower salinities. However, an assessment of these two assumptions is needed for a wide array of stenohaline organisms from different taxa to exclude a taxonomic reason for these findings, since all stenohaline organisms in this study were sea urchins. Lastly, the euryhaline *Mytilus* species complex investigated in study 3 demonstrated a high plasticity regarding their gene expression patterns of osmoregulatory pathways. Whether this is an ability of euryhaline species, would have to be investigated by expanding such studies to stenohaline species.

Another mechanism to cope with future environmental salinity changes is genetic adaptation. Ultimately, tolerant phenotypes could become fixed in the population [74, 170]. Moderate levels of phenotypic plasticity can compensate some effects of salinity changes and may drive evolution [268, 74], while high levels of phenotypic plasticity may reduce intensity of selection and thus limit adaptation rate [268, 74, 81]. Adaptation via natural selection to environmental changes requires time, and is regarded to limit the response capacity, especially for long-lived organisms [284]. However, there is emerging evidence for the potential of rapid adaptation over timescale of anthropogenic climate change [298, 153].

The Baltic Sea with its pronounced salinity gradient and locally adapted populations is an ideal set-up to simulate future selection gradients [283, 384]. Indication of local adap-

tation in the genomes of Baltic Sea organisms has been detected in a number of fish species such as cod, herring flounder or stickleback [168, 109, 177, 19]. Previous studies have assessed the transgenerational plasticity and standing genetic variation in oysters from the Gulf of Mexico and found ample genetic variation for evolutionary adaptation to low salinity conditions, whereas transgenerational plasticity as well as epigenetics did not appear as a primary mechanism for low salinity acclimation [106]. While mussels show a large genetic difference between populations, this was not found for sea stars [308]. It is thus likely, that similar to isopods and barnacles, that show little differences between populations [384, 386], the main mechanism for salinity tolerance capacity in sea stars lies in their phenotypic plasticity. Yet, little is known about other Baltic key organisms. This shows that underlying response mechanisms towards environmental stress can be quite species-specific.

The large differences in gene expression between the two Baltic mussel populations and phenotypic traits such as osmolyte concentrations and mortality, in combination with the known differences in allele frequencies, indicates a local adaptation of Baltic *Mytilus* sp. populations. It seems that these species had a high potential for adaptation which was exploited over the last thousands of years to adapt to the low salinity conditions of the Baltic Sea. This is indicated by the extremely wide range of salinities tolerated by Baltic *Mytilus* sp.. The Baltic Sea thus offers the possibility to study organisms that are now adapted to living at their physiological limit. Research on the tolerance capacities of populations along this salinity gradient could give insight into the evolutionary processes involved in achieving euryhalinity. With respect to future projections it seems likely that these species are already adapted to low salinity conditions and thus a passive shift of species with the salinity gradient in the Baltic Sea will take place. However, with decreasing salinities in other areas (e.g. estuaries), eastern Baltic Sea organisms could migrate to such regions and have the potential

to successfully inhabit such areas in the future.

5.10 Future research

In the course of this project the systematic review revealed that there are still large gaps of knowledge with respect to CVR, especially regarding differences between phyla and osmolytes. Future studies should broaden their scope to analyse the whole palette of organic compounds as well as inorganic ions in parallel. The best-practice guidelines give clear indications of what to consider when designing an osmolyte analysis study. Furthermore, current efforts in the workgroup pursue the establishment of a protocol with a fluorescent tracer (FITC-inulin) to estimate extracellular space in invertebrates instead of using radioactive tracers, as has been achieved in recent studies on mammals [26, 363], to render measurement of extracellular volume more accessible (Bauer, Podbielski, Melzner, ongoing work). Inulin is a compound that is not incorporated into the cells and distributed throughout the extracellular space after injection into body fluids without being eliminated from it (e.g. by excretion, storage or decomposition) [161]. Thus, by attaching a tracer to it the extracellular volume can be quantified.

Furthermore, to assess the potential for acclimation and rapid adaptation under persistent low salinity stress and distinguish which mechanism are in place, multiple populations of target species would have to be assessed, preferably in multi-generational common-garden experiments on a physiological and genomic level [224].

Other research areas such as the generation of desalination scenarios, other functions of osmolytes, or changes in biotic interactions as a result of changing environmental salinity are closely related to osmolyte research and will require more attention in the future as these are relatively sparsely investigated and will gain more relevance with future salinity

changes.

The first research area that is interesting for future research are desalination scenarios. While desalination is already observed in the polar regions [241] salinity changes are also predicted in other areas such as the Baltic Sea, the Gulf of Mexico, shallow Australian coral reefs and the Arctic [27, 107, 113, 341]. It is thus crucial to obtain more knowledge on potential regions affected by desalination and to achieve a higher precision in forecasts. In the specific case of the Baltic Sea, multiple studies predict a desalination of 1.5-2 until 2100 [107, 209], however models are still very coarse and results depend significantly on the models applied. A recent study showed the large degrees of uncertainty of future predictions [208]. By incorporating sea level rise into their model, increases in seawater and freshwater input could potentially cancel each other out and thus no desalination would take place in the Baltic Sea [208]. However, it was shown that the results depend highly on the applied Earth System Models. A change between global models that are incorporated into the regional model of the same study supported previous literature and also found a decrease in salinity, thus emphasizing the uncertainty we still face [208]. The natural variability of physical parameters of the Baltic Sea hampers precise estimations of the magnitude of change. Despite these uncertainties, desalination in the Baltic Sea is still very likely to happen (pers. communication U. Gräwe). Thus, further research is needed to increase the predictive capacity of future projections.

On a biochemical level, osmolytes might have additional effects, for instance as a chemical defense against predators, or potentially act as attractant in marine invertebrates [396], but this needs further research . It is known that amino acids stimulate activity in prawns [54]. Furthermore, homarine was shown to inhibit growth of epibionts and predation in corals[344, 206, 322]. Organic compounds in algae are known to possess abilities that serve as wound-induced chemical defense, or as antifouling defense [294]. DMSP is widespread

in marine microalgae and may serve to repel grazers [383, 359, 294]. Proline is known as a deterrent of herbivores brown algae [294]. And betaines of beta-alanine, proline and hydroxyproline are speculated to fulfill a role as chemical defense against herbivores and pathogens in terrestrial plants [114]. Future research on potential additional chemical effects of osmolytes would be interesting with regards to their effectiveness when concentrations are reduced under low salinity. Would future desalination potentially diminish chemical defense of organisms and thus further affect marine invertebrates? How would this affect predator-prey interactions and benthic ecosystems?

On an ecological level, not much is known how predator-prey interactions might be affected by changes in salinity conditions. Predator-prey interactions of blue mussels and a major predator *Carcinus maenas* have been shown to be affected by low salinity [156]. Salinity has been shown to affect shell stability, which could favor crab predation, the main predator of blue mussels in the Baltic Sea, which effectively controls their population [143, 156]. Also prey preference of crabs has been demonstrated to shift at low salinity [156]. Moreover, salinity changes will likely cause changes in benthic community composition due to expected range shifts of foundation species and affiliated species in a future climate [367]. Vuorinen et al. [367] assume that the cascading effects of these changes would be extensive in the Baltic Sea. There is thus a large need to study the effect of climate change stressors on species interactions and to investigate how this could affect benthic communities in a desalinated environment.

Lastly, multiple climate change stressors influence the capacity for salinity tolerance such as temperature, pH, pollution, food supply, etc. [43, 342, 242, 52, 406]. Especially the interaction of temperature and salinity was shown by multiple studies to have severe effects on mortality, metabolism, osmoregulation, condition index and stress responses of marine invertebrates [86, 17, 362, 138, 229, 121]. Thus, climate change-induced increases in

warming, ocean acidification and desalination can exacerbate the negative effects on marine species and aggravate survival in changing oceans. This should be considered in future research when estimating the effects of salinity on individuals, populations and benthic communities of marine invertebrates.

6

Conclusion

My thesis contributed to a better understanding of the underlying mechanisms of CVR in osmoconformers and established complete osmolyte budgets by using untargeted methods. It further incorporated organismal physiological parameters to assess salinity tolerance. I investigated what determines successful salinity tolerance in osmoconformers.

The first part of this thesis generated a baseline of current osmolyte research and revealed the diversity of osmolytes utilized by marine osmoconformers. The meta-analysis high-

lighted that inorganic as well as organic osmolyte concentrations are significantly reduced under persistently low salinities. Furthermore, it was shown that significant differences between phyla exist. Yet, it became apparent that large gaps in the current literature exist due to the application of targeted methods for organic osmolyte analysis and taxonomic bias. The best-practice guidelines that resulted from my analysis suggest an improved approach for future osmolyte research.

Within the second part I analysed the osmolyte systems in multiple Baltic Sea species after long-term acclimation to low salinity. This approach used a variety of analytical tools to include multiple types of organic and inorganic osmolytes and thus establish a comprehensive osmolyte budget. This study could corroborate findings from the meta-analysis, namely i) that taxonomic differences in organic osmolyte pools exist between molluscs and echinoderms, ii) that inorganic ions and organic osmolytes are utilized to acclimate to salinity changes, iii) that both, methylamines and FAAs are important in cellular osmoregulation, iv) that alanine, betaine, glycine, taurine, sodium and chloride are the main osmotic effectors. The analyses of osmolyte systems could be further linked to other phenotypic biomarkers for individual fitness and CVR capacity. This integrative approach used a fine-scale monitoring at the lower salinity threshold. Together these results helped to determine a critical salinity range of each of the organisms and highlighted their phenotypic plasticity. These two studies thus successfully established a better understanding of underlying mechanisms of CVR and salinity tolerance of multiple species.

The third study used transcriptomics to better understand mechanisms of CVR and cellular pathways that are activated during prolonged salinity stress. It tested the capacity in salinity tolerance of two locally adapted *Mytilus* sp. populations living at distinctly different native habitat salinities ($S = 5 - 8$ vs. $S = 1220$). This study showed a divergent transcriptomic response between moderately and highly low-salinity tolerant mussels from

the *Mytilus* species complex. It identified genetic marker genes involved in CVR. The gene expression study further highlighted gene expression variation between locally adapted populations. Whether this gene expression variation is heritable, needs to be determined in future studies.

With ongoing climate change, geographic range shifts are predicted for many species. Range shifts will be the consequence, if organisms are not able to acclimate through high levels of phenotypic plasticity or rapidly adapt through genetic assimilation or natural selection of tolerant genotypes from standing genetic variation. This study is thus an essential contribution to determine capacity of Baltic Sea key species to tolerate predicted changes in salinity and to increase our understanding of their phenotypic plasticity via the underlying mechanisms of CVR.

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Author list & contribution statement

The following authors contributed to the three chapters of the doctoral thesis of Imke Podbielski entitled *Towards a mechanistic understanding of the biochemical, physiological and transcriptomic adaptation to salinity changes in benthic osmoconformers*. The contribution of the PhD candidate to each chapter are listed below:

Chapter 1: The following authors contributed to Chapter 1: Imke Podbielski (I.P.), Dr. Lara Schmittmann (L.S.), Dr. Trystan Sanders (T.S.), PD Dr. Frank Melzner (F.M.).

I.P. designed the search string, conducted the database search, examined titles, abstract and full-texts for inclusion criteria. I.P. transferred data and metadata from included study to a data base. I.P. conducted the meta-analysis and analyzed results. I.P. wrote the paper. I.P. and F.M contributed to the interpretation of the data. I.P., L.S., T.S. and F.M. contributed to the finalization of the manuscript.

Chapter 2: The following authors contributed to Chapter 2: Imke Podbielski (I.P.), Dr. Claas Hiebenthal (C.H.), Dr. Mithra-Christin Hajati (M.H., Dr. Christian Bock (C.B.), Prof. Dr. Markus Bleich (M.B.), PD Dr. Frank Melzner (F.M.).

I.P. and F.M. designed research; C.H. contributed to experimental set-up, I.P. performed research; M.B. and C.B. contributed analytic tools; I.P. and M.B. developed IC protocols for tissue samples; I.P. analyzed data; M-C.H. and I.P. programmed R-Scripts; and I.P. wrote the paper. I.P., C.B., M.B. and F.M contributed to the interpretation of the data and I.P., C.H., M-C.H., C.B. and F.M. contributed to the finalization of the manuscript.

Chapter 3: The following authors contributed to Chapter 3: Imke Podbielski (I.P.), Pierre de Wit, PhD (P.d.W.), Dr. Trystan Sanders (T.S.), Dr. Uwe John (U.J.), PD Dr. Frank Melzner(F.M.).

T.S.and F.M. designed the experiment. T.S. conducted the experiment and RNA sample preparation; U.J. contributed analytic tools; I.P. and P.d.W. designed the transcriptomic research; I.P. performed bioinformatic analysis of RNAseq data and conducted statistical analysis; I.P. wrote the manuscript. I.P., P.d.W. and F.M contributed to the interpretation of the data and I.P., P.d.W. and F.M. contributed to the finalization of the manuscript.

Eidesstattliche Erklärung

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