Molecular Stress Responses In Estuarine Fish

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Summary

Estuaries cover only about 6% of the global coastal areas but are among the most biologically productive ecosystems. They have a massive influence on carbon cycle, serve as nurseries, migration routes, and sources for fish populations. However, they are also among the ecosystems most affected by direct human interventions and anthropogenically caused climate change. Despite their high ecological importance and the ecosystem services they provide, only one-tenth of a percent of all climate change research is conducted through observational studies in estuaries. Global models used for predicting the effects of climate change often fail in these understudied systems. Some of the strongest influencing factors include oxygen deficiency, particularly from anthropogenic nutrient input, rising temperatures, extreme events such as heatwaves, altered salinity patterns due to changes in precipitation and sea level rise, as well as increases in sediment load due to construction or altered flow patterns.

Fish populations in estuaries have a significant impact on the ecosystem services provided, from the transport and storage of carbon to being a direct food source, yet they are increasingly exposed to environmental stressors. The multiple stressors that can simultaneously affect an organism in wild habitats can have unexpected interactive effects on the organism itself and its interaction with associated microbiota, the holobiont. This aspect has been little explored in laboratory experiments and even less in field studies, but it is important for understanding and predicting changes in ecosystems and providing a foundation for protection and restoration.

Multicellular organisms have a highly conserved cellular stress response that directly reacts to stressors, protecting macromolecules, degrading damaged macromolecules, halting the cell cycle, or inducing controlled cell death to protect the entire organism in case of severe damage. Vertebrates like fish also have additional stress responses controlled by stress perception in the brain and hormone release, which allow responses on various levels. The organismic and cellular response to one stressor generally limits the capacity to respond to additional stressors. The universal mechanism underpinning physiological stress responses is the adjustments of abundance in messenger RNA that can be determined and described with high precision via transcriptome studies. As the costs of holistic omics methods continue to decrease, large-scale applications for identifying stressors, stress interactions, and previously unconsidered biotic interactions become feasible.

In the first part of this work (**Chapter 2**), the impact of abiotic gradients along the estuarine stressor mosaic on tissue-specific stress responses in immunologically important gill tissues and metabolically important liver tissues of a top-predatory freshwater species, *Sander lucioperca* L., was investigated using differential expression and abundance analyses alongside network-based approaches. As an approach to assess the holobiont's reaction, bacterial composition patterns on the gills were determined in parallel. The results showed turbidity-induced starvation in fish and increased cellular stress and immune responses in reaction to hypoxia and eutrophication coincided with the increase in abundances of various opportunistic bacteria. The work also served for method validation and demonstrated that the different omics methods (mRNA and bacterial 16S gene sequencing) complement each other well, and the differential analyses support the network results.

To track movement patterns, habitat use, and spatial connectivity, we conducted stable isotope analyses of δ13C and δ15N on the most dominant fish species in the study system (**Chapter 3**). For potamodromous ruffe, it was shown that they remain in the estuary throughout their life cycle, as there were hardly any marine or fluvial isotope signatures observed. This suggests a brackish water population of this species that is spatially distinct from upstream freshwater populations. Additionally, a high degree of site fidelity was observed, suggesting limited short-term movement along the salinity gradient. The stable isotope patterns of ruffe and smelt were then combined in the next chapter (**Chapter 4**) with spatially and seasonally extended gill mucus bacterial composition data. Gills are a multifunctional organ in constant and direct exchange with the surrounding water, making them a

potential entry point for pathogens. The composition of the microbial community can therefore be an important indicator of fish health. In the site-faithful ruffe, the composition of the microbiota was heavily influenced by spatial conditions. In anadromous smelt particularly during spawning migration a gradual adaptation of bacterial composition was detected. The stable isotope data and bacterial patterns largely overlapped, providing mutual insights into migration patterns. In both fish species, a stable core microbiome comprising few but highly abundant bacterial taxa was identified, which diminished in both fish species under prolonged hypoxia and eutrophication conditions. This effect was accompanied by an increase in opportunistic taxa (*Acinetobacter, Shewanella, Aeromonas*).

To capture the fish responses, we further integrated 340 tissue-specific transcriptome datasets from livers and gills of smelt and ruffe with the bacterial data, isotopes, fish abundances, physiological and abiotic data to unravel the interactive effects of dominant stressors on a large scale (**Chapter 5**). In both fish species, rising water temperatures in spring were accompanied by the occurrence of opportunistic Flavobacteriaceae and a strong increase in adaptive and innate immune responses across tissues. Persistent hypoxia in summer in the upper river, on the other hand, was associated with rising abundances of opportunistic *Acinetobacter* taxa alongside cellular stress responses, particularly regarding cell cycle control and cell death. In anadromous smelt especially, generalized additive models showed a parallel decline in immune responses, indicating energy reallocation-driven immunosuppression, potentially making the fish more susceptible to infections. Population genetic analyses of both species showed high connectivity among populations but functional diversity and signs of selection in genes important for adaptation to high temperatures and hypoxia, related to the HIF transcription factor and pathway genes, as well as the immune system.

Finally, based on a 40-year time series, we examined changes in the abundances and composition of life cycle guilds of fish in the Elbe estuary (**Chapter 6**). While there was an improvement in habitat quality with increasing water quality throughout the 1990s, since 2010, catches have been declining again, and total fish density has decreased by 92% in the last decade. Drivers identified included recurring hypoxia situations, high concentrations of suspended particulate matter (SPM), and the reduction of nursery habitats.

In summary, this Phd-project provides spatiotemporal insights into the molecular stress responses of dominant fish species in conjunction with host-associated bacteria as an estimate of the holobiont response in an estuarine system for the first time. Various methods for large-scale integration of omics techniques were evaluated and established. The use of different methods particularly enabled the validation of individual results. A minimally invasive, non-lethal monitoring of fish-associated bacteria, supported by point investigations of molecular stress responses within the host organism, could represent a cost-efficient extension of current long-term monitoring methods. The current results can already be used to guide ecosystem recovery efforts. The data show a need for action, as the decline in fish abundance is expected to be accompanied by a genetic bottleneck, reducing genetic diversity and stripping populations of adaptive capacity, especially considering increasing stressors with ongoing climate change (e.g., hypoxia, temperature). However, the longer the population collapse lasts, the stronger these effects can manifest themselves due to genetic drift.

Zusammenfassung

Ästuare bedecken nur etwa 6 % der globalen Küstengebiete, gehören jedoch zu den biologisch produktivsten Ökosystemen. Sie haben Einfluss auf den Kohlenstoffffluss, dienen als Brutstätten, Migrationsrouten und Quellen für Fischpopulationen. Allerdings gehören sie auch zu den Ökosystemen die am stärksten von menschlichen Eingriffen und dem menschengemachten Klimawandel beeinflusst werden. Trotz ihrer hohen ökologischen Bedeutung und der erbrachten Ökosystemdienstleistungen finden nur 0.1% aller Klimawandelforschung in Form von Beobachtungsstudien in Ästuaren statt. Globale Modelle zur Auswirkung des Klimawandels versagen in diesen Systemen häufig. Zu den stärksten Einflussfaktoren zählen Sauerstoffmangel, insbesondere durch anthropogen verursachten Nährstoffeintrag, steigende Temperaturen, Extremereignisse wie Hitzewellen, veränderte Salzgehaltsmuster aufgrund von veränderten Niederschlägen und Meeresspiegelanstieg sowie Zunahmen in der Sedimentlast durch Bauarbeiten oder veränderte Strömungsverhältnisse.

Fischpopulationen in Ästuaren haben einen großen Einfluss auf die erbrachten Ökosystemdienstleistungen, vom Transport und der Speicherung von Kohlenstoff bis hin zur direkten Nahrungsversorgung, sind jedoch immer stärker zunehmendem Umweltstress ausgesetzt. Die multiplen Stressoren, die in Wildhabitaten gleichzeitig auf einen Organismus einwirken, können unerwartete interaktive Effekte auf den Organismus selbst und sein Zusammenspiel mit assoziierten Mikrobiota, dem Holobionten, haben. Diese Aspekte wurden bisher nur wenig in Experimenten und noch weniger in Freilandstudien untersucht, sind jedoch wichtig für das Verständnis und die Vorhersage von Veränderungen in Ökosystemen und stellen eine Grundlage für den Schutz dar.

Vielzellige Organismen besitzen eine konservierte zelluläre Stressantwort, die direkt auf das Einwirken von Stressoren reagiert und Makromoleküle schützt, beschädigte Makromoleküle abbaut oder den Zellzyklus stoppt bzw. den Zelltod einleitet, um den Gesamtorganismus bei Schädigungen zu schützen. Fische als Vertebraten haben darüber hinaus weitere stresswahrnehmende Mechanismen im Gehirn und hormonausschüttungsgesteuerte Stressantworten, die Reaktionen auf verschiedenen Ebenen ermöglichen. Die organismische und zelluläre Antwort auf einen Stressor begrenzt dabei die Kapazität für die Reaktion auf weitere Stressoren. Die universelle Anpassung von Genexpressionsmustern als Antwort auf Stressoren lässt sich mit molekularen Methoden mit hoher Präzision beschreiben. Durch die stetig sinkenden Preise holistischer Omics-Methoden wird der Einsatz zur Ermittlung von Stressoren und unbekannter biotischer Interaktionen möglich.

Im ersten Teil dieser Arbeit (**Kapitel 2**) wurde über den Einsatz von differentiellen Expressions- und Abundanzanalysen parallel zu netzwerkbasierten Ansätzen untersucht, wie sich abiotische Gradienten entlang des Ästuaren-Stressormosaiks auf die gewebespezifische Stressantworten in immunologisch bedeutsamen Kiemen- und besonders metabolisch bedeutsamen Lebergeweben einer top-prädatorischen Süßwasserart, *Sander lucioperca* L., auswirken. Als Ansatz zur Abschätzung der Reaktion des Holobionten wurden parallel Muster der bakteriellen Zusammensetzung auf der Kieme ermittelt. Die Ergebnisse zeigten besonders durch Trübung bedingte Hungersymptome im Fisch und erhöhte zelluläre Stress- und Immunantworten als Reaktion auf Hypoxie und Eutrophierung, die mit dem Anstieg der Abundanzen verschiedener opportunistischer Bakterien zusammenfielen. Die Arbeit diente unter anderem der Methodenvalidierung und ergab, dass sich die verschiedenen Omics-Methoden (mRNA und bakterielle 16S Gen-Sequenzierung) in hohem Maße ergänzen, und die differentiellen Analysen die Netzwerkergebnisse stützen.

Um die Bewegungsmuster, Habitatnutzung und räumliche Verbundenheit zu verfolgen, haben wir stabile Isotopenanalysen von δ13C und δ15N an den dominantesten Fischarten im Untersuchungsgebiet durchgeführt (**Kapitel 3**). Bei potamodromen Kaulbarschen zeigte sich, dass diese sich während ihres gesamten Lebenszyklus im Ästuar aufhalten, da sich kaum marine oder fluviatile Isotopensignaturen zeigten. Dies deutet auf eine Brackwasserpopulation dieser Art hin, die sich von flussaufwärts lebenden Süßwasserpopulationen in der räumlichen Nutzung abgrenzt.

Außerdem zeigte sich eine hohe Standorttreue, die darauf schließen lässt, dass die Tiere sich nur wenig kurzfristig entlang des Salinitätsgradienten bewegen. Die ermittelten stabilen Isotopenmuster von Kaulbarschen und Stinten wurden im nächsten Kapitel (**Kapitel 4**) mit räumlich und saisonal erhobenen Bakteriendaten des Kiemenschleims kombiniert. Kiemen sind ein multifunktionales Organ, das im konstanten und direkten Austausch mit dem Umgebungswasser steht und eine potenzielle Eintrittspforte für Pathogene darstellt. Die Zusammensetzung der mikrobiellen Gemeinschaft kann ein wichtiger Indikator für die Fischgesundheit sein. Bei den standorttreuen Kaulbarschen wurde die Zusammensetzung der Mikrobiota stark von den lokalen Bedingungen beeinflusst, während anadrome Stinte besonders bei der Herbstmigration eine schrittweise Anpassung der bakteriellen Zusammensetzung zeigten. Die stabilen Isotopendaten und bakteriellen Muster überlappten und gaben beide Auskünfte über die Wandermuster. In beiden Fischarten zeigte sich zudem ein stabiles Kernmikrobiom aus wenigen, stark dominanten Arten, das sich in beiden Fischarten lediglich unter Hypoxie und Eutrophierung im Flussoberlauf im Sommer reduzierte. Dabei kam es zur Übernahme von opportunistischen bakteriellen Taxa (*Acinetobacter, Shewanella, Aeromonas*).

Um die Reaktionen der Fische zu erfassen, haben wir im weiteren Prozess 340 gewebespezifische Transkriptomdatensätze von Lebern und Kiemen von Stinten und Kaulbarschen mit den bakteriellen Daten, Isotopen, Fischabundanzen, physiologischen und abiotischen Daten integriert und interaktive Effekte dominanter Stressoren großskalig untersucht (**Kapitel 5**). In beiden Fischarten ging der Anstieg der Wassertemperatur im Frühjahr mit dem Auftreten opportunistischer Flavobacteriaceae und einem starken Anstieg gewebeübergreifender adaptiver und angeborener Immunantworten einher. Fortgesetzte Hypoxie im Sommer im Oberlauf hingegen zeigte das Ansteigen opportunistischer *Acinetobacter*-Taxa zusammen mit zellulären Stressantworten (Regulierung des Zellzyklus und des Zelltods). Besonders in anadromen Stinten zeigten die generalized additive models ein paralleles Abfallen von Immunantworten, was auf eine durch Energieumverteilung bedingte Immunsuppression hindeutet und die Tiere anfälliger für Infektionen machen könnte. Populationsgenetische Analysen beider Arten ergaben eine hohe Konnektivität der Populationen. Jedoch zeigten sich funktionelle Diversität und Anzeichen für Selektion in Genen, die wichtig für die Anpassung an hohe Temperaturen und Hypoxie rund um den HIF-Transkriptionsfaktor und verwandte Signalwege sowie Immun-Anpassungen sind.

Abschließend untersuchten wir anhand einer 40-jährigen Zeitreihe Veränderungen in den Abundanzen und Zusammensetzungen der Lebenszyklusgilden der Fische im Elbe-Ästuar (**Kapitel 6**). Generell kam es zu einer Verbesserung des Habitats mit steigender Wasserqualität im Laufe der 1990er Jahre, seit 2010 sind die Fänge aber wieder rückläufig und die gesamte Fischdichte ist in der letzten Dekade um 92 % gesunken. Als Treiber wurden wieder auftretende Hypoxie-Situationen zusammen mit hohen Konzentrationen an suspendierten Partikeln sowie eine Reduzierung von Jungfischhabitaten identifiziert.

Diese Arbeit ermöglichte zum ersten Mal räumlich-zeitliche Einblicke in die molekularen Stressantworten von dominanten Fischarten im Zusammenspiel mit wirtsassoziierten Bakterien als Abschätzung der Holobionten-Reaktion in einem Ästuarsystem (**Kapitel 7**). Es wurden verschiedene Methoden zur Integration von Omics-Methoden evaluiert. Ein minimal-invasives, nicht-letales Monitoring von fischassoziierten Bakterien, gestützt durch punktuelle Untersuchungen molekularer Stressantworten im Wirtsorganismus, könnte dabei eine kosteneffiziente Erweiterung aktueller Langzeit-Monitoringmethoden darstellen. Die aktuellen Ergebnisse können bereits genutzt werden, um Bestrebungen zur Renaturierung des Ökosystems zu leiten. Die Daten zeigen dabei Handlungsbedarf, da der Rückgang der Fischabundanzen erwartungsgemäß mit einem genetischen Flaschenhalseffekt einhergehen wird, der die genetische Diversität reduziert und die Populationen besonders im Hinblick auf zunehmende Stressoren durch den fortschreitenden Klimawandel (z. B. Hypoxie, Temperatur) ihrer Anpassungsfähigkeit beraubt. Je länger der Populationseinbruch andauert, desto stärker können sich diese Effekte aufgrund von genetischer Drift manifestieren.

Chapter 1 General Introduction

1.1. Estuaries

As transition ecosystems between marine and freshwater, estuaries represent complex mosaic environments (Lauchlan & Nagelkerken, 2020) that experience high spatiotemporal variation in physico-chemical conditions (i.a. salinity, turbidity, temperature, dissolved oxygen, habitat availability) (Eagleson & Ippen, 1966; M Elliott, 2006). Yet, due to year-round primary biomass production (phytoplankton, benthic algae, marsh grass), continuous mixing of the water column by tides, high nutrient input and recycling (due to microorganisms, filter feeders) they are prominent for belonging to the most biologically productive ecosystems on the planet (Day et al., 2013). Estuarine systems provide a wide range of ecosystem services as waterways (Wetzel et al., 2013), carbon storage (Ricart et al., 2020), and serve as nurseries and population sources for fish via recruitment and replenishment, making them crucial for maintaining the diversity and productivity of large marine ecosystems. Thus, changes in these systems have major impacts on adjacent ecosystems, making estuaries priorities for resource management (Baptista et al., 2014; Beck et al., 2003; Dahlgren et al., 2006; Gillanders, 2002; Gillanders et al., 2003; Seitz et al., 2014; Vasconcelos et al., 2011; Vasconcelos et al., 2010).

1.1.1. Fishes in estuaries

Due to interaction with adjacent biomes, fish assemblages in estuaries result from marine, estuarine, diadromous and freshwater species (Potter et al., 2015). They generally comprise few dominant stress-tolerant and well-adapted species associated with r-selected (small and short-lived; r-K- selection model (MacArthur & Wilson, 1967) or opportunistic traits (short generation time, high reproductive effort, low batch fecundity, low investment per offspring (W&R model (Winemiller & Rose, 1992)) (King & McFarlane, 2003; Pianka, 1970). The environmental conditions in estuaries should promote low functional diversity due to convergence of life-history traits and feeding habits related to primary source of food production (Baptista et al., 2014; Elliott & Quintino, 2007; Teichert et al., 2017). However, spatial heterogeneity in local conditions along estuaries determines species distributions through environmental filtering (Attrill, 2002; Mouillot et al., 2007; Whitfield et al., 2012) resulting in a high taxonomic turnover supporting overall diversity by species changes along estuarine gradients (Basset et al., 2013; Teichert, Lepage, Chevillot, et al., 2018). The entrance width and annual discharge influence overall biodiversity with an increase in tidal range enhancing hydrodynamics, reducing eutrophication and hypoxia increasing functional diversity, while the opposite effect occurs with increasing turbidity (Nicolas et al., 2010; Pasquaud et al., 2015; Teichert et al., 2017).

Classification of estuarine fish into life cycle guilds based on their migration patterns creates an understanding of habitat exploitation at different life stages and shows the spatio-temporal distribution of fish within estuaries (Elliott & Quintino, 2007). In 47 estuaries of the Northeast Atlantic, 148 species have been described from various types of life history strategies of which 31% belong to the marine straggler guild (sporadic in estuaries, stenohaline, restricted to lower reaches (Potter et al., 2015)), 20% are marine migrants, 8% are diadromous, 27% belong to the estuarine resident species guild, and 15% belong to the freshwater species guild (Teichert, Pasquaud, et al., 2017). Estuarine species were shown to be mainly distributed along opportunistic-periodic gradients of life history strategies (W&R-model) (Teichert, Pasquaud, et al., 2017). Estuarine resident guilds reveal mixed opportunistic-equilibrium attributes (small body size, short generation time, low fecundity but high spawning frequency and parental care (Pérez-Ruzafa, Marcos, Pérez-Ruzafa, & Pérez-Marcos, 2013; Vila-Gispert, Moreno-Amich, & García-Berthou, 2002), i.a. Gobiidae, Lotidae, Callionymidae) (Teichert, Pasquaud, et al., 2017). Multi spawning increases the likelihood of progeny encountering favourable conditions (Franco et al., 2008; Pérez-Ruzafa et al., 2013), r-selected traits suiting productive but unpredictable habitats (Winemiller, 2005). Equilibrium species (long-generation time, low fecundity, i.a. Rajidae, Topedinidae) were shown to be restricted to euryhaline open water. Periodic species (large

size, later maturity, long life span, high fecundity (Pérez-Ruzafa et al., 2013) i.a. catadromous *Anguilla anguilla* & marine migratory Sparidae, Gadidae, Clupeidae) are characterized by migration habits depending on life-stage requirements taking advantage of seasonal productivity i.a. as nurseries for juveniles (Maes, Taillieu, Van Damme, Cottenie, & Ollevier, 1998; Teichert, Pasquaud, et al., 2017; Winemiller & Rose, 1992).

1.1.2. Climate change and immediate human impact on fishes in estuaries

Structure and functioning of coastal and estuarine environments have dramatically changed over the last century due to human interference (over-exploitation, habitat fragmentation, chemical pollution) and species invasion (Lotze et al., 2006). Due to the input of nutrients, hypoxia has become a widespread phenomenon (Le Moal et al., 2019) and hypoxic dead zones have doubled every decade since the 1950s (Diaz & Rosenberg, 2008). Depth limitation also gives these ecosystems little inertia to temperature fluctuations (Dulvy et al., 2008; Rutterford et al., 2015). The cumulative effects are expected to magnify due to climate change-driven shifts in temperature, sea levels, precipitation, wind patterns (Baptista et al., 2014; Crain, Kroeker, & Halpern, 2008; Halpern et al., 2008), associated with a reduction in biomass and biodiversity of inhabiting fauna that potentially effecting overall ecosystem functioning (Altieri & Gedan, 2015; Diaz & Rosenberg, 2008; Statham, 2012). However, only 0.1% of total climate change related research is directed towards *in situ* observations in estuaries. Long-term studies on climate change impact on estuaries that support modelling approaches and the development of mitigation plans, are largely missing to this date (Biguino et al., 2023).

Results from 30 French estuaries indicate associations between local human disturbances and decrease in overall fish abundance, species richness and reduced functional redundancy (Teichert, Lepage, & Lobry, 2018). In such cases, generalists that are better adapted to disturbed environments replace specialists with unique functional roles in such cases (Clavel et al., 2011). Over the last three decades, 80% of the overall fish abundance vanished within the coastal and estuarine nursery habitats of the east English Channel in the bay of Somme. Fish bearing r-selected functional traits (i.a *Pleuronectes platessa, Solea solea, Sprattus sprattus, Clupea harengus*), generally favoured in theses habitats, appear highly impacted by warming leading to a regime shift towards K-selected functional traits (*Dicentrarchus labrax, Scophthalmus maximus*) (McLean et al., 2019). The latter appear often less responsive to climate change (Lynam et al., 2017; Simpson et al., 2011), which might however only appear with a substantial time lag due to longer lifespans (McLean et al., 2019). High environmental responsiveness makes r-selected species vulnerable to warming in the short term (Perry et al., 2005; Schweiger et al., 2008; Simpson et al., 2011). Habitats rendered unfavourable may be shifted towards more suitable ones, especially shifting poleward (Fossheim et al., 2015; McLean et al., 2018; Montero-Serra et al., 2015). In the long-term, however, a high capacity for evolutionary adaptation is expected given rapid generation times (Rijnsdorp et al., 2009).

Summarizing the current status, climate warming and immediate human impact profoundly alter the functional structure in estuarine and marine nurseries and thereby the stability of large marine ecosystems. Lower-latitude ecosystems dominated by r-strategists were shown to decline in cold-water species (Auber et al., 2017), loose overall fish abundance, species richness and functional redundancy (Teichert, Lepage, & Lobry, 2018) and shift towards alternative functional groups with higher trophic level (McLean et al., 2019; Pecuchet et al., 2017). Poleward ecosystems are experiencing an increase in r-selected species (McLean et al., 2018; Nicolas et al., 2011). Ecological mismatches between nekton and critical resources at important time points within the life cycles of estuarine fish (Lauchlan & Nagelkerken, 2020) like a temporal mismatch between primary production and food availability for larval fishes (Houde, 2016) or altered timing of preferred conditions for migration between marine and freshwater environments (Davis et al., 2014) are expected. Reorganizations of fish functional structure are likely to go along with changes in nutrient cycling, energy transfer and

biomass turnover having major implications for ecosystem functioning and services provided (Blanchard et al., 2011; Houk et al., 2018; Rooney et al., 2006).

1.1.3. The Elbe estuary

As one of the largest European estuaries, the tidal Elbe belongs to the coastal-plain estuaries characterized by partially mixing with strong tidal action and absence of a marked halocline (Elliott & Hemingway, 2008; Thiel & Potter, 2001). The tidal Elbe is strongly anthropogenically impacted by channelization, inlet of sewage, water supply and abstraction of power plant coolant influencing tidal range, current velocity and reducing sub- and intertidal shallow water areas (Kerner, 2007; Thiel et al., 2003). Between Hamburg and Cuxhaven, 61 fish species were classified in 2010, composed of 40% marine life cycle category (Potter et al., 2015), 40% freshwater, 13% anadromous, and 5% estuarine species (Eick & Thiel, 2014). However marine species contributed only 0.4% to fish abundance, 1,4% were freshwater, < 0.1% estuarine while 98% belong to the anadromous life cycle category with European smelt *Osmerus eperlanus* L. accounting for 96% alone. Lower reaches characterized by high salinity changes show higher species diversity. In upstream direction the percentage of marine species and total abundance decline (Eick & Thiel, 2014; Thiel & Potter, 2001). Freshwater species increase from meso- to oligohaline areas with *Sander lucioperca* L. (< 1% abundance) and *Gymnocephalus cernua* L. (**GC**) (1%) being most abundant. Anadromous (i.a. *O. eplerlanus* (**OE**), *G. aculeatus*), freshwater (i.a. *S. lucioperca* (**SL**)*, G. cernua*) and marine estuarine-opportunist (i.a. *Platichthys flesus* (**PF**)) ecological guilds build up a major part of species abundance throughout the whole tidal Elbe estuary while marine stragglers seem not to pass the mesohaline boundary (Eick & Thiel, 2014) (**Fig. 1.1**).

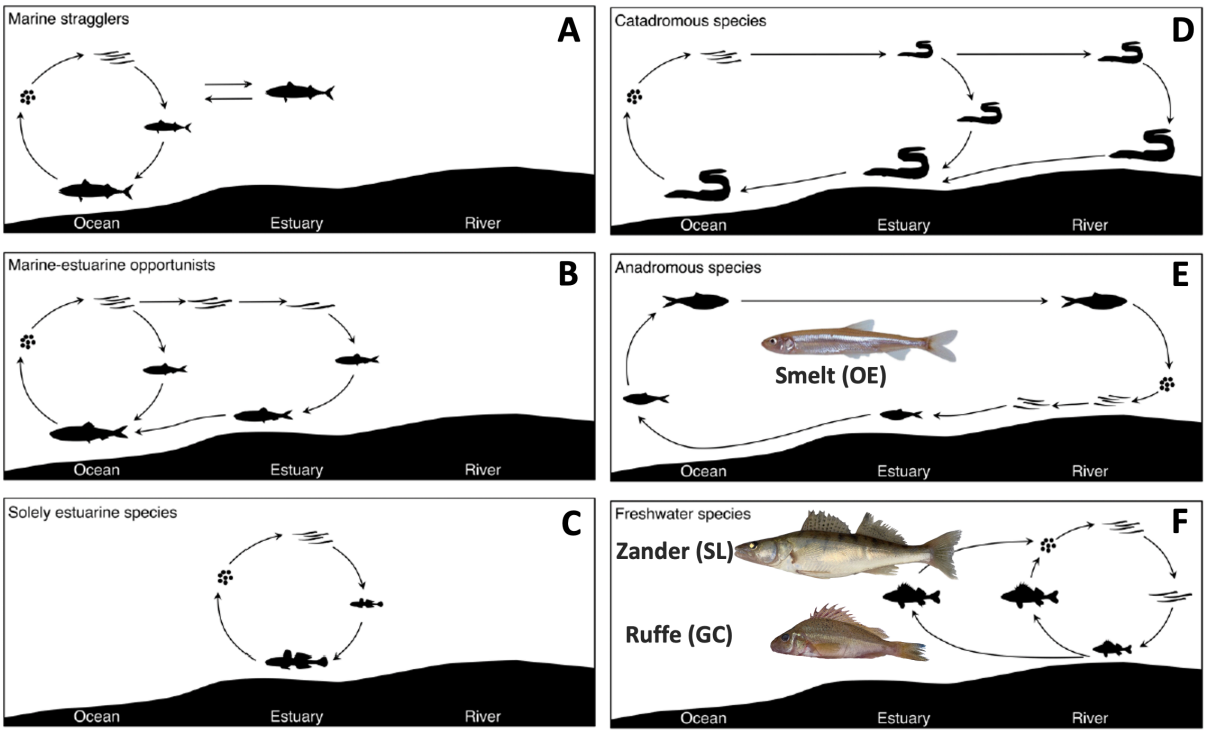


Fig. 1.1 Dominating life-history guilds in the Elbe estuarine system. Shown are migration patterns and habitat usage of the six dominant life-history guilds in the Elbe estuary and the integration of the three target species in this thesis modified from (Hauten, 2024) based on (Thiel, 2011; Whitfield et al., 2022). Marine stragglers (A): marine fish species that occur irregularly and in low densities in the river mouth with highest salinities; Marine-estuarine-opportunists (B) (e.g., herring *Clupea harengus*): marine species more frequently occurring in higher densities, especially in the juvenile life stage; Solely estuarine species/estuarine residents (**C**) (e.g., common goby *Pomatoschistus microps*); Catadromous species (**D**) (e.g., European eel *Anguilla anguilla*) reproduce in the sea, migrate to rivers and estuaries until sexual maturity. Anadromous species (**E**) (e.g.,

smelt): predominantly in the ocean and migrate to limnic waters to spawn; Freshwater species/freshwater migrants (**F**) (e.g., zander & ruffe): usually restricted to limnic habitats, but also occur in higher densities at higher salinities.

Cyclical annual and interannual changes in the species composition are described for the tidal Elbe estuary, attributable to variation in sequential migration of marine opportunist and diadromous species and movements of freshwater species during winter and spring (Eick & Thiel, 2014; Thiel & Potter, 2001). Seasonal fish abundance is strongly affected by juvenile life stages whose abundance is controlled by seasonal water temperatures (Thiel et al., 1995) that can cause high mortality at very low temperatures in winter (Costa et al., 2007). Oxygen concentration levels were shown to affect fish distribution in the tidal Elbe (Eick & Thiel, 2014), i.e. smelt biomass is correlated with reduced dissolved oxygen content in the estuary (Thiel et al., 1995). Dissolved oxygen levels below 7.5 mg/l coupled with temperatures higher than 15 °C may act as barriers for fish movement in estuaries (Pomfret et al., 1991). Water temperature and top water drain are known to influence spawning activity in species like smelt (Köhler, 1981; Lillelund, 1961). Increased turbidity is associated with enhanced mortality by bonding and damaging gill structures of larval till adult fish (Costa et al., 2007; Costello et al., 2007; Hess et al., 2015; Maitland, 2000), reducing egg survival (Selleslagh et al., 2009) and impacting predation success (Liljendahl-Nurminen et al., 2008).

Since 2011 a reduction in smelt biomass has been observed attributed to combined effects of anthropogenically induced reduction of shallow reaches (i.e. in the Mühlenberger Loch), cooling water withdrawal, increased maintenance works (hopper dredger and water injection) enhanced turbidity and reappearing hypoxia situations constituting multiple stressors for the population (Scholle & Schuchardt, 2020). During the last 20 year measuring period, dissolved oxygen deficiency situations are reappearing from May to August within the oligohaline reaches from Hamburg to Grauerort with concentrations below 2 – 4 mg/L over weeks to month. A general temperature increase is not registered and hypoxia situations are not described to increase during the last years, however there is a tending decrease in oxygen levels (falling below 8 mg/l) in the seasonal time frame between March and April since 2000 (Scholle & Schuchardt, 2020). A trend towards reduced surface water drain has been observed in spring over the last two decades, which in turn led to increased maintenance work and turbidity levels at several sites since 2011, associated with a tendency for salinity increase (Scholle & Schuchardt, 2020). Numerous pollutants i.a. PBDE, PAH, HCB, TBT and mercury exceed threshold values of the Water Framework Directive in Europe in the Elbe near Hamburg. Metals, drugs, antibiotics and hormones such as estrogen and diclofenac show elevated values in the Elbe (Blondzik et al., 2017) and could have an impact on the physiology and behaviour of fish (Brodin et al., 2014; Wetzel et al., 2013).

1.2. Stress & stress response in fishes

Confronted with changes in their environment, species have a repertoire of response options through I. shift of distribution, II. acclimation or III. adaptation through genetic selection (Hoffmann & Sgró, 2011; Munday et al., 2013; Vagner et al., 2019; Williams et al., 2008). Acclimation and adaptive capacity result from phenotypic plasticity (Lande, 2009) divisible into levels of organization from molecular to social and dependent on life-stage specific capacities and requirements (Dahlke et al., 2020; Madeira et al., 2020; Vagner et al., 2019). The stress response in fish, the physiological and behavioural response triggered by environmental challenges (stressors), is a complex series of biochemical, physiological and behavioural adjustments enabling maintenance of internal homeostasis and survival under a range of conditions (Barton, 2002; Petitjean et al., 2019; Schreck & Tort, 2016; Wendelaar Bonga, 1997). To connect the overlapping concepts of stress and phenotypic plasticity, one might assume stress response as the pathway to acclimation within the phenotypic plasticity concept.

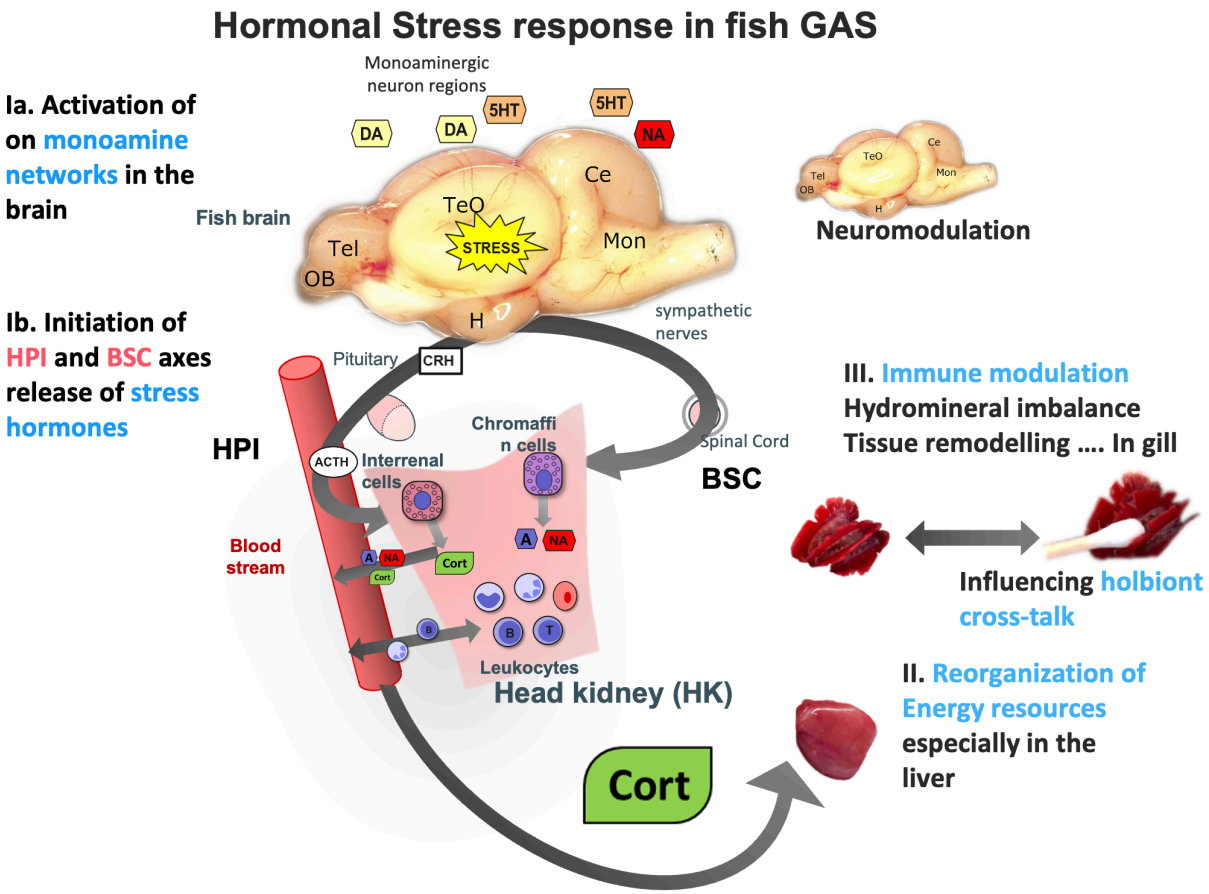


Fig. 1.2 Overview of the hormonal stress response in fish (general adaptation syndrome GAS). Primary response activated the brain-sympathetic-chromaffin (BSC) axis triggering the release of catecholamines (adrenaline A, Noradrenaline NA) from head-kidnes (HK) cells into the bloodstream. In parallel, activation of the hypothalamic–pituitary–interrenal (HPI) axis causes the release of corticotropin-releasing hormone (CRH) and adrenocorticotropic hormone (ACTH) into the blood that triggers the release of cortisol (CORT) from HK-interrenal cells into the bloodstream. Secondary responses (reorganization of energy resources, altered blood pressure, release of glucose and lactate) and tertiary responses (impairment of immune system, behavior, growth, reproduction). Modified form and (Koll et al., 2025; Martorell Ribera et al., 2020).

The energy-demanding active stress response process (allostasis) (McEwen & Wingfield, 2003) can have negative long-term consequences depending on level/duration/number of stressors (Balasch & Tort, 2019; Schreck & Tort, 2016; Schulte, 2014). Prolonged exposure or severe stressors lead to allostatic overload where the adaptive stress response fails, homeostatic regulations remain disturbed and a situation of distress arises in which pathologies and disease occur as a result of exhaustion (Gorissen & Flik, 2016; Korte et al., 2007; Madaro et al., 2015; McEwen & Wingfield, 2003; Schreck, 2010). Stress response is frequently described as hormetic (biphasic dose responses (Calabrese & Baldwin, 2002), where mild stressful experiences improve performance enhancing stress resilience through mechanism of habituation and stress hardening (Schreck, 2009; Schreck et al., 1995).

Stressors can either be perceived by involving the brain, which leads to a coordinated reaction mediated by stress hormones (Wendelaar Bonga, 1997) (**Fig. 1.2**), while others like environmental temperatures can directly induce molecular changes in cellular macromolecules or induce epigenetic changes (Feil & Fraga, 2012; Iwama et al., 1998; Kültz, 2005; Rey et al., 2016; Schulte, 2014).

At the **organismal level**, stress responses like fight-or-flight and general adaptation syndrome (Selye, 1950) are controlled by hormone release. The general stress response falls into three consecutive steps. The primary, constituting a neuroendocrine cascade (catecholamines by brain-sympathetic-chromaffin (BSC) axis (Feldman, 1985) within seconds and cortisol by hypothalamus-pituitary-interrenal (HPI) (Wendelaar Bonga, 1997) axis within minutes upon perception of a stressor

with the main function of immediate energy supply via glycogenolytic effects of adrenaline. A secondary cascade of mostly reversible (Eissa & Wang, 2016) cardiovascular, respiratory, cellular metabolic and immune responses (Barton & Iwama, 1991; Mommsen et al., 1999) with sustained hypoglycemia via cortisol that primarily targets gills, liver, intestinal epithelia and the brain (Gorissen & Flik, 2016). A tertiary level relating to performance and behavioral changes (Barton, 2002; Stehfest et al., 2017) that may result in destructive and potentially irreversible consequences with prolonged activation (Schreck & Tort, 2016) by energy reallocation away from growth, reproduction and immunity towards stress response (Huntingford et al., 2006). Complex behaviors have an important role in stress response and coping with a dynamic environment. Conscious perception of the environment, memory, learning, appraisal of aversive and non-aversive stimuli and prediction of the outcome of stressful situations have a fundamental role in allostasis (Gorissen & Flik, 2016; McEwen & Wingfield, 2003). Central brain regions in the processing of input and key regulators in dealing with stress, the mammalian limbic system (i.a. hippocampus, amygdala) and cortex (McEwen et al., 2015) have been identified in equivalent form in the teleostean telencephalon (Mueller, 2012; Mueller et al., 2011; Vargas et al., 2009) enabling fish to show higher cognitive functions (Broglio et al., 2015; Bshary & Brown, 2014).

On **cellular level** (next chapter) the expression of stress-related genes on primary, secondary and tertiary stress response levels are altered (Faught et al., 2016). These effects are well studied for short-term exposure to single stressors under artificial rearing condition in model species (Gorissen et al., 2012; Pottinger, 2010; Remen et al., 2012).

1.2.1. The cellular stress response level

Besides hormonal mediated cellular responses, cells themselves comprise an interlinked cellular stress response (CSR) which constitutes an universal, unspecific and evolutionary highly conserved defence reaction assessing and counteracting stressors potentially damaging macromolecules (Kültz, 2005; Logan & Buckley, 2015). Outside the limits of elastic cell homeostasis, the consecutive or parallel responses include **I.** prevention and repair of damage, **II.** control of the cell cycle, **III.** reallocation of energy and finally **IV.** cell death (Kültz, 2020). The CSR depends on the minimal stress proteome comprising i.a. redox regulation, DNA damage sensing an repair, macromolecular stabilization, energy metabolism and is species and tissue dependent (Colin et al., 2016).

Several main components mitigating cellular damage comprise chaperones repairing denaturated proteins and maintaining integrity of the protein pool (i.a. heat shock proteins HSPs) (D Madeira, 2012; Feder & Hofmann, 1999; Narum & Campbell, 2015) or ubiquitin targeting of irreversibly damaged protein for degradation in the ATP-dependent ubiquitin-proteasome system (UPS) (Bard et al., 2018). High protein demand or stressor mediated impact on folding activate the unfolded protein response (UPR) associated with the immediate endoplasmatic reticulum (ER)-associated degradation (ERAD) of proteins (Hetz, 2012; Hwang & Qi, 2018). All these highly interrelated mechanisms aim at mitigating proteotoxicity from aggregation of damaged proteins.

Also, reactive oxygen species (ROS) scavenging enzymes (SODs, CAT, GPXs, PRXs) and antioxidants (glutathione, ascorbic acid, tocopherol, carotenoids) (Sulmon et al., 2015), neutralizing ROS and oxidation products (e.g. lipid peroxides). These are universally generated with stress exposure as byproduct in enhanced metabolism, by the disruption of the mitochondrial electron transport chain under oxygen deficiency, during activation of the immune system to kill pathogens or by xenobiotic-induced malfunction of proteins (Birnie-Gauvin et al., 2017).

1.2.2. The interactive nature of stressors

Tolerance to stressors depends on the species, the life history of an individual and on the kind of stressor itself. Against temperature organisms usually show a bell shaped tolerance (Erofeeva, 2021)

(**Fig. 1.4 B**), while showing monotonically increasing or decreasing curves against oxygen and pollution (Vos et al., 2023). Co-working of multiple stressors may narrow the capacity for compensation (**next chapter**) and the diverse, multiple stress factors in wild habitats lead to complex interactions reaching from additive to synergistic to reversing effects (**Fig. 1.3**) (Birk et al., 2020; Colston & Jackson, 2016; Crain et al., 2008). Unexpected interactive effects might occur due to shared physiological pathways involved under multi-stress conditions (Petitjean, Jean, Côte, Larcher, et al., 2020; Petitjean et al., 2019). To stay with the last example, ROS accumulate during multiple-additive/synergistic stressor interactions. For instance, both temperature and immune challenges produce ROS increasing oxidative damage (Preynat-Seauve et al., 2003; Vinagre et al., 2012). If ROS production exceeds the defence mechanisms, macromolecules get damaged (Lushchak, 2011). High DNA damage result in genotoxicity (Birnie-Gauvin et al., 2017; Finkel & Holbrook, 2000) increasing cancer risk (Gandar, Laffaille, Marty-Gasset, et al., 2017; Hagenaars et al., 2013; Topal et al., 2014), potentially cascading from effects on the survival of the individual extending into the dynamics of populations (Anitha et al., 2000; Cheng et al., 2018; Malev et al., 2010).

A paucity of information on general physiology of fish in nature and especially on stress response to chronic and multiple stressors is described (Hoem & Tveten, 2019; Madaro et al., 2015; Schreck & Tort, 2016). Information on which is needed to understand and project direct anthropogenic or climate-related effects on fish populations (Petitjean, Jean, Côte, Larcher, et al., 2020; Petitjean et al., 2019).

Some examples from the few recent studies incorporating multiple stressor interactions within their study design give first hints on the complexity: Temperature and infection affect behaviour on opposing angles, a usual increase in activity, exploration and foraging fostered by increased metabolism in ectotherms is prevented during infection (Petitjean, Jean, Côte, Lamarins, et al., 2020). Individual exposure to temperature or parasitism can have limited effect on cellular level, but combined with metal contamination the increased bioaccumulation during higher temperatures lead to high levels of cellular damage and growth impairment (Petitjean, Jean, Côte, Larcher, et al., 2020). An additional level of complexity results from intraspecific variability, as different populations influenced by different life histories can show varying effects in stress outcomes and interaction shown in fish (Petitjean et al., 2021). Therefore, considering the conservation of species also involves understanding responses across distributions to determine influences of local environments and genetic factors, as previous exposure and genomic variation may be linked with adaptations to local conditions (Crozier & Hutchings, 2014). All these factors need to be known and understood to project trajectories for ecosystem responses to climate change or recovery from human perturbations (Vos et al., 2023).

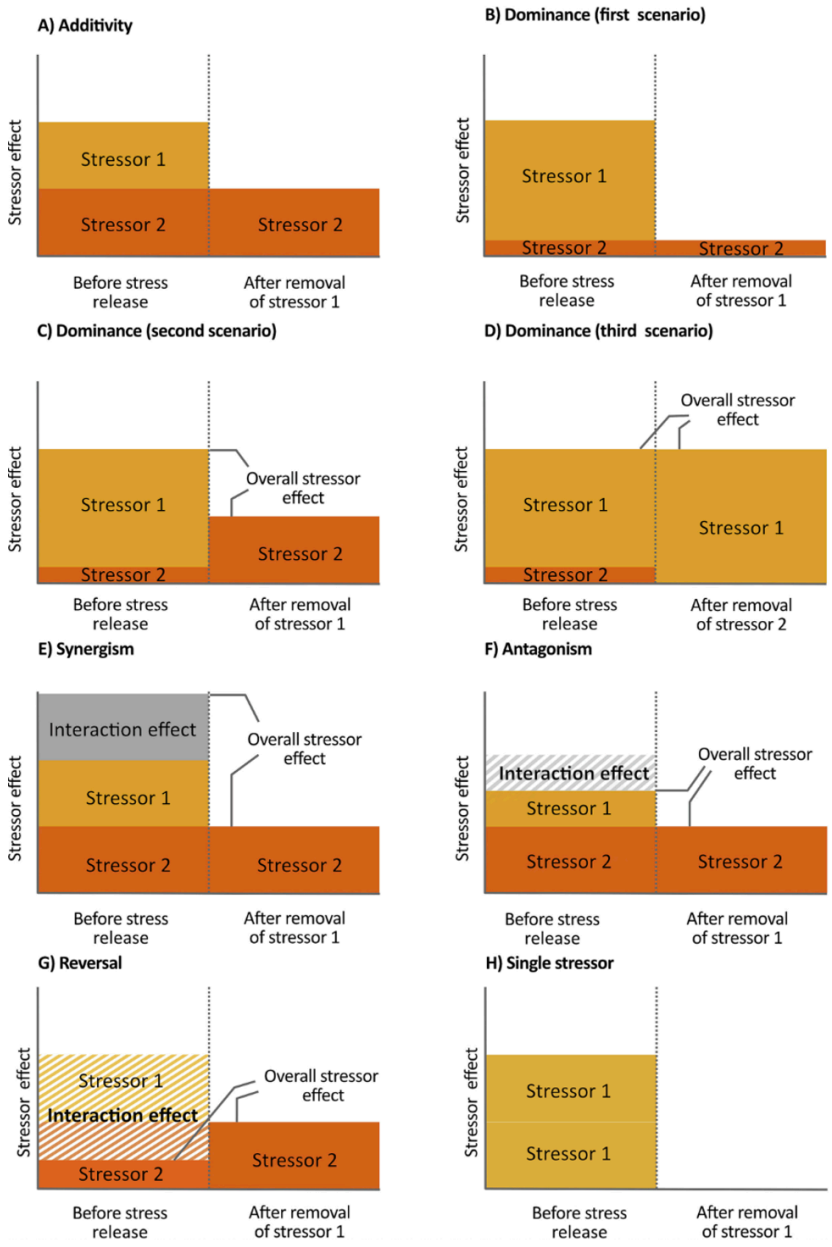


Fig. 1.3 Stressor interaction and the net effects when releasing species, populations or ecosystems from single stressor in a multi-stressor environment taken from (Vos et al., 2023).

1.2.3. Stress response strategies

With energy supply and allocation being key in successful stress coping, metabolism is central to stress response across levels of biological organization from molecular to the whole individual level (Petitjean et al., 2019). A conceptual framework based on metabolism and energy management (McEwen & Wingfield, 2010; Sokolova et al., 2012) characterizes effects of moderate vs. strong or multiple stressor exposure. According, the former leads to a compensation strategy reallocating energy from reserves and routine behaviours towards physiological defences (Bonneaud et al., 2003; Pi, Li, Zhang, & Li, 2016). In the latter, the energetic demand is expected to exceed aerobic capacity resulting in metabolic depression (Guppy & Withers, 1999). Energy is conserved, physiological defences are inhibited and cell damage is expected to result in detrimental effects for fish survival on the long run (Sokolova, 2013; Sokolova et al., 2012) eventually transmitted across generation impacting evolutionary trajectories of wild populations (Petitjean et al., 2019).

The framework is supported by several laboratory studies on *Carassius auratus* L. indicating decreased energy consumption, amino-acid metabolism and stress refence but increased cellular lesions under combined thermal and toxic stressors (Gandar, Laffaille, Canlet, et al., 2017; Gandar, Laffaille, Marty-Gasset, et al., 2017; Jacquin et al., 2019). Also multiple stressors exposure in sea bream lead to blockage of cortisol responses and increased anaerobic metabolism (Bermejo-Nogales et al., 2014). Studies from wild fish in support are however scarce.

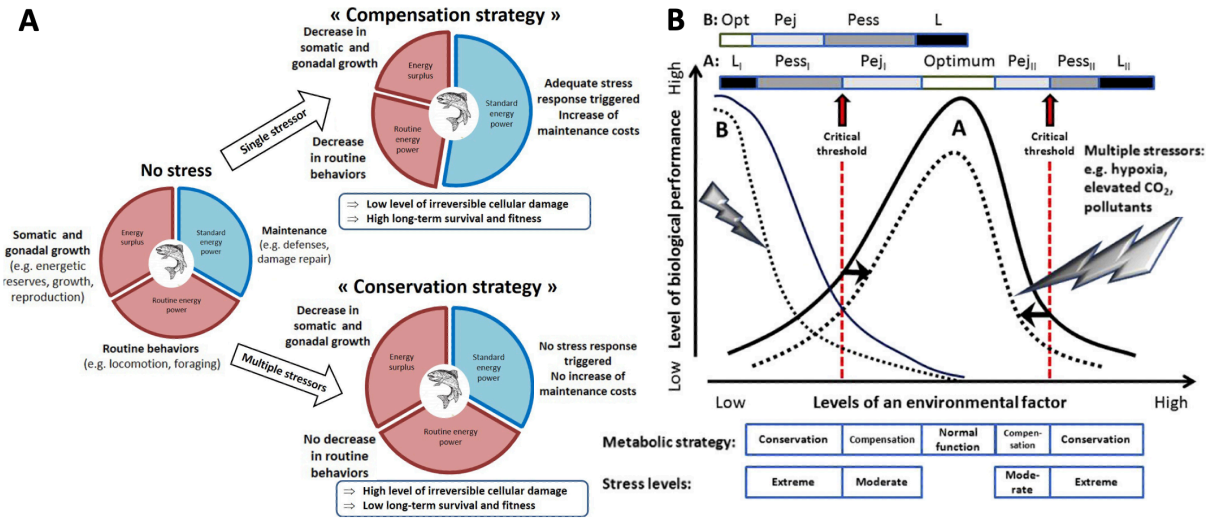


Fig. 1.4. Organismal stress response strategies. A schematic hypothesis how fish deal with single and multiple stressors (A) modified from (Petitjean et al., 2019) indicating an increase in energy investment under single stressor compensation strategy opposed to no changes in energy reallocation but deleterious cellular damage under multiple stressors. This is based on framework for assessing limits of environmental stress tolerance founded in energy homeostasis in invertebrates (B) modified from (Sokolova et al., 2012). Solid lines indicate the biological performance proportional to the aerobic scope in reaction to a single environmental factor (Temperature (A), or pollutant (B) between lethal to optimum range. At the pessimism a metabolic switch is expected from compensation to conservation. The capacity is lowered under the influence of multiple stressors (hypoxia, pollution) (dotted lines).

1.2.4. The holobiont concept

Rather recently the ubiquitous nature of host-associated microbes and their role in ecology, evolution and adaptation to environmental change has led to a spread and integration of the holobiont concept into scientific fields (Matthews, 2024). The stress response described above is not restricted to the host, rather the interaction between host and microbiota and their response to environmental drivers is unified in one entity: The holobiont (Bordenstein & Theis, 2015). While holobiont research is still in its infancy, and there is relatively little insight in the understanding of which microbiota are required to ensure viability of host populations in wild animals and plants, effective conservation of macro-organisms requires the consideration of how to also conserve associated bacteria, viruses and fungi (Carthey et al., 2020; Limborg et al., 2024).

In fish, from the moment of hatching, diverse microbial communities establish themselves on all mucosal surfaces (Legrand et al., 2020). During osmotic fluctuation in the environment for example, iono regulatory processes were recently shown to be in part modulated by the nature of the microbial biofilms lining mucosal tissue (Sylvain et al., 2023). Environmental changes, such as variations in water quality, were shown to cause compositional imbalances that might convert harmless commensals into harmful pathogens (**next chapter**) (Kelly & Salinas, 2017).

1.2.5. Immune system in fish

The immune system in fish is relatively similar organized to other vertebrates splitting into innate and adaptive components. The first of which acts as first defence line to repel, detects and fight infections un-specifically via physical barriers, cellular factors and humoral components (Riera Romo et al., 2016). The adaptive immune components instead allow for a highly discriminating and long lasting immune response orchestrated by T- and B-cells creating cell mediated immunity and immunoglobin production, respectively (Mashoof & Criscitiello, 2016).

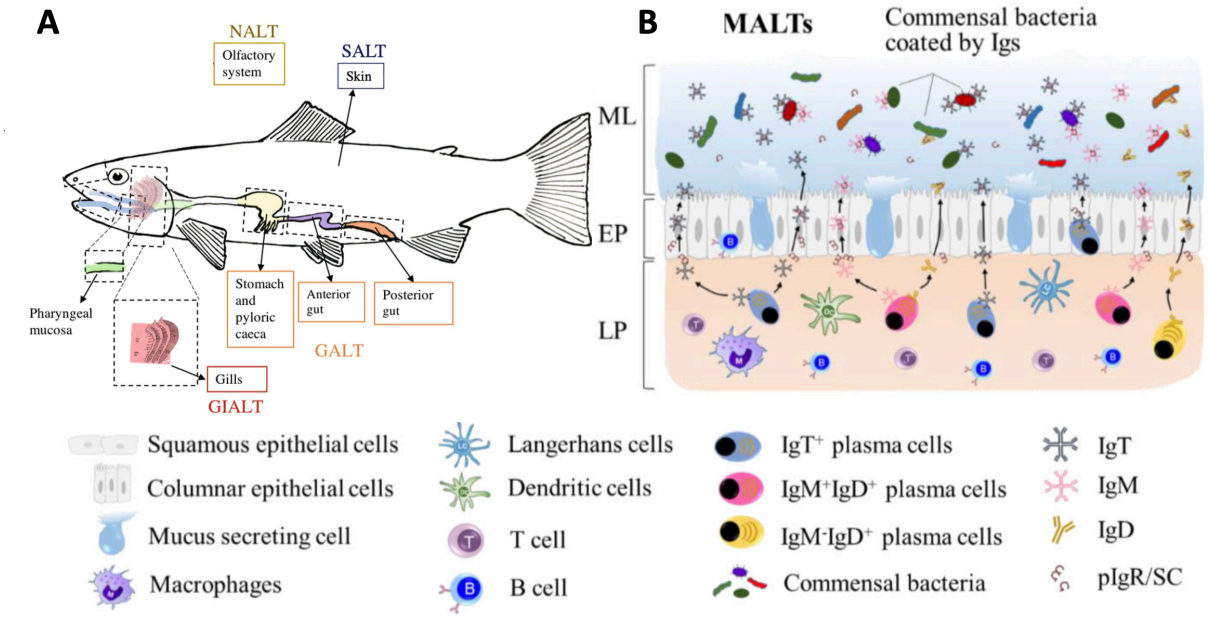


Fig. 1.5. Mucosal-associated lymphoid tissues in fish (MALT). Fish owe various MALTs, especially important in constant exchange with the environment are the gill- (GIALT) and skin- (SALT) associated lymphoid tissues (**A**) modified from (Salinas et al., 2022). The cellular structure, position of immune cells in the mucus layer (ML), epidermis (EP) and lamina propria (LP) as well as the cross-talk between host and microbiota at the mucosal surfaces like the GIALT are shown on the right side (B) modified from (Yu et al., 2020).

With the lack of lymph nodes and bone marrow, lymphatic tissues around the kidney/head-kidney and the mucosal-associated lymphoid tissues (MALT) have special importance in fish (Ángeles Esteban, 2012; Mokhtar et al., 2023). MALTs are extremely complex tissues that are in case of the gill- (GIALT) and skin- (SALT) associated lymphoid tissues in direct and constant exchange with the surrounding environment while keeping homeostasis in the mucosa via combined functioning of innate and adaptive immune cells (**Fig.1.5 A**) (Salinas, 2015).

The first line is a mucus layer mainly composed of mucins responsible for pathogen entrapment, physical protection and signalling created by secretory goblet cells (GCs) (Cone, 2009). The matrix contains a variety of innate humoral substances inhibiting microorganisms growth or leading to neutralization (lysozyme, proteases, lectins) (Magnadóttir, 2006) as well as adaptive secretory immunoglobins (Igs) (Xu et al., 2016). The epidermis and lamina propria beneath are populated by innate immune cells (macrophages, neutrophils, eosinophilic granule cells, natural killer cells and dendritic cells) and lymphocytes (Ángeles Esteban, 2012; Rességuier et al., 2020). High densities of lymphocyte populations comprising cytotoxic CD8+ and CD4+ T helper cells (Th) & B cells (Nakanishi et al., 2015) that are associated with dominant IgM and IgD and IgT/T Ig isotypes (Smith et al., 2019) have been identified in the GIALT. Igs are the main constituents against pathogens in fish (Uribe et al., 2011) and enable an efficient cross-talk between the host and its microbiome (Yu et al., 2021).

1.2.6. Bacteria and the fish immune system

B and T cells discriminate between beneficial bacteria and opportunists. One mechanism involved is the coating of commensal bacteria on the mucosal surfaces in Igs like IgT isotypes (Xu et al., 2013; Zhang et al., 2010). Depletion of these was shown associated with increase in bacterial abundances in the gill surface and translocation of commensal taxa indicating various functions in keeping microbiota homeostasis (Xu et al., 2020) (**Fig.1.5 B**).

Such immune functions are however energetically costly and highly affected by energy reallocation during stress response in fish (Tort, 2011). Numerous studies, especially in the realm of aquaculture, have shown weakened immune responses and increased pathogen proliferation under conditions of low oxygen levels (Abdel-Tawwab et al., 2019; Boutin et al., 2013; Leeuwis et al., 2024) and increased temperature (Ghosh et al., 2022; Matanza & Osorio, 2018; Morshed & Lee, 2023; Yang et al., 2022). Similarly, the presence of suspended sediment has been found to cause microbial shifts favouring pathogenic communities (Hess et al., 2015) similar to sublethal nitrite exposures (Ortiz et al., 2023; Yu et al., 2021).

This environmental sensitivity highlights the external mucus composition as a biomarker for fish health (Legrand et al., 2018; Sehnal et al., 2021; Sylvain et al., 2020). The fish microbiome may be built by a stable community of core taxa of low number but shared over a large range of conditions and a variable community strongly influenced by environmental conditions (Sehnal et al., 2021). Investigating changes in the core microbiome may deliver indication for deep reorganization and dysbiosis (Carding et al., 2015), while analysis of variable taxa might deliver potential indicators for specific or multiple stressors (Sehnal et al., 2021).

1.3. Molecular tools in wild fish studies and monitoring

Omics refers to the comprehensive analysis and measurement of complete sets of biological molecules, exploring how these molecules contribute to the structure, function, and dynamics of an organism or a group of organisms (Yamada et al., 2021). These tools are increasingly considered in the context of biomonitoring and conservation biology, as they enable a deep understanding of processes at all levels of biological organization and of previously overlooked interactions (Connon et al., 2018; Miranda et al., 2023; Oomen & Hutchings, 2017). As explained above co-working of multiple stressors is expected to lead to unforeseen synergistic or antagonistic effects in organisms (Petitjean, Jean, Côte, Lamarins, et al., 2020; Petitjean et al., 2019), sublethal impacts of which can contribute to population declines over generations (Connon et al., 2018). Molecular adjustments precede high level responses thereby probably allowing earlier detection of adverse events or situations (Bahamonde et al., 2016). Hormonal controlled similar to unspecific cellular stress responses are based on the reprogramming of gene expression in all consecutive steps (Faught et al., 2016). Real-world transcript-omics and proteomics studies indicate molecular mechanisms used by fish to cope with adverse effects of a multi-stress environment and potentially allow for disentangling individual stressors or interactive effects. Reversely they inform about the underlying environmental conditions themselves allowing for the identification of unexpected stressors as shown in recent studies on range expansion in invasive species (Jeffrey et al., 2019), xenobiotic response in estuarine flounder (Laurent et al., 2023), or in anthropogenically salinized riverine populations (Escobar-Sierra et al., 2024). Previous studies by our working group on wild caught Elbe ruffe and flounder indicated that gills are a pronounced side of cellular stress responses to hypoxia. The comparison between laboratory experiments mimicking the real world to immediately wild caught animals further showed that responses in a set of marker genes (oxygen carrying genes and apoptosis markers) were stronger regulated in the wild (Tiedke et al., 2014). This indicates additive or synergistic interaction of multiple stressors in the habitat that were not covered under laboratory conditions.

The energetic investments and physiological adaptations required to achieve a homeostatic or new allostatic state (Sterling, 1988) represent a measurable quantity that increases with each stress response, thereby limiting the capacity for further stress responses (Balasch & Tort, 2019; Tort, 2011). By examining metabolic processes and different components of the stress response, it is possible to gain insights into the stress load and transition between response strategies, helping to determine the limits of an organism and identify conditions or habitats that can support a population (Sokolova et al., 2012).

1.3.1. Population differentiation & signatures of selection

As explained above, population structure and history of stress exposure can largely affect the tolerance and outcome of a stress response. Genetic diversity at functional gene loci might be crucial for the adaptive potential of wild populations. Low levels of immune gene diversity could lead to increased susceptibility to pathogens in a population (Magid et al., 2022; Spielman et al., 2004) or decreased capacity to adapt to described stressors like hypoxia (Xiao & Wang, 2024). Omics tools allow for the determination of population structure and nucleotide variability for example via the determination of single nucleotide polymorphisms (SNPs) from genomic or transcriptomic sequencing (Bugg et al., 2023; Jeffries et al., 2019; Thorstensen et al., 2020).

1.4. Objectives

This doctoral thesis aimed at gaining comprehensive insides on estuarine wild fish stress and holobiont responses over spatial and seasonal scale. To this end a framework for large-scale integration of wild fish monitoring data over different levels of biological organization of the host organism via holistic omics tools and physiological end-point measurements matched with bacterial composition data was developed (**Chapter 2**). Next, we put a spotlight on the estuarine food web position of the main fish species via stable isotope analysis to gain insights on feeding habits, habitat exploitation and especially migration patterns (**Chapter 3**). To fill a gap in the literature, we aimed at determining seasonal fluctuations in bacterial composition on external fish mucus communities along the estuarine salinity gradient that correspond to fish migration patterns identified by stable isotope analysis, and at establishing potential bacterial biomarkers for fish movement and health monitoring (**Chapter 4**). To further gain insight into the holobiont responses, we created large-scale tissue-specific transcriptomic datasets matching the bacterial data from the most abundant fish species in the study habitat (**Chapter 5**). These were further used to evaluate the stress-response strategies applied by estuarine wild fish from different life-history guilds under prevailing conditions, model cellular stress and immune responses in reaction to dominant abiotic and bacterial stressors and finally to determine population structure, genetic variability and local adaptations. Finally, we analysed the changes in fish community and abundance over the last 40 years within the Elbe estuarine habitat to evaluate recent alterations and inform protective strategies from a continuous fish monitoring (**Chapter 6**).

1.5. References: General Introduction

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Chapter 2 Network-based omics integration

Network-based integration of omics, physiological and environmental data in real-world Elbe estuarine Zander

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Network-based integration of omics, physiological and environmental data in real-world Elbe estuarine Zander

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Abbreviations:

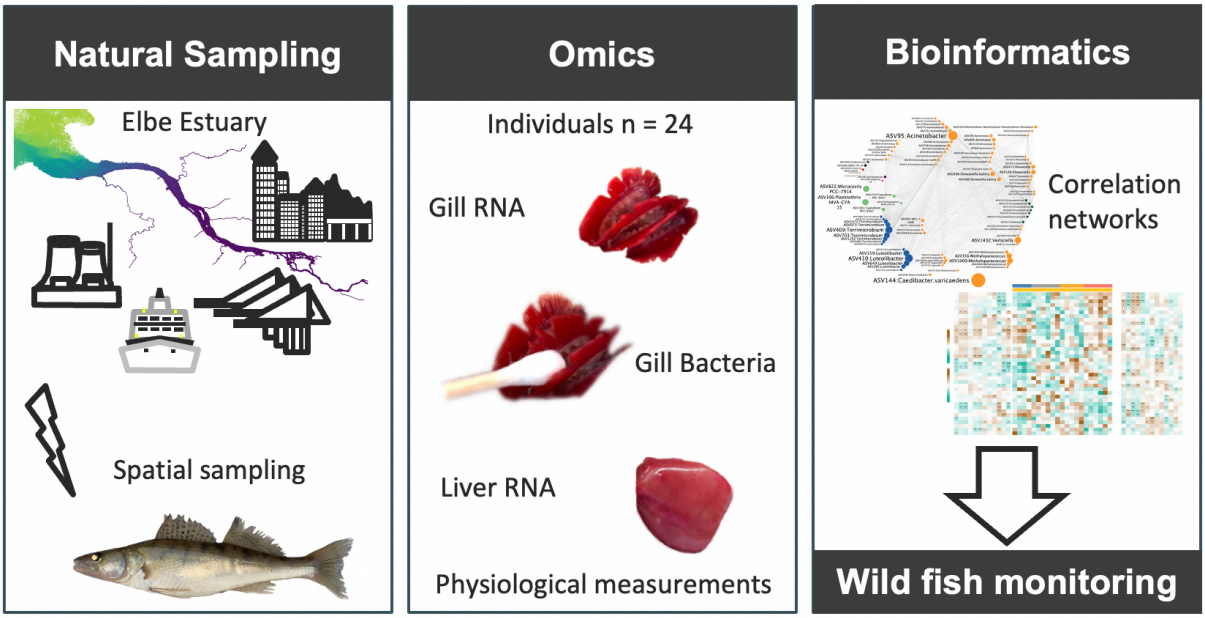
DEG differentially expressed genes,
DEA differential expression analysis,
SSU Small subunit rRNA,
WGCNA Weighted gene correlation network analysis,
DO dissolved oxygen,
ER endoplasmatic reticulum,
TCA tricarboxylic acid cycle,
TPM million mapped reads
FDR false discovery rate

1. Abstract

Coastal and estuarine environments are under endogenic and exogenic pressures jeopardizing survival and diversity of inhabiting biota. Information of possible synergistic effects of multiple (a)biotic stressors and holobiont interaction are largely missing in estuaries like the Elbe but are of importance to estimate unforeseen effects on animals' physiology. Here, we seek to leverage host-transcriptional RNA-seq and gill mucus microbial 16S rRNA metabarcoding data coupled with physiological and abiotic measurements in a network analysis approach to decipher the impact of multiple stressors on the health of juvenile *Sander lucioperca* along one of the largest European estuaries. We find mesohaline areas characterized by gill tissue specific transcriptional responses matching osmosensing and tissue remodeling. Liver transcriptomes instead emphasized that zander from highly turbid areas were undergoing starvation which was supported by compromised body condition. Potential pathogenic bacteria, including *Shewanella*, *Acinetobacter*, *Aeromonas* and *Chryseobacterium*, dominated the gill microbiome along the freshwater transition and oxygen minimum zone. Their occurrence coincided with a strong adaptive and innate transcriptional immune response in host gill and enhanced energy demand in liver tissue supporting their potential pathogenicity. Taken together, we show physiological

responses of a fish species and its microbiome to abiotic factors whose impact are expected to increase with consequences of climate change. We further present a method for the close-meshed detection of the main stressors and bacterial species with disease potential in a highly productive ecosystem.

2. *Graphical abstract*



3. *Introduction*

Coastal and estuarine environments have undergone significant changes in the past century due to overexploitation, habitat fragmentation, pollution, and species invasion (Lotze et al., 2006). Estuaries, as transitional ecosystems, exhibit intense variations in physicochemical conditions such as hydrology, salinity, turbidity, temperature, oxygen levels and habitat availability (Elliott & Quintino, 2007). Global change is expected to increase pressures on fish communities in estuaries, with a particular focus on the increasing frequency and intensity of oxygen minimum zones (OMZ), which have critical impact on fish physiology and distribution (Breitburg et al., 2018; Cottingham et al., 2018; Diaz & Rosenberg, 2008; Lauchlan & Nagelkerken, 2020; Sampaio et al., 2021).

Adding to this, the tidal Elbe as one of Europe's largest estuaries faces substantial pressure from centuries of construction projects including diking and improvement of access to the Hamburg harbor. Deepening and maintenance dredging of navigation channels resulted in the relocation of ca. 23 million m^3 sediment in 2021 (WSV.de, 2023) of which a proportion was resuspended into the water column (Kappenberg & Grabemann, 2001). Low dissolved oxygen levels, including instances of hypoxia (< 2mg/L) in upstream areas, have been reported since the 1980s (Schöl et al., 2015). During summer, the otherwise well-mixed estuary (Middelburg & Herman, 2007) experiences stratification, particularly in the port area and deepened navigation channel. Under these conditions it is common to observe oxygen depletion and ammonium accumulation in the hypolimnic waters (Pein et al., 2021). Limited efforts have been made to address these issues.

Fish respond to changes in their environment through various mechanisms, including behavioral, morphological, physiological, and molecular adaptations, with severe stress ultimately leading to mortality (Richards, 2009). Avoidance reactions in fish (Roberts et al., 2009; Thiel et al., 1995) can be hindered by factors such as oxygen minimum zones, which restrict access to deeper, oxygen-rich areas (Diaz & Rosenberg, 2008). The first timeseries studies on one anadromous fish species (*Osmerus eperlanus*) in the Elbe estuary have revealed massive declines in abundance over the last

decade attributed to multiple stressors comprising habitat loss, cooling water extraction, dredging, increased turbidity and recurring hypoxia (Scholle & Schuchardt, 2020).

In this study we use juvenile zander *Sander lucioperca* (Linnaeus, 1758) (0+ cohort) sampled in late summer 2021 from the Elbe estuary as a model organism to explore gene expression patterns and holobiont interaction linked to biometric parameters indicative for physiological condition in response to steep abiotic gradients. The zander is among the most abundant fish in the ecosystem and among the top predatory species in the Elbe exerting a strong top-down control on other species (Eick & Thiel, 2014). In contrast to their embryonic stages, which require a salinity of less than 7 psu for their development (Klinkhardt & Winkler, 1989), juvenile and adult zander tolerate mesohaline conditions and strong osmotic fluctuations (Brown et al., 2001) allowing them to inhabit the entire estuary. Life history strategies of the population comprise either brackish residency or freshwater-brackish water strategy in which reproduction and larval growth occur in freshwater with later dispersal to more saline habitats (Kafemann et al., 2000). Larvae typically hatch between April and June and switch to piscivorous feeding within their first year (Vinni et al., 2009), with *O. eperlanus* being primary prey in the tidal Elbe (Kafemann & Thiel, 1998). The summer period is characterized by intensive growth, the juveniles sampled here spent 4 to 5 months in the estuary reaching 16 ± 2 cm with high metabolic activity susceptible to be altered by the multiple prevailing stressors (Laurent et al., 2023).

Despite a well-found knowledge of the abiotic and biotic changes occurring within the estuary, many spatio-temporal factors (i.e. temperature, salinity, DO levels) are confounding and make it difficult to determine the precise mechanisms underlying observed losses in fish biomass. Omics are increasingly considered in the context of biomonitoring and conservation biology, as they enable a deep understanding of processes at all levels of biological organization and of previously overlooked interactions (Connon et al., 2018; Miranda et al., 2023). Co-working of multiple stressors is expected to lead to unforeseen synergistic or antagonistic effects in organisms (Petitjean et al., 2019), sublethal impacts of which can contribute to population declines over generations (Connon et al., 2018). Molecular adjustments precede high level responses thereby probably allowing earlier detection of adverse situations (Bahamonde et al., 2016). Real-world omics studies indicate molecular mechanisms used by fish to cope with adverse effects of multi-stress environment. Reversely they can inform about the environmental conditions themselves allowing the identification of unexpected stressors as shown in recent studies (Jeffrey et al., 2019; Laurent et al., 2023).

Biotic interaction with microbiota on mucosal surfaces of teleost fish play a crucial role in host metabolism, growth and health (Kelly & Salinas, 2017). The gill mucosal surface constitutes a special habitat for host-associated microbes due to its unique functions in waste excretion, gas exchange and immune activity (Pratte et al., 2018; Salinas, 2015). Local immune cell populations composed of adaptive and innate parts form the gill associated lymphoid tissue (GIALT) (Salinas, 2015) and enable fish to discriminate between beneficial and pathogenic bacteria thus keeping microbiome homeostasis (Yu et al., 2021). Changes in the environment however, including water conditions, seasonal changes and physiological stress can lead to compositional disturbances that turn commensals into pathogens (Kelly & Salinas, 2017). Several studies, particularly in aquaculture, have indicated an impaired immune response and spread of pathogens under low-oxygen conditions (Abdel-Tawwab et al., 2019; Boutin et al., 2013; Leeuwis et al., 2024). Likewise, suspended sediment was indicated to cause microbial shifts towards pathogenic communities (Hess et al., 2015). This sensitivity to environmental parameters makes the external mucus composition a biomarker for fish health built by a stable community of core taxa of low number but shared over a large range of conditions and a variable community strongly influenced the environment (Sehnal et al., 2021).

The objective of this study was to design an unsupervised network-based dimension reduction method based on real-world holobiont omics data from RNA and 16S rRNA libraries to precisely identify tissue-specific gene profiles and dynamics in bacterial composition. This allows us to determine driving stressors in the ecosystem and, by describing the microbiome and linking it to the immune

response in the host, to provide a first basis for the use of microbial biomarkers for the evaluation of fish health in the estuarine ecosystem.

4. Material and methods

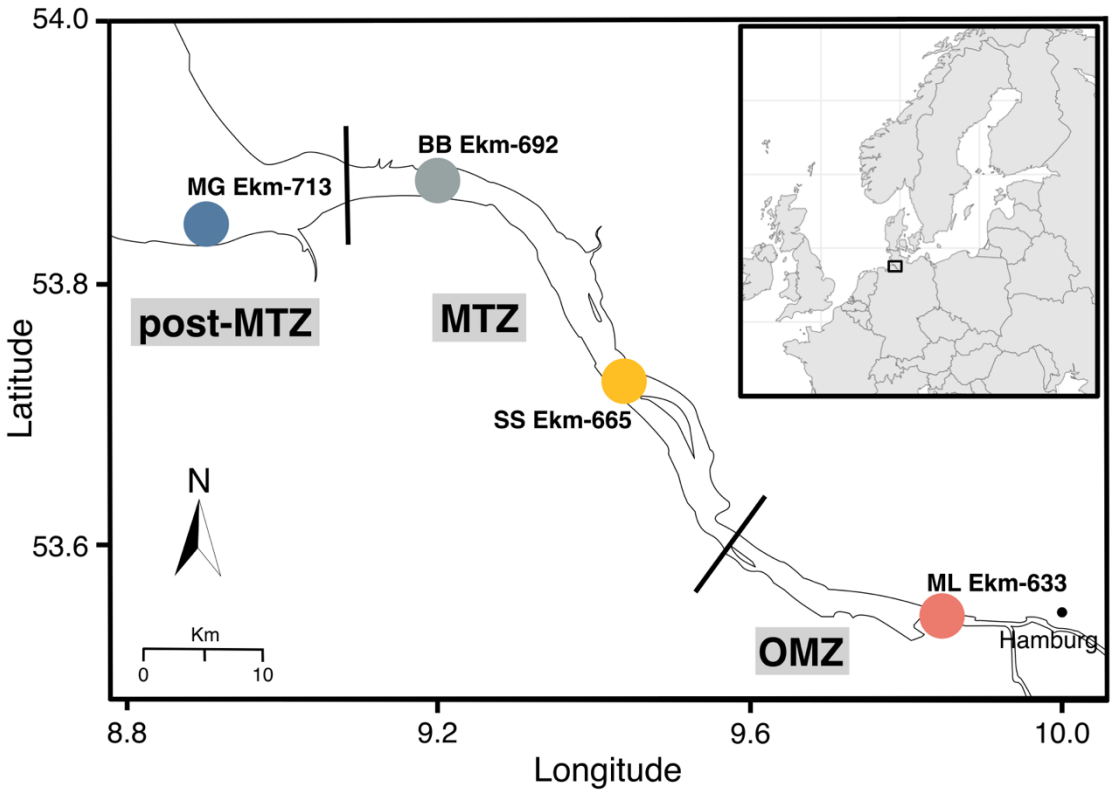


Fig. 1 Elbe estuary with section classification following (Amann et al., 2012): Oxygen minimum zone (OMZ), maximum turbidity zone (MTZ), and post-MTZ and sampling locations Mühlenberger Loch (ML), Schwarztonnensand (SS), Brunsbüttel (BB) and Medemgrund (MG) with Elbe river kilometer (Ekm).

4.1. Sample Collection

Fish were caught with a stow-net fishing vessel (opening area of 135 m², mesh size of 10 mm at the cod end) at four stations along the Elbe estuary (Fig. 1) in the summer period from 25 – 29.08.2021. Sampling stations were chosen within different estuarine sections classified by dominating abiotic drivers (Amann et al., 2012): Station ML-Elbe kilometer 663 within oxygen minimum zone (OMZ) (stream km 620 – 650, low oxygen, summer < 2 mg/L, salinity < 0.5 psu), stations SS-Ekm 665 and BB-Ekm-692 within the maximum turbidity zone (MTZ) (stream-km 650 – 705, high loads of suspended matter, salinity > 0.5 < 5 psu) and station MG-Ekm 715 within post-MTZ (Ekm 705 – 730, transition full marine, salinity > 1 < 20 psu). See Fig. 1 for sampling overview map and Fig. 2 C for abiotic conditions in the water column at sampling times. Sampling procedures followed the standards described in the German Animal Welfare Act (§4 TierSchG). Exemptions to the ordinances on nature reserves were obtained (**see Permits**). 3102 individuals of age 0+ were caught at the sampling stations (200 at MG, 134 at BB, 208 at SS and 2560 at ML). Six individuals from each ebb haul (n = 24, total length 16 ± 2 cm) were randomly chosen and processed immediately on board. Trends in physiological measures (length, weight, body condition) are representative in the subsets when compared to the larger sample size (see Fig. A.1, (Amieva Mau, 2022)). Physiological and abiotic sample data are supplied in Appendix C. Sample processing was conducted in the following standardized manner: After being brought on board, the fish were stunned with a blow to the head, before being killed with a heart stab. Samples were cooled to 4° until sampling immediately afterwards to ensure best RNA quality.

Fish were measured and weighed before recovering gill bacteria with sterile cotton swabs from the middle part of the second and third arch. The same part of the gill tissue as well as the right most edge of the liver were resected for preservation. All samples were kept on dry ice on board and moved to -80°C until further procession. Tissue samples were first placed in tubes with RNAlater (Macherey-Nagel) for one hour before they were moved to dry ice. Abiotic conditions (oxygen & salinity levels, Secchi depth, temperature, pH) were measured at start and end of each haul using a multi-probe (Hanna HI 9829 and Secchi disc) at the water surface. Water samples (n = 4) obtained from the water column were vacuum filtered onto 0.2 µm polycarbonate membranes (Whatman™ Nucleopore™ Track-Etch). Whole fish samples were frozen at -20°C and further analyzed in the lab: body indices were determined (Fulton's body condition (FultonK), hepato-somatic - (HSI) and spleno-somatic indices (SSI) see (Petitjean et al., 2020)), stomach content was weighed and age determination from otoliths and scales was performed.

4.2. Transcriptome

RNA extraction

Total RNA extraction from gill and liver tissues was performed via sample homogenization (45 seconds) with ceramic beads in peqGOLD Trifast (Peqlab), RNA dissolving in chloroform and mixing with 70% ethanol before immobilization on a silica membrane (Crystal RNA Mini Kit, BiolabProducts). On-column DNA digestion with RNase free DNase I (Qiagen) was performed for all samples. RNA quantity and integrity were controlled by spectroscopy (Nanodrop, Thermo Fisher) and on denaturing agarose gels and Bioanalyzer (Agilent Technologies) (avg. RIN 9.6 ± 0.6 over 48 samples). For every individual (n=24) liver and gill mRNA was isolated using either (NEBNext Poly(A) Magnetic Isolation Module (New England BioLabs) followed by stranded cDNA library creation (NEBNext Ultra II Directional RNA Library Preparation kit (New England BioLabs) or the Illumina RNA stranded mRNA kit including polyA enrichment. Individually barcoded libraries were 2x150 paired-end sequenced on NovaSeq6000 (Illumina) (300 cycles, v1.5 reagents, S4 FlowCell) on three different lanes resulting in 3.4 billion reads (avg. ca. 25 ± 6.8 million). To account for possible batch effects from library preparation and sequencing runs, biological replicates from sampling location are spread over runs.

Transcriptome analysis

Transcriptome analysis was performed on the University Hamburg Hummel HPC. Read files were quality controlled using FASTQC (v 0.11.9) and MultiQC (1.12) followed by removal of adapter sequences, clipping of first and last base pairs, quality filtering bases lower Phred score 20 and discarding reads lower than 20 base pairs in length via TrimGalore (v 0.6.10) wrapper of cutadapt (Martin, 2011) and FastQC (Andrews, 2010). Clean reads were mapped to the genome of *Sander lucioperca* SLUC_FBN_1.2 (Nguinkal et al., 2019) using Hisat2 (v.2.2.1) (D. Kim et al., 2015) (overall mapping rate 99 ± 1%) followed by expression determination via featureCounts (subread v.2.0.2) (Liao et al., 2014) (assignment rate 51 ± 5.4 %; 18 ± 5 million assignments). All downstream analyses were performed in R (v.4.3.0) using visualization packages ggplot2 (v.3.4.2) and cowplot (v.1.1.1). Genome wide annotation databases org.XX.eg.db (v.3.17.2) (Carlson, 2019) for human, mouse, and zebrafish as well as AnnotationHub (v3.17) (Morgan, 2023) entries for zander were used to translate between different gene identifiers, names and symbols. CDS regions from the reference genome were annotated using diamond (v.2.0.15) blast against UniProt, UniRef90, TrEMBL, RefSeq-Protein and Human EMBL-EBI reference proteome databases. Blast results were filtered for E-value < 1e-6 and bit score higher 50, for multiple hits to the same transcript, the one with the lowest E-value was retained. Wherever human genes could be assigned to transcripts on gene-level the human annotation was used for pathway analyses.

DEA & Functional term enrichment

Differential expression analysis was performed to verify network analysis results via DESeq2 (v. 1.41.4) (Love et al., 2014) (significance when FDR adjusted p value < 0.05 & Log2FoldChange (LFC) 0.05 or 1) contrasting sampling locations along the estuary. Genes failing to meet expression threshold of transcripts per million mapped reads (TPM) of 1 in at least three samples were excluded from downstream analyses (Lataretu & Hölzer, 2020). Genes were aggregated and hierarchical clustered using in ComplexHeatmap (v.2.17) (Gu et al., 2016). Functional enrichment of heatmap clusters was performed via overrepresentation analyses as described in section 2.3. For results see Fig. A 2 & A 3.

4.3. Microbiome

DNA extraction

Swabs were incubated in a mix of lysozyme, mutanolysin and lysostaphin before the cells were physically disrupted in a bead mill (Travis et al., 2020). Proteinase K digestion in CTAB buffer was followed by chlorophorm-phenol extraction. Nucleic acids were precipitated in PEG 6000 using LPA-carrier and recovered and washed repeatedly with 95% and 70% ethanol before resuspension in water (Neubauer et al., 2021). QC was performed via spectroscopy (Nanodrop, Thermo Fisher) and gel electrophoreses.

DNA Sequencing

The V3-V4 variable regions of the 16S rRNA gene were amplified in a one-step PCR using the primer pair 341F-806R (dual-barcoding approach (Kozich et al., 2013); primer sequences: 5'-CCTACGGGAGG-CAGCAG-30 and 5'-GGACTACHVGGGTWTCTAAT-30). After verification of the presence of PCR-products by gel electrophoresis, normalization (Sequal Prep Normalization Plate Kit; Thermo Fisher Scientific, Waltham, USA) and equimolar pooling was performed. Sequencing was conducted on the MiSeq platform (MiSeqFGx; Illumina, San Diego, USA) with v3 chemistry (2x300 bp). The settings for demultiplexing were 0 mismatches in the barcode sequences.

DNA Bioinformatics

Sequence data trimming, amplicon sequence variant (ASV) prediction and taxonomic identification were performed using cutadapt, FastQC and DADA2 (v.1.29.0) (Callahan et al., 2016). Quality profiles of paired reads were inspected and truncated at 270 and 190 for forward and reverse reads. ASV inference was performed with pooling method and taxonomy assignment used the SILVA SSU v138 taxonomic database (Quast et al., 2013). Accuracy of DNA extraction, sequencing until taxonomic assignment was validated via inclusion of mock community samples (ZYMO research). ASV table and sample data were parsed to phyloseq (v 1.45.0) (Mcmurdie & Holmes, 2012) removing ASVs taxonomically assigned to non-bacteria. Low abundance taxa with sum of counts lower than 0.005% of total sum of all counts were filtered reducing the number of zeros in the dataset by 50% to improve interpretability and minimize the risk of spurious correlations. A total of 1285 bacterial ASVs were included in further analyses, minimum sampling depth was ca. 7000, (avg. ca. 21000 ± 13000) clean sequences for fish samples. Alpha diversity measures were calculated via vegan package (v. 2.6.4) (Oksanen et al., 2022). The core microbiome was determined from relative abundance data via microbiome package (v. 1.23.0) (Lahti & Shetty, 2017) with filtering detection threshold to 0.0001% within samples and 90% prevalence. Centered log-ratio (CLR) transformation to the ASV matrix was applied following best practices for handling of compositional data (Gloor et al., 2017) using the microbiome package (Lahti & Shetty, 2017). The transformed data were used for visualization and network analyses. Permutation multivariate analysis of variance was used to test differences in community structure between fish and bacterioplankton as well as between fish sampling groups in vegan followed by post hoc pairwise t-test via pairwiseAdonis (P. Martinez Arbizu, 2020). Detailed results in Tab. A 2. Negative binominal GLM fitting and Wald statistics in DESeq2 (v. 1.41.4) (Love et

al., 2014) were used to identify taxa with significantly changing abundances between conditions used to verify the network analysis results. For results see Tab. A 3 and Fig. A 5 F.

4.4. Holobiont analyses: WGCNA

A Mantel test based on distance matrices was used as an initial approach to reveal overall matrix correlations (Fig. A 4). Weighted gene co-expression network analysis (WGCNA) (v.1.77-1) (Langfelder & Horvath, 2008) was then used for network inference using the following steps and parameters: 1. Signed network construction using biweight midcorrelation measure (Wilcox, 1997). Weighted adjacency is created via soft-thresholding, multiplying the co-expression matrix by a power that leads to approximate scale-free topology. Signed Topological Overlap Measure (TOM) is then used to measure interconnectedness accounting for indirect interactions and assignment of genes and bacterial clusters was performed via hierarchical clustering. Three samples were identified as outliers in both tissue RNAseq datasets by PCA and Euclidean distance clustering and excluded for network analysis and DEA. Parameter list: networkType = "signed", TOMType = "signed", corType = "bicor", minModuleSize = 30/5 (RNA/SSU), reassignThreshold = 0, deepSplit = 3, mergeCutHeight = 0.25. Our parameters were set to allow highest reproducibility between the different datasets in this study and upcoming samplings. Minimum module sizes of 30 and 5 for transcriptomic and SSU (Small subunit rRNA) datasets were chosen to facilitate interpretability. The first principal components of each module (Eigengene/EigenASV) are considered representative for the gene expression or bacteria abundance profile. Functional roles were assigned to the network modules by overrepresentation analyses against the KEGG- (Kanehisa & Goto, 2000) and GO-databases (Aleksander et al., 2023) using ClusterProfiler (v.4.9.0) (Wu et al., 2021). A subset of genes with correlation between gene expression profile and module eigengene (module membership) higher 0.8 was used for function annotation to increase interpretability. From each module, nodes with highest intramodular connectivity from the adjacency (hubs) were inspected as they are considered and shown functionally important (Langfelder et al., 2013). Module eigengenes/ASVs where correlated between the different networks and to physiological traits of the fish (Fulton`s body condition, weight, length, stomach content weight, HSI, SSI) and external abiotic factors (DO levels, salinity, Secchi depth) via Pearson correlation. An integrated heatmap analysis approach was used to visualize host responses and holobiont interaction following a workflow developed by Strand et al., 2021.

Principal component analysis

Principal component analyses (PCA) were performed on variance stabilizing transformed counts for RNAseq data or CLR transformed counts for 16S data using PCAexplorer and PCAtools (Blighe, 2023; Marini & Binder, 2019). Evaluation of clustering in PCA-plots indicated no obvious batch effects between sequencing runs. Nevertheless, ComBat-seq (Y. Zhang et al., 2020) batch effect adjustment tool was applied on the datasets but lead to an overall non-batch effect related reduction in variance and was discarded for further analyses.

5. Results

To gain insight into fish response and fish-microbiota interactions under steep abiotic gradients in an estuarine habitat, we applied an integrative approach of tissue specific RNA-seq and gill epithelial mucus 16S rRNA amplicon sequencing collected from juvenile zander from Elbe estuary during late summer. The sampling sites spanned mesohaline to freshwater habitats (Kennish, 2002), with DO levels, turbidity and salinity showing variation between the discreet estuarine areas (Amann et al., 2012): The post-maximum turbidity zone (post-MTZ, station: MG), the maximum turbidity zone (MTZ, stations: SS, BB) as well as the oxygen minimum zone around the Hamburg harbor area (station ML) (Fig. 1).

5.1. Network analysis

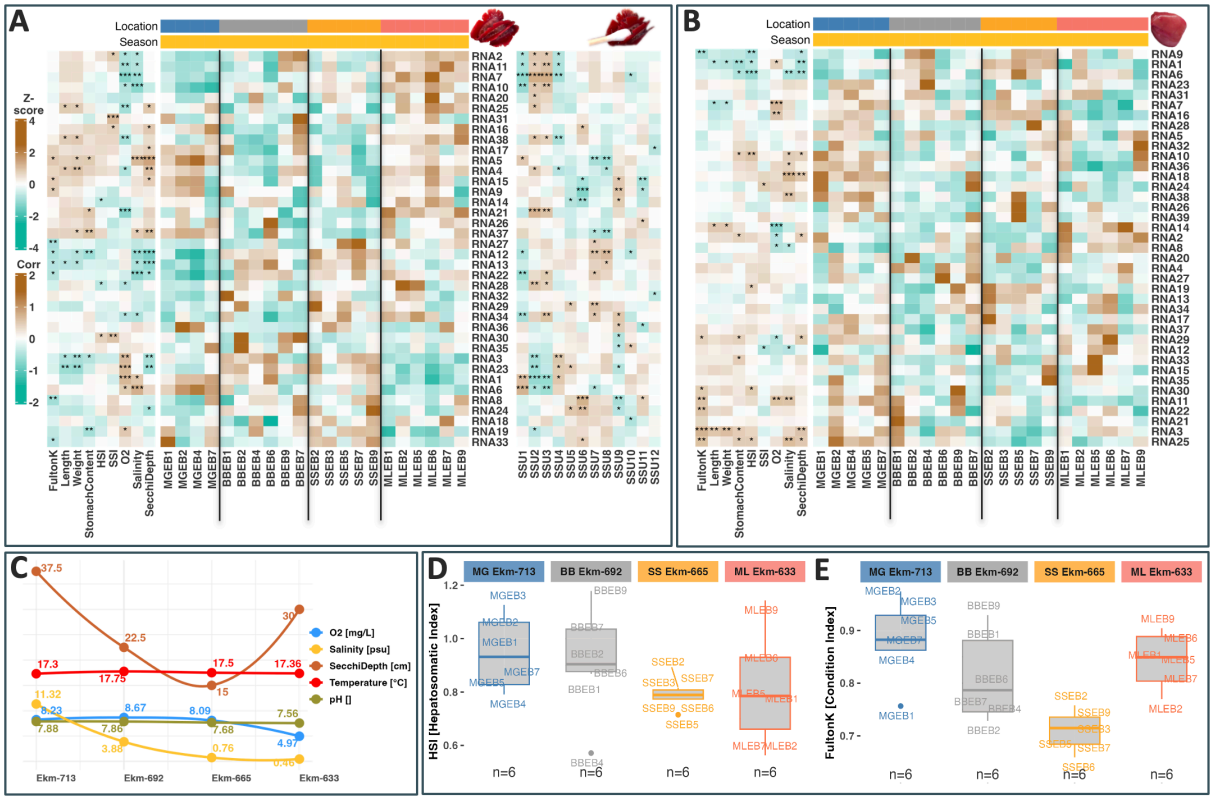


Fig. 2 Relationship between gene expression, bacterial composition, biometric fish data and abiotic conditions for Elbe estuarine zander. Integrated heatmap of gill network analysis (A) showing correlation between host gene expression module eigengenes (middle panel: Gill-RNA0-38), physiological traits (left panel: FultonK, StomachContent, HSI (hepatosomatic index)) and relevant abiotic factors (left panel: O2, salinity, Secchi depth) as well as microbiome abundance module eigenASVs (right panel SSU0-12). The middle panel shows Z-score of eigengene values per module as rows for individual fish ordered for sampling location in upstream direction as columns. The left panel shows Pearson correlation strength between eigengene and host/external traits, the right panel shows Pearson correlation to SSU eigenASVs (brown representing positive, zero white and green negative correlation) and statistical significance indicated by stars: * p < 0.05, ** p <= 0.01, *** p <= 0.001. Integrated heatmap of liver network analysis of matching fish (B) with host module eigengenes (right panel: Liver-RNA0-39) correlated to physiological traits and abiotic factors (left panel). Abiotic factors measured in the water column are depicted for sampling stations (C) as well as selected physiological measurements of HSI (D) and Fulton condition (E).

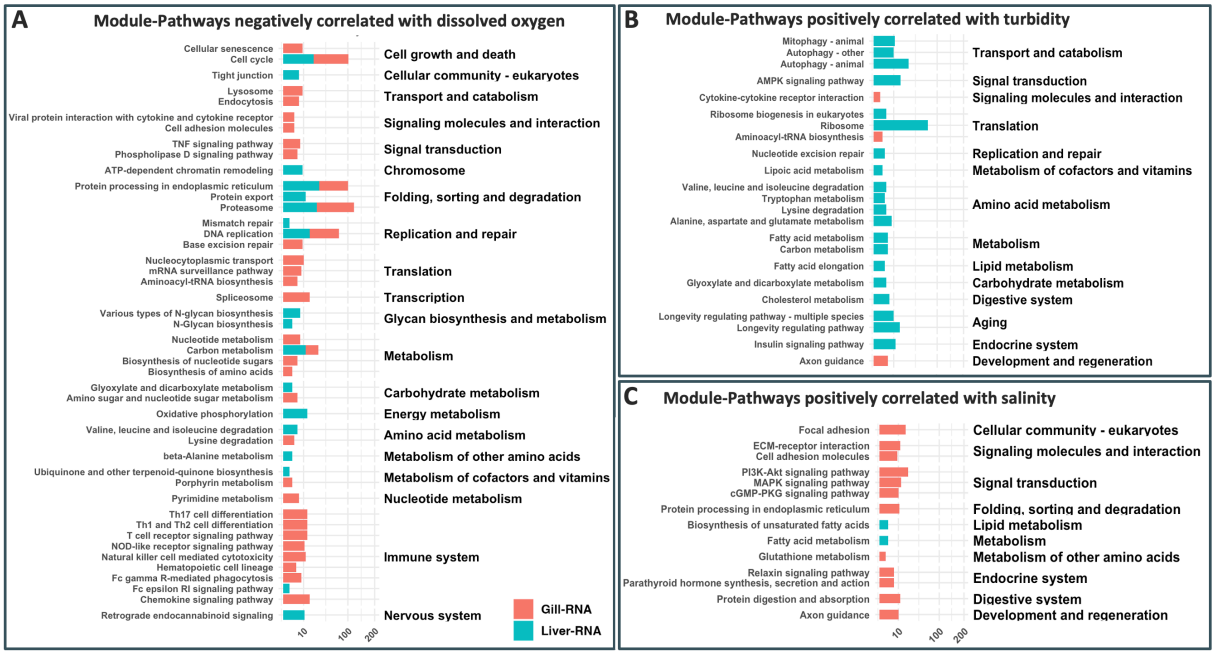


Fig. 3 Tissue specific gene regulation in relation to estuarine sections. KEGG pathway enrichments in modules correlated with abiotic traits from tissue specific network analyses are shown. Modules with negative correlation to dissolved oxygen levels (A) and positive correlation to turbidity (B) and salinity (C) are indicative for gene expression patterns within the oxygen minimum zone, the maximum turbidity zone and the post MTZ. In each plot KEGG-level 1 (left y Axis) and KEGG- level 2 (right y Axis) against the number of genes within each module with > 0.8 Module membership are depicted.

5.1.1. Post-maximum turbidity zone – salinity adaptation

The post-MTZ was characterized by mesohaline conditions ranging from 9.0 – 14.9 psu and stable DO levels between 8.2 – 8.5 mg/L (see Fig. 2 C). Only a few RNAseq modules positively correlated to salinity levels exhibited functional enrichment. Gill-RNA-1 (753 genes with module membership MM > 0.8, Pearson's correlation R = 0.54 and p value 0.01 to salinity levels) was enriched in cellular communication and signaling (focal adhesion, extracellular matrix (ECM)-receptor interaction, cell adhesion), signal transduction (PI3K-AKT, MAPK, cGMP-PKG) and axon guidance (compare Fig. 2 A & B & Fig. 3 C). Module hub genes were transmembrane protein 204 (*tmem204*) and protein tyrosine kinase 2 (*ptk2aa*). Genes in Gill-RNA-5 (144 genes, salinity R = 0.67, p 7.8E-04) were involved in ER protein processing and Gill-RNA-37 (22 genes, salinity R = 0.46, p 3.4E-02) in glutathione metabolism (*gpx7*, *mgst3a*). The liver tissue-specific response in RNA-Liver-10 (22 genes, salinity R = 0.49, p 0.022) was related to fatty acid metabolism (*elovl5*, *scdb*).

5.1.2. Maximum turbidity zone – enhanced energy metabolism

The maximum turbidity zone was marked by high particle load (Secchi depth of 10 – 30 cm), salinity fluctuation between 5 – 0.6 psu (SS: 0.9 – 0.6 psu, BB: 1.6 – 5.2 psu) but stable DO levels 7.6 – 8.8 mg/L (see Fig. 2 C). Gill-RNA modules with significant negative correlation to Secchi depth values comprised Gill-RNA-3 (1469 genes, Secchi depth R = -0.55, p 9E-3) enriched in ribosomal genes of both subunits and Gill-RNA-13 (50 genes, Secchi depth R = -0.69, p 5E-4) that contained genes involved in axon guidance (compare Fig. 2 A & B & Fig. 3 C). Liver-RNA-1 (754 genes, Secchi depth R = -0.57, p 5E-3) comprised genes related to autophagy (AMPK, mTOR, Insulin, FoxO, adipocytokine), mitophagy and peroxisome with autophagy related hub gene *bnip4*. Liver-RNA-6 (118 genes, Secchi depth R = -0.56, p 6.6E-3) was composed of ribosomal subunit genes. Liver-RNA-9 (138 genes, Secchi depth R = -0.45, p 3.6E-2) was highly enriched in metabolic pathways comprising fatty and amino acid degradation (tryptophan metabolism, valine, leucine and isoleucine degradation, lysine degradation, alanine, aspartate and glutamate metabolism).

5.1.3. Oxygen minimum zone – cellular stress and immune reactions

The oxygen minimum zone was characterized by low DO levels between 4.6 – 5.0 mg/l (46 – 52 % saturation) and stable salinity levels (0.46 psu). Seven Gill-RNA modules were negatively correlated to oxygen levels in the surrounding milieu, five of them were also negatively correlated to salinity (see Fig. 2 A & B). Gill-RNA-Module 2 (455 genes, DO R = -0.52, p 0.015) was highly enriched in replication and repair pathways (DNA replication, base excision repair, mismatch repair, nucleotide excision repair), cell growth and death (cell cycle, apoptosis, necroptosis), folding, sorting and degradation (proteasome), immune system (antigen processing and presentation) as well as signal transduction (NF-kappa B signaling pathway). Hub genes comprised proteasomal subunits (*psmb9a*, *psma6l*, *psmb10*, *psme1*), innate immunity related *irf1b* and *erap2*. Gill-RNA-4 (326 genes, DO R = -0.51, p 0.017) showed enrichment in protein procession (proteasome, protein processing in ER, Protein export), genetic information processing (nucleocytoplasmic transport and spliceosome). Hub genes were *psmd2*, *ups5*, *usp14* involved in protein processing as well as cell proliferation related *ppp5c*. The immune response in gill tissue was divided between two modules. Gill-RNA-7 (123 genes, DO R = -0.79, p 2.6e-05) included innate immune response parts (chemokine signaling and viral protein interaction with chemokine). Hub genes were microbicidal oxidase system of phagocytes (*cyba*, *nox1*) and MHC class II paralogues. Three variants encoding for eosinophil peroxidase (*epx*-like) and mucin-2 like

isoforms were most correlated to DO levels (R-0.85) and highly upregulated in the Hamburg area (LFC > 1, see Fig. A 2). Gill-RNA-11 (213 genes, DO R = -0.59, p 4.5e-03) was enriched in adaptive immune system processes specifically T cell receptor signaling, T-helper cell differentiation, NK cell mediated cytotoxicity and general chemokine signaling. Hub genes include the protein tyrosine phosphatase *ptprc* and lymphocyte cytosolic protein 2 *lcp2a*. Gill-RNA-21 (64 genes, DO R = -0.77, p 4.6e-05) was enriched in porphyrin metabolism (hubs: *rhd* and *alas2*) and strongly upregulated hemoglobin subunit alpha-2-like and beta-2-like. Gill-RNA-25 (19 genes, DO R = -0.6, p 4e-03) shows enrichment in lysine degradation (*kmt2d*, *ezh2*, *kmt2bb*). The liver specific response was less pronounced compared to gill, Liver-RNA-2 (2327 genes, DO R = -0.45, p 0.03) was enriched in genetic information processing pathways (proteasome, protein processing in ER, DNA replication, spliceosome, base excision repair), cellular processes (cell cycle) and metabolism (carbon metabolism, N-glycan biosynthesis, TCA cycle). Hub genes include again *usp14* and *ppp5c*. Liver-RNA-8 (81 genes, DO R = -0.49, p 0.02) was enriched in energy metabolism (oxidative phosphorylation), metabolism (of amino acids, cofactors, vitamins), mismatch repair as well as protein procession (proteasome, protein export, N-glycan biosynthesis).

5.2. Gill microbial community structure

Approximately 630,000 trimmed, filtered and merged reads across 24 zander and four water samples resulted in 42.000 ASVs. Filtering for ASVs contributing at least 0,005% of overall abundance reduced sparsity in the dataset by 50% and retained 1285 ASVs for further analysis (complete ASV list in Appendix B). Species accumulation analysis indicated that the rare microbiome was not sufficiently captured in the dataset (see Fig. A 5 A) but overall composition was when excluding rare taxa (Fig. A 5 B).

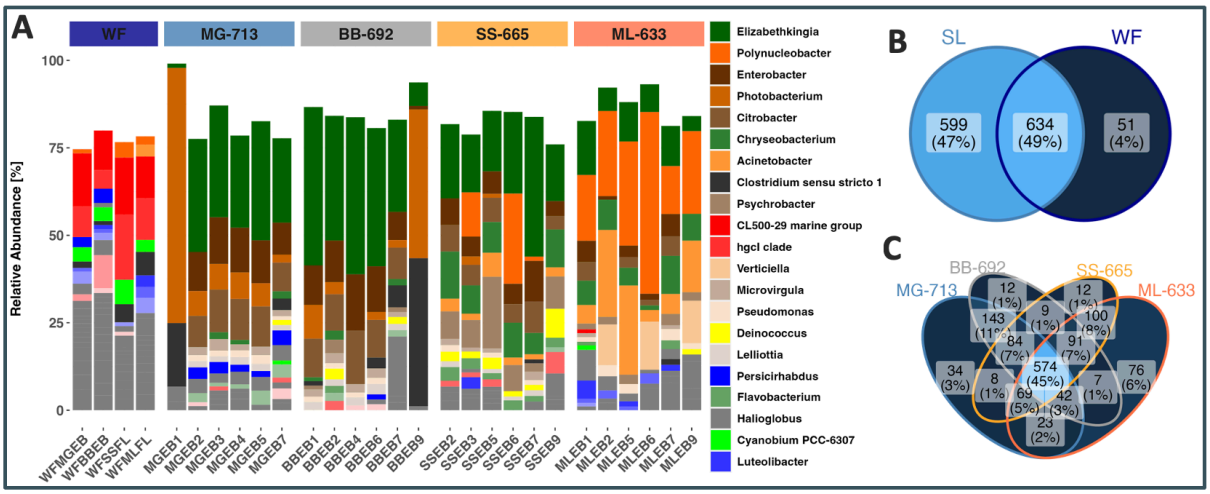


Fig. 4 Bacterial composition fish gill mucus and bacterioplankton. Compositional barplot (A) shows genus accumulated relative abundance (> 1%) per sample. Venn Diagrams depict presence/absence of bacterial ASVs found in water samples (WF) and on fish (SL) (B), and in fish swabs along the curse of the estuary (C).

5.2.1. Host-associated differ from free-living bacterial communities

We identified seven bacterial phyla associated with the fish microbiome and eight phyla in the surrounding water column (Tab. A 1). There was a significant difference between the microbiome of the fish and that of the surrounding water column (DF 1, F 7.5686, p 0.001) (Fig. A 5 C, Tab. A 2). Of the 1285 ASVs identified 51 were found only in the water column while 600 were unique to the fish gill microbiome (Fig. 4 B). The water column was characterized by a large abundance of Actinobacteriota (25% in water samples, 1 – 6% in fish), Proteobacteria (38%, fish: 53 – 78%), Bacteroidota (15%, fish: 14 – 32%), Verrucomicrobiota (8%, fish: 1 – 3%) and several phyla not

represented in fish samples at all: Cyanobacteria (4%), Nitrospirota (3%), Acidobacteriota (2%), Planctomycetota (1%). Firmicutes, Deinococcota and Desulfobacterota in the contrary were only recovered from fish gills.

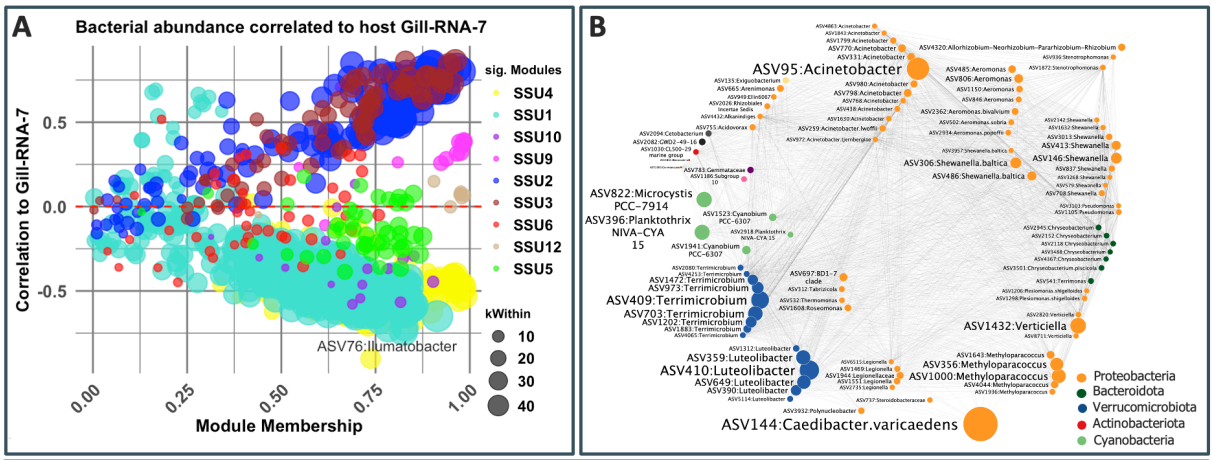
5.2.2. Host-associated communities diverge along the course of the estuary

We found a significant site-dependent difference in the bacterial community composition of the fish gill (DF 3, F 3.5533, p 0.001) between all sampling locations (Fig. A 5 C, PC2, Tab. A 2). 45% of ASVs occurred in at least one fish from each sampling site and there were few site-specific taxa (1 - 6%) (Fig. 4 C). The community was dominated by Proteobacteria followed by Bacteroidota and Actinobacteria which were the only phyla present on all individuals. The ratio of abundances between Proteobacteria and Bacteroidota was subjected to strong changes (MG: 62/21%, BB: 53/20 %, SS: 54/32%, ML: 78/14%). Enterobacterales (32%), Flavobacteriales (19%), Burkholderiales (13%), Pseudomonadales (9%) and Clostridiales (6%) make up 80% of the relative bacterial abundance over the estuary. Enterobacterales (49 % in most downstream – 12% in most upstream sampling station) steadily decrease over the course of the estuary with Photobacterium (27% – 0%), Citrobacter (8 – 3%), Enterobacter (7 – 2.5%), Lelliottia (4 – 2%) being the most abundant genera. Burkholderiales (3 – 36%) with the genera Polynucleobacter (0 – 28%) Verticiella (0 – 7%) and the Pseudomonadales (5 – 17%) with the genera Acinetobacter (0 – 14%) and Psychrobacter (0 – 2%, SS 8%) show increasing relative abundance in fish gill swabs in upstream direction. Flavobacteriales show differing patterns with the genera Elizabethkingia (16 – 8%) steeply decreasing over estuary and the genus Chryseobacterium (0 – 6%, SS 9%) increasing with a peak in the middle of the estuary. Alpha diversity slightly changes along the course of the estuary, observed richness steadily increased from 364 to 502 ASVs (sampling group means) in upstream direction with slight changes in Shannon index: MG: 3.9; BB: 3,6; SS: 4.3; ML: 4.0.

5.2.3. The core microbiome community of estuarine zander gills

The core microbiome comprised 43 ASVs from 11 orders predominated by roughly even relative abundance of Proteobacteria and Bacteroidota (Fig. A 5 D & E): Proteobacteria (Enterobacterales (18%), Pseudomonadales (1%), Burkholderiales (1%), Caulobacterales, Sphingomonadales), Bacteroidota (Flavobacteriales (16%), Chitinophagales (1%), Sphingobacteriales), Actinobacteriota (Microtrichales (1%), Corynebacteriales, Micrococcales), Deinococcota (Deinococcales) & Verrucomicrobiota (Chthoniobacterales). While the relative proportion of core taxa in the total microbiome declined along the course of the estuary (56 to 28%), the relative proportions within core taxa stayed overall stable (Bacteroidota/Proteobacteria from 55/40 to 60/35%). The Hamburg harbor area has the largest impact on the core microbiome, with slight increases in Bacteroidota. CL500-29 marine group was the only taxon shared in large amounts (> 1%) between core fish gill microbiome and surrounding water column.

5.2.4. Potential pathogens correlate to host immune response



C

Phylum	Order	Lowest Taxonomic level	Relative Abundance [%]	SSU Module	Module Membership	Correlation to Gill-RNA-7	Correlation to Gill-RNA-11
Proteobacteria	Enterobacterales	ASV306: Shewanella.baltica	Avg 0.05; max 0.53	2	0,90	0.85	0.61
		ASV413: Shewanella	0.04; 0.42	2	0.98	0.80	0.50
		ASV415: Shewanella	0.07; 0.44	3	0.89	0.78	0.60
		ASV221: Shewanella putrefaciens	0.03; 0.36	3	0.85	0.72	0.69
		ASV485: Aeromonas	0.02; 0.16	2	0.80	0.78	0.56
		ASV158: Aeromonas	0.07; 0.76	3	0.83	0.73	0.59
		ASV846: Aeromonas	0.01; 0.03	2	0.82	0.63	0.63
		ASV1298: Plesiomonas shigelloides	0.01; 0.08	2	0.91	0.69	
	Pseudomonadales	ASV95: Acinetobacter	0.15; 1.09	2	0.90	0.75	0.50
		ASV972: Acinetobacter.tjernbergiae	0.01; 0.09	2	0.86	0.74	
		ASV259: Acinetobacter.lwoffii	0.02; 0.14	2	0.89	0.69	
		ASV977: Acinetobacter.johnsonii	0.02; 0.17	3	0.91	0.77	0.50
		ASV1105: Pseudomonas	0.01; 0.10	2	0.86	0.74	
		ASV4432: Alkanindiges	0.01; 0.10	2	0.86	0.69	
	Xanthomonadales	ASV1872: Stenotrophomonas	0.01; 0,17	2	0.84	0.70	
	Methylococcales	ASV1000: Methyloparacoccus	0.08; 0.61	2	0.83	0.79	
		ASV272: Methylococcaceae	0.30; 1.11	3	0.96	0.87	
	Legionellales	ASV1944: Legionellaceae	0.02; 0.08	2	0.89	0.76	
	Burkholderiales	ASV15: Verticiella	1.14; 11.51	3	0.83	0.86	
		ASV25: Polynucleobacter	3.02; 38.54	3	0.84	0.77	0.64
Verrucomicrobiota	Chthoniobacterales	ASV973:Terrimicrobium	0.06; 0.42	2	0.90	0.84	0.63
Bacteroidota	Flavobacteriales	ASV3488: Chryseobacterium	0.01; 0.08	2	0.80	0.68	
		ASV3501: Chryseobacterium.piscicola	0.01; 0.04	2	0.87	0.64	0.58
Planctomycetota	Gemmatales	ASV140: Gemmataceae	0.15; 0.71	3	0.95	0.84	0.53

Fig. 5 Specific bacterial strains highly correlated with innate and adaptive immune response in host gill. Bacterial abundance correlated to host gill innate immune response Gill-RNA-7 module eigengene (A). ASV module membership on X-axis and Pearson correlation on Y-Axis, point size indicates intramodular connectivity, color core depicts module membership. The co-occurrence network module Gill-SSU-2 (B) highly correlated with host immune response is shown where point size indicates average relative abundance ASVs with highest correlation to host immune response modules Gill-RNA-7 and 11 (C). Relative abundance values over all samples (Avg.) and for the highest sample (max.) are shown. Only the first strain per lowest taxonomic level with Module Membership > 0.8 and Pearson correlation > 0.6 is depicted.

The bacterial network modules Gill-SSU-2 (Fig. 5 B) and 3 were correlated with the host gill immune modules (Gill-RNA-7 & 11) (Fig. 5 A and Fig. A 6), comprising 17 genera with Pearson correlation (> 0.6) and intramodular connectivity (> 0.8) (Fig. 5 C). Of these *Verticiella* (4 ASVs), *Shewanella spp.*, *S. baltica* and *S. putrefaciens* (9 ASVs), *Aeromonas* (8 ASVs), *Acinetobacter spp.*, *A. johnsonii*, *A. tjernbergiae*, *A. lwoffii* (24 ASVs), *Polynucleobacter* (4 ASVs), *Plesiomonas* (2 ASVs) and *Chryseobacterium spp.*, *C. piscicola*, *C. haifense* (13 ASVs) were with almost all strains absent from the bacterioplankton. In the contrary, Methylococcaceae (4 ASVs), Gemmataceae (4 ASVs), *Luteolibacter* (5 ASVs), *Terrimicrobium* (9 ASVs), *Cyanobium* (3 ASVs) and *Legionella* (7 ASVs) were abundant in both water and fish samples.

6. Discussion

In this study we sought to understand, through OMICs, how the predatory fish zander responds to changing abiotic and biotic (microbiome) conditions along an estuary, as a means for understanding existing and future paradigms of environmental change and fish biomass losses. Here we were able, using a global molecular approach, to demonstrate how fish physiology is impacted in a tissue-specific manner, both by different environmental impacts (salinity, turbidity and oxygen availability) and by changes in the relative contribution of core and potentially pathogenic bacteria on the fish gills.

6.1. Salinity adaptation

While zander larvae are stenohaline bound to freshwater and oligohaline areas, juveniles acquire the capacity to adapt to a wider range of osmotic fluctuations. Osmoregulation requires synergistic action of a complex sensing and signal transduction network that could be achieved by enhanced osmosensing in the juvenile zander via mitogen-activated protein kinase (MAPK) pathway (Kültz & Avila, 2001) and PI3k-Akt regulating involved salinity stress response (Zhao et al., 2021). Further, we found cell-cell/ECM interactions related to gill remodeling processes during salinity fluctuations (Jeffries et al., 2019; Lam et al., 2014; Zhou et al., 2022) and protein tyrosine kinase 2 (here ptk2aa) as hub gene in gill remodeling (Y. Zhang et al., 2023). Major epithelial cells in fish gills, pavement and mitochondria-rich cells, play complementary roles in ion transport (Evans et al., 2005). Their growth and differentiation may be organized by axonal guidance contributing to functional plasticity in estuarine fish (Lai et al., 2015). In accordance, axonal guidance was enriched in zander gill from mesohaline till oligohaline areas. Whilst osmoregulation is among the most energy-intensive metabolic activities in teleosts (Bœuf & Payan, 2001), mesohaline zander in this study showed highest biometric values and gene expression pattern related to hepatic fat accumulation (Perera et al., 2020).

6.2. Starvation in murky waters

The mid estuary maximum turbidity zone is marked by liver specific elevated metabolic pathways, autophagy and regulation of ribosomal genes. These correlate with lowest biometric data (length, weight, HSI, and compromised body condition) indicative for nutritional stress (Echevarría et al., 1997). During periods of nutrient deprivation, fish exhibit shifts in energy utilization, depleting liver glycogen (De Pedro et al., 2003; Yang et al., 2019) and relying on lipids as major energy source (Bar, 2014). Lipid droplets are broken down to free fatty acids via lysosomal-autophagy pathway (J. Wang et al., 2018). In mammalian cells, starvation causes disruption of mitochondrial structure (Domenicali et al., 2001) thus increases ROS and induces autophagy via AMPK pathway (L. Li et al., 2013). Likewise, fish show increased ROS (Varju et al., 2018) and induced autophagy and mitophagy (Pan et al., 2022). Autophagy is a key pathway mediating damage control and metabolic adaptation (Kroemer et al., 2010) triggered i.a. by hypoxic conditions involving BNIP3 and Beclin1 signaling while nutrient depletion activates AMPK-dependent ULK1 and Beclin1 (Kim et al., 2022) in mammals. Zebrafish ortholog genes include hypoxia-induced bnip3a, closest to human BNIP3, and hypoxia-independent bnip3 and bnip4 (Feng et al., 2011). Here we identified *bnip4* and *sirt5*-like as hub genes related to autophagy. Sirtulin enzymes, such as mitochondria-specific SIRT5, play pivotal role controlling lipid metabolism, mitophagy and apoptosis possibly via AMPK pathway (Guedouari et al., 2017; Hong et al., 2020). These could be indicative for nutrient depletion driven autophagy activation in zander liver.

The upregulation of ribosomal genes appears seemingly counterintuitive as ribosome biogenesis is most energy-consuming (Kraft et al., 2008) and downregulated in starving fish (Salem et al., 2007). However, severely reduced translation might be detrimental for acclimatization responses (Zhu & Dai, 2019), especially in a multi-stress real-world setting. Ribosomes occupy large amounts of amino acids that can be tapped by autophagy during starvation delivering fuel for proteome remodeling (Rabinowitz & White, 2010). However, autophagic tissue degradation has to be balanced by macromolecule synthesis to maintain homeostasis (Rabinowitz & White, 2010).

The cause of the starvation situation remains unclear. Turbidity, in general exerting negative effects on prey capture (Ortega et al., 2020), did not affect foraging success in juvenile zander across a range of turbidity levels (Ljunggren & Sandström, 2007). We did not investigate potential influences on prey availability or altered food spectra, observed in other estuarine predatory fish during increased turbidity (Lowe et al., 2015). As tissue fat content is a crucial factor for overwinter survival of juvenile zander (Rennert et al., 2005), the low weight, HSI and body condition particularly detected in the upstream part of the turbidity zone together with fat and other energy source degradation pathways

activation can be expected to affect survival rates of MTZ zander. Sediment load was shown to damage gill tissue in different species (Hess et al., 2015; Lowe et al., 2015), however an anticipated immune response linked to turbidity was not evident here. The relationship between starvation and immune regulation is complex and might relate to suppression of tissue (Salem et al., 2007) and external mucus immune functions (Soltanian & Gholamhosseini, 2019). We cannot rule out starvation related immunosuppression masking underlying processes.

6.3. Cellular stress in oxygen minimum zone

Gills are the primary organ for physiological exchanges and first response to stressors like hypoxia (Evans et al., 2005). Low DO acclimatization involves optimizing uptake and distribution, reflected in enhanced erythrocyte and hemoglobin concentrations (Richards et al., 2009). Identified genes, including hemoglobin subunit beta-2-like *hbb2*, linked to oxygen transfer and upregulated in OMZ, mirror findings in other percid species like Elbe estuarine ruffe and yellow croaker (Mu et al., 2020; Tiedke et al., 2015). Hypoxic conditions also induce epigenetic modulation by KMT2 family genes (Tsai & Wu, 2014), proposed as adaptive coping mechanism in rainbow trout (Léger et al., 2021) and identified here as module hubs (*kmt2d* and *kmt2bb*).

The upregulation of carbon and amino acid metabolism pathways together with TCA and OXPHOS in juvenile zander liver from the estuarine OMZ indicates a locally enhanced energy demand. Remodeling of energy metabolism is an effective strategy in fish to compensate increased demand during hypoxic conditions (Ding et al., 2020) or infection (Xiong et al., 2022). The liver plays a pivotal role controlling energy reserves utilizing amino acids as primary fuel (S. Jia et al., 2021) with upregulated metabolism (Gracey et al., 2001; Ni et al., 2014) observed during hypoxic episodes. Prolonged hypoxia can increase dominance of aerobic TCA and OXPHOS (Ding et al., 2020).

Local response was further marked by different aspects of cellular stress response (Kültz, 2005) including cell cycle arrest and DNA repair accompanied by affected proteostasis in both tissues. Halting energy-intensive genome duplication processes leverages time for macromolecular repair and allows for energy redirection (Kültz, 2020). Recovery from stressors on the other hand is accompanied by sustained repair processes (Mustafa et al., 2015; Y. Zhang et al., 2021). In our study, *ppp5c* is a cross-tissue hub gene involved in cell cycle arrest and DNA damage repair (Hinds & Sánchez, 2008) shown dysregulated in fish under severe stress (Bertucci et al., 2020). The ATP-dependent ubiquitin-proteasome system (UPS) mitigates proteotoxicity from damaged proteins (Bard et al., 2018) resulting i.a. from disrupted endoplasmic reticulum (ER) (Hetz, 2012). In both tissues we found moderate hypoxia correlated with UPS and endoplasmic reticulum (ER) stress response (Hwang & Qi, 2018) characterized by luminal chaperone and ER-associated degradation (ERAD) activation. Besides ubiquitin-proteasome subunits, deubiquitinase genes (DUBs) *usp5* & *usp14* showed highest intramodular connectivity linking enhanced protein turnover with DNA repair (Nakajima et al., 2014), immune response (P. Jia et al., 2020) and autophagic processes (D. Wang et al., 2021). Enrichment in apoptosis and necrosis pathways specific in gill indicates stress exceeding cellular tolerance levels (Kültz, 2005) in the exposed tissue.

Adaptive modification of the ubiquitin-proteasome composition by disassembly into the 20S unit increases recognition capacity for oxidatively damaged proteins (Grune et al., 2011). Inflammation as well as oxidative stress induce transcriptional upregulation of *PSMB8*, *PSMB9*, *PSMB10*, encoding specialized beta subunits in the 20S proteasome core creating the so-called immunoproteasome (Früh et al., 1994). Immunoproteasomes are central to processing antigenic peptides presented by major histocompatibility complex class (MHC) I molecules, clearance of oxidized proteins and protection of cells from inflammation induced damage (Johnston-Carey et al., 2016). In mammals, transcription of the inducible B-type subunits is suspected to be controlled by the Interferon regulation factor-1 (IRF-1) signal transduction pathway (Foss & Prydz, 1999). In line, we identified B-type subunits (*psmb8a* & *b*, *psmb9a*, *psmb10*) together with *irf1b* as hub genes in the largest oxygen correlated gill module. So

far there is relatively little research focusing in depth on proteasomal gene expression in fish. Enhanced expression of *psmb* (8, 9, 9-L, 10) was shown during antibacterial and antiviral response (Kasthuri et al., 2014) used as discriminative markers for infection monitoring (Miller et al., 2017). The expression and high intramodular connectivity of a full set of 20S proteasome, proteasome activator and inducible immunoproteasome indicate elevation of oxidized proteins due to oxygen stress and elevated ROS-levels and increased activity of immunologically active cells in the gill tissue of Hamburg harbor fish coordinated by *irf1b* signaling.

6.3.1. Gill immune response

Both, the co-expression networks and the differential gene expression analyses identified specific marker genes for T and B cell signaling upregulated in Hamburg area. Adaptive immune module hubs *ptprc* as essential regulator of T and B cell antigen receptor (Barashdi, 2021) and T cell signaling related LCP2 (*lcp2a*) were also identified as immune hubs in bacteria challenged flounder (L. Wang et al., 2022). The latter was also found regulated upon infection with eukaryotic parasites (Syahputra et al., 2018) and bacteria (Z. Li et al., 2018) indicating cross-species and cross-tissue importance in teleost immune response. Immune cells use reactive oxygen species (ROS) for destruction of pathogen cells involving CYBA and NOX2 encoded phagocyte NADPH oxidase multiprotein complex (Bedard & Krause, 2007). In mucosal immunity NOX1 is hypothesized to replace NOX2 (Geiszt et al., 2003), activity of which requires CYBA, NOXO1 and NOXA1 stimulated by IFN-γ (Kuwano et al., 2006). Phagocyte NADPH oxidase has key regulatory function in innate immune response via ROS mediated signaling in mammals (Thomas, 2018), matching identification of this gene set as innate immune module hubs in juvenile zander. The highly upregulated eosinophil peroxidase variants in Hamburg area indicate involvement of eosinophils in anti-microbial immune response (Mickenberg et al., 2014). Eosinophils are associated with parasitic infections, controlling inflammation and maintaining epithelial barrier (Gleich, 2000) but have only been studied in a limited number of teleosts including zebrafish (Balla et al., 2010), turbot (Noia et al., 2021) and flounder (Choi et al., 2021). All variants show a strong negative correlation with DO, indicative for eukaryotic infestation in the OMZ. Strong upregulation of Mucin-2 like isoforms involved in GIALT physical barrier in Hamburg area further support the local immune response. These variants have already been found enhanced in skin mucus of salmonids under physiological stress going along with immune suppression and overgrowth in bacteria (Tacchi et al., 2015).

6.4. The zander gill microbiome

Juvenile zander host a complex gill microbiome distinct from that of the surrounding water column which was clearly impacted by the sampling location along the estuary. Although influenced by the bacterioplankton (Pratte et al., 2018), gill structure and function support a unique and highly diverse bacterial composition in wild fish (Minich et al., 2020). Differing tolerances of inhabiting taxa to physiochemical gradients influence the composition of fish mucus communities (Lokesh & Kiron, 2016). The strong variation in bacterial composition along the estuary identified in this study is accompanied by only a slight increase in observed taxa with decreasing salinity. The significant differentiation between sampling sites combined with relatively high similarity of bacterial composition on individuals from the same location might be indicative for low movement patterns of juvenile zander within the course of the estuary. Overall, the oxygen minimum zone and freshwater transition in the Hamburg harbor area were found most influential for the variable composition as well as for the core microbiome. This is indicative for a microbial reorganization and possible disease in these animals (Sehnal et al., 2021). On the one hand the core species, assumed to serve beneficial roles in the host, decline: *Asinibacterium and Enterobacter taxa are considered to inhibit pathogen growth* (Halet et al., 2007; Schubiger et al., 2015), *Lelliottia* is described as a critical part in juvenile percides gut microbiomes (Gallo et al., 2023). *Elizabethkingia* is a well-known component in mucus communities in freshwater and in marine fish (Jacobs & Chenia, 2011; M. Zhang et al., 2022). On the other hand,

with the emergence of known freshwater taxa like *Polynucleobacter* (Jezbera et al., 2011) and *Verticella*, a variety of opportunistic pathogenic taxa gain abundance in the OMZ.

6.4.1. The zander holobiont in the Elbe estuary

Host-microbe and microbe-microbe interaction are gaining more and more attention due to their tremendous importance for fish health (Kelly & Salinas, 2017). A few recent studies focused on the bacterial composition on gills in wild fish (Pratte et al., 2018) assessing pathogen load from the presence of specific genera and species (Itay et al., 2022; Minich et al., 2020). Here, we aimed to describe the holobiont incorporating bacterial abundance with tissue specific gene expression patterns in the host. Stressful conditions are expected to affect the interplay (Yu et al., 2021), oxygen deficiency in aquaculture for example has been linked to immunosuppressive effects (Abdel-Tawwab et al., 2015; Gallage et al., 2017) and increased abundance of potentially pathogenic taxa (Boutin et al., 2013). As such, a substantial number of suspected or confirmed fish pathogens on zander gill aligned with an intensified host immune response in the oxygen minimum zone. Taxa with suspected opportunistic pathogenic functions comprise *Pseudomonas* (Duman et al., 2021), *Chryseobacterium* (Loch & Faisal, 2015), *Acinetobacter* (Kozińska et al., 2014), *Psychrobacter* (Hisar et al., 2002) and *Aeromonas* (8 ASVs), the latter known for causing multi-tissue damage in freshwater fish (Nielsen et al., 2001). Kidney specific transcriptome studies indicated OXPHOS and proteasome to be strongly activated upon Aeromonas infection followed by cellular senescence and apoptosis pathways (Xiong et al., 2022) matching our results. *Shewanella* strains (16 ASVs), including the species *S. putrefaciens* and *S. baltica*, showed highest correlation to the immune response and abundances of up to 3.5% in fish while being scarce in bacterioplankton. *Shewanella* is known for its role in decomposing organic matter under hypoxic conditions (Nealson & Scott, 2006) and various strains have been found in gill mucus communities of marine fish (Itay et al., 2022; Pratte et al., 2018) some of which resemble potentially pathogenic species (Minich et al., 2020). Besides repeated detection in diseased fish (Cocchi et al., 2018; Paździor et al., 2023; Rusev et al., 2016), a recent study found high pathogenicity of *S. putrefaciens* strains with infection rates peaking when the DO values fall below 5 mg/L (Esteve et al., 2017).

The oxygen minimum zone in the Elbe estuary shows persistent DO levels below 5 mg/L during the summer month. Nevertheless, the largest numbers of juvenile zander hatching in freshwater or oligohaline waters reside in this region. The observed cellular stress response, the immune response, the impaired core microbiome and high levels of potentially pathogenic bacteria are not directly correlated with compromised biometrics, but may indicate a burden on the health situation of the fish that precedes these physiological markers. The effects shown here are also likely to have impact on other fish species that hatch and develop in this region and for which a decline in biomass has already been observed in the Elbe estuary (Scholle & Schuchardt, 2020). Immediately downstream from the OMZ, zander show signs of starvation and reduced biometric data. Migration to the lower parts of the estuary, where high biometric values were observed, would require crossing of the OMZ and MTZ. The prevailing conditions could represent a migration barrier due to avoidance behavior (Roberts et al., 2009; Thiel et al., 1995) and thus weaken parts of the population. This would affect the nutrient flow and carbon sequestration in the estuary which is strongly influenced by the presence of top predatory fish species (Atwood et al., 2015; McLean et al., 2019).

Although we focused on the responses to the three dominant environmental factors, these must be regarded as proxies for an environment with numerous biotic and abiotic pressures. The Elbe estuary has a long history of organic compound and heavy metal pollution with toxicity values expected to affect fish communities especially in the harbor area (Wetzel et al., 2013) that were not measured here. Contaminant and pathogen defense both affect the same physiological traits (Petitjean et al., 2019) and stimulate oxidative stress response that was already triggered by the lowered oxygen levels in the OMZ interacting with the overall response of zander from this region.

With this exploratory study, we were able to create a global overview of potential drivers of fish health in this ecosystem and interpret these against the background of experimental data from the literature. With the relatively large amount of high-quality data and independent bioinformatic approaches, we were able to identify patterns based on high numbers of correlated gene regulations. Many findings are consistent with laboratory experiments (e.g. gill remodeling processes in response to salinity and lowered DO values), but for some correlations, such as the interaction of opportunistic bacteria, environmental stressors and the immune response in the host, the study is limited and more in-depth investigations will be required. Especially in the light of recent research findings related to deoxygenation and suppression of the adaptive immune components in fish (Leeuwis et al., 2024), our findings here will need to be validated in large-scale multi-stress experiments across an extended range of methods.

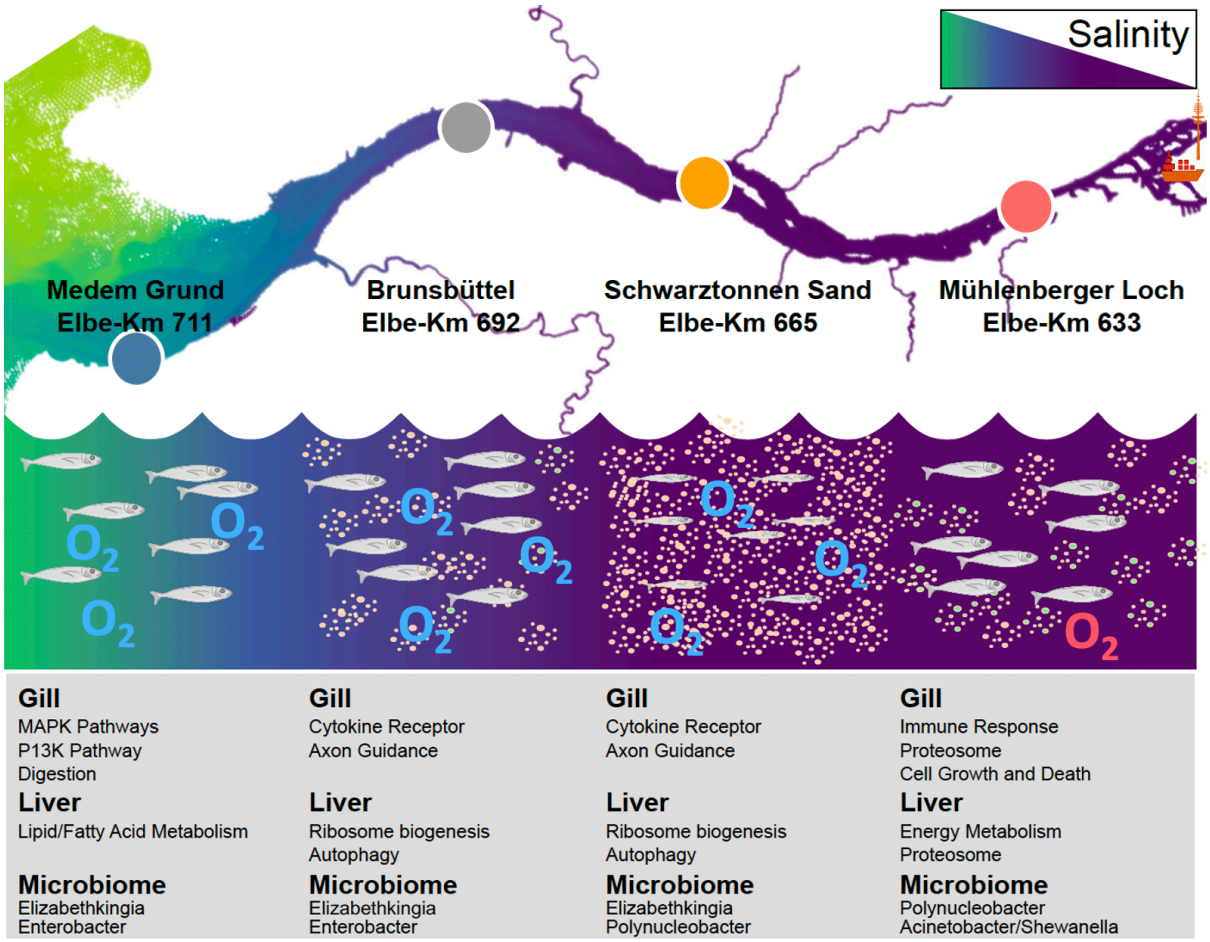


Fig. 6 Graphical summary. The main tissue specific gene expression pattern and most important gill mucus bacteria are shown in the course of the estuary correlated with the dominant environmental drivers (salinity, turbidity, oxygen levels) used as proxies for multiple stressors.

7. Conclusion

This explorative study is one of the first using an unsupervised analysis approach of the microbiome in combination with tissue-specific gene expression in an estuarine wild-fish population. Our results provide deeper insight into prevailing stressors than biometric and abundance data alone, improve the ability to assess the health of fish stocks and to understand population declines. Our findings (Fig. 6) reveal that juvenile zander gene expression is significantly impacted by turbidity, related to signs of starvation, and reduced oxygen levels triggering robust immunological and cellular stress responses. The disruption of the core microbiome and the rise of potential pathogens under

low-oxygen conditions highlight the pressing need to understand and address the responses of the holobiont. As oxygen deficiency events are projected to increase due to climate change, this study underscores the potential escalation of disease risks, such as shewanellosis. Mowing forward, future studies should consider multi-stress experiments in conjunction with OMICS-based time-series monitoring across additional, likely more vulnerable life stages to validate these results and anticipate how fish populations in highly productive estuarine nursery areas will develop under climate change.

8. Data availability

Sequence data are deposited in the ENA Sequence Read archive under study PRJEB71116. We made the full analysis available in stepwise R markdown files and illustrated HTMLs, including metadata and supplementary lists under https://github.com/vollkorrn/ElbeEstuarineZander.

9. Permits

Sampling procedures were according to the standards described in the German Animal Welfare Act (§4 TierSchG). The implementation of the stow-net fishing for scientific purposes is approved in accordance with § 10 Regulation for the implementation of the Hamburg Fisheries Act in the Elbe estuary by the Authority for the Environment Climate, Energy and Agarwirtschaft (A132-Supreme Fisheries Authority, Stadthausbrücke 12, 20355 Hamburg), by the State Fisheries Office Bremerhaven (Fischkai 31, 27572 Bremerhaven) according to § 10 of the Lower Saxony Coastal Fisheries Ordinance and by the State Office for Agriculture, Environment and Rural Areas of Schleswig-Holstein (Department 3, Fisheries, Hamburger Chaussee 25, 24200 Flintbek). Exemptions to the ordinances on nature reserves Mühlenberger Loch/Neßsand (Amt für Naturschutz, Grünplanung und Bodenschutz, Abteilung Naturschutz, Neuenfelder Strasse 19, 21109 Hamburg) as well as a nature conservation permit to conduct research fishing in protected areas in the NSG "Rhinplate und Elbufer südlich Glückstadt"/FHH area DE 2393-393 "Schleswig-Holsteinisches Elbästuar mit angrenzenden Flächen" from the Office of Environmental Protection (Department of Nature Conservation, Langer Peter 27a, 25506 Itzehoe).

10. Author contributions

RK: Conceptualization, Methodology, Investigation, Formal analysis, Data curation, Validation, Writing- original draft, Visualization, Software, Project administration. JT: Investigation, Writing - review & editing. EH: Investigation, Visualization, Writing - review & editing. JW: Supervision, Validation, Writing - review & editing. AF: Funding acquisition, Conceptualization, Supervision, Validation, Writing - review & editing, Project administration. RT, CM: Conceptualization, Funding acquisition, Resources, Writing - review & editing.

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12. Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work RK used DeepL/translate/write in order to improve language of the manuscript. After using this tool, RK reviewed and edited the content as needed and takes full responsibility for the content of the publication.

13. Appendix

Appendix A: Supplementary data

Appendix B: Full list of ASVs with taxonomy, sequence data, relative abundance and counts per sample. Abundance filtered (0.005% overall abundance)

Appendix C: Sample Data

14. References

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Chapter 3 Habitat exploitation & migration

Tracking life cycle strategies of flounder and ruffe in the Elbe estuary

Manuscript in preparation

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1. Abstract

Estuaries are favoured by certain fish species, as they offer a high food supply and thus suitable feeding habitats for various life stages. However, the challenging conditions and salinity gradients in estuaries limit the ability of certain fish species to exploit these habitats. Therefore, fishes developed a variety of strategies within their life cycles to optimize their habitat exploitation. Flounder *Platichthys flesus* and ruffe *Gymnocephalus cernua* are eponymous and thus defining species for the lower river regions of northern Europe, but information on the role of estuarine habitats on these species is still scarce. However, this knowledge is essential to uncover spatiotemporal habitat use preferences and to better understand population dynamics of flounder and ruffe. Here we show the annual habitat exploitation and migrations of flounder and ruffe from the Elbe estuary using stable isotopes δ13C and δ15N. Our study offers new insights into population structures, overwintering strategies, spatial connectivity and site fidelity of flounder and ruffe. According to these findings, juvenile flounder show a high migration radius overall, low site fidelity and movements to upper freshwater areas in winter. Ruffe completes its entire life cycle in the study area with low spatial connectivity throughout the year, potentially indicating a distinct brackish population in the Elbe estuary. Our outcomes demonstrate how migratory flounder and ruffe optimize their habitat use during their life cycle in estuaries. Our study further illustrates that migration patterns of fish using estuaries within their life cycle are far more complex than previously assumed with salinity acting as a main driver of movements. Our study should therefore serve as a starting point to further unravel annual habitat use to improve the conservation of fishes in estuaries.

Key words: Elbe estuary, fish community, fish migration, stable isotope analysis

2. Introduction

Estuaries are highly productive ecosystems that offer a variety of benefits for aquatic consumers, especially fishes. These benefits include high food supply and low predation pressure, supporting immense biomasses and fast growth, especially in early life stages (Elliot and Hemingway 2002, Day et al. 2013). However, estuaries are also challenging environments for aquatic biota due to rapidly changing physico-chemical parameters (e.g., oxygen, temperature) and especially strong salinity gradients (Pasquaud et al. 2007, Wetzel et al. 2013). As a result, species richness is usually low and species are usually generalist feeders (Whitfield and Harrison 2020, Mosman et al. 2023).

Estuarine conditions limit the movements and distributions of certain fish species (Whitfield 2021), a phenomenon, known as the barrier effect (Whitfield et al. 2022). This effect leads to behavioral (e.g., migration, life cycle) and metabolic adaptations (e.g., osmoregulation) in some fishes, which incur additional physiological and energetic costs to exploit the estuary for nursery, feeding, spawning and as migration routes (Elliot and Hemingway 2002, Whitfield et al. 2022). Estuarine fish species have therefore evolved various life history strategies based on their scope to optimize their habitat exploitation (Whitfield et al. 2021).

Salinity particularly shapes the fish assemblage in estuaries, causing a decrease in the number of marine species from the river mouth towards inland, while the number of limnic species shows a reverse trend (Thiel and Potter 2001). This in turn leads to a special composition of the fishes, as both limnic, brackish and marine species occur in the same habitat (Maes et al. 2005, Whitfield 2015), which is also reflected in the term *ruffe-flounder region*, referring to these areas of northern European rivers (Hölker and Temming 1996; Carstens et al. 2004). Both ruffe (*Gymnocephalus cernua*) and flounder (*Platichthys flesus*) are therefore characteristic fish species for these regions and occur regularly in high abundances and at different life stages, thus each fulfilling important ecological roles (Eick and Thiel 2014).

Life cycle movements of fish comprise a series of movements that often occur at regular intervals (e.g., daily, tidal, seasons) (Pittman and McAlpine 2001). These include home range movements (e.g., tidal, daily, seasonal), ontogenetic shift or migrations (non-spawning, spawning), which can differ significantly in their distance and duration (Pittman and McAlpine 2001). The term *migration* is often used in association with spawning migrations or the traveling of long distances from one area to the next (e.g., for feeding) by fishes (Morais and Daverat 2016). In particular, the classification of life history guilds refers mainly to migration patterns based on different reproductive strategies (e.g., Thiel and Potter 2001).

Flounder follows a facultative catadromous migration pattern (Daverat et al. 2012) with a high number of juveniles living in temperate estuaries benefitting from higher temperatures, high food supply and low predation risk especially in the freshwater sections (Gibson 1994, Andersen et al. 2005).

Ruffe, on the other hand, occurs in both freshwater and brackish water but has better growth rates in estuaries due to high food availability (Hölker and Thiel 1998). Recent studies of 16S metabarcoding and stable isotope analyses revealed stationary behavior of resident ruffe via gill mucus microbiome composition in the Elbe estuary (Koll et al. 2024, chapter 7). However, detailed information on the movement patterns of these species is largely lacking, especially in the Elbe estuary, one of the largest estuarine ecosystems in northern Europe (Schwentner et al. 2021). Understanding migrations as part of their life cycle and population dynamics of ruffe and flounder in the Elbe estuary can serve as a basis for sustainable management. In addition, knowledge on preferred habitats of different life stages of these species in estuaries regarding prevailing environmental conditions (e.g., salinity, food supply) are essential to understand their role in the trophic food chain and to further validate nutrient and energy fluxes and transport in the Elbe estuary.

To track ruffe and flounder movements, we used stable isotope analysis which has proven to be an effective approach to investigate habitat connectivity and migrations of limnic, estuarine and marine fish (Morais and Daverat 2016). The low and stable trophic enrichment of carbon isotopes (trophic enrichment factor (TEF) ca. 0.8 - 1.1‰) enable identification of the origin of carbon sources from the base of the food web to higher trophic levels (DeNiro and Epstein 1978, Doi et al. 2005). The isotope ratios ($\delta^{13}C$) of primary producers are linked to the surrounding geochemical (e.g., climate) and biochemical processes (e.g., degradation, photosynthetic pathways), resulting in local isotopic signatures, which in turn can be retrieved in the consumer tissue (Peterson and Fry 1987, Post 2002). Carbon sources derived from freshwater sources are generally depleted in their $\delta^{13}C$ whereas those of marine origin have high $\delta^{13}C$ values (Peterson and Fry 1987). Nitrogen isotope ratios ($\delta^{15}N$) enrich stepwise per trophic level (TEF 2-4‰) and are appropriate for determining trophic positions of organisms in a food web (McCutchan et al. 2003, Nielsen et al. 2018).

Here, we used stable isotope analysis to evaluate how ruffe and flounder exploit the estuarine resources during their distinct life cycles. Specifically, we analyzed seasonal stable isotope data along the salinity gradient of the Elbe estuary to resolve this information temporally and spatially. This study aims to improve our understanding of seasonal migration patterns and habitat exploitation of fish by answering the following questions: Can we determine life cycle strategies of flounder and ruffe using spatial and temporal stable isotope information? Which areas of the estuary are particularly important for the species studied? The results of our study promote a detailed understanding of life cycle patterns and habitat exploitation in fish to draw conclusions about ecosystem functioning, population dynamics and species conservation.

3. Material and methods

3.1. Study area

The Elbe estuary is a temperate, tidal influenced and well-mixed periodically stratified estuary in northern Europe (Middelburg and Herman 2007, Amann et al. 2015, Pein et al. 2021) that extends over 140 km from the weir of Geesthacht to Cuxhaven, where it ends up in the North Sea (Francescangeli et al. 2021). The area is known as a hot spot of anthropogenic activities due to its economic importance of the port of Hamburg, Germany´s largest seaport (Schwentner et al. 2021). Due to both riverine inflow from inland and the connection to the North Sea, the estuary provides a transitional zone of both fresh- and marine water forming a steep salinity gradient and an overall highly dynamic system (Schwentner et al. 2021, Spiekermann et al. 2022).

3.2. Sample collection and processing

We conducted fishing using a commercial fishing vessel in spring (May/June), summer (August), autumn (November), winter (March) and spring (May/June) from 2021 to 2022 at five stations located at the lower, middle and upper section along the salinity gradient of the Elbe estuary (compare with Magath and Thiel 2013) (Fig. 3.1). Salinity conditions were measured using a multiprobe (Hanna instruments ©) before and after each haul.

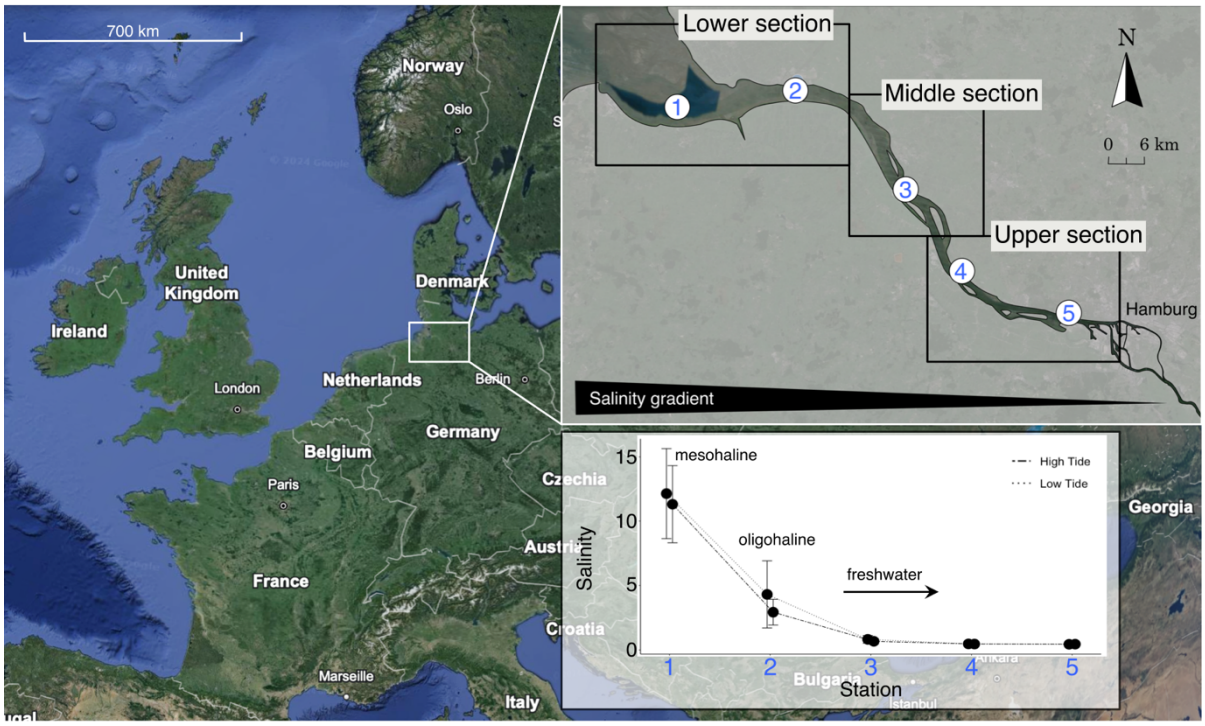


Fig. 3.1 The Elbe estuary in Northern Europe with sampling stations (1) Medemgrund, (2) Brunsbüttel, (3) Schwarztonnensand, (4) Twielenfleth and (5) Mühlenberger Loch and sections reflecting the salinity gradient of the main channel towards the city of Hamburg (map was created using Google Earth, 2024).

Sampling was conducted with a stow net that has an opening of 135 m^2 and 10 mm meshes at the cod end. We sampled for 3-4 hours at each station covering ebb and flood events. Individuals of both species were measured and weighed on board. The sample size was reached after normality of the length distribution was achieved, although this was not always possible due to the local absence of length classes during the sampling period (see supplement Tab S3.1).

We divided specimens into juvenile and adult life stage based on total length measurements. As ruffe reach sexual maturity at 2-3 years of age in northern areas often with a total length of 11-12 cm (Kolomin 1977, Gutsch and Hoffman 2016), we referred to ruffe <11 cm as juvenile (6.2 - 10.9 cm) and above as adult (11 - 22.6 cm). For flounders, we assume maturity at a total length of 20 cm, based on other studies (e.g. Mendes et al. 2020), so individuals with a total length below this limit are termed as juvenile (5.8 - 19.5 cm) and above as adult (20 - 39 cm).

We took white muscle tissue samples for stable isotope analysis of $\delta^{13}C$ and $\delta^{15}N$ from the dorsoventral side of the body, rinsed them with distilled water and stored them at -80°C for further analysis. A ring net with an opening of 60 cm and a mesh size of 1000 µm was used to collect macroinvertebrates samples, which were treated similarly to the fish samples. These samples were used to further predict distinct spatial $\delta^{13}C$ signatures to verify isotopic limits of the estuary and to predict fish movements.

In the laboratory we freeze-dried the samples for 24 hours and ground them to fine powder using a MM440 Cell Lyser by Retsch©. 0.8 – 1.0 mg of each sample were filled into tin capsules and folded. The UC Davis Stable Isotope Facility in California measured $\delta^{13}C$ and $\delta^{15}N$ of the samples using a continuous flow isotope ratio mass spectrometer (IRMS) PDZ Europa ANCA-GSL elemental analyzer interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK). International standards VPDB (Vienna Pee Dee Belemnite) and atmospheric air were used for carbon and for nitrogen. Isotope ratios were expressed using the delta notation:

$$\delta X = [(\frac{R_{Sample}}{R_{Standard}}) - 1] \quad \textbf{Eq. 3.1}$$

with X being the stable isotope ratio of $\delta^{13}C$ and $\delta^{15}N$ in per mille (‰), and R the relation of the mass ratio of the heavy and light isotopes ($^{13}C/^{12}C$ and $^{15}N/^{14}N$) of the sample divided by the ratio found in international standards.

Habitat exploitation and migration

To improve the interpretation of spatial habitat use and migration behavior of resident fish in the isotope data we predicted distinct $\delta^{13}C$ values of estuarine fish using local $\delta^{13}C$ values (see Guelinckx et al. 2006) of mysid shrimps *Neomysis integer* and *Mesopodopsis slabberi*, which we caught year-round in the study area. These species are known to be important prey organisms for fish in the study area (Sepúlveda et al. 1993, Fockedey and Mees 1999). Due to minor seasonal variation in the mysid isotopic data, we pooled the $\delta^{13}C$ values by station and estimated the means and standard deviations (SD) of the most downstream station 1 and most upstream station 5 (Fig. 3.1), that further represent the limits of a year-round estuarine isotope ratio. $\delta^{13}C$ above and below these limits are defined as marine and riverine signals, respectively, whereas ratios within these ranges are further defined as estuarine signal.

Ontogenetic niche shift

We verified the possible ontogenetic niche shifts of flounder and ruffe using generalized additive modeling (GAM) between isotopic data of $\delta^{13}C$ and $\delta^{15}N$, respectively, and fish total length (TL) using the equation:

$$\text{Response} = \beta + s(V_i) + \ldots + s(V_n) + \varepsilon \quad \text{(Eq. 3.2)}$$

Where β is the intercept, s the smoother function of predictors V_i and V_n and ε the error term (Casini et al. 2012).

We evaluated normal Q-Q-Plots, Cook´s distances to determine potentially critical outliers and histograms to check for normality of residuals. Then, we ran the models with different predictor values and chose the best fitting model based on lowest Aikaike´s Information Criterion (AIC) with empirical criteria >=2 (Aikaike 1976) (see supplement Tab. S3.2, S3.3)

Due to the high variance in flounder data, the conducted GAMs were not appropriate for describing the relationship between total length and isotopes (see supplement Tab. S3.3). We concluded that the isotope values of flounder reflected migration rather than ontogenetic shift, so that we split the data seasonally. Therefore, we continued with 95% CI ellipses of $\delta^{13}C$ per total length of juvenile and adult flounder to describe isotopic shifts during ontogeny on a seasonal scale using the ranges of the isotopic limits as described above.

Spatial connectivity

To analyze spatial differences in diet-derived carbon isotopes on a seasonal scale to further detect spatial connectivity and site fidelity of flounder and ruffe, we assigned $\delta^{13}C$ values of each species to upper, middle, and lower sections based on their catch location according to season. We verified the normal distribution of the samples for each season through descriptive statistics and checked for homogeneity of variance using Levene's test. Subsequently we performed either a one-way ANOVA or a Kruskal Wallis test to determine significant differences in $\delta^{13}C$ between local groups per season. Using pairwise post-hoc tests with Bonferroni p value adjustment, we evaluated whether significant differences between the sections occurred.

Interspecific niche differences

To investigate isotopic niche widths and overlaps use of ruffe and flounder, we first tested for normality and variance homogeneity using Shapiro-Wilk and variance tests. Then, we analyzed $\delta^{13}C$ and $\delta^{15}N$ of both life stages per season and section using Wilcoxon rank test to distinguish significant differences between their $\delta^{13}C$ and $\delta^{15}N$ values ($p < 0.05$). We pooled the stable isotope data of juvenile and adults of flounder as we found no significant differences in the data. For ruffe we used data from adult life stage only. Data with a low sampling size per section ($n < 3$) was removed from the analysis. We calculated standard ellipse areas (SEA) with 40 % confidence interval (CI) to estimate isotopic niche width and overlap of individuals caught at the three sections to distinguish spatial habitat exploitation of fish on a seasonal basis using SIBER package (version 2.1.6) in R (Stable Isotope Bayesian Ellipses in R) (Jackson et al. 2011) (supplement Tab. S3.4). Therefore, we used corrected standard ellipse area (SEA_c) for small sample sizes of the isotope data. Additionally, we estimated Layman metrics (Layman et al. 2007) per season between lower, middle, and upper sections.

All statistical analyses were performed using R studio version 4.0.4 (R Core Team, 2024) using packages, cowplot (version 1.1.1), DHARMa (version 0.4.6), dplyr (version 1.1.2), forcats (version 1.0.0), ggarrange (version 0.4.5), ggmap (version 4.0.0), ggplot2 (version 3.4.2), ggpubr (version 0.6.0), gridExtra (version 2.3), hrbrthemes (version 0.8.7), mgcv (version 1.8-39), nlme (version 3.1-155), plyr (version 1.8.8), reshape2 (version 1.4.4), SIBER (version 2.1.6), tidyverse (version 1.3.1).

4. Results

4.1. Annual habitat exploitations by flounder and ruffe

Flounder and ruffe were caught at juvenile and adult life stages in the Elbe estuary during all seasons. The $\delta^{13}C$ isotope values provided information on marine, estuarine and riverine carbon sources derived in individuals (Fig. 3.2). The flounder population was dominated by juvenile specimens, especially in winter when the adults were almost absent. We observed primary marine and estuarine $\delta^{13}C$ signals in both life stages, with a shift to riverine signals starting in autumn, maximizing in winter and ending in spring in only juvenile flounder. Adult flounder were nearly absent in our winter catches. Ruffe, meanwhile, occupied primarily the estuarine isotopic ranges during the entire season, with juveniles tending to shift into the $\delta^{13}C$ range derived from the lower river/estuarine origins. In winter we caught less individuals containing lowest isotopic variability in both juvenile and adult ruffes. However, we found two outliers in juvenile ruffe in spring that contained a riverine (2021) and a marine (2022) $\delta^{13}C$ signal.

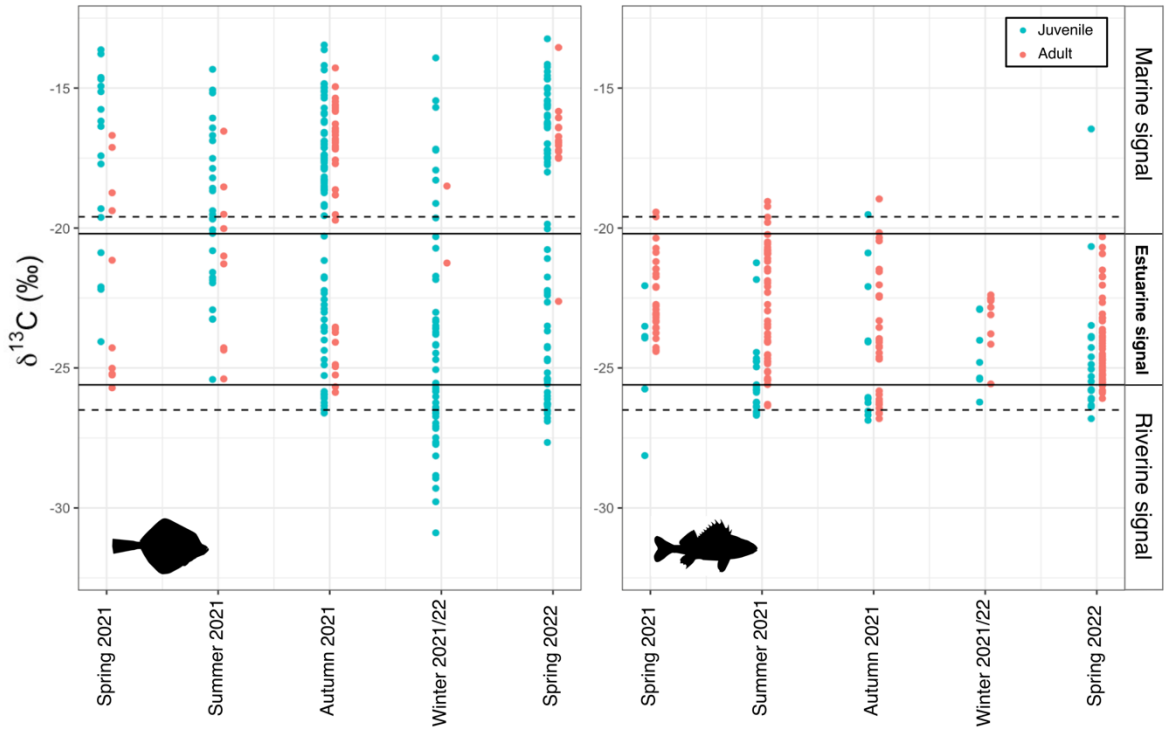


Fig. 3.2 $\delta^{13}C$ of European flounder and ruffe at different life stages (juvenile, adult) from spring 2021 to spring 2022. Black horizontal lines show predicted estuarine $\delta^{13}C$ signal in fish and SD (dashed lines) estimated from the $\delta^{13}C$ of mysids caught at station 1 (border to the sea) and station 5 (border to the river).

4.2. Ontogenetic niche shift

We did not find a clear relationship between fish length and isotope values in the flounders. For clarity, however, we split the $\delta^{13}C$ data at 20 cm total length. We observed a shift in isotope values based on seasonal ellipses of juvenile flounder in particular, which revealed information on temporal migration behavior (Fig. 3.3). The overall length distribution of flounder showed that juvenile fish with peak values between 10 cm and 14 cm were predominant in our samples, while adult fish (> 20 cm) were caught less frequently (Fig. 3.3A). Seasonal 40 % CI ellipses of juvenile flounder $\delta^{13}C$ were used to determine potential effects of size on isotope values (Fig. 3.3B). Our analysis revealed that the $\delta^{13}C$ of the smallest flounder shifted from the marine to the estuarine range with increasing fish length in summer and autumn. In winter, $\delta^{13}C$ values change to lower riverine $\delta^{13}C$ values, with stable isotope ratios increasing again with fish growing in spring. In general, adult flounder did not show a clear isotopic scheme overall, except for autumn when we observed two different $\delta^{13}C$ patterns with either estuarine or marine isotopic ratios. High $\delta^{13}C$ values, reflecting marine derived carbon sources, dominated in adults in spring with no $\delta^{13}C$ ratios detected in the lower riverine isotope range.

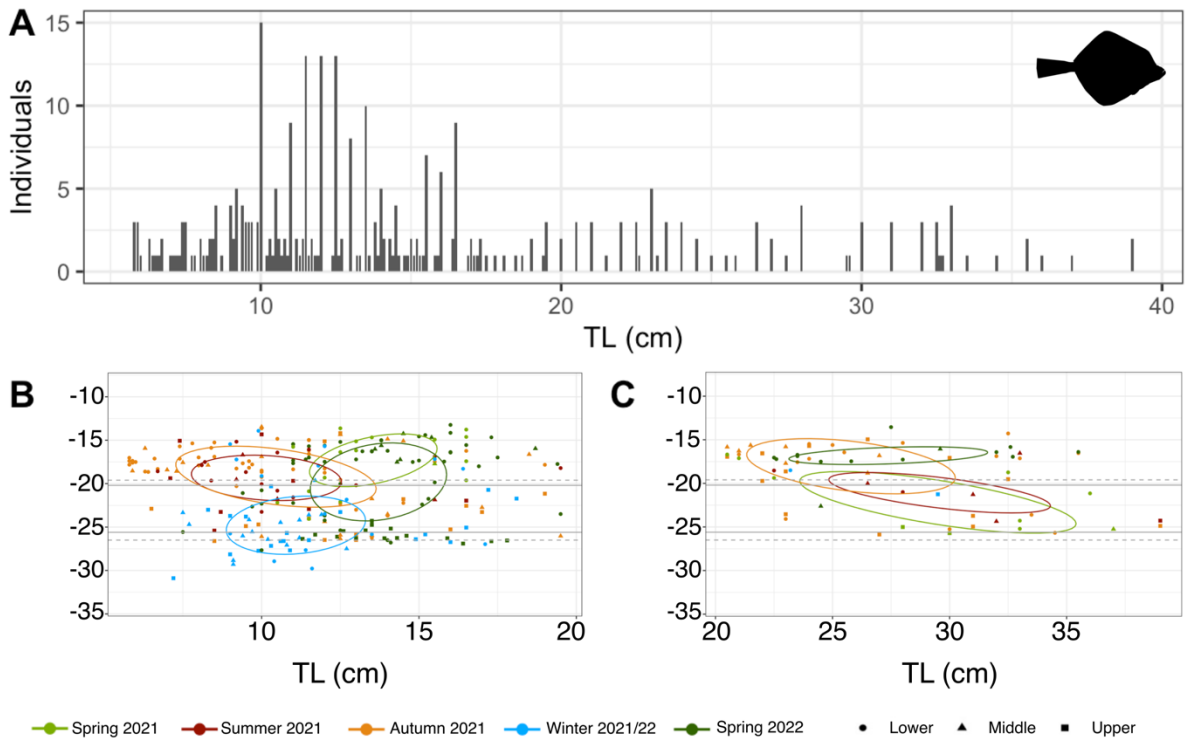


Fig. 3.3 (**A**) Visualization of measured total length (TL) in cm of flounder per number of caught individuals (see Tab. S3.1). (**B**) $\delta^{13}C$ ratios vs. TL of juveniles and (C) adults with ellipses (~40 CI) per season. Grey horizontal lines show predicted estuarine $\delta^{13}C$ signal in fish and SD (dashed lines).

The ruffe population included individuals with total lengths between 6.2 and 22.6 cm, with a peak between 10 and 18 cm (Fig. 3.4A). GAM revealed a significant linear relationship of $\delta^{13}C$ and $\delta^{15}N$ values as a function on length (Fig. 3.4B, C). Based on the AIC, we included section and season as predictor variables to describe both stable isotopes (Tab. S.3.2). For $\delta^{13}C$, differences in isotope values and total length (R^2 adjusted = 0.43, F = 21.0, df = 227) occurred between sections, but with less effect on seasonal variation. Only the two spring samplings differed significantly from each other in their isotope values ($p < 0.05$).

The $\delta^{15}N$ ratios increased significantly with enlarging ruffe size (R^2 adjusted = 0.59, F = 104.9, df = 227) to a higher extent than $\delta^{13}C$. No differences were found between the lower and middle section; however, a significant effect was observed between all seasons.

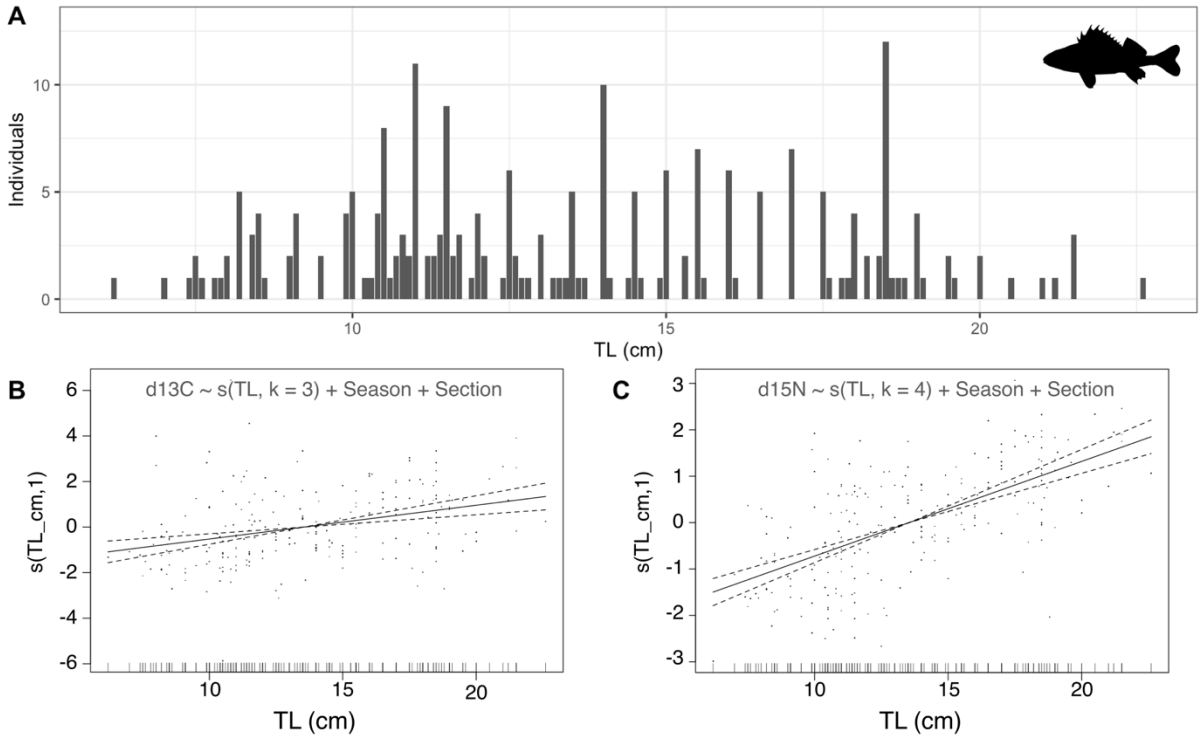


Fig. 3.4 Size-specific differences in stable isotope ratios in ruffe. (**A**) Total lengths (TL) in cm of caught ruffe during seasonal samplings. (**B, C**) Frequency distribution of TL and the relationships between TL and $\delta^{13}C$ as well as $\delta^{15}N$ based on GAM formula displayed above, respectively. The lines represent the linear effects of TL on the isotope values, the dashed lines the smoothing function for each isotope variable and the dots the distribution of the individual data.

4.3. Spatial connectivity and site fidelity using $\delta^{13}C$

We further compared $\delta^{13}C$ values by section at each season using ANOVA and Kruskal-Wallis (KW) tests to determine whether there are differences between local $\delta^{13}C$ signatures that further reflect spatial habitat use and site fidelity. For flounder, the statistical analysis revealed significant differences in $\delta^{13}C$ ratios between estuarine sections in spring (2021: $p < 0.05$, $F = 3.99$, $n = 27$, 2022: $p > 0.0001$, KW-chi-squared = 33.40, $n = 86$) and autumn ($p < 0.001$, KW-chi-squared = 9.99, $n = 36$). Subsequent post-hoc and Wilcox tests showed that significant differences occurred regularly between the more saline lower and the fresher upper section. Differences between the upper and middle section could only be detected in spring 2022. No spatial difference in $\delta^{13}C$ ratios were found for flounder in summer ($p > 0.05$, $F = 2.18$, $n = 36$) and winter ($p > 0.05$, KW-chi-squared = 6.18, $n = 54$).

The statistical analysis of ruffe data indicated significant differences in the $\delta^{13}C$ ratios between the sections of the estuary in summer ($p < 0.001$, $F = 3.03$, $n = 67$), autumn ($p < 0.001$, $F = 22.06$, $n = 43$) and spring (2022) ($p < 0.001$, KW-chi-squared = 14.44, $n = 68$). Post-hoc tests indicated significant differences between the lower and upper areas of the Elbe estuary. In summer, all sections differed significantly in their $\delta^{13}C$ values. In autumn, individuals from the upper and middle sections as well as in spring 2022 from the lower and middle section differed significantly from each other. No differences were found in spring 2021 ($p > 0.05$, $F = 3.05$, $n = 34$) and winter ($p > 0.05$, $F = 0.64$, $n = 16$).

4.4. Interspecific niche differences

Overall, flounder and ruffe exhibited variable spatio-temporal isotope patterns, each reflecting the distinct migration patterns of the two species (Fig. 3.5; see also supplement Tab. S3.3). We found the highest isotopic overlaps in flounder between estuarine sections in summer and autumn ranging from 26.1% to 41.9%. During winter the lower section yielding the largest isotopic range (SEA_c

17.3‰^{2}, TA = 39.3), while flounder from the middle and upper sections largely overlapped (i.e. 67.4%). In spring, flounder formed a separate isotope group, while individuals from the lower and middle section overlapped with 16.4% using 40% CI. We determined the smallest isotope ranges in the middle and upper sections with SEA_c of 3.1‰^{2} and 4.0‰^{2}.

In contrast, in ruffe we observed that sections increasingly separate from spring to autumn with overall narrow isotopic ranges. Highest overlap of 23.9% were estimated between middle and upper sections. In summer and autumn, we noticed a similar isotopic pattern in ruffe with ellipses shifting from low $\delta^{13}C$ values in the upper sections to higher $\delta^{13}C$ values in the lower section. We only captured the ruffe in winter at the middle and upper sections in low abundances (n = 8) with the largest overlap of 49.1% for this species, but the lowest SEA_c between 2.1‰^{2} and 1.9‰^{2}.

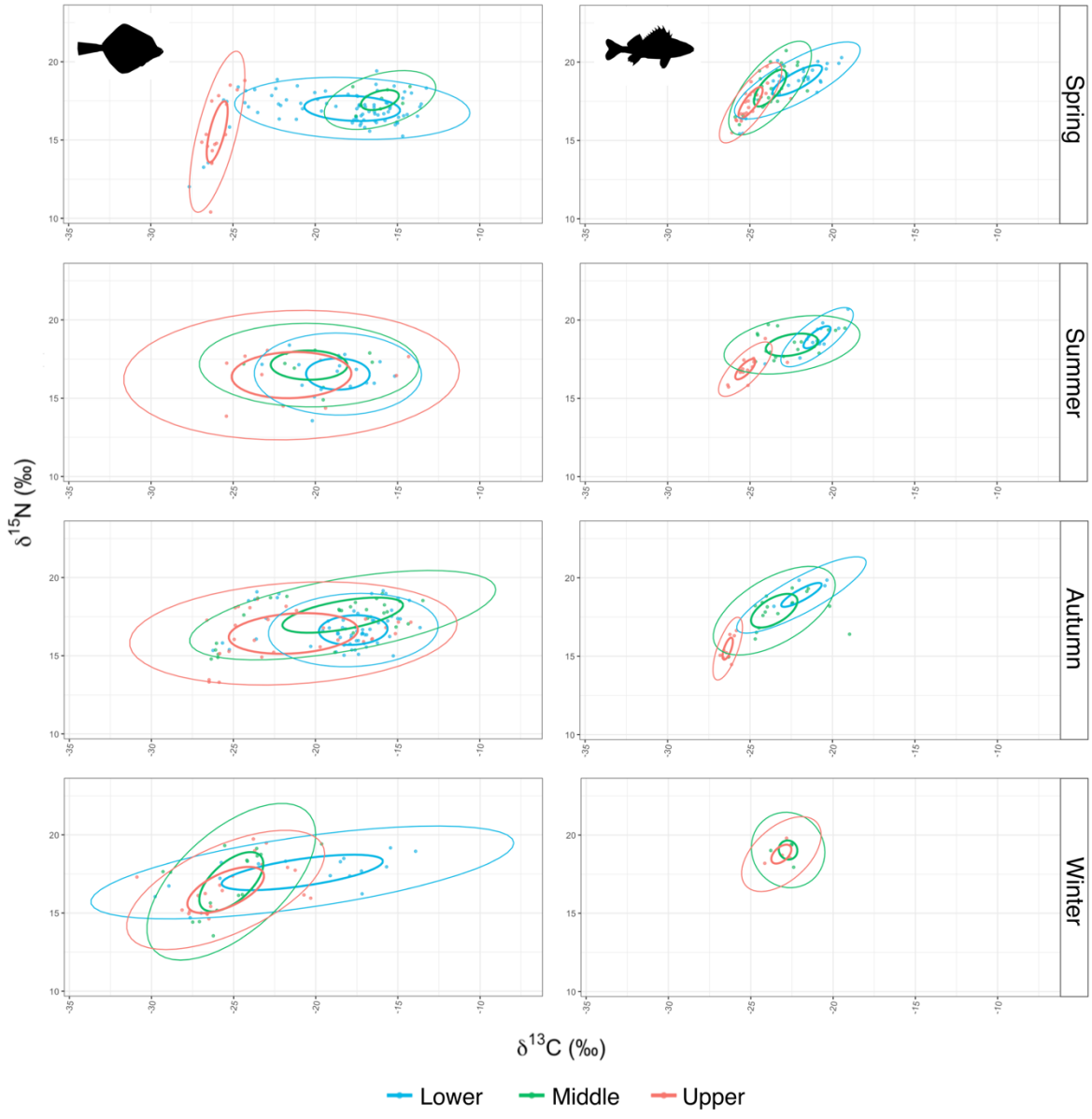


Fig. 3.5 Overlaps of isotopic ranges of $\delta^{13}C$ and $\delta^{15}N$ of flounder (left) and ruffe (right) caught at lower (blue), middle (green) and upper (red) sections during the different seasons. Outer and inner ellipses represent 95 % and 40 % confidence intervals, respectively.

5. Discussion

5.1. Life cycle guild classification of flounder in the Elbe estuary

We observed high dispersion of $\delta^{13}C$ data in flounder that demonstrates the utilization of diverse habitats and resources from marine to riverine origins by mainly juvenile individuals caught in the Elbe estuary. This high variability in $\delta^{13}C$ values further reflected the high migration activity of flounder between the different estuarine habitats. Our study therefore confirms the outcomes of other studies that flounders use the estuary mainly during their juvenile life stage to benefit from the prevailing higher temperatures, low predation pressure and rich food availability, thus juveniles occurring here in high abundances (Gibson 1994, Andersen et al. 2005).

However, there is no universal definition describing the migration pattern of flounder, which has led to controversial discussions in the scientific community in the past. Some authors have described the migration pattern as *catadromous* which is still widely accepted today (Summers 1979, Borsa et al. 1997, Minier et al. 2000, Marchand et al. 2003, Franco et al. 2008, Daverat et al. 2012). Franco et al. (2008) claimed flounder to belong to the *marine migrants* as flounder representing a marine species with juveniles entering the system for nursery and migrating back during their sub-adult phase. In the Elbe estuary flounder has been characterized as *marine estuarine opportunist* (MEO) with particularly juveniles entering the estuary in high numbers for nursery but use also adjacent coastal marine and other habitats for this purpose (e.g., Eick and Thiel 2014, Potter et al. 2015, Thiel 2011). Daverat et al. (2012) observed a dependency of young flounder life stages on estuarine habitats as nursery habitat in the Seine, Gironde and Minho estuary, and therefore used the term *facultative catadromous* to describe their life cycle that is linked to the estuarine habitat. This variety of definitions underlines the adaptability of flounder populations in different ecosystems and habitats to optimize their exploitation.

Even though flounder of the Elbe estuary have been considered MEO in the past, some studies indicate a certain dependency of the occurring flounder. For example, some authors described selective tidal transport of flounder larvae into the estuary and a tendency to enter areas with freshwater conditions (e.g., Bos 1999, Bos and Thiel 2006). Our results underline the importance of the estuary as an essential nursery area, which would suggest a dependence of the population(s) present. Therefore, we used the definition *facultative catadromy* by Daverat et al. (2012) in this study to describe flounder migration in the Elbe estuary.

5.2. Potamodromous life cycle in resident ruffe

Ruffe in contrast mostly stayed within estuarine isotopic limits. This narrow distribution of $\delta^{13}C$ data reveals a limitation for its migrations. Although ruffe is known to tolerate a wide range of environmental parameters (Svirgsden et al. 2015), the upper salinity tolerance is 10 - 12 ppt (Kottelat and Freyhof 2007). In our study, individuals were most caught within the oligohaline to freshwater parts (stations 2-5) of the Elbe with salinities between 0.3 and 6.1 and were nearly absent at the mesohaline station 1 (Medemgrund) (Tab S3.4). In estuaries ruffe benefit from the rich food supply, which can cause an improvement of individual growth rates compared to freshwater habitats (Hölker and Thiel 1988). However, as a freshwater species that potentially suffers from osmotic pressure due to fluctuating salinity levels (Svirgsden et al. 2015), ruffe must adapt to the prevailing environmental conditions in the estuary. To exploit estuarine advantages permanently, brackish water populations exhibit adaptations that are absent in freshwater populations. These include the production of larger eggs with higher yolk lipids and proteins (Svirgsden et al. 2015), greater salinity tolerances of embryos and the ability to utilize spawning sites with salinities of up to 6-8 ppt (Vetemaa and Saat 1996).

Seasonal $\delta^{13}C$ ranges of the ruffe we studied indicated only a rarely use of riverine food sources implying an uncrossed upstream barrier. Therefore, our results tend towards the existence of a brackish water ruffe population distinct from an upstream freshwater population. To support this suggestion further analyses of population genetics and physiological adaptations of ruffe in the Elbe River are required.

5.3. Annual fish migration and local habitat exploitation of flounder

Stable isotope approaches enable the tracking of movements and the habitat exploitation of fish species (Morais and Daverat 2016). Our findings uncover distinct life cycle strategies of flounder and ruffe on a spatiotemporal scale in the Elbe estuary.

For flounder, we could follow the facultative catadromous migration pattern during the juvenile and adult life stages (Fig. 3.6). Although larvae are not covered in our study, previous studies showed upstream movements via flood tide transport, whereby individuals are carried within the water body during high tide events and sink to the bottom during ebb tide (Bos et al. 1995, Bos 1999, Andersen et al. 2005, Trancart et al. 2012). Aquarium experiments with early life stages showed that larvae and juvenile flounders prefer low salinities or freshwater, which explains their migration behavior into freshwater areas during these life stages (Bos and Thiel 2006). In the Elbe estuary, flounder can thus migrate further upstream until they reach their retention area shown to be at Hahnöfer Nebenelbe and the Mühlenberger Loch (station 5), particularly between March and May (Möller 1984). This region is known as an important nursery area for fish in the Elbe estuary (Thiel 2011), where flounder larvae mainly feed on calanoid copepod *Eurytemora affinis* and oligochaete *Tubifex* spec. (Ladiges 1935, Bos et al. 1995). Bos et al. (1995) further suggested that the straightened water way of the main channel with reduced shallow areas leads to a limitation in larvae´s mobility. After further development, juveniles continue their migration towards upstream through the Hamburg port (Bos et al. 1995).

During our sampling, we caught the smallest flounder in summer and autumn, which is consistent with the observations of Möller (1984), who reported developed juvenile flounders of age group (AG) 0 first appearing in July in the Elbe estuary. Souza et al. (2013) observed a similar trend in autumn where 30% higher densities of juvenile flounder compared to other seasons shifted upstream to the tidal freshwater area of the Minho estuary, Portugal. Our flounder caught during that period previously exploited marine and estuarine sources as shown by our higher $\delta^{13}C$ values. Thus, we assume that AG0 had either not yet migrated further upstream or that individuals who had migrated had not yet returned. In contrast, $\delta^{13}C$ values of winter caught flounders were depleted, indicating intensive consumption of freshwater food sources. This riverine $\delta^{13}C$ signal therefore revealed a return migration of juvenile flounder from the upstream riverine area back to our study area. Previous studies also showed that young flounder frequently exploit upstream feeding areas in estuaries as low $\delta^{13}C$ values indicating their dependency on a freshwater-derived diet in early juvenile life stages (Selleslagh et al. 2015, Mendes et al. 2020). However, the food supply is limited during winter, which leads to a reduction in the food intake of juvenile flounder from daily rations > 20% of body weight to < 5% (Thiel 2001, 2011). Further, somatic condition and growth are limited due to low salinities (Gutt 1985, O´Neill et al. 2011, Mendes et al. 2020). However, this area provides less fluctuations in salinities which is generally known to be positively correlated with development and growth of young flounder (Andersen et al. 2005).

Juvenile flounders migrated downstream again by the end of winter, where they were mainly feeding on estuarine and marine food sources. The mechanisms behind this backwards movement remain unknown but understanding them may be crucial for comprehending understand the full life cycle of flounder in the Elbe estuary.

Even if our data indicate overwintering behavior, this result is not consistent with the findings of Bos et al. (1995), according to which juvenile flounders already occur in June upstream of the port of Hamburg as far as the Geesthacht weir. Accordingly, the upstream areas could not only be wintering areas, but also generally important nursery areas for flounder. In our spring sampling in 2022, we also found some individuals with riverine $\delta^{13}C$, but in a much smaller proportion than the juveniles caught in winter.

We conclude from this that some juvenile flounders may already be in the area upstream in spring, but that the majority mainly use the habitat in winter. As the studies by Bos et al. (1995) were carried out 30 years ago, recent detailed studies on the spatio-temporal habitat use of juvenile flounders are necessary to proof our assumptions.

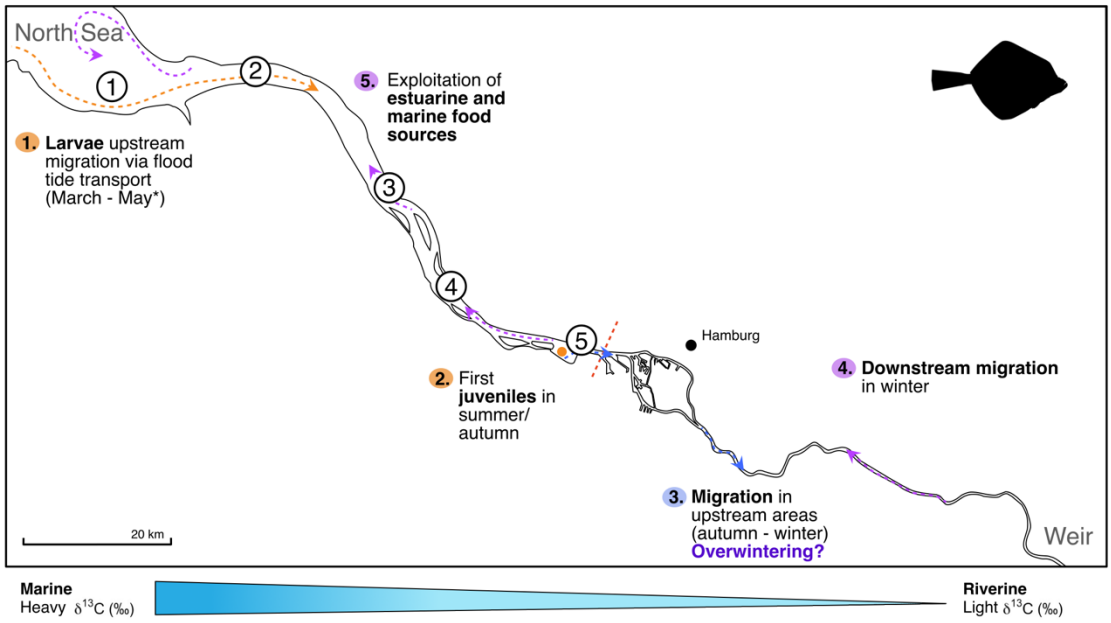


Fig. 3.6 Life cycle reconstruction of flounder in the Elbe estuary based on stable isotope data of seasonal $\delta^{13}C$ and literature (* Möller, 1984; Bos et al., 1995). Arrows point towards migration direction of fish, starting during early life stages in late winter/spring from the river mouth to Mühlenberger Loch (orange) Bos et al. (1995), potentially overwintering migration into riverine sections upstream of the city of Hamburg (blue), and backwards movement while exploiting estuarine to marine food sources (purple). Fish were caught during seasonal cruises at locations: Medemgrund (1), Brunsbüttel (2), Schwarztonnensand (3), Twielenfleth (4), Mühlenberger Loch (5).

Adult flounders used the estuary mainly between spring and autumn for feeding. During winter mature fish were nearly absent in the estuary due to spawning activities in the adjacent North Sea between January and April (Thiel 2011, Eick and Thiel 2014). The stable isotope ratios of adult flounder displayed that they fed on either predominantly marine or estuarine associated food sources, especially in spring and autumn. Hence, we observed two distinct strategies, and additionally two size classes of AG0 reaching the estuary (see section above) (Bos et al. 1995), which suggests diverging migratory behavior and could further indicate two flounder populations exploiting the Elbe estuary. This would further support the assumption of authors of former studies according to which two North Sea populations with distinct hatching areas use the estuary for nursery and feeding (Campos et al. 1994; Bos et al. 1995).

5.4. Habitat exploitation of resident ruffe

By contrast, ruffe showed a completely different isotopic pattern than flounder thus revealing different movement patterns and habitat exploitation. During their life ruffe undergo plenty of

movements that can vary by season and life stage but on shorter distances with higher site fidelities compared to flounder. Tracing precise migration patterns as shown above for flounder is not possible for the ruffe of the Elbe estuary, as the increased site fidelity requires a higher spatial resolution of the isotope baseline signals (e.g., Harrod et al. 2005), which our analysis not covered. Generally, juveniles conduct daily vertical migrations (DVM) from deeper to shallow inshore habitats in summer and overwinter in deeper waters (Gutsch and Hoffman 2016). Adults undertake, besides DVM, spawning and seasonal movements, mainly forced by abiotic conditions (e.g., current velocity, temperature, oxygen, salinity) and food availability (Gutsch and Hoffman 2016). Stable isotopes offer information about fish migration as mentioned above, but individual movements cannot be identified in high resolution by our analysis.

Since ruffe retreat to deeper areas during the winter months, their absence during our winter sampling could indicate that they migrate to the Hamburg harbor area and other deep waters. This behavior is typical for potamodromous Percidae, which usually overwinter in waters with great depths and weak currents (Chemagin 2023).

5.5. Fish migration and spatial connectivity

To characterize movements of flounder and ruffe, we analyzed spatial differences in $\delta^{13}C$ values of the two species. No significant difference in $\delta^{13}C$ would indicate a strong spatial connectivity and higher migration frequency of fish between the sections of the Elbe estuary. Our analyses already demonstrated an intensive migration behavior and wide habitat range of flounder in the Elbe estuary. These outcomes are also reflected by higher isotopic variations during the year. High spatial connectivity in flounder suggest high migration frequencies, especially during summer, when there is a high overlap of SEA_c and no significant differences of $\delta^{13}C$ between the sections. Flounders caught at the upper and lower sections had lowest connectivity to each other, indicating moderate migrations within the estuary. During springtime flounders from the upper section were isotopically isolated from flounders caught further downstream. From this we conclude that flounders inhabiting the transition area also use the adjacent Wadden Sea and salt marshes for feeding (e.g., Selleslagh and Amara 2008) which further can prevent intraspecific competition (Mendes et al. 2020).

In contrast, ruffe showed a markedly smaller isotope range with distinct local isotope ratios over time, caused by an overall smaller migration range and higher site fidelity than flounder. Especially in summer and autumn, the spatial connectivity of ruffe from the upper, middle and lower sections was low. This result can be confirmed by former studies, whereby ruffe have a generally narrow home range and only undertake larger migrations during the spawning season (e.g., Popova et al. 1998). The high connectivity in spring 2021, thus could be associated with the spawning season, which takes place in the Elbe estuary between March and May (Thiel and Thiel 2015). As the spawning activities of ruffe are temperature triggered (Thiel and Thiel 2015), the timing is subject to a certain variability, explaining why we could not detect spatial connectivity in the following spring 2022.

5.6. Ontogenetic niche shift

We observed increasing $\delta^{13}C$ and $\delta^{15}N$ values with increasing total length of generalist ruffe, reflecting a switch in prey from lower to higher trophic levels. Beside this ontogenetic shift, we found weak seasonal variations but a strong spatial effect, demonstrating the high site fidelity of ruffe in the Elbe estuary. Previous studies showed that juvenile ruffe of age groups 0 - 2 increasingly feed on zooplankton, mainly calanoid *Eurytemora affinis* and small mysids (Hölker and Thiel 1998). With increasing body size, brown shrimps *Crangon crangon* and small fish such as juvenile smelts (*Osmerus eperlanus*) and gobies (*Pomatoschistus* spec.) are also part of the diet (Hölker and Hammer 1994), which was visible by a larger diversity in stable isotope values during our study. Insects such as Chirinomidae or Trichoptera play a subordinate role in the diet, but small macroinvertebrates such as

Mysidae and Amphipoda are consumed in large quantities by ruffe in the Elbe estuary (Hölker and Thiel 1998).

Due to the large dispersion of the isotope data of the migratory flounder, we could not detect an ontogenetic shift isotopically, as the isotope values rather reflected the diverse local food sources and less the trophic shift of the prey organisms. However, other studies on feeding ecology also showed a switch from zooplankton to larger prey such as Crustacea and small fish (Thiel and Thiel 2015). The low site fidelity and high migration frequencies as well as different behavior of two flounder populations that arrive in the estuary with a time delay, hamper a more precise analysis of feeding behavior. Further studies with detailed baselines per site and supplementary genetic analyses to delineate different populations could further enlighten ontogenetic effects in estuarine flounder.

6. Conclusion

In this study, we were able to track annual migrations and habitat exploitation using stable isotope analysis in the Elbe estuary that was never conducted before. It turned out that the annual migration along the salinity gradient throughout the year, considering the total length of fish, is appropriate to track fish movements, but small-scale migrations can only be detected to a limited extent. However, our findings show that the migration patterns of flounder and ruffe are far more complex than previously assumed. These results should therefore contribute to a better understanding of population dynamics and to deriving better conservation strategies for the future.

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9. Supplements

Tab. S3.1 Number of juvenile and adult individuals of ruffe and flounder caught at lower (LS), middle (MS) and upper section (US) per season

Season	Ruffe						Flounder					
	Juvenile			Adult			Juvenile			Adult		
	LS	MS	US	LS	MS	US	LS	MS	US	LS	MS	US
Spring 2021	1	4	1	15	11	2	16	1	0	7	1	2
Summer 2021	0	1	19	14	19	14	15	4	8	2	5	2
Autumn 2021	1	1	9	6	17	9	47	21	15	15	7	14
Winter 2021/22	3	4	0	1	4	4	16	16	20	1	0	1
Spring 2022	2	14	2	15	15	20	49	7	16	12	2	0

Tab. S3.2 Model diagnostics of generalized additive models (GAM) of isotopic values $\delta^{13}C$ (family = gaussian, link function = identity) of flounder P. flesus that were chosen based on AIC, n = 322

Formula: $\delta^{13}C$ ~ s(TL_cm, k = 3) + Season + Section				
Coefficients	Estimate	Standard Error	t value	p-value
Intercept	-18.78075	0.68836	-27.283	0.000 ***
Summer 2021	0.72765	0.92564	0.786	ns
Autumn 2021	1.03616	0.77430	1.338	ns
Winter 2021/22	-2.96128	0.89186	-3.320	0.001 **
Spring 2022	-0.01139	0.78868	-0.014	ns
Middle section	-1.60571	0.53308	-3.012	0.001 **
Upper section	-4.40364	0.50026	-8.803	0.000 ***
R_2 (adj.) = 0.318		F- value = 2.547	GCV = 12.744	p -value = ns
Deviance explained = 33.5%		edf = 1.813	Scale est. = 12.395	
Formula: $\delta^{15}N$ ~ s(TL_cm, k = 6) + Section				
Coefficients	Estimate	Standard Error	t value	p-value
Intercept	16.9021	0.1010	167.429	0.000 ***
Middle section	0.3608	0.1971	1.831	ns
Upper section	-0.6046	0.1838	-3.289	0.001 **
R_2 (adj.) = 0.0477		F- value = 0.799	GCV = 1.8563	p -value = ns
Deviance explained = 5.66 %		edf = 1	Scale est. = 1.8333	

ns = not significant

Tab. S3.3 Model diagnostics of generalized additive models (GAM) of isotopic values $\delta^{13}C$ (family = gaussian, link function = identity) of ruffe *G. cernua* that were chosen based on AIC, n =227

Formula: $\delta^{13}C$ ~ s(TL_cm, k = 3) + Season + Section				
Coefficients	Estimate	Standard Error	t value	p-value
Intercept	-22.2559	0.3062	-72.694	0.000 ***
Summer 2021	0.1678	0.3398	0.494	ns
Autumn 2021	-0.2011	0.3639	-0.553	ns
Winter 2021/22	-0.2048	0.4718	-0.434	ns
Spring 2022	-0.6542	0.3292	-1.987	0.01 *
Middle section	-1.0570	0.2766	-3.821	0.000 ***
Upper section	-2.4611	0.3172	-7.759	0.000 ***
R_2 (adj.) = 0.426		F- value = 20.96	GCV = 2.4525	p -value = 0.000 ***
Deviance explained = 44.4 %		edf = 1	Scale est. = 2.3661	
Formula: $\delta^{15}N$ ~ s(TL_cm, k = 4) + Season + Section				
Coefficients	Estimate	Standard Error	t value	p-value
Intercept	19.1360	0.1885	101.532	0.000 ***
Summer 2021	-1.5118	0.2092	-7.228	0.000 ***
Autumn 2021	-1.6250	0.2240	-7.254	0.000 ***
Winter 2021/22	-0.6516	0.2904	-2.244	0.01 *
Spring 2022	-1.6836	0.2027	-8.308	0.000 ***
Middle section	0.1055	0.1703	0.620	ns
Upper section	-0.5541	0.1953	-2.838	0.001 **

R_2 (adj.) = 0.585	F- value = 104.9	GCV = 0.92943	p -value = 0.000 ***
Deviance explained = 59.8 %	edf = 1	Scale est. = 0.89667	

ns = not significant

Tab. S3.4 Isotopic niche width of flounder and ruffe using $\delta^{13}C$ and $\delta^{15}N$ from lower, middle, and upper section per season with n = number of individuals, TA = total area, SEA = standard ellipse area and SEA_c = corrected standard ellipse area. Overlaps were estimated using SIBER package in R with ~40 % CI.

Species	Season	Section	n	TA	SEA (‰²)	SEAc (‰²)	Overlap (%) Lower	Middle	Upper
P. flesus	Spring								
		Lower	84	63.1	12.6	12.8	/	16.4	0
		Middle	8	4.6	2.7	3.1		/	0
		Upper	16	9.4	3.7	4.0			/
	Summer								
		Lower	17	21.2	7.9	8.4	/	39.8	34.5
		Middle	9	13.7	8.4	9.5		/	41.9
		Upper	10	33.3	19.3	21.8			/
	Autumn								
		Lower	62	42.8	10.4	10.6	/	33.9	26.1
		Middle	28	36.7	14.2	14.7		/	32.7
		Upper	29	37.0	18.1	18.7			/
	Winter								
		Lower	17	39.3	16.2	17.3	/	19.2	26.0
		Middle	16	28.5	12.5	13.4		/	67.4
		Upper	21	31.7	12.0	12.7			/
G. cernua	Spring								
		Lower	30	13.2	4.1	4.3	/	17.8	1.2
		Middle	26	10.1	3.3	3.4		/	23.9
		Upper	22	7.3	2.3	2.4			/
	Summer								
		Lower	14	4.5	1.7	1.9	/	21.3	0
		Middle	19	9.0	4.0	4.2		/	≥ 0
		Upper	14	5.1	1.8	1.9			/
	Autumn								
		Lower	6	2.4	1.9	2.4	/	12.4	0
		Middle	17	18.2	6.7	7.1		/	0
		Upper	9	1.0	0.6	0.7			/
	Winter								
		Middle	4	1.0	1.4	2.1	/	/	49.1
		Upper	4	1.0	1.3	1.9			/

Tab. S3.5 Occurrence of ruffe, reflected as number of caught individuals (n) compared to salinity measured at each sampling season and station at different tides.

Station	Tide	Season									
		Spring 21	n	Summer 21	n	Autumn 21	n	Winter 21/22	n	Spring 22	n
1	Ebb	3.7 – 9.6	/	9.0 - 13.6	1	9.1 – 14.1	/	4.9 – 14.3	/	9.7 – 14.6	/
	Flood	4.2 – 11.1	/	10.7 – 15.0	/	9.0 – 15.1	/	4.5 – 11.0	/	9.2 – 13.41	/
2	Ebb	1.3 – 1.4	10	2.5 – 5.2	9	1.4 – 5.4	2	1.1 – 1.8	1	2.5 – 6.1	15
	Flood	0.56 - 1.4	6	1.6 – 4.4	4	1.4 – 4.6	5	0.69 – 1.6	3	2.2 – 3.7	2
3	Ebb	0.49	10	0.65 – 0.86	14	0.66 – 0.71	7	0.4 – 0.47	5	0.63 - 1	1
	Flood	0.47 - 0.5	/	0.6	6	0.63 – 0.68	11	0.46 -0.48	3	0.53 – 0.8	3
4	Ebb	0.39	2	0.48 – 0.53	4	0.48 – 0.58	1	0.38 – 0.43	1	0.45 – 0.49	13
	Flood	0.37	/	0.45 – 0.52	4	0.47 – 0.56	2	0.38	/	0.44 – 0.47	15
5	Ebb	0.31 - 0.32	/	0.46 – 0.46	10	0.44 – 0.45	5	0.28 – 0.38	1	0.44	9
	Flood	0.31	1	0.46 – 0.47	15	0.44 -0.45	10	0.41- 0.42	2	0.44 -0.45	9

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Chapter 4 Seasonal fish microbiota

Spatio-temporal plasticity of gill microbiota in estuarine fish

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Spatio-temporal plasticity of gill microbiota in estuarine fish

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Key words: 16S, Metabarcoding, Network analysis, Hypoxia, Holobiont

Abbreviations:

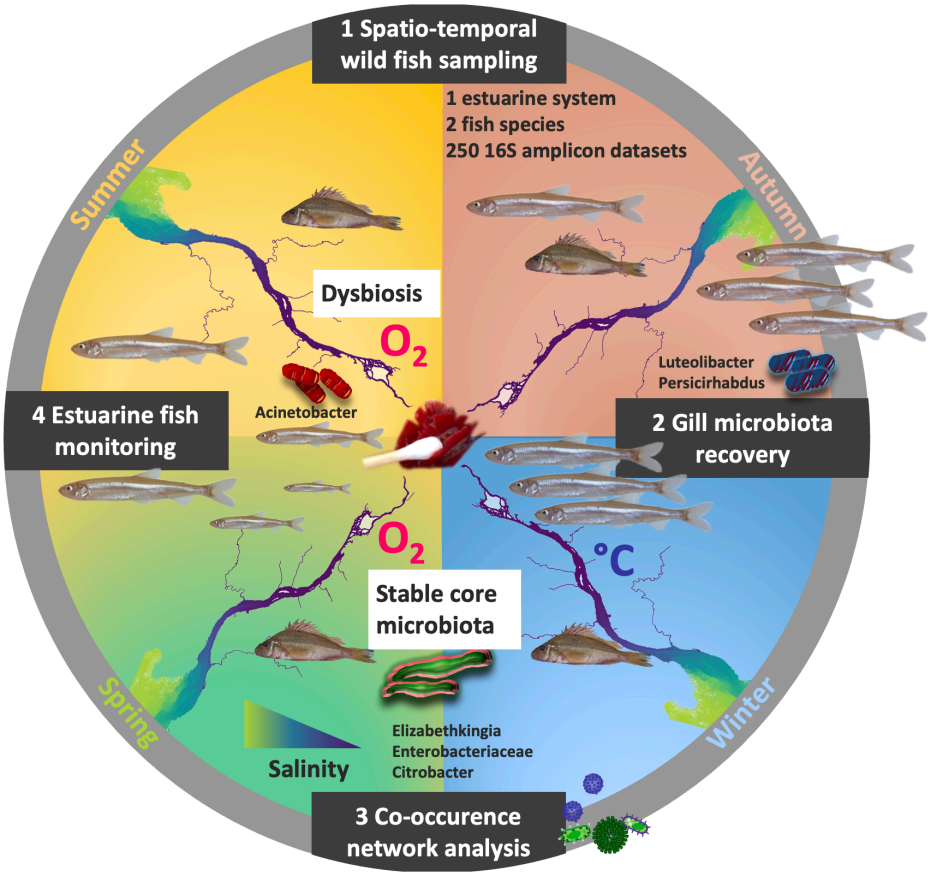
SSU Small subunit rRNA**,**
WGCNA Weighted gene correlation network analysis,
DO dissolved oxygen, TOC total organic carbon, SPM suspended particulate matter
HSI hepato-somatic index, SSI spleno-somatic index,
FCF Fulton's condition factor
OE *Osmerus eperlanus,* GC *Gymnocephalus cernua,* WF water filter = bacterioplankton

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1. *Abstract*

Coastal marine and estuarine systems are subject to enormous endogenous and exogenous pressures, particularly climate change, while at the same time being highly productive sources and nurseries for fish populations. Interactions between host and microbiome are increasingly recognized for their importance for fish health, with growing evidence indicating that increasing environmental pressures impact host resilience and favor the raise of opportunistic bacterial taxa. The microbial composition of the gill mucus reflects environmental conditions and represents an entry route for pathogens into the fish body. High-throughput sequencing of prokaryotic populations from 250 samples of two fish species with highly different habitat preferences, as well as seasonal and spatial distributions in the Elbe estuary system, allowed us to describe the variation of the microbiota along a salinity gradient and under fluctuating environmental conditions. The analysis of estuarine fish core microbiota in relation to variable bacterial components indicated dysbiotic states under sustained hypoxia and high nutrient loads largely driven by increased prevalence of facultatively aerobic (*Acinetobacter*) and anaerobic heterotrophs (*Shewanella*, *Aeromonas*). By correlating bacterial abundances with environmental and physiological parameters in a co-occurrence network approach, we describe plasticity in microbiota composition, identify potential biomarkers for fish health monitoring and reconstruct movement patterns of the fish. Our results can help to shape future minimal-invasive and cost-effective monitoring programs, and identify factors that need to be controlled in the estuary to promote fish and stock health.

2. *Graphical abstract*



3. Introduction

Communities of microbes colonize the mucosal surfaces of fish from hatching on, influencing the health of their host (Legrand et al., 2020) and responding as holobiont to seasonal variation in nutrients, salinity and temperature. Physiological stress however, impacting the multidirectional interaction between host and microbiome, might create opportunists or pathogens from otherwise commensal species (Kelly & Salinas, 2017). Changes in salinity (François-Étienne et al., 2023), temperature (Ghosh et al., 2022; Matanza & Osorio, 2018; Morshed & Lee, 2023; S. Yang et al., 2022) and dissolved oxygen (DO) (Boutin et al., 2013; Fan et al., 2020; Shi et al., 2021; Song et al., 2023; Wang et al., 2021) have been shown to impact both the host physiology and the microbiota composition. Studies indicating hypoxia-induced immune suppression make it difficult to assess the impact on fish populations in the light of climate change (Abdel-Tawwab et al., 2019; Leeuwis et al., 2024). Globally, increased frequency of extreme events and shifts in climate patterns are associated with impaired host resilience and disease outbreaks related to microbial imbalances and raise of opportunistic taxa in aquaculture (Samsing & Barnes, 2024), marine (Egan & Gardiner, 2016), freshwater (Esteve et al., 2017) and estuarine systems (Suzzi, Stat, Gaston, Siboni, et al., 2023).

Estuarine ecosystems constitute important nursery areas and population sources for fish (Pasquaud et al., 2015; Seitz et al., 2014; Tournois et al., 2017), and are characterized by intense fluctuations in these physicochemical conditions (Elliott & Quintino, 2007). Climate change is expected to increase frequency and intensity of heatwaves, oxygen minimum zones (OMZ) and change salinity regimes affecting inhabiting fish fauna (Breitburg et al., 2018; Cottingham et al., 2018; Diaz & Rosenberg, 2008; Lauchlan & Nagelkerken, 2020; Little et al., 2017; Sampaio et al., 2021). The Elbe estuary in Germany, is one of the largest in Europe and has long been exposed to endogenous pressures. For example, over the last decades the Elbe has experienced increasing turbidity due to dredging (Heininger et al., 2015; Reese et al., 2019; van Maren et al., 2015), expansion of oxygen minimum zones (OMZ) (Colombano et al., 2021), intensified stratification of the well-mixed water column and eutrophication (Pein et al., 2021). The combined effect of these various stress factors is associated with a drastic decline in fish biomass in the system of more than 90% over the last decade (Koll et al., 2024; Scholle & Schuchardt, 2020; Theilen et al., 2024).

Knowledge on fish associated microbiota is largely biased towards farmed fish and gastrointestinal communities (Egerton et al., 2018). Only a few studies, all but one dealing with internal microbiota, have focused on the highly productive intersection between land and marine ecosystems in estuaries (de Macedo et al., 2024; Koll et al., 2024; Suzzi et al., 2022; Suzzi, Stat, Gaston, & Huggett, 2023; Suzzi, Stat, Gaston, Siboni, et al., 2023; Wei et al., 2018). Although the external microbial composition, with its immediate exchange with the environment, has a huge potential for monitoring, studies including external microbiomes of wild fish are mostly focused on marine or freshwater habitats (Amill et al., 2024; Itay et al., 2022; Minich et al., 2020, 2022; Pratte et al., 2018; F. É. Sylvain et al., 2020; Varela et al., 2024). The fish gill, with critical roles in respiration, osmoregulation and waste exchange (Evans et al., 2005), forms not only a unique habitat for the microbes but as mucosal surface further constitutes a route for pathogen invasion (Pratte et al., 2018; Salinas & Magadán, 2017). A gill associated lymphoid tissue (GIALT) (Salinas, 2015) composed of adaptive and innate immune cell populations enables multidirectional interaction between microbes and host (Gomez et al., 2013; Kaetzel, 2014) and discrimination between bacterial taxa keeping the microbiome in homeostasis (Nakanishi et al., 2015; Yu et al., 2021).

In this study, we aimed to determine the spatio-temporal variation in bacterial composition in a seasonal time-series of estuarine fish gill microbiota. Here we focus on two fish species with (1) highly different life-styles and habitat usage and (2) high abundance and key function to create a larger picture on the underlying dynamics in the estuarine system. Anadromous smelt (*Osmerus eperlanus* L.) is a key species in food webs of a number of European riverine ecosystems (Illing et al., 2024) and

constitute up to 96% of the fish abundance in the Elbe estuary (Eick & Thiel, 2014; Theilen et al., 2024). They begin annual spawning migrations to estuarine freshwater sites at the end of their second year of life (Thiel & Thiel, 2015) gathering in autumn at the mouth of the estuary and migrating upstream as temperatures drop to 3 - 6°C (Freyhof & Kottelat, 2007). Within a month they reach spawning grounds upstream Hamburg and spawn until march (Borchardt, 1998; Eick, 2015; Thiel & Thiel, 2015). Hatched larvae drift to shallow habitats downstream of the port, where oxygen deficiency can increase mortality (Diercking & Wehrmann, 1991; Thiel et al., 1995; Thiel & Thiel, 2015). Adults avoid low-oxygen areas and accumulate where oxygen exceeds 5 mg/l (Möller & Scholz, 1991). In spring, all life stages occupy the estuary before the majority of adults migrate to the ocean (Thiel & Thiel, 2015). The ruffe (*Gymnocephalus cernua* L.) on the other hand, is a rather undemanding bentho-pelagic freshwater species common in oligo- to mesohaline (0 - 12 psu) and eutrophic estuaries (Gutsch & Hoffman, 2016). It is the third most common fish in the estuary (Eick & Thiel, 2014) and important benthic predator of invertebrates (Hölker & Thiel, 1998). Ruffe migrate to shallow waters in summer and deeper areas in winter (Gutsch & Hoffman, 2016). They mature at 1 - 3 years and spawn in early and possibly again in late summer in warm waters (> 12°C) where embryos require high oxygen levels (Gutsch & Hoffman, 2016; Ogle, 1998).

Recent studies demonstrated the special importance of time-series in contrast to single measurements to gain overview of biotic interaction and drivers that shape it (Minich et al., 2020; Scheifler et al., 2022). We generate here a comprehensive spatio-temporal dataset of the bacterial community via 16S rRNA sequencing linked to environmental conditions and physiological fish data. The objective is to study the responsiveness and plasticity of the microbiota under multiple pressures, especially along a salinity gradient and under deoxygenation and eutrophic conditions. Therefore, we determine core taxa and variable components in the microbial composition and relate them to driving forces as means of determining disruptive situations and biomarkers for life history and potentially the overall health situation of fish stocks in this estuarine habitat.

4. Material and methods

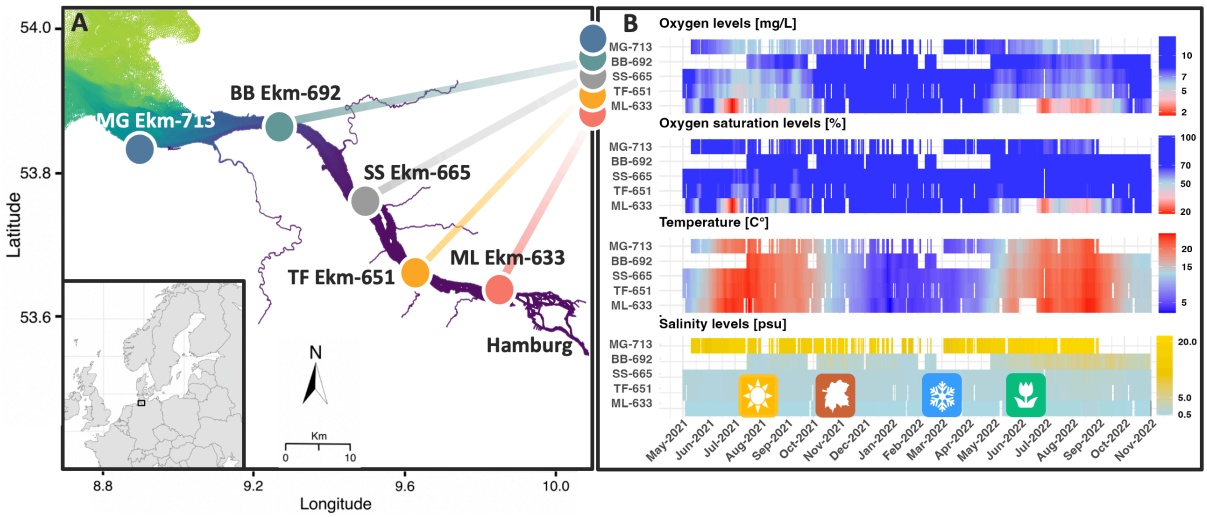


Fig. 6 Overview map of sampling locations along the course of the estuary (A) and abiotic conditions (dissolved oxygen levels, oxygen saturation levels, temperature and salinity) in the water column in a seasonal and spatial view from automated monitoring systems. Sampling stations are marked by colored dots while seasonal sampling periods are indicated by symbols.

4.1. Sample Collection

Fish were caught with a stow-net fishing vessel (opening area of 135 m², mesh size of 10 mm at the cod end) at five stations along the Elbe estuary (Fig. 1) at all four seasons. Sampling stations were chosen within different estuarine sections classified by dominating abiotic drivers (Amann et al.,

2012): Station ML-Elbe kilometer 663 and TW-Ekm 651 within the OMZ (stream km 620 – 650, low oxygen, summer < 2 mg/L, salinity < 0.5 psu), stations SS-Ekm 665 and BB-Ekm-692 within the maximum turbidity zone (MTZ) (stream-km 650 – 705, high loads of suspended matter, salinity 0.5 ~ 5 psu) and station MG-Ekm 715 within post-MTZ (stream km 705 – 730, transition full marine, salinity 1 ~ 20 psu). Sampling procedures followed the standards described in the German Animal Welfare Act (§4 TierSchG). Exemptions to the ordinances on nature reserves were obtained (see Permits). Seven individuals per species (when possible) from ebb and flood hauls at each station (OE n = 129, GC n = 90) were processed immediately on board in the following standardized manner: Fish were measured and weighed before recovering gill bacteria with sterile cotton swabs from the middle part of the second and third arch. White muscle tissue from the dorsoventral site of each individual was dissected and rinsed with distilled water for latter stable isotope measurements. Bacterioplankton samples (n = 20, 500 ml) obtained from the water column were vacuum filtered onto 0.2 µm polycarbonate membranes. All samples were kept on dry ice on board until they were moved to -80°C until further processing. Whole fish samples were frozen at -20°C and further analyzed in the lab: body indices (Fulton's body condition (FCF), hepato-somatic - (HSI), spleno-somatic (SSI) and gonado-somatic indices (GSI)) were determined (Petitjean et al., 2020), stomach content was weighed and age determination from otoliths and scales was performed. Abiotic conditions (oxygen, salinity, Secchi depth, temperature, pH) were measured at start and end of each haul using a multi-probe (Hanna HI 9829 and Secchi disc) at the water surface. In addition, abiotic data (PO_4, NH_4, NO_3, NO_2, suspended particular matter (SPM), O_2, pH) from continuous national measurement programs (www.fgg-elbe.de) were downloaded for appropriate times and stations close by where the fishing took place and included in the analyses.

4.2. DNA extraction & sequencing

DNA extraction, sequencing and bioinformatics were performed as explained elsewhere (Koll et al., 2024). In short CTAB/chlorophorm/phenol extraction was performed followed by amplification of the V3-V4 variable regions of the 16S rRNA gene in a one-step PCR using the primer pair 341F-806R (dual-barcoding approach (Kozich et al., 2013); primer sequences: 5'-CCTACGGGAGG-CAGCAG-30 and 5'-GGACTACHVGGGTWTCTAAT-30). PCR-products were verified via gel electrophoresis, normalized (Sequal Prep Normalization Plate Kit; Thermo Fisher Scientific, Waltham, USA), equimolar pooled and sequenced on a MiSeq platform (MiSeqFGx; Illumina, San Diego, USA) with v3 chemistry (2x300 bp). The settings for demultiplexing were 0 mismatches in the barcode sequences.

4.3. DNA Bioinformatics

Microbiota analysis was performed on the University Hamburg Hummel high performance cluster. Read files were quality controlled, adapter, quality and length filtered via TrimGalore (v 0.6.10) (Krueger, 2015). All downstream analyses were performed in R (v.4.3.0) using visualization packages ggplot2 (v.3.4.2) and cowplot (v.1.1.1). For Amplicon sequence variant (ASV) prediction and taxonomic identification DADA2 (v.1.29.0) (Callahan et al., 2016) was used. Quality profiles of paired reads were inspected and truncated at 270 and 190 for forward and reverse reads. ASV inference was performed with pooling method and taxonomy assignment used the SILVA SSU v138 taxonomic database (Quast et al., 2013). To ensure contamination free and accurate analyses, mock community samples (ZYMO research) were added along the whole process from DNA extraction until taxonomic assignment (Fig. A. 7). ASV table and sample data were parsed to phyloseq (v 1.45.0) (Mcmurdie & Holmes, 2012) removing ASVs taxonomically assigned to non-bacteria. Low abundance taxa in each of the individual fish and bacterioplankton datasets were filtered at a value reducing the number of zeros in the datasets by ca. 50% while minimally reducing the number of overall counts (Fig. A. 1) to improve interpretability and minimize the risk of spurious correlations. The filtering value was determined as sum of counts lower than 0.005% of total sum of all counts. Alpha diversity measures were calculated via vegan package (v. 2.6.4) (Oksanen et al., 2022) and the core microbiota were determined from

relative abundance data via microbiome package (v. 1.23.0) (Lahti & Shetty, 2017) with filtering detection threshold to 0.0001% within samples and 90% prevalence. Centered log-ratio (CLR) transformation to the ASV matrix was applied following best practices for handling of compositional data (Gloor et al., 2017). The transformed data were used for visualization and network analyses.

4.4. Stable isotope analysis

Additional stable isotope analysis of $\delta^{13}C$ was conducted to determine movement directions of investigated fish. Tissue samples were freeze-dried for 24 hours and grinded to powder using a cell lyser. Samples were weighed (0.8 – 1.2 mg) and folded into tin capsules (Hekatech). $\delta^{13}C$ ratios in permille (‰) were measured by the UC Davis Stable Isotope Facility of the University of California using a continuous flow isotope ratio mass spectrometer (IRMS) PDZ Europa ANCA-GSL elemental analyzer interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK). Ratios are expressed relative to the international standard VPDB (Vienna Pee Dee Belemnite) for carbon using the delta notation (δ^{13}) (Fry, 1988, 2013). We predicted estuarine $\delta^{13}C$ ranges for resident fish using stable isotope information of prey specimens of mysid shrimp from MG-Ekm 713 and ML-Ekm 633, which inhabit distinct $\delta^{13}C$ ratios that enables spatial determination of food origin (Guelinckx et al., 2006). Stable isotope ratios of mysid shrimp were pooled, and median and standard deviations were calculated. Due to the carnivorous feeding preferences of mysids in estuaries (Modéran et al., 2012), we assume similar trophic levels as in fish, thus trophic enrichment factors on the data could be neglected during our analysis. $\delta^{13}C$ above and below these limits indicated riverine and marine derived sources that was stored in the flesh of the consumer.

4.5. Statistical analyses

First, we visualized the gill bacterial community according to the fish species, sampling season and location using stacked barplots (Fig. 1). Then we computed PCoAs based on pairwise averaged subsampled Bray-Curtis dissimilarity between all gill mucus and bacterioplankton samples (N = 245) to study how the sample cluster according to origin and season. The distance matrix was further used to test for significant differences in community structure between the aforementioned factors via permutational analyses of variance (PERMANOVA) with 999 permutations. R vegan package and post hoc pairwise t-test pairwiseAdonis (P. Martinez Arbizu, 2020) were applied.

Secondly, we identified bacterial biomarkers on ASV level discriminant for bacterioplankton or gill mucus assemblage, the host species identity, the seasonal influence as well as selected locational effects. We used the *multipatt* function from the *IndicSpecies* package (v 1.7.14) (De Cáceres & Legendre, 2009) with indicator value > 0.7 and P-value < 0.05 after 999 permutations based on the read abundance of the ASV table with internal correction for group size inequality. Relative abundance (%) of these biomarkers are shown in the supplementary material Tab. A. 4, relative abundance of overall ASV counts were visualized as heatmap in Fig. A. 2B.

Thirdly, we analyzed the response of the fish mucus communities to seasonal and spatial gradients and in relation to physiological measurements first by computing average Bray-Curtis distance-based redundancy analyses (dbRDA) (Legendre & Anderson, 1999) via vegan *capscale* function following the workflow described in (F.-É. Sylvain et al., 2022). Environmental and physiological variables were selected by stepwise model building for constrained ordination (*ordistep*) and assessment of multicollinearity between variables by measuring variance inflation factors (VIF) keeping *ordistep* selected parameters with VIF < 10 as explicative variables. Association strength between explicative variables and bacterial assemblage was then measured by fitting environmental vectors on the reduced model ordination using vegan *envfit* (Tab. A. 3).

Finally, Mantel test was used on the distance matrices to gain initial overview of association between fish mucus assemblages, bacterioplankton communities and environmental and

physiological measures (Fig. 4E, Fig. A. 4B). We than applied a weighted gene co-expression network analysis (WGCNA) (v.1.77-1) (Langfelder & Horvath, 2008) to infer networks of co-abundant bacterial taxa and relate them to physiological and environmental data in an integrated heatmap analysis approach (Koll et al., 2024; Strand et al., 2021) (Fig. 5, Fig A 6 & 7). Parameter list: networkType = "signed", TOMType = "signed", corType = "bicor", minModuleSize = 3, minKMEtoStay = 0.5, deepSplit = 2/3 (for fish mucus / water filter WF respectively), mergeCutHeight = 0.15/0.3 (Fish/WF), maxPOutliers_value = 0.05/0.1 (Fish/WF). Initial module detection by hierarchical clustering is controlled by deepSplit parameter (1 - 4, last being most sensitive). The minModuleSize defines the minimum size for the initial module where ASVs with correlation to the module eigenmode (KME) smaller than minKMEtoStay are removed. Different modules whose eigennodes correlate higher than 1 – mergeCutHeight are merged. The parameters chosen here seek to detect modules with highly correlated nodes but not losing interesting profiles represented by only a few taxa. The parameters are adjusted to account for the different sampling sizes. Module eigengenes/ASVs were correlated between the different networks and to physiological traits of the fish and external abiotic factors via Pearson correlation and corrected for multiple testing via FDR.

5. Results

Approximately 15 million trimmed, filtered and merged reads (mean 58,000 reads) across 139 *O. eperlanus* (OE), 89 *G. cernua* (GC) gill mucus and 20 bakterioplankon (WF) samples resulted in 42.000 ASVs. The datasets were divided for filtering, retaining 1552, 1393, 2364 ASVs for the individual datasets. Species accumulation analysis indicated that by the exclusion of rare taxa the microbiota were sufficiently captured in the datasets (see Fig A.1).

5.1. Bacterial gill communities deviate from bacterioplankton

Gill mucus communities were dominated by six phyla (> 1% overall abundance) including Proteobacteria (54%), Bacteroidota (27%), Actinobacteriota (7%), Firmicutes (5%), Verrucomicrobiota (4%), Deinococcota (2%) at similar proportions between species (Tab. A.1). The bacterioplankton in contrast was composed of additional phyla Desulfobacterota (1.5%), Acidobacteriota (2,3%), Cyanobacteria (1,4%), Nitrospirota (1,9%), Gemmatimonadota (1,4%), Chloroflexi (1,2%) but lacked high abundances of Firmicutes (0.3%) and Deinococcota (0.1%).

Analyses of alpha diversity showed significantly higher richness and Shannon indices in free-living bacteria samples compared to the fish mucus communities, but no overall significant difference between the two fish species (Fig A. 3A & B)

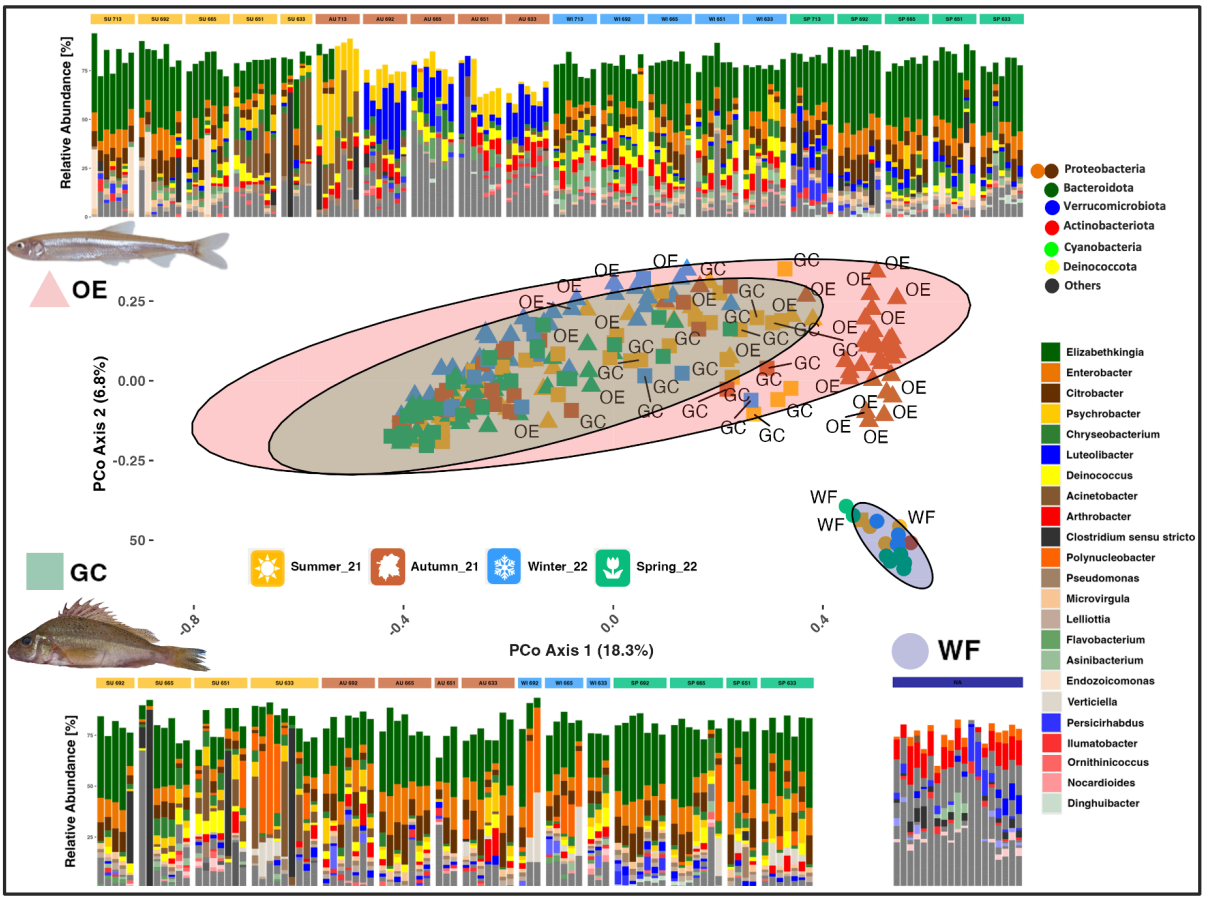


Fig. 7 Overview of spatio-temporal patterns in bacterioplankton and bacterial gill mucus communities. Principal coordinates analyses (PCoA) of gill bacterial community based on averaged subsampled Bray-Curtis dissimilarity (middle panel). Ellipses display the sample kinds (OE, GC, WF), coloration by season of sampling. Stacked barplots (top & bottom panel) depict relative abundance of the most abundant genera within the three biomes (OE upper panel, GC & WF bottom panels). Samples are ordered by season and faceted sampling station along the course of the estuary (left = most downstream, right = most upstream). Coloration follows a phylum code (top right legend) and individual colors by genus (right legend, grey resembles unspecified taxa). The 34 overall most abundant genera are shown.

The principal coordinates analysis in Fig. 2 showed a strong distinction between the bacterioplankton and the fish gill mucus community along Axis 1 and 2 summarizing 18.3 und 6.8% variance. PERMANOVA indicated a significant distinction between fish (OE: $F_{1, 143} = 28.775$, $R^2 = 16\%$, $p = 0.001$, GC: $F_{1, 103} = 33.134$, $R^2 = 25\%$, $p = 0.001$) and bacterioplankton samples.

Presence-absence analyses (Fig. A 2A) indicated that roughly half of the detected ASVs (> 1600) are unique to the bacterioplankton while about 20% (ca. 500) are shared between both fish and surrounding water. The shared taxa make up about 50 ± 5% in relative abundance on all three biomes. Taxa with the highest overlap between the biota (> 0.5% relative abundance in both) comprise *Luteolibacter*, *Persicirhabdus*, *Flavobacterium*, *TRA3-20*, *Ilumatobacter* and *Halioglobus*, adding *Polynucleobacter* between GC and bacterioplankton (Tab. A. 2). Between free-living and fish-associated biota 1447 and 385 bacterial biomarkers at ASV level were determined, for the latter however the strains with significant abundances (> 0.2% relative abundance) constituted all core taxa in the fish mucus (see next chapter).

Although significantly distinct ($F_{1, 224} = 5.8978$ $R^2 = 2.6\%$ $p = 0.001$), the two fish species showed a strong overlap with largest distinction indicated by the seasonal sampling along Axis 1. OE samples collected in autumn formed a prominently distinct cluster. Presence-absence analyses indicated that both species share more then 1000 ASVs while about 474 were unique to OE and 326 were unique to GC. Comparing the seasonal samplings for each species, relatively low amounts of ASVs (< 5%) were

unique to single seasons. Comparing the fish species, no indicator taxa were specific to OE, and only strains of *Verticiella*, *Polynucleobacter* and *Candidatus Megaira* were determined specific for GC at relevant abundance levels (> 0.2%). In the temporal-spatial comparison within each fish species, few taxa appeared significant, only *Acinetobacter* strains were determined indicative for summer conditions in the estuary in both fish associated communities (see Tab. A. 4).

5.2. Stable estuarine core gill microbiota

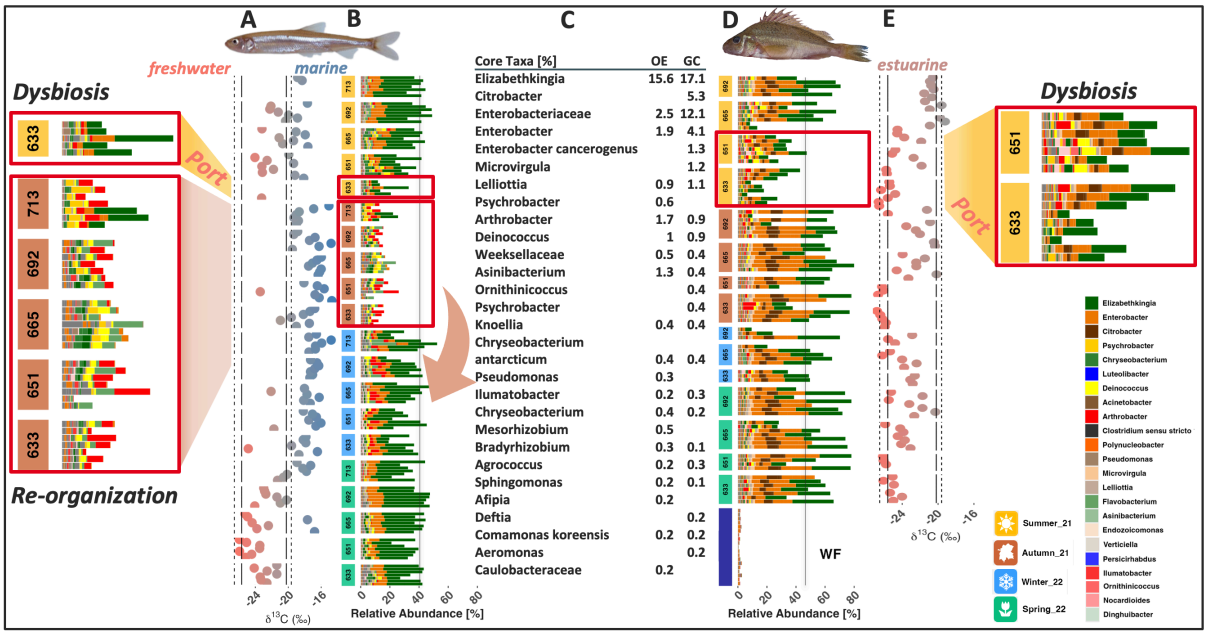


Fig. 8 Core microbiota of Elbe estuarine fish. Microbial taxa present in 90% of the samples per species respectively and their contribution to the overall microbial community per sample are shown on the stacked barplots (**B** & **D**). Sample groups are ordered by season and facets within season by sampling location (mesohaline Ekm 713 to freshwater Ekm 633). The right (**D**) panel also contains the relative contributions to the bacterioplankton from seasonal and spatial sampling (dark blue). Areas marked by red squares and enlarged depict sampling groups with deviating abundance in bacterial core taxa. The most abundant taxa on lowest determined taxonomic level and their overall relative contribution (> 0.2% relative abundance) to the respective datasets are shown in list (**C**) for both species respectively. The stable isotope signal for δ^{13} C from fish muscle tissue (**A** & **E**) indicates in which region inside and outside the estuary the individual animals fed. The limits of the estuary are -20.2 ± 0.6 (lower reaches) and -25.6 ± 0.9 (upper reaches). Values above this come from the marine, below this from the freshwater reaches upstream.

The core microbiota (determined by prevalence) over seasonal and spatial samplings comprised 27 ASVs from 13 orders (21 genera) accounting for 30 ± 12% in OE and 64 ASVs from 11 orders (30 genera) accounting for 50 ± 22% of the overall relative microbiota abundance in GC (Fig. 3). Only a fraction of these taxa was present in the bacterioplankton (33% OE, 20% GC) accounting for < 1% of the bacterioplankton relative abundance (Fig. 3D). *Elizabethkingia*, the dominant bacterial taxon in the fish microbiota accounts for only 0.06% of the relative abundance in the free-living community. The gill mucus core microbiota of both fish species were composed by only four phyla, in GC Proteobacteria (almost entirely composed of Enterobacterales) made up 56% followed by Bacteroidota (almost entirely represented by Flavobacterales) with 36% and Actinobacteria 5% and Deinococcota 2%. In OE Bacteroidota made up 60% followed by Proteobacteria 27%, Actinobacteria 9%, Deinococcota 3%. OE showed a strong seasonal variation in autumn samples driven by the low abundance of Enterobacterales and *Elizabethkingia*. Most prominent, both fish species showed a strong decline in the abundance of core taxa in freshwater and Hamburg Port area only in summer (Ekm 651 – 633) (OE 18%, GC 22%) (Fig. 3B & D, highlighted).

5.3. Drivers in gill mucus bacterial composition

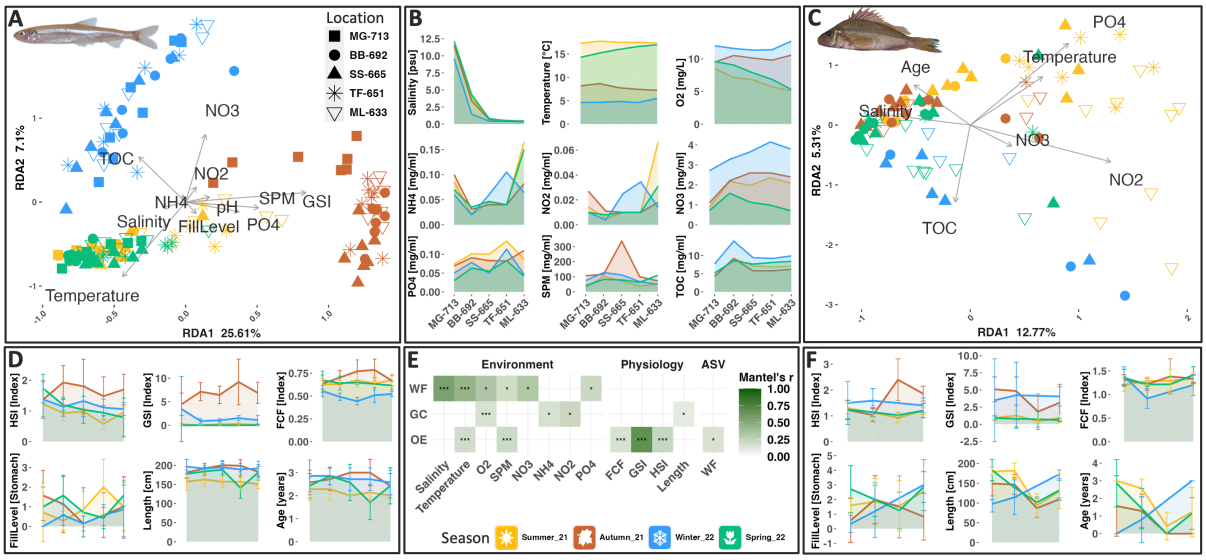


Fig 9 Environmental and physiological drivers in gill mucus bacterial composition. Average Bray-Curtis distance-based redundancy analyses (dbRDA) on gill mucus bacterial community in two host species *O. eperlanus OE* (**A**) and *G. cernua GC* (**C**). Datapoints indicate 16S rRNA gill mucus microbiota samples colored for season of sampling while the shape indicates sampling location along the course of the estuary. Physiological and environmental samples were selected by stepwise model building for constrained ordination (ordistep) and VIF < 10. Abiotic data from water column in spatio-temporal course are depicted (**B**). TOC stands for total organic carbon (mg/L) and SPM (suspended particular matter). Oxygen levels were removed from dbRDA by VIF control from both analyses for multicollinearity with temperature and nutrient loads. Biometric data sampled per fish species in spatio-temporal course (**D** & **F**). GSI (gonado-somatic index), HSI (hepato-somatic index), FCF (Fulton's condition factor). Global matrix correlation between fish gill microbiota and bacterioplankton to environmental and biometric data as well as between bacterioplankton ASV matrix (WF) and the fish gill microbiota matrices (OE, GC) tested by Mantel test (**E**). Significance levels are depicted as < 0.05 (*), < 0.01 (**) and < 0.001 (***).

Redundancy analysis revealed that bacterial composition in the anadromous species *O. eperlanus* was strongly influenced by seasonal effects in environmental and biometric measures while a more pronounced spatial pattern appeared in the stationary species *G. cernua* (Fig. 4 A & C). The variances explained by the first two RDA dimensions varied considerably between the two species (OE 32% and GC 17%). Fitting environmental variables (envfit results Tab. A. 2) showed significant effects of temperature, salinity, PO_4, NO_3, NO_2 and TOC on the gill mucus microbial communities in both species. OE samples showed a strong seasonal clustering for autumn along SPM, PO_4 and GSI values, winter samples aligned along TOC and a combined cluster of spring and summer samples group along temperature where samples from the upper estuary (ML-633, TW-651) deviated with PO_4. GC samples instead showed a stronger overlap for seasonal effects, again summer samples from the upper estuary (ML-633, TW-651) deviated strongest along with PO_4, NO_2 and Temperature axes. Interestingly physiological parameters other than age in GC and GSI in OE, were not significantly correlated to the bacterial composition in the parametric analysis. In addition, we evaluated the association between bacterial sample distances and physiological and environmental data as well correlation between bacterioplankton and fish mucus communities via individual non-parametric Mantel tests (Fig. 4 E, all results Tab. A 3). The results showed associations (r = 0.20 – 0.55) between bacterioplankton and environmental parameters (salinity, temperature, O_2, SPM, NO_3, PO_4), most pronounced for the first two. For both fish species however association to environmental data were weaker and distinct. GC was correlated with O_2, NH_4 and NO_2, while OE was correlated with Temperature and SPM. Related to physiological data OE was most strongly correlated with GSI (r = 0.67) and associated FCF and HSI, while in GC there was a weak correlation with length measurements. There was no global association between the bacterioplankton community and GC, and a slight overall trend between OE and free-living bacteria.

5.4. Movement patterns & microbiota plasticity

The stable isotope ratios of $\delta^{13}C$ from fish muscle reflects their long-term feeding preferences of several weeks to months depending on metabolic activity of the tissue and growth rates of the fish (Buchheister & Latour, 2010) that further enables the reconstruction of migration. These data indicated that OE gathering in the estuary autumn just arrived from the North Sea with $\delta^{13}C$ ‰ values above -20 (Fig. 3A). As described above, the bacterial core taxa in this group were rare (Fig. 3B), while spatio-temporally resolved mantel tests indicated a significant association to the bacterioplankton communities (Fig. A. 4B). Analyzing the bacterial taxa shared with the estuarine bacterioplankton while excluding the core taxa in the fish mucus community, showed a steady increase in abundance in upstream direction from 15% in the estuarine mouth to 26 – 29% in the middle section until 47% in the harbor area (Fig. A. 4A). OE caught in winter (3 months after the autumn animals) completed the spawning process (as indicated by low GSI indices) but still showed a strong marine isotope signal. The core microbiota and abundance of shared taxa with bacterioplankton however aligned to summer and spring animals (Fig. 3 A & B). In contrast, the stable isotope signal range in GC samples was within the boundaries of the estuary the whole year (between -20 $\delta^{13}C$ ‰ in lower estuary and -24 $\delta^{13}C$ ‰ in upper estuarine areas) and showed a strong spatial pattern for the sampling stations along the course of the estuary (Fig. 3E).

5.5. Bacterial network response

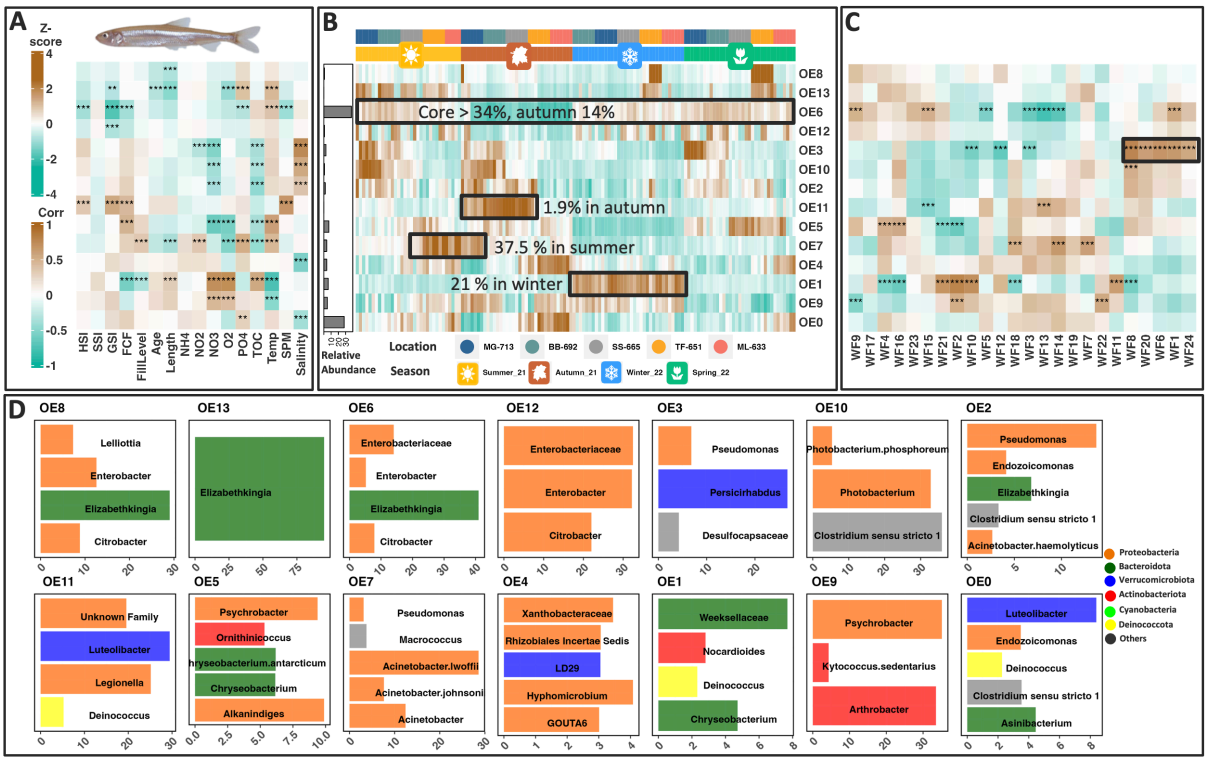


Fig. 10 Relationship between bacterial co-occurrence networks, biometric measurements and abiotic conditions for Elbe estuarine smelt OE. Integrated heatmap of gill bacterial mucus network analysis showing correlation between ASV module eigengenes (**B**: OE-0-13), physiological traits (**A**: HSI, SSI, GSI, FCF, fill level, age, length) and relevant abiotic factors (**A**: NH_4, NO_2, NO_3, O_2, PO_4, TOC, temperature, SPM, salinity) as well as bacterioplankton module eigenASVs (**C** WF1-23). **B** shows Z-score of eigengene values per module as rows for individual fish (n = 129) ordered by season and faceted within season by sampling location in upstream direction as columns. Overall relative abundance per module is shown in the barplot on the left side. **A** and **C** show FDR corrected Pearson correlation strength between eigenASVs and host/external traits, the right panel shows FDR corrected Pearson correlation to WF-SSU eigenASVs (brown representing positive, zero white and green negative correlation) and statistical significance indicated by stars: * P < 0.05, ** P <= 0.01, *** P <= 0.001. Only correlations r > 0.3 are shown. Taxa with highest intra-modular abundance (%) are shown in barplots (**D**) colored by phylum identity. For network analysis on ruffe GC see Fig. A. 5.

We performed separate network analyses on the centered-log ratio (CLR) transformed bacterial data from the gill mucus of both fish host species and water samples to explore patterns in interacting taxa and relate sub-networks (modules) to environmental drivers. The role of individual taxa in mediating environmentally driven functions was explored via intra-network connectivity (K_{in}) and abundance correlation to prevailing pressures. For the analysis we focus on the main factors determined by RDA in section 4.3. WGCNA clustered the gill mucus taxa into 13 and 16 sub-networks for OE and GC, respectively, and 24 for the bacterioplankton (Fig. 5C, Fig A. 6). Both fish mucus assemblage networks resulted in highly overlapping sub-networks correlated to the dominant environmental drivers.

The prevalence determined core-taxa (section 4.2) were also reflected within networks of both species in the dominant modules OE6 and GC8 (Fig. 5 & Fig. A. 5), overlapping by 70.5 and 90.1% in abundances with prevalence determined taxa shown in Fig. 3, respectively. They are also highly similar between the fish species by 80 – 96% overlapping taxa. OE6 summarized 33 – 37% in overall abundance in summer, spring and winter but only 14% in autumn while GC8 summarizes 36 – 60% over all seasons. OE6 is dominated by *Elizabethkingia* (40.9% within module abundance), Enterobacteriaceae (24.8%), *Enterobacter* (11%), *Citrobacter* (10.8%), identical to GC8 with *Elizabethkingia* (37.4%), Enterobacteriaceae (27.2%), *Enterobacter* (12.3%), *Citrobacter* (12.5%).

Uncorrelated taxa (OE0 & GC0) make up 20% of the overall microbial abundance in both species driven by *Luteolibacter*, *Asinibacterium*, *Clostridium sensu stricto 1* strains and *Rhodobacteraceae* in GC. In migrating autumn smelt the amount of uncorrelated taxa raised to 49%.

5.5.1. Salinity

Freshwater conditions in the estuary are correlated with smelt network module OE4 (r -0.54 to salinity, P ***, 2.5 – 17% in upstream direction) showing uprises in *Rhizobiales Incertae Sedis* (7.8% module abundance proportion), *Xanthobacteraceae* (4.5%) and nitrobacteria *Ellin6067* (8%), *Hyphomicrobium* (7.5%) and *Gaiella* (5.9%). In ruffe, GC11 (r -0.36 to salinity, P **, 3 – 20% bacterial abundance in upstream direction) is characterized by *Polynucleobacter* (54%), *Verticiella* (27%) and *Candidatus Megaira* (10%).

The mesohaline conditions on the contrary were correlated with OE3 (r 0.72, P ***, 17 – 0% in upstream direction) comprising *Persicirhabdus* (33.9%), *Ilumatobacter* (10.5%) and *Halioglobus* (10.4%). Less abundant but highly correlated, GC7 (r 0.77, P ***, 3 – 0.1% in upstream direction) is composed of *Halioglobus* (17.6%), *Persicirhabdus* (8.1%), *Ilumatobacter* (6.7%), *Candidatus Symbiobacter* (4.9%) and Luteolibacter (4.6%).

5.5.2. Temperature

Elevated temperatures were highly correlated with smelt module OE5 (r 0.61, P ***, 6.5% overall abundance) composed of *Chryseobacterium* (22.3% module abundance proportion), *Alkanindiges* (9.9%), *Psychrobacter* (9.5%), *Deinococcus (*7.8%) and *Paracoccus* (6.5%). Likewise, ruffe GC5 (r 0.4, P ***, 7.1% overall abundance) consisted of *Chryseobacterium* (12.8%), *Paracoccus* (10.4%), *Deinococcus* (10.4%), *Ornithinicoccus* (6%). In both fish species the same strains of *Flavobacterium* (r 0.7) and *Ornithobacterium* (r 0.6) showed the highest correlations to elevated temperature values.

On the contrary, lowered temperatures were highly correlated to OE1 (r -0.77, P ***, 21.2% in winter) dominated by *Chryseobacterium* (17.1%), *Flavobacterium* (13.6%), *Deinococcus* (9.3%). GC6 (r -0.63, P ***, 6% in winter) consists of *Escherichia-Shigella* (6.9%), *Sphingomonas* (9.1%), *Thermomonas* (5.2%), *Deinococcus* (9.3%), Weeksellaceae (4.8%), *Flavobacterium* (8.9%).

5.5.3. Desoxygenation

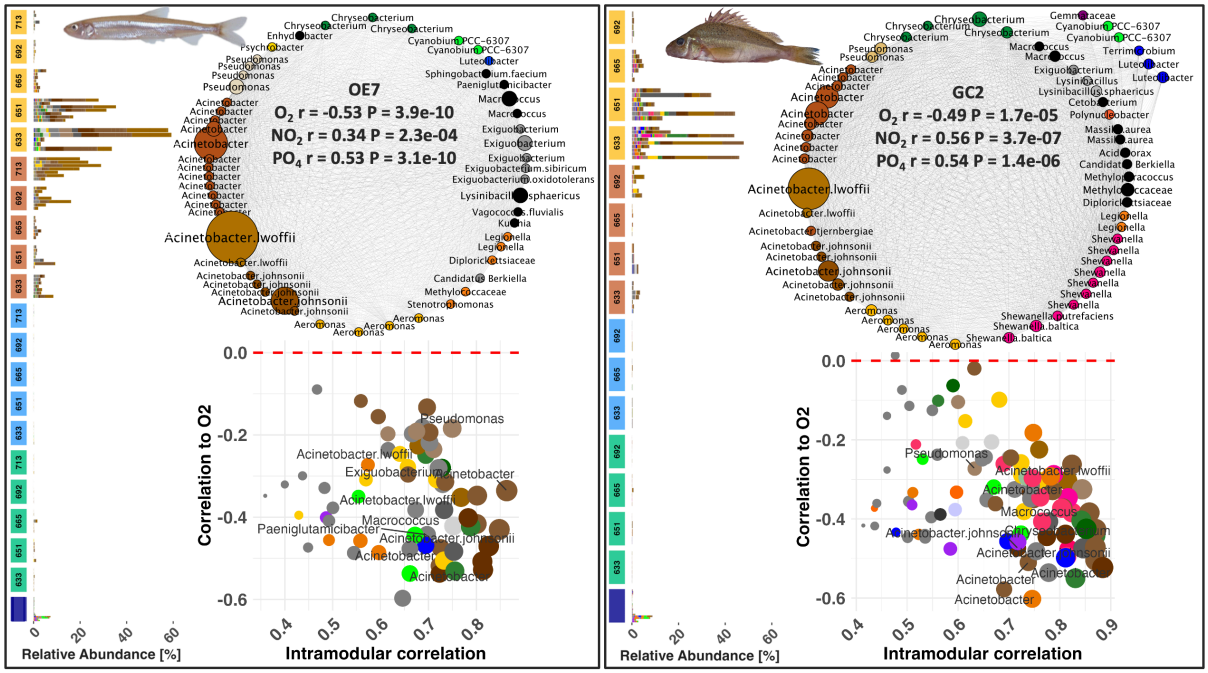


Fig. 11 An/-Aerobic heterotrophs with opportunistic pathogenic potential thrive under prolonged hypoxia in the Hamburg Port area (Ekm 651 – 633). Dominant bacterial species in smelt OE and ruffe GC network modules with highest negative correlation to oxygen values are shown as network modules OE7 and GC2. Stacked barplots depict relative abundance of these taxa in spatio-temporal course. Dotplots indicate individual correlation of ASVs to DO levels and intra-module correlation.

In both fish species, highly similar modules correlated with deoxygenation and nutrient levels (OE7: DO -0.53***, PO_4 0.53***, NO_2 0.34 *** and GC2: DO -0.49***, PO_4 0.54***, NO2 0.56***) overlapping in 63% of the ASVs. OE7 was largely dominated by *Acinetobacter* (66.4%, *A. lwoffii* 31.1%, *A. johnsonii* 10.17%), *Exiguobacterium* (5.5%), *Macrococcus* (4.6%) and *Pseudomonas* (4.4%). Similarly, GC2 was composed by *Acinetobacter* (54.2%, *A. lwoffii* 20.6%, *A. johnsonii* 10.7%), *Macrococcus* (7.9%), *Shewanella* (5.4%, *S. baltica* 2%, *S. putrefaciens* 0.6%), *Chryseobacterium* (4.6%), *Aeromonas* (4.5%) and *Pseudomonas* (3.8%). While these taxa account for only 4% in overall microbiota abundance in both species, sampling groups from the upper estuary (Ekm 651 – 633) in late summer reached 31.7% and 22.2% with individual samples peaking above 60% relative abundance.

6. Discussion

Through amplicon sequencing of gill mucus bacteria in two highly different estuarine key fish species, we aimed to gain insights into the plasticity of the host associated microbiota, movement patterns of the fish and developing indicators for stressful conditions in the host. Many studies have dealt with the influence of abiotic stress on the gut and skin microbiota in fish, usually under artificial rearing conditions (Bell et al., 2024). However, despite its huge potential for health monitoring, gill compositions are largely overlooked (Sehnal et al., 2021; Xavier et al., 2024), especially along physicochemical gradients in an estuarine system.

6.1. Life history strategies & microbiota plasticity

Albeit surrounded by water, a clear distinction existed between bacterioplankton and gill mucus microbiota communities suggesting selective colonization (Koll et al., 2024; Legrand et al., 2018; Minich et al., 2020; Pratte et al., 2018; Rosado et al., 2021). Though many taxa are shared between the biomes, a few core taxa, rare in the water column, dominate the fish gill mucus. The spatial and temporal resolution of the dataset revealed insights into the dynamics of microbiota plasticity. In autumn, anadromous *O. eperlanus* migrate to the estuary. Their microbiota composition

showed a diminished amount of estuarine core but increased estuarine bacterioplankton taxa, suggesting a two-step microbiome adaptation: an initial increase of bacterioplankton-shared taxa that compete best for niches in the host environment at a given salinity (Schmidt et al., 2015) rising with residence time during the one-month ascent process (Borchardt, 1998). In this context, strains of Luteolibacter, a genus found in both fresh and salt water (Ji et al., 2021; Xie et al., 2022), develop high abundances. Although not extensively studied, strains of this genus have been found correlated with expression of immune pathways in an estuarine fish (Koll et al., 2024) and are hypothesized to antagonize pathogen growth and being involved in epithelial tissue repair (Mondal et al., 2022; Nakatani & Hori, 2021; Wu et al., 2022). A potential role in the extensive restructuring of the microbiota composition in migratory smelt however requires further investigation. In the second, slower step, the estuarine core taxa gained in abundance and became dominant along the entire salinity gradient. The indicator analysis showed overall no loss or gain of abundant taxa but a shift in abundance ratios as underlying mechanism.

In contrast, *G. cernua's* stable isotope data reflect a stationary lifestyle indicating residence for several weeks to month dependent on metabolic activity (Buchheister & Latour, 2010; Grønkjær et al., 2013) matching with microbiota composition mainly influenced by spatial drivers. In accordance with the stable isotope signal, a few bacterial indicator taxa (*Verticiella, Polynucleobacter* and *Candidatus Megaira*) are specific for freshwater residency in *G. cernua*. In *O. eperlanus* decreasing salinity correlated with nitrogen-metabolizing taxa (*Hyphomicrobium, Gaiella, Ellin6067, TRA3-20, GOUTA6, Rhizobiales, Xanthobacteraceae*) (Chen et al., 2022; Xiao et al., 2022; C. Yang et al., 2023), potentially preventing toxic ammonia buildup at the gills (Legrand et al., 2018; van Kessel et al., 2016) in the anadromous species less adapted to high loads of nitrogen compounds in estuarine freshwater areas. In the bacterioplankton these conditions were associated with an increase in nitrogen metabolizing *Nitrospira* and *TRA3-20* while in ruffe gill mucus all these taxa were omnipresent. Mesohaline indicators (*Persicirhabdus, Ilumatobacter, Halioglobus*) co-occur in fish and bacterioplankton and have been found in association with various marine organisms, although their function is poorly understood (Amin et al., 2022; Han et al., 2021; Scheifler et al., 2023).

6.2. Estuarine gill core microbiota

There are various definitions and interpretations of the core microbiome. While functional associations based on taxonomic core groups are limited and likely related to microbial gene sets instead of taxa, exploring patterns in microbial frequencies across populations builds a foundation to study ecological processes and evaluate community stability (reviewed in Risely, 2020). The core taxa identified here (defined by occurrence and abundance in the mucus of fish species in estuarine habitats) reflect the common and partially temporal taxonomic core that remains stable across spatial and seasonal fluctuations.

The dominant taxa detected, *Elizabethkingia* (Jacobs & Chenia, 2011), *Enterobacteriaceae*, *Lelliottia* (Salgueiro et al., 2020), *Arthrobacter* (Tsoukalas et al., 2023), *Deinococcus* (Amill et al., 2024), *Asinibacterium* and *Knoellia* (Zou et al., 2023) are all well described from external fish microbiota. *Asinibacterium* and *Enterobacter* taxa have been associated with the inhibition of pathogen growth (Halet et al., 2007; Schubiger et al., 2015). The genus *Citrobacter* contains pathogens of marine and freshwater fish (Liu et al., 2024; Sato et al., 1982), but was found here as a constant compartment of the gill microbiota in both fish species. This is in line with recent studies showing that potential pathogens may well be part of the core community of apparently healthy individuals (Itay et al., 2022; Yajima et al., 2023).

Overall, the core microbiota of the two fish species were almost identical and highly concordant with another top predatory fish species (*Sander lucioperca* L.) in the estuarine system (Koll et al., 2024). These data indicated that the gill mucus core microbiota of estuarine fish were similarly shaped regardless of trophic level, life history and habitat use, and the estuarine-specific signal was acquired

within a time frame of less than three months in migratory fish. This suggests that the strong fluctuations in physiochemical conditions in the estuarine system allowed colonization of only a small number of generalist species or strong host selection for critical ecological functioning (Luan et al., 2023; Roeselers et al., 2011; Sharpton et al., 2021). The size of the core microbiota with ~30 genera appeared to be low in estuarine fish compared to hundreds in other studies (Lorgen-Ritchie et al., 2022; Sharpton et al., 2021), but at 30 – 50% high in terms of abundance similar to gut microbiota studies (Wilkes Walburn et al., 2019).

Stability in the bacterial community is proposed to be important for the health of the host (Zaneveld et al., 2017). We determined here the average core abundance proportions in this larger dataset as means to identify animals with strong deviation which might be indicative for profound reorganization or dysbiosis. Such groups of animals with low core abundance were found in the migration autumn smelt, described in detail above, and in both fish species under prolonged lowered oxygen conditions in the upper part of the estuary in summer (Fig. 3, next chapter).

6.3. Deoxygenation & dysbiosis in estuarine fish

Dysbiosis, the disturbance of microbiome homeostasis (abnormal taxonomic structure and metagenomic function) by uprise of opportunistic taxa (Egan & Gardiner, 2016; Levy et al., 2017), affects microbiome functionality and host physiology and is associated with disease states in fish (Legrand et al., 2020; Mougin & Joyce, 2023). Understanding these processes and identifying biomarkers for dysbiotic processes before physical signs of infection are important not only in fish farming (Mougin & Joyce, 2023; Xavier et al., 2024), but also for monitoring the health of wild fish populations and ecosystems.

While dysbiosis is often defined as a reduction in diversity and microbial richness, measurements of alpha diversity show contradicting results even in controlled conditions in fish with clear disease signs (Karlsen et al., 2017; Liu et al., 2024; Mougin & Joyce, 2023; Vasemägi et al., 2017; Xavier et al., 2024; X. Zhang et al., 2018). Changes in Proteobacteria to Bacteroidetes (P:B) ratios were also suggestively linked to disease states in different fish microbiomes (Xavier et al., 2024). The P:B ratio in estuarine fish gill mucus was at 3:1 higher than in marine taxa (10:1) (Legrand et al., 2018) and decreased slightly during periods of deep reorganization (autumn smelt 7:1) and prolonged oxygen depletion in late summer (OE 4.6:1, GC 4:1). Overall, however, we did not identify clear patterns in alpha diversity measures associated with environmental factors in agreement with recent reviews (Xavier et al., 2024).

We observed a strong increase in *Acinetobacter* coinciding with the decrease in the abundance of core microbiota in both fish species under persistent hypoxia (< 5mg/L) and elevated nutrient loads (nitrates, nitrites and phosphates). Particularly noteworthy, this pattern was not seen in spring when oxygen levels just start declining at similar nutrient loads. Increases in abundance of *Acinetobacter,* albeit less prevalent (< 1%) were observed in the surrounding water column matching described functions in nitrification and nitrogen as well as phosphorous compound assimilation (Carr et al., 2003; Zhong et al., 2023). *Acinetobacter* are repeatedly described as part of the core microbiota of marine fish (Lorgen-Ritchie et al., 2022; Varela et al., 2024), although they can occur as pathogens in a number of freshwater fish (Kozińska et al., 2014; Malick et al., 2020; Visca et al., 2011; M. Zhang et al., 2023). Several strains of *A. johnsonii* and *A. Iwoffii* were shown to cause lesions and hemorrhage especially in gill and liver tissues (Bi et al., 2023; Cao et al., 2018). Notably co-infections of *Acinetobacter* species (Malick et al., 2020), similar to *Shewanella* (Erfanmanesh et al., 2019) and *Aeromonas* (Chandrarathna et al., 2018; Wise et al., 2024) can significantly influence the severity and course of diseases in fish through synergistic interactions like immunosuppressive effects (Kotob et al., 2016; Okon et al., 2023).

We detected a strong co-occurrence of different *Acinetobacter* strains (*A. iwoffii*, *A. johnsonii*) with *Aeromonas* and *Pseudomonas* and in GC also with *Shewanella* (*S. baltica* & *S. putrefaciens*) in

both fish species independently becoming a significant component of the microbiota. As a limitation in this study, no physiological measurements (i.e. lesions) of gills or other tissues were performed and no correlations with biometric markers (FCF, HSI, SSI) were detected. Thus, no direct consequences for fish health can be determined in the present data set. Against the background of wild sampling, however, these physiological endpoint makers are highly variable and do not allow conclusions to be drawn about the influence on health with the sample sizes available here. In a previous study however, we identified strong correlations of the immune response and cellular stress response in the gill and liver of another estuarine fish species under the low oxygen regime going along with an increase in *Shewanella* and *Acinetobacter* in the gill mucus (Koll et al., 2024).

7. Conclusion

Our data showed variable and stable compartments in gill mucus microbiota that could be used for minimally invasive long-term monitoring of estuarine fish in the context of changes occurring due to multiple anthropogenic stressors. Combined with a previous study, we have been able to show, for three species of fish in the Elbe Estuary, an easily identifiable core microbiome associated with apparently healthy fish. Further, for each species, we showed changes in these core microbiota, either as a consequence of migration or increased environmental stressor levels. The latter was related to increased abundancies of potentially opportunistic taxa which may impact fish survivability. Whilst this is an initial study and multi-year time series and further targeted studies would be required to clarify a pathogenic role of these strains and the ability of the gill microbiome to recover once stressful conditions are mitigated, these results are of global significance and concern. Estuaries should be considered protected spawning and nursery areas, ensuring marine fish stocks are well maintained, though our data and others suggest this is not the case. However, increased monitoring of bacterial compositions on estuarine fish and the water column, might provide an additional tool by identifying areas of reduced impact that can be restored or conserved, ensuring that estuaries continue to provide this much needed ecosystem function.

8. Data availability

The sequence data are deposited in the ENA Sequence Read Archive under the study PRJEB77621. The complete analysis is available in stepwise R markdown files, including metadata and supplementary lists as well as visualizations as HTMLs at DOI: 10.5281/zenodo.12819246

9. Funding

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10. Permits

Sampling procedures were according to the standards described in the German Animal Welfare Act (§4 TierSchG). After being brought on board, the fish were stunned with a blow to the head, before being killed with a heart stab. The implementation of the stow-net fishing for scientific purposes is approved by the Authority for the Environment Climate, Energy and Agarwirtschaft, by

the State Fisheries Office Bremerhaven and by the State Office for Agriculture, Environment and Rural Areas of Schleswig-Holstein. Exemptions to the ordinances on nature reserves Mühlenberger Loch/Neßsand as well as a nature conservation permit to conduct research fishing in protected areas in the NSG "Rhinplate und Elbufer südlich Glückstadt"/FHH area DE 2393-393 from the Office of Environmental Protection were obtained.

11. Author contributions

RK: Conceptualization, Methodology, Investigation, Formal analysis, Data curation, Validation, Writing- original draft, Visualization, Project administration. EH: Investigation, Visualization, Formal analysis, Data curation, Review & Editing. JT: Investigation, Permit acquisition, Review & Editing. CB: Data curation, Resources, Review & Editing. MB: Investigation, Data curation. RT & CM: Resources, Review & Editing. JW: Supervision, Validation, Review & Editing. AF: Funding acquisition, Conceptualization, Supervision, Validation, Review & Editing, Project administration.

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13. Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work RK used DeepL/translate/write in order to improve language of the manuscript. After using this tool, RK reviewed and edited the content as needed and takes full responsibility for the content of the publication.

14. Appendix A.

Supplementary data

15. References

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Chapter 5 Spatio-temporal holobiont

Advancing molecular wild fish monitoring: Spatio-temporal holobiont dynamics in an estuarine system
Manuscript in preparation

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Key words: RNAseq, 16S, Metabarcoding, Network analysis, Hypoxia, Anthropogenic stressors, Holobiont, Monitoring, Climate change, SNP, adaptation, population structure

Abbreviations:

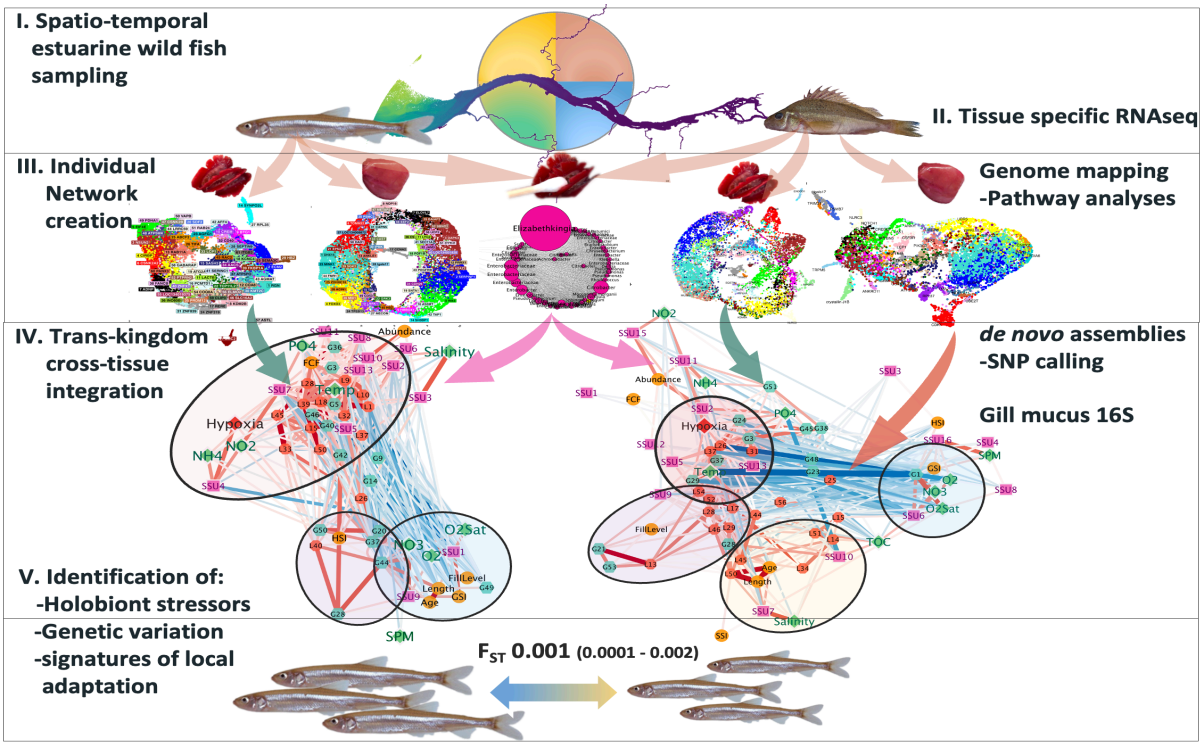
SSU Small subunit rRNA**,**
WGCNA Weighted gene correlation network analysis,
DO dissolved oxygen, TOC total organic carbon, SPM suspended particulate matter
HSI hepato-somatic index, SSI spleno-somatic index,
FCF Fulton's condition factor
OE *Osmerus eperlanus,* GC *Gymnocephalus cernus*
SNP single nucleotide polymorphism

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1. *Abstract*

Estuaries are highly variable stressor mosaics for inhabiting fauna but are among the biologically productive ecosystems on the planet. However, one of the largest European estuaries is currently experiencing a massive decline in fish biomass attributable to direct anthropogenic influences, expected to intensify with climate change. So far, there is a lack of studies investigating the influence of spatial gradients and seasonality on the molecular level stress response in hosts and their associated microbiota as holobionts. Here we investigate the acclimatory stress response of two key fish species using 340 tissue-specific transcriptome data sets with complementary 16S microbiota analyses. Both species show compensatory stress responses rather than energy conservation strategies. Immune responses in gill and liver were particularly strongly associated with increasing temperatures and abundances of opportunistic bacteria (Flavobacteriaceae, *Acinetobacter*). Under continued hypoxia in the systems upper reaches close to the port of Hamburg, signs of immunosuppression occur in parallel with increased cellular stress responses (heat shock, apoptosis, cell cycle control). In pelagic smelt in particular, adaptive immune responses decline with lowered oxygen levels above values usually considered hypoxic, whereas in benthopelagic ruffe these are maintained even under continued hypoxia. Both populations show high genetic exchange over time and space, but signs of selection especially in key genes within immune and cellular stress response (HIF, GPX, HSP, MR1 and HLA) highly upregulated in summer and upstream areas. With the current population collapse of > 92% abundance, a genetic bottleneck is to be expected. The loss of genetic variation especially in these key factors for climate change adaptation will increase via genetic drift the longer no measures for ecosystem restoration are taken. Prolonged hypoxia in the port region was a stressor hotspot in both species, but we also determined unexpected antiviral-, xenobiotic and in ruffe liver innate immune responses correlated salinity fluctuation. Generalized additive models indicated high predictive potential of simple microbiota composition analyses for fish stress responses, which might therefor add a valuable monitoring tool in urban estuarine systems. Overall, the data show alarming sub-lethal effects of prevailing estuarine multi-stress conditions that can be used to guide ecosystem restoration and monitoring efforts.

2. *Graphical abstract*



3. Introduction

Estuaries are highly productive aquatic transition zones (Ducrotoy et al., 2019), yet they rank among the most anthropogenically altered habitats (Pein et al., 2023). Human-induced changes in the Elbe estuarine study system in northern Germany include hydromorphological interventions via deepening and canalization leading to a doubling of the tidal range (Hein et al., 2021) and increase in suspended particular matter (SPM) (Heininger et al., 2015; Reese et al., 2019). Together with a reduction in shallow reaches, intensified stratification and eutrophication in the system, oxygen minimum zones (OMZ) expand (Pein et al., 2021). Fostered by the deepening of the harbor, nitrogen compounds, primarily from anthropogenic sources (Kerner, 2000), are decomposed in the freshwater area of the estuary (Amann et al., 2012; Sanders et al., 2018; Schöl et al., 2015). Nitrification alone has been estimated to contribute up to 30% to oxygen depletion (Schöl et al., 2015), coinciding with critical oxygen concentrations below 4 mg/L for weeks to months annually (Fig. 1).

In the Anthropocene hypoxia has become particularly widespread due to the input of nutrients (Le Moal et al., 2019) and the consequences of climate change, and exceeds the impact of other stressors on aquatic organisms (Rosenfeld & Lee, 2022; Sampaio et al., 2021). Definitions for what is considered as hypoxia vary greatly, often described is a drop to 50% dissolved O_2 (DO) of the ecosystem mean (Breitburg, 2002; Hughes et al., 2015), 50% of maximum DO saturation (Breitburg, 2002) or DO values between 2 - 2.5 mg/L (Gray et al., 2002). Meta-analyses of freshwater and marine fish species however found a critical non-lethal impact on growth and nutrient uptake between 4.5 - 6 mg/L DO (Hrycik et al., 2017; Rosenfeld & Lee, 2022), far above most values mentioned before.

In a recent study by our working groups extending over a 40-year time series we identified significant changes in fish populations within the Elbe Estuary. In addition to a population loss of 91% over the last decade to an unprecedented low, these include massive changes in life cycle guilds, the decline of key taxa and increase in marine species related to the reappearing hypoxic situations and increased turbidity (Theilen et al., 2025, under review).

In addition to anthropogenic influences, estuarine ecosystems are also exposed to exogenous pressures from climate change to which they have only limited resilience due to their depth limitation (Dulvy et al., 2008; Rutterford et al., 2015). Frequency and intensity of heatwaves, oxygen minimum zones (OMZ) and changes in salinity regimes are expected to increase and to greatly affect inhabiting biota like fish (Breitburg et al., 2018; Cottingham et al., 2018; Lauchlan & Nagelkerken, 2020; Little et al., 2017; Sampaio et al., 2021). In several other estuarine systems, alterations in species abundance and life strategy compositions have already been observed, affecting nutrient cycles and overall ecosystem functioning (Auber et al., 2017; McLean et al., 2019; Teichert et al., 2017). At this moment, direct anthropogenic interventions appear to surpass the climate related factors in the Elbe system, though the latter are expected to intensify in near future.

Projecting trajectories for the degradation, recovery, or shift to a new state in ecosystems is particularly challenging under multiple stress conditions. Models like the Asymmetrical Response Concept capture stress tolerance, dispersal, and biotic interactions as determinants for stress response and provide a conceptual basis for understanding ecosystem recovery (Vos et al., 2023). These models consider direct and indirect effects through environmental variables and interactions with species under multiple stress scenarios, crucially however is an identification of all environmental variables and stressors exceeding species tolerance levels. Organisms experience abiotic stressors (e.g., temperature, dissolved oxygen, salinity levels), biotic stressors (e.g., pathogens, parasites, predators), and anthropogenic stressors (the ones named before surpassing natural levels (Piggott et al., 2015; Vos et al., 2023), habitat degradation and pollution) (Schulte, 2014). In wild habitats, these diverse, multiple-stress factors lead to complex interactions that can range from additive to synergistic to reversing effects (Birk et al., 2020; Crain et al., 2008).

Chapter 5 Spatio-temporal holobiont

The organismal stress response comprises a wide range of physiological and behavioral acclimatory processes (Wendelaar Bonga, 1997) that are highly dependent and limited by the energy available to an organism, the capacity of its procurement or mobilization, determining the adaptive strategy used (Sokolova, 2013; Sokolova et al., 2012). Up to a threshold, stressors are expected to be compensated, resulting in a trade-off between maintenance and fitness-related functions, extreme stress however might disturb the energy balance to such an extent that conservation of metabolic resources can occur which prevents the long-term survival of an organism or population. The energetic investments and physiological adaptations to regain a homeostatic state or new allostatic state are referred to as allostatic load (Sterling, 1988), a measurable quantity that increases with each stress response and limits the capacity for further stress response (Balasch & Tort, 2019; Tort, 2011). By measuring metabolic processes and different components of the stress response, it is possible to gain insight into the load and the transition between response strategies and thus determine limits of the organism and conditions/habitats that can support a population (Sokolova et al., 2012). At the cellular level, stressors affect macromolecules, damage their integrity directly or are associated with the generation of chemical radicals that attack cellular molecules (Kültz, 2005). Outside the limits of elastic cell homeostasis, an evolutionarily highly conserved cellular stress response takes effect that includes I. prevention and repair of damage, II. control of the cell cycle, III. reallocation of energy and IV. finally cell death (Kültz, 2020).

Holistic OMICS approaches such as RNAseq, which provide approximations of gene expression (Buccitelli & Selbach, 2020), can provide insights into cellular responses to stressors that are not yet well understood (Semeniuk et al., 2022). The molecular mechanisms by which organisms cope with the multi-stress environment potentially allow to disentangle individual influences or the interaction of driving forces and conversely inform about the prevailing conditions themselves allowing the identification of unexpected stressors as has been demonstrated recently in freshwater systems (Connon et al., 2018; Escobar-Sierra et al., 2024; J. Jeffrey et al., 2023; J. D. Jeffrey et al., 2019) as well as in estuarine habitats (Jeffries et al., 2019; Koll, Theilen, et al., 2024; Laurent et al., 2023).

An often-overlooked aspect in such studies is that organisms exist as holobionts (Bordenstein & Theis, 2015), where the interaction between microbiota and their host in response to environmental drivers is unified. Considering holobionts is essential for modern monitoring and conservation, as bacterial influences are critical for fish health (Kelly & Salinas, 2017) and microorganisms as part of biodiversity should be recorded, given their potentially overlooked disappearance along with host organisms (Limborg et al., 2024). One attempt to record bacterial diversity is based on 16S amplicon sequencing. In a previous study, we described the gill-associated bacterial data of the two estuarine fish species in focus of this study over time and space and linked them to migration patterns of the fish species as well dysbiotic states correlated with hypoxia and eutrophication in the Elbe estuary (Koll, Hauten, et al., 2024). The study identified an estuarine-specific host associated core microbiome that was characteristic of both fish species despite different life cycle categories and only appeared to be disturbed under high abiotic stressor levels (prolonged oxygen depletion and eutrophication).

In this continued study, we are now investigating the associated stress responses in the host organisms to provide a comprehensive understanding of holobiont stress responses in estuarine fish. The target species caught over the course of the estuary and over seasons are critical for the estuarine food web. Smelt (*Osmerus eperlanus* L. - OE) is a key species in the food webs of many European riverine ecosystems (Illing et al., 2024) and occupies the habitat with several life stages at once throughout the year (Thiel & Thiel, 2015). The ruffe (*Gymnocephalus cernua* L. - GC) on the other hand is a rather undemanding benthic predator adapted to eutrophic estuaries (Eick & Thiel, 2014; Gutsch & Hoffman, 2016; Hölker & Thiel, 1998). Both species were shown drastically decreasing in abundances in the system over the last decade (Theilen et al., 2025, under review).

To provide a holistic view of molecular responses, we generated tissue specific gill and liver transcriptomes from statistically robust sample sizes (n = 340) and recorded the bacterial composition

of each fish's gill mucus, complemented with further physiological endpoint measurements. Besides utilizing gene expression data in trans-kingdom network approaches, we created *de novo* transcriptome assemblies and performed population genetic analyses as means to identify the population structure in both fish species and identify potential adaptational processes. The study specific aims were to determine **I.** species and tissue specific responses to abiotic gradients in relation to the bacterial composition on the fish, **II.** infer the allostatic load and stress response strategy chosen by the individuals under the prevailing conditions allowing for estimations into future conditions, **III.** determine hotspots of stressor interaction and potentially unknown stressors as means of informing ecosystem restoration efforts and lastly **IV.** to evaluate ongoing adaptive processes in the populations. The workflows, datasets and results are made openly available with the hope of advancing the integration of molecular methods into species monitoring and ecosystem conservation.

4. Material and methods

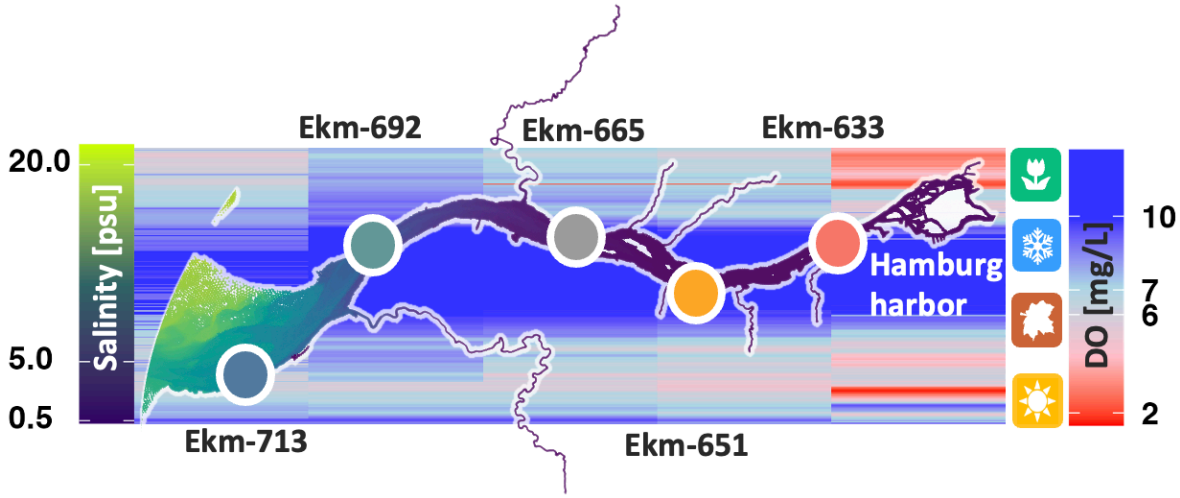


Fig. 12 Sampling design. Map of the Elbe estuary, Germany, colored by salinity gradient. Sampling stations along the course of the tidal Elbe (Ekm = Elbe-Kilometer) as well dissolved oxygen (DO) levels at seasons of sampling in August 2021 (Summer, yellow), November 2021 (Autumn, brown), March 2022 (Winter, blue) till June 2022 (Spring, green) along the course of the estuary.

4.1. Sample Collection

Sample collection has been explained in detail elsewhere (Koll, Hauten, et al., 2024). In short, a stow-net fishing vessel was used capture fish along the estuarine course four seasons. The process can be viewed in detail in the linked video material: https://www.biologie.uni-hamburg.de/en/forschung/grk2530/blog/2023/0905-fish-in-estuarine-carbon-cycle-video.html. When possible seven individuals per location and time point were processed immediately on board (OE n = 81, GC n = 90) including the extraction of tissue samples (gill & liver) as well as recovery of gill mucus. Tissue samples were first preserved in RNAlater, later moved to dry ice then -80°C together with mucus swabs until further processing. Sampling procedures followed the standards described in the German Animal Welfare Act (§4 TierSchG). Whole fish were preserved as -20°C to allow for determination of body condition (FCF), hepato-somatic - (HSI), spleno-somatic (SSI) and gonado-somatic indices (GSI)), stomach content and age via otoliths and scales in the lab. Abiotic data were measured on board using a multi-probe (Hanna HI 9829) and matched with measurements from continuous national measurement programs (www.fgg-elbe.de) (PO_4, NH_4, NO_3, NO_2, suspended particular matter (SPM), O_2, pH).

4.2. DNA extraction, sequencing & processing

The bacterial composition data were analyzed and presented in an individual publication (Koll, Hauten, et al., 2024). In short, the V3-V4 variable regions of the 16S rRNA gene were amplified in a one-step PCR using the primer pair 341F-806R (dual-barcoding approach (Kozich et al., 2013); primer sequences: 5'-CCTACGGGAGG-CAGCAG-30 and 5'-GGACTACHVGGGTWTCTAAT-30) and sequenced on a MiSeq platform (MiSeqFGx; Illumina, San Diego, USA) with v3 chemistry (2x300 bp). Demultiplexing was conducted with 0 mismatches in the barcode sequences. Read files were quality controlled via TrimGalore (v 0.6.10) (Krueger, 2015) and processed in R (v.4.3.0) via DADA2 (v.1.29.0) (Callahan et al., 2016) using the SILVA SSU v138 taxonomic database (Quast et al., 2013). ASV tables were filtered in phyloseq (v 1.45.0) (Mcmurdie & Holmes, 2012) removing non-bacteria and low abundant taxa with sum of counts lower 0.005% reducing matrix sparsity by 50%. Centered log-ratio (CLR) transformation to the ASV matrix was applied for integration in network analyses.

4.3. RNA extraction & sequencing

Tissue specific transcriptomic data were gathered and processed similar as explained in detail in (Koll, Theilen, et al., 2024). In short, total RNA from tissue samples were extracted via silica membrane kits (Crystal RNA Mini Kit, BiolabProducts) including on-column DNA digestion via RNase free DNase I (Qiagen). RNA quantity and integrity were controlled by spectroscopy (Nanodrop, Thermo Fisher) and on denaturing agarose gels and Bioanalyzer (Agilent Technologies) (avg. RIN 9.6 ± 0.4 over 336 samples, no sample lower RIN 7 was used). For both species, matching tissue specific RNAseq and 16S datasets were created for 89 animals of GC leading to 178 individual tissue-specific RNAseq datasets and 81 specimens of OE leading to 158 RNAseqs. For OE no samples from autumn sampling were processed as bacterial composition and stable isotope data showed that these were migrating fish just recently entering the estuary from the North Sea for reproduction (Koll, Hauten, et al., 2024). mRNA was isolated using either (NEBNext Poly(A) Magnetic Isolation Module (New England BioLabs) followed by stranded cDNA library creation (NEBNext Ultra II Directional RNA Library Preparation kit (New England BioLabs) or the Illumina RNA stranded mRNA kit including polyA enrichment. Individually barcoded libraries were 2x150 paired-end sequenced on NovaSeq6000 (Illumina) (300 cycles, v1.5 reagents, S4 FlowCell) on five different lanes resulting in 7.1 billion reads (avg. ca. (GC Gill 30 ± 10, GC Liver 28 ± 11, OE Gill 27 ± 7, OE Liver 32 ± 12 million, no dataset < 12 mio. reads was used in the analyses). To account for possible batch effects from library preparation and sequencing runs, biological replicates were spread over runs and clustering was inspected via principal component analysis (PCA).

4.4. Transcriptome analysis

The University Hamburg high performance cluster Hummel was used for all sequence data processing. Adapter sequences, first and last base pairs, reads with Phred score < 20 or sequence length < 20 bp were filtered via TrimGalore (v 0.6.10). For OE clean reads were mapped to the recently published genome of *Osmerus eperlanus* fOsmEpe2.1 (Sander Institute Date: 2023/12/11) using Hisat2 (v.2.2.1) (Kim et al., 2015) (overall mapping rate was 99 ± 0.03%) followed by expression determination via featureCounts (subread v.2.0.2) (Liao et al., 2014) (assignment rate 45 ± 10%; 44 ± 17 mio. reads). With a lack of a reference genome, clean reads of GC were mapped to the closely related genome of *Sander lucioperca* SLUC_FBN_1.2 (Nguinkal et al., 2019) resulting in high mapping rates (98.5 ± 0.6%) and assignment rates (55 ± 6 %; 17.8 ± 7.7 mio. assignments). All downstream analyses were performed in R (v.4.3.0) using visualization packages ggplot2 (v.3.4.2) and cowplot (v.1.1.1). Genome wide annotation databases org.XX.eg.db (v.3.17.2) (Carlson, 2019) for human, mouse, and zebrafish as well as AnnotationHub (v3.17) (Morgan, 2023) entries for OE and zander were used to translate between different gene identifiers, names and symbols. Blast annotation of the reference genomes CDS regions was performed via diamond (v.2.0.15) against UniProt, UniRef90, TrEMBL, RefSeq-Protein and Human EMBL-EBI reference proteome databases. Blast results were filtered for E-value < 1e-6 and bit score higher than 50, for multiple hits to the same transcript, the

one with the lowest E-value was retained. Wherever human genes could be assigned to transcripts, these annotations were used for downstream pathway analyses.

4.5. SNP calling

A subset of samples representing the seasonal, spatial and tissue specific variation in the datasets was used to create de novo transcriptome assemblies following a more stringent quality filtering (> q30, min length 36bp). The overall workflow followed the approaches described by (Bugg et al., 2023; Thorstensen et al., 2020).

First, several De novo assemblies with differentiating settings were created using Trinity (v.2.15.1) (Grabherr et al., 2011) and RNAspades (v.3.15.5) (Bushmanova et al., 2019) and compared via N50 and Nx50 values as well as single-copy orthologs via BUSCO (v.5.3.1) (Manni et al., 2021) (**Fig.S.14**). Reads were first multi-mapped to each transcriptome separately via Bowtie2 (v2.4.4) (Langmead & Salzberg, 2012), bam files were then subsampled to 10% of the prior amount individually to be processed together in the SuperTranscripts pipeline (Davidson et al., 2017) clustering transcript into genes in Corset (v.1.07) (Davidson & Oshlack, 2014) and into a linearized representation via Lace (v1.14.1). Star (v. 2.7.11b) (A Dobin, 2013) was then used in a 2-pass mode to splice-aware align trimmed reads to the reassembled transcriptomes. Lace annotated junctions were provided with the new transcriptome and a sjdbOverhang of 149 bp read length was chosen (mapping rates 72.8 ± 6.4% GC_G & 73.4 ± 3.7% OE_G **Fig.S.14**). STAR-aligned bam files were cleaned, read-groups were added and duplicates marked using Picard (v 3.1.1) (Broad Institute, 2019) before reads containing Ns in cigar ends were split via GATK4 (v 4.4.0.0) (Van der Auwera et al., 2013).

Secondly, using STACKS (v.2.65) (Catchen et al., 2013) loci for SNP calling were created (gstacks) and SNPs were called (populations) with a minimum minor allele count of 3 and at least 80% of the data present. Inspections showed a strong influence of different sequencing facilities on the SNP outcome. Therefore, analyses were re-run containing only data from one facility leading to a reduction in samples to 48 in GC and 63 in OE resulting in 96,860 SNPs and 131,117, respectively. To account for paralogous loci biasing population genetics measures, loci with heterozygosity ≤ 0.7 and read ratio deviations ≤ 7 were filtered out using HDplot (McKinney et al., 2017). VCFtools (v. 0.1.16) (Danecek et al., 2011) was used to filter biallelic SNPs of genotype and site quality ≥ 20 retaining 93,492 SNPs for GC and 127,163 for OE.

Thirdly, the SNPs datasets obtained were used in two ways, (a) to identify the population structure using putatively neutral SNPs and (b) to conduct outlier analyses and identify putative adaptive processes. For (a), PLINK2 (Chang et al., 2015) was used to prune SNPs for linkage disequilibrium, filter Hardy–Weinberg equilibrium and missingness (0.1 in GC, 0.05 in OE) with 20 SNPs window-size and r^2 of 0.2 using the super-clusters as chromosomes. A set of 5,138 in GC and 8,841 in OE of putative neutral SNPs was used in a combination of exploratory unsupervised analyses as means of assessing the populations structure in the two estuarine species. Ancestry coefficients were identified between K 1 – 5 groups and evaluated via cross validation error groups in Admixture (v.1.3.0) and visualized pophelper (v2.3.1) (Alexander et al., 2009; Francis, 2017) followed by Weir & Cockerham's F_{ST} calculation with 95% confidence intervals generated by 1000 bootstrap iterations in Hierfstat (v.0.5-11) (Goudet, 2005) between identified groups. In parallel adegenet (v. 2.1.10) (Jombart & Ahmed, 2011) was used for cluster identification from successive K-means via find.clusters. Bayesian information criterion (BIC) was used on 40 intial PCs to determine most well-supported number of clusters of which F_{ST} was calculated again.

For (b) an unsupervised approach via pcadapt (v.4.4.0) (Luu et al., 2017) was used to test for outlier SNPs accounting for the likely occurrence of admixed populations. Informative PCs were selected by Cattell's rule and issues with false positive results (Foll & Gaggiotti, 2008) were addressed by restricting the interpretation to transcripts with ≥ 2 outlier SNPs and a q-value of 0.05.

Transcript IDs of significant SNPs were related to the putative function by annotating the transcriptomes via Trinotate (v3.2.2). In short transcripts and predicted ORFs (TransDecoder v.5.5.0) were annotated using diamond against UniProt, UniRef90, TrEMBL, RefSeq-Protein, Human EMBL-EBI Reference Proteome databases and HMMER (v.3.2.2) homology search against Pfam databases. Blast results were filtered as described before and human genes were used in enrichment analyses. The chosen assembly in OE contained 125693 Trinity genes clustered into 90516 Corset-clusters, annotation predicted 33076 proteins of which 21778 were aligned with the *Osmerus eperlanus* [fOsmEpe2.1] genome used in the WGCNA analysis. In GC 88508 Trinity genes resulted in 82691 clusters, 25481 predicted proteins and 20233 aligned with the *Sander lucioperca* [SLUC_FBN_1.2] genome used in WCGNA analysis.

4.6. Statistical analyses

First, global trends in the transcriptomic datasets in relation to bacterial, abiotic and physiological measurements were assessed via distance-based RDA and unsupervised individual Mantel tests to justify and validate the network approach. The exact procedure and results are presented and described in the supplement section 1 (Fig. S.1).

Secondly, we applied weighted gene co-expression network analyses (WGCNA) (v.1.77-1) (Langfelder & Horvath, 2008) to infer individual networks from tissue specific gene expression patterns and co-abundant bacterial taxa relating them to physiological and environmental data (Koll, Theilen, et al., 2024; Strand et al., 2021) (**Fig. 2 & 3, Fig.S.6 & 7**). The parameters were chosen in such a way that highly correlated nodes are recognized but also small specific patterns can be displayed in order to take into account the complex spatio-temporal structure of the data sets. Module eigengenes/ASVs where correlated between the different networks and to physiological traits of the fish and external abiotic factors via Pearson correlation and corrected for multiple testing via FDR before visualizing edge-weight spring-embedded networks in Cytoscape (v.3.10.1) (Shannon et al., 2003). Functional roles of sub-networks were assigned by retaining genes with intramodular correlation of > 0.8 for overrepresentation analysis against the KEGG- (Kanehisa & Goto, 2000) and GO-databases (Aleksander et al., 2023) via ClusterProfiler (v.4.9.0) (Wu et al., 2021).

The relationship between module eigengenes of selected immune and cellular stress responses and prevailing abiotic conditions and bacterial eigenASVs or CLR transformed abundances was assessed via Gaussian generalized additive models (GAMs) mgcv (v.1.9-1) (Wood et al., 2016). A model-theoretical approach employing Akaike Information Criterion (AIC) was utilized for model selection to identify best-fitting models. Model diagnostics were performed via gratia (v.0.9.2) (Simpson, 2024) and DHARMa (v.0.4.7) (Hartig & Hartig, 2017), all model diagnostics can be found in the supplement (**Fig. S.8-13**).

5. Results

The spatio-temporal gene expression patterns in both fish species and tissues were predominantly influenced by temperature and dissolved oxygen content (**Fig. S.1**) and split into four main responses: Tissue remodeling, metabolic adaptations, cellular stress responses and, most evident in their finely tuned patterns, immune responses. While these are closely interlinked, we focus on the patterns of immune and cellular stress responses, which fall into the following main categories: **I** and **II** responses to high and low temperatures, closely related to oxygen saturation and loads of nitrogen compounds; **III** antiviral defenses and **IV** salinity-triggered responses. While trends were similar across species and tissues, there were especially distinct tissue-specific patterns.

5.1. I: Temperature & Hypoxia

5.1.1. GC

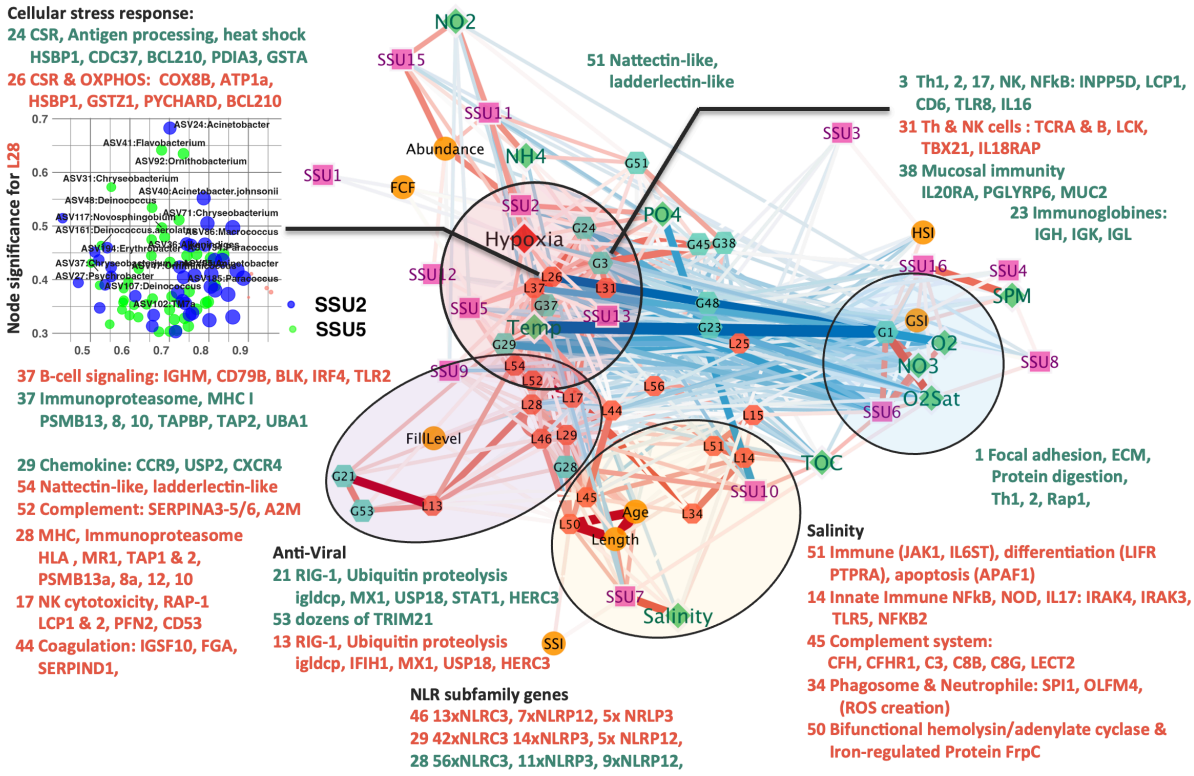


Fig. 2 Edge-weight spring-embedded network visualization of Pearson correlations between GC tissue specific (gill = turquoise, liver = red, numbers indicate the individual network module (e.g. turquoise 24 = G24 = Gill network module 24)) and bacterial (gill mucus SSU1-13) WGCNA eigen-genes/ASVs and physiological and abiotic measurements. The networks are based on gill and liver RNAseq as well as 16S datasets from 89 animals. Continuous line colors and width represent the Pearson correlation coefficients (red, thick = 1, blue, thick = -1, white, thin ± 0.3). Only significant adjusted correlations > ±0.3 are depicted. Hypoxia is coded as binary variable (0 = > 6mg/L), Abundance indicates the whole catch number during the fishing trips. Ellipses include coordinated response patterns: heat/hypoxia, viral response, salinity and cold stress. The labels describe pathways that are enriched within the modul genes (> 0.8 intramodular correlation) or functions determined from the hub genes listed. The network was reduced to nodes enriched in immune and cellular stress responses without changing network structure, the entire networks and the individual histories of all nodes can be found in the supplement **Fig. S.6.** The dot plot depicts the correlation of individual bacterial taxa abundance and selected module Eigengene (y-axis) as well as the individual intramodular correlation (x-axis & point-size).

The strongest temperature (r > 0.5 Temp) and oxygen saturation (r < -0.3 O_2Sat) related responses in both GC tissues include structural tissue modifications via ECM-receptor interaction, focal adhesion, protein digestion & axon guidance (GC_L1, GC_G4, 52, 6, 7). Further identical modifications in oxygen transport encompassing hemoglobin assembly (HBA1-like, hbaa1, HBB2, TMOD4) (GC_L19, GC_G41, r -0,5 O_2Sat) in both tissues and liver specific ROS detoxification (peroxisome, xenobiotic response) comprising well known stress marker gens (*CAT, SOD1, GSTA1, GSTK1, ALDH8A1, ADH1, FMO5*) and metabolic adaptations (carbon & amino acid metabolism) (GC_L4) as well as increased insulin-, FOXO - adipocytokine- & glucacon signaling (*PPTC7*) (GC_L5).

These are accompanied by a strong increase in the immune response in both tissues (**Fig. 2**), in gill summarized in one major module (GC_G3) enriched in various adaptive immune pathways (Th differentiation, T-cell & B-cell receptor signaling, till NFκB signaling) coordinated by the negative regulator of myeloid cell proliferation *INPP5D*. While this module itself is moderately upregulated under high temperature (r 0,47), hypoxia (r -0,44 O_2Sat) and elevated ammonia levels (r 0,36 NH_4) in the upper part of the estuary, it is centrally correlated with all other immune patterns in the gill tissue. Specific responses comprise mobilization of immune cells to inflammatory sites (*CCR9* & *CXCR4*) (GC_G29, r -0.59 O_2Sat), as well as cellular stress responses (GC_G24, r -0.45 O_2Sat)) including heat shock response (*HSBP1, HSPE1, CDC37*), ROS scavenging (*GSTA, SUMO2a, SUMO3a*), apoptosis (*BCL2L10, PYCARD* and with kME < 0.8 also *CASP3, CASP8, CASP11, PDCD6, CFLAR*), antigen processing

(*PDIA3, PDIA6, PSME1, PSME2, CALR*) and further hubs related to cytoskeleton reorganization (*CFL1, ARPC3, TUBA1A* & *ARHGDIA*).

The matching responses in the liver are divided into several smaller modules organized around the B-cell receptor signaling pathway (GC_L39) comprising membrane bound IgM antibody (*IGHM*), the signal transduction moiety (*CD79a*) and polymeric immunoglobin receptor encoding gen as hubs. This is on one side highly correlated with a complex cellular stress response pattern (GC_Liver26, r – 0,67 O_2Sat) strongly aligned with the gill specific one (r 0.73) composed of crucial genes for cellular respiration & energy metabolism (*ATP5IF1, COX6B, NDUFA13*), as well as hubs in mitochondrial protein translation (*MRRF*) heat shock response (*HSBP1*), DNA repair (*SWI5, RDM1*), detoxification (*GSTZ1, ESTD, TST*), as well as different genes with opposing functions in immune activation and apoptosis (different *PYCARD* variants, *BCL2L10, CARD8*) and different *SERPINB1* variants and *SERBP1* regulating proteinase activity and suppressing caspase-recruitment domains (CARD). On the other hand, with various protease inhibitor variants (*SERPINA3/5/6*) and *A2M* protecting tissue during inflammation (GC_L52) as well as specific C-lectin variants (nattectin-like, ladderlectin-like) (GC_L54). An identical C-lectin module (GC_G51) exists in the gill tissue, however with temperature but strongly nitrite and ammonia (r 0.44 NH_4) levels correlated pattern.

To a lesser degree (r 0.3 – 0.5 temp), both tissues show a HIF-1 (*HK1, HIF1a*) response (GC_L8, GC_G31) accompanied by DNA maintenance & splicing (GC_L23) & OXPHOS (GC_L40) in liver, as well as spliceosome (GC_G41), ribosome (GC_G9) & glycolysis (GC_G10) enrichment in the gill.

With specific correlation to elevated ammonia levels and lowered oxygen saturation, there are almost identical *CDK1* coordinated modules enriched in cell cycle processes, mismatch repair & xenobiotics response (*TK1, RRM1, RRM2, DUT*) (GC_L10, GC_G12) and xenobiotic response in the liver (*CYP2A13, GSTT1, UGT1A1, CYP2F1, ALDH3A2*) (GC_L21), further highly correlated (r 0.8) with the temperature driven xenobiotic response in GC_L4.

5.1.2. OE

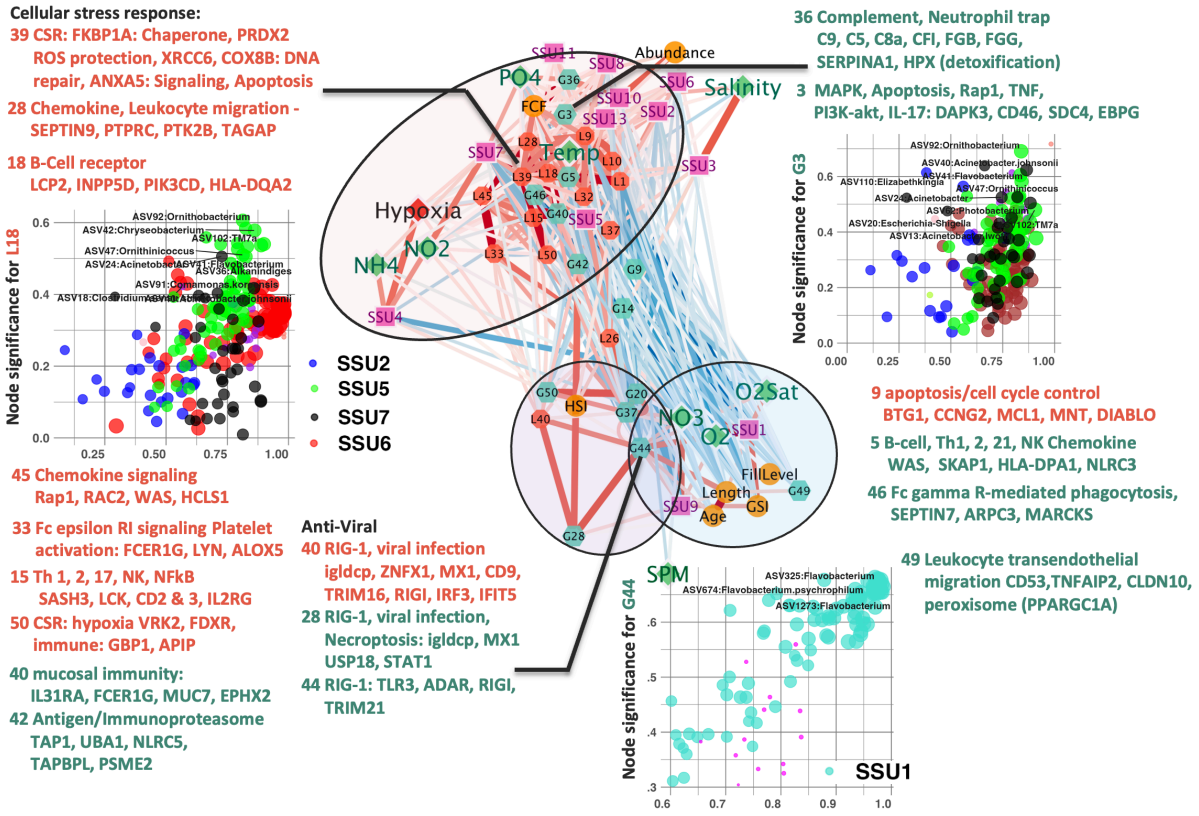


Fig. 3 Edge-weight spring-embedded network visualization of Pearson correlations between GC tissue specific (gill = turquoise, liver = red, numbers indicate the individual network module (e.g. red 39 = L39 = Liver network module 39)) and bacterial (gill mucus SSU1-13) WGCNA eigen-genes/ASVs and physiological and abiotic measurements. The networks are based on gill and liver RNAseq as well as 16S datasets from 81 animals. Continuous line colors and width represent the Pearson correlation coefficients (red, thick = 1, blue, thick = -1, white, thin ± 0.3). Only significant adjusted correlations > ±0.3 are depicted. Hypoxia is coded as binary variable (0 = > 6mg/L), Abundance indicates the whole catch number during the fishing trips. Elipses include coordinated response patterns: heat/hypoxia, viral response, salinity and cold stress. The labels describe pathways that are enriched within the modul genes (> 0.8 intramodular correlation) or functions determined from the hub genes listed. The network was reduced to nodes enriched in immune and cellular stress responses without changing network structure, the entire networks and the individual histories of all nodes can be found in the supplement **Fig. S.7.** The dot plots depict the correlation of individual bacterial taxa abundance and selected module Eigengenes (y-axis) as well as the individual intramodular correlation (x-axis & point-size).

The most pronounced temperature driven responses (r > 0.5 Temp, r < 0.3 O2Sat) in OE liver, include the complement system, lipid, amino acid and carbon metabolism, HIF-1 and xenobiotic responses (OE_L1). Hub genes of this major seasonal response are beneath regulatory and structural genes (regucalcin, *HSPG2*, keratin 94) also major ROS protecting and detoxification genes (Glutathione S-transferase rho (*GSTR*), *GSTM3, UGT2B20*).

Highly correlated but specifically enriched during prolonged hypoxic conditions and high nitrite levels in Hamburg harbor were cellular stress responses comprising chaperones (*FKBP1A*), ROS protection (*PRDX2, COX8B*) & DNA repair (*XRCC6, OGG1*) (OE_L39). Also apoptosis and cell cycle control (*BTG1, CCNG2, MCL1, MNT, DIABLO*) (OE_L9, OE_L10), HIF-1a & TNF signaling (*TNFRSF5, PTGS2, HIF1Aa, HK1*) (OE_L32), ROS stress (*FDXR, VRK2* isoform1 (Blanco et al., 2007)) and immune signaling (*GBP1, APIP*) (OE_L50) (**Fig. 3**).

Further broad temperature responses include restructuring of the extracellular matrix, focal adhesion (OE_L2), as well as OXPHOS and TCA (OE_L43). In overlap and strongly correlated between tissues, the gill response contains protein digestion, ECM receptor interaction & focal adhesion (OE_G2, OE_G12), hub genes related to detoxification (*AS3MT*) and cell cycle (*CDK19*) (OE_G16).

Most upregulated immune components in gill comprise large modules enriched in NK-cell cytotoxicity, B-, T-cell receptor signaling, chemokines (*WASP, NLRC3, CD3G, HLA-DPA1, TRIM2, INPP5D*) (OE_G5) as well as CSR and innate immune parts: MAPK, Rap1, TNF, bacterial-viral infection & apoptosis (*DAPK3, CD46, SDC4, EBPG*) (OE_G3) and Fc gamma R-mediated phagocytosis (*SEPTIN7, ARPC3, MARCKS*) (OE_G46). Corresponding but lower expressed responses in liver were adaptive B- & T-cell receptor signaling (*LCP2, INPP5D, PIK3CD, HLA-DQA2*) (OE_L18) and chemokine signaling (*SEPTIN9, PTPRC, PTK2B, TAGAP*) (OE_L28).

Specifically enriched in summer in gill tissues were complement components (*C9, C5, C8a, CFI, FGB, FGG, SERPINA1*), neutrophil trap and detoxification (*HPX*) (OE_Gill36), hubs ins innate immunity (*SCARF2, ANTXR1, COLEC12*) and angiogenesis (*PDGFRB, ZNF521, PDGFRB*) as well as cell adhesion (OE_G39). Related to increased nitrite and lowered oxygen saturation in upper estuarine areas also protein processing, stabilization and degradation (*SEC11A, PDIA, HSP90B1*) (OE_G41).

More ubiquitous related to elevated temperatures in spring and summer are ribosomal assembly (OE_L27), lysosome and peroxisome (OE_L12), p53 cell cycle signaling (OE_L8, OE_L21), hemoglobine synthesis (OE_L29), TCA & energy metabolism (OE_L34). Also, hubs in Inflammation (*IL31RA, C5AR1, APCS, CCR12A*), immunoglobin receptor (several *FCER1G*), detoxification (*EPHX2*), mucins (*MUC7, MUC2*) (OE_G40), TNF signaling (OE_G9) and antigen processing (OE_G42) in gill. With slight correlation to lowered DO, similar genes as in GC related to hemoglobin synthesis (hemoglobin subunits alpha 1, 2, D & beta, 1, 2) and cell shape integrity (*TMOD4, SPTB*) were found specifically upregulated in the lower estuarine sections (OE_L29, OE_G45).

5.1.3. Spatio-temporal immune responses

The cross-tissue trans-kingdom network correlation identified distinct clusters in fish immune responses and bacterial components at high temperatures and low oxygen during spring and summer. In the following we compare spatial and especially the seasonal variation in these patterns.

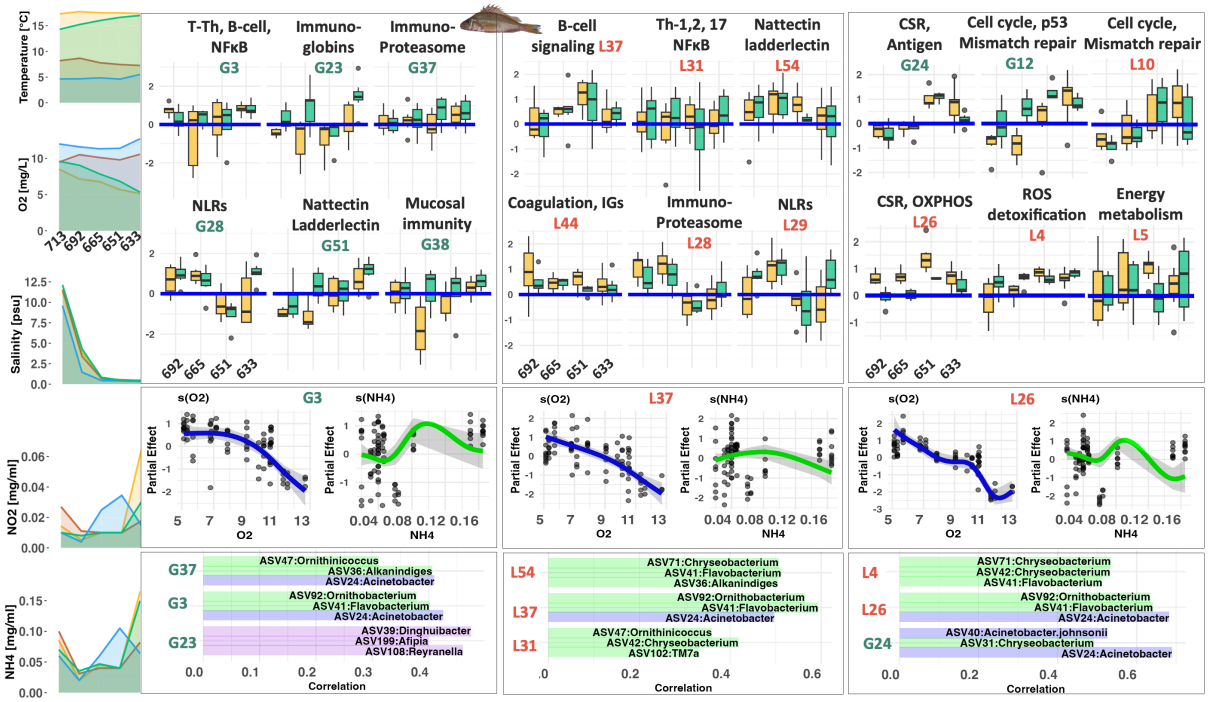


Fig. 4 Immune response in estuarine *Gymnocephalus cernua* (GC). Boxplots module Eigengenes of selected immune responses for gill (left), liver (middle) as well as cellular stress responses in both tissues (right) over the course of the estuary during summer and spring. In the lower panels smoothers for significant predictors in generalized additive model for the module eigengenes are shown explaining substantial amounts of variance (GC_G3: 60%, GC_L37: 50%, GC_L26: 87%). Barplots in the bottom panels depict the three bacterial ASVs witch highest corrected Pearson correlation to gene expression Eigengenes.

In GC gill tissue, core components of the immune response, T-cell and B-cell and NFκB signaling, immunoproteasomes and factors involved in pathogen recognition (IGs, NLRs, C-type lectins, *PGLYRP, PINLYP* (GC_G38)), showed strong upregulation in the spring increasing in upstream direction (**Fig. 4**, left upper panel). During summer, most of these responses were lowered, especially the expression of NLRs, immunoproteasomes and immunoglobins with a highly expressed (average TPM > 2000) Ig mu chain C region membrane-bound form as module hub (GC_G23). Adaptive immune components showed however little variation between seasons in gill and liver. In the latter, B-cell signaling was slightly higher in the upper estuary while T-cell and NFκB signaling form standing immunity (GC_L31). Also, C-type lectins (GC_L54), NLCs, immunoproteasome and coagulation cascades show little difference between seasons and were rather enriched in lower estuarine reaches in liver indicating tissue specific response patterns.

Cellular stress responses and cell cycle control were specifically high in summer in the upper estuary in both tissues while ROS detoxification and energy metabolism pathways where equal between seasons (**Fig 4**, right upper).

The GAMs indicate a significant increase in adaptive immune and cellular stress responses with declining oxygen levels over the full range (5 – 13 mg/L) and a slightly increasing but saturating effect of ammonia with higher values (**Fig 4**, middle panels).

NLRs in gill and liver and gill IGs were correlated (r > 0.5) with strains of *Dinghuibacter*, *Reyranella*, *Sediminibacterium* & *Afipia* (GC_SSU10) that are specifically abundant in spring fish constituting around up till 1 % relative abundance each. The B- and T-cell signaling,

immunoproteasomes (r > 0.3 – 0.5) and most strongly the cellular stress responses (r > 0.5) displayed high correlation with either the whole bacterial modules GC_SSU2 and GC_SSU5 EigenASVs and/or specifically with the abundances of several *Acinetobacter, Flavobacteria, Ornithinicoccus, Ornithobacterium, Chryseobacterium* strains (**Fig 4**, bottom panels).

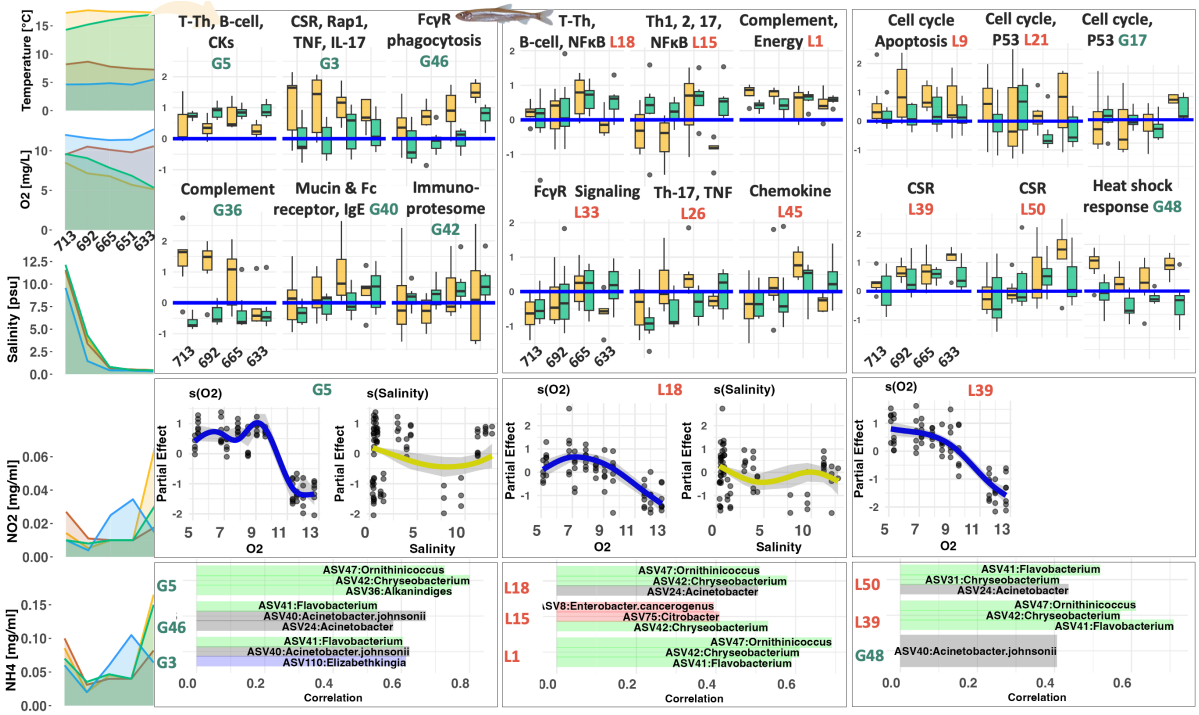


Fig. 5 Immune response in estuarine *Osmerus eperlanus* (OE). Boxplots module Eigengenes of selected immune responses for gill (left), liver (middle) as well as cellular stress responses in both tissues (right) over the course of the estuary during summer and spring. In the lower panels smoothers for significant predictors in generalized additive model for the module eigengenes are shown explaining substantial amounts of variance (OE_G5: 90%, OE_L18: 58%, OE_L39: 75%). Barplots in the bottom panels depict the three bacterial ASVs witch highest corrected Pearson correlation to gene expression Eigengenes.

Similar to ruffe, core gill immune responses in OE were highly upregulated in spring especially in the upper part of the estuary. In summer however, adaptive immune components decline in the upper estuary (OE_G5), while the cellular stress response (OE_G3) and innate components (FcγR phagocytosis, complement system activation) strongly increase (**Fig. 5**, left upper panel).

The OE liver likewise shows a strong increase in immune components spring and in upstream direction, however in summer all immune components drop, especially the adaptive T- and B-cell signaling (OE_L18, OE_L15). Instead, cellular stress responses, heat shock response and cell cycle control and apoptosis increase especially in the upper estuary (**Fig. 5**, right upper panel). GAMs depict a strong impact of oxygen levels on the adaptive immune and cellular stress responses, however while the effect peaks between 7 – 9 mg/L and declines again at low oxygen levels (< 6 mg/L) for the former, the lowest oxygen levels have the highest positive impact on the cellular stress module eigengene (**Fig. 5**, middle row, Report **Tab.1**). In high overlap with the ruffe, adaptive and innate immune components and especially cellular stress responses are highly correlated with either the whole bacterial modules or specific strains of *Acinetobacter* (OE_SSU7) or *Flavobacteria*, *Ornithinicoccus*, *Ornithobacterium*, *Chryseobacterium* (OE_SSU5) (**Fig. 5**, bottom row).

5.1.4. Cellular stress response prediction by bacterial abundances

The previous analyses showed some of the strongest associations between certain aspects of the cellular stress response and the occurrence of certain individual bacterial taxa or bacterial modules. Although the bacterial networks were generated individually by the respective gill mucus

communities, the taxa and modules in relation to the cellular stress and immune response are almost identical in both fish species. The *Flavobacteriaceae*-dominated modules (GC_SSU5 & OE_SSU5) increase strongly in the course of the estuary in spring and summer, but collapse in the upper reaches in summer (**Fig. 6** & **7**). The second cluster around *Acinetobacter* (GC_SSU2 and OE_SSU7) increases in abundance in both species strongly in summer in the harbor area of the estuary.

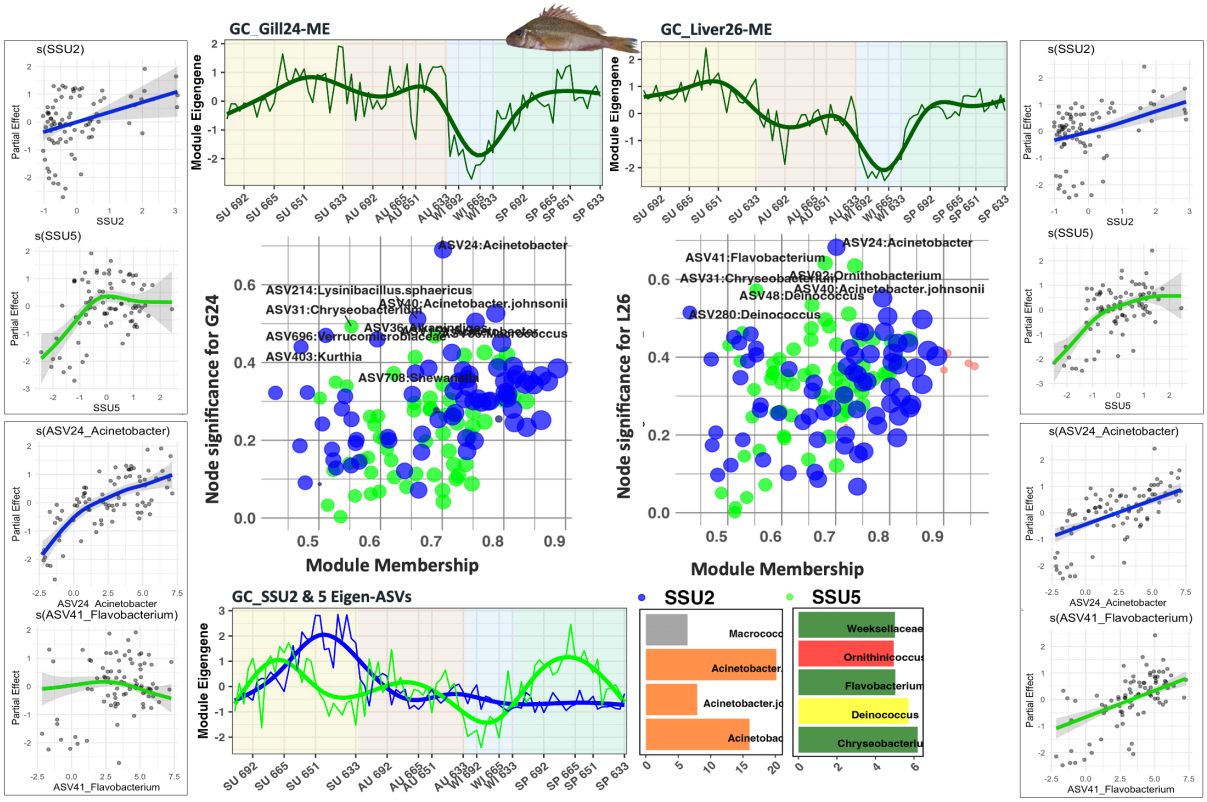


Fig. 6 Cellular stress response and potentially opportunistic pathogens. Line plots depict the Eigengenes (ME) of a gill and liver modules enriched in components of cellular stress response (CSR) (GC_G24, GC_L26) as well as those of the bacterial modules that show highest module correlation (GC_SSU2 & 5) along the course of the estuary (left = downstream, right = upstream) and over seasons. Central dot plots depict the correlation of individual bacterial taxa with CSR MEs (y-axis) as well as the individual intramodular correlation (x-axis & point-size). Barplots for SSU2 & 5 show dominant taxa in the modules by abundance. The outermost plots visualize the partial effects of the bacterial module eigen-ASVs (upper) and the dominant taxa by abundance (lowest) on CSR MEs (gill = left side, liver = right side) determined by generalized additive models. Model results in section **Tab.1**, model validation in **Fig.S.8.**

EigenASVs (GC_SSU2 & 5) explain substantial parts of the variance (41% & 55%) in the GC gill (GC_G24) and liver (GC_L26) CSRs, respectively. With increase in GC_SSU2 EigenASV the partial effect in both tissues CSR response increased indicating a direct and consistent influence over the range of the variables. SSU5 EigenASV showed a more complex relationship with a linear range at low values and a flattened slope at higher values indicating that high SSU5 loads do not significantly affect the CSR response further from a threshold on. The models predicting the effect of individual ASVs (ASV24_Acinetobacter (SSU2) and ASV41_Flavobacterium (SSU5)) explained 58% (gill) and 62% (liver) wherein liver showed significant association to both strains while in gill only *Acinetobacter* significantly affected the module eigengene.

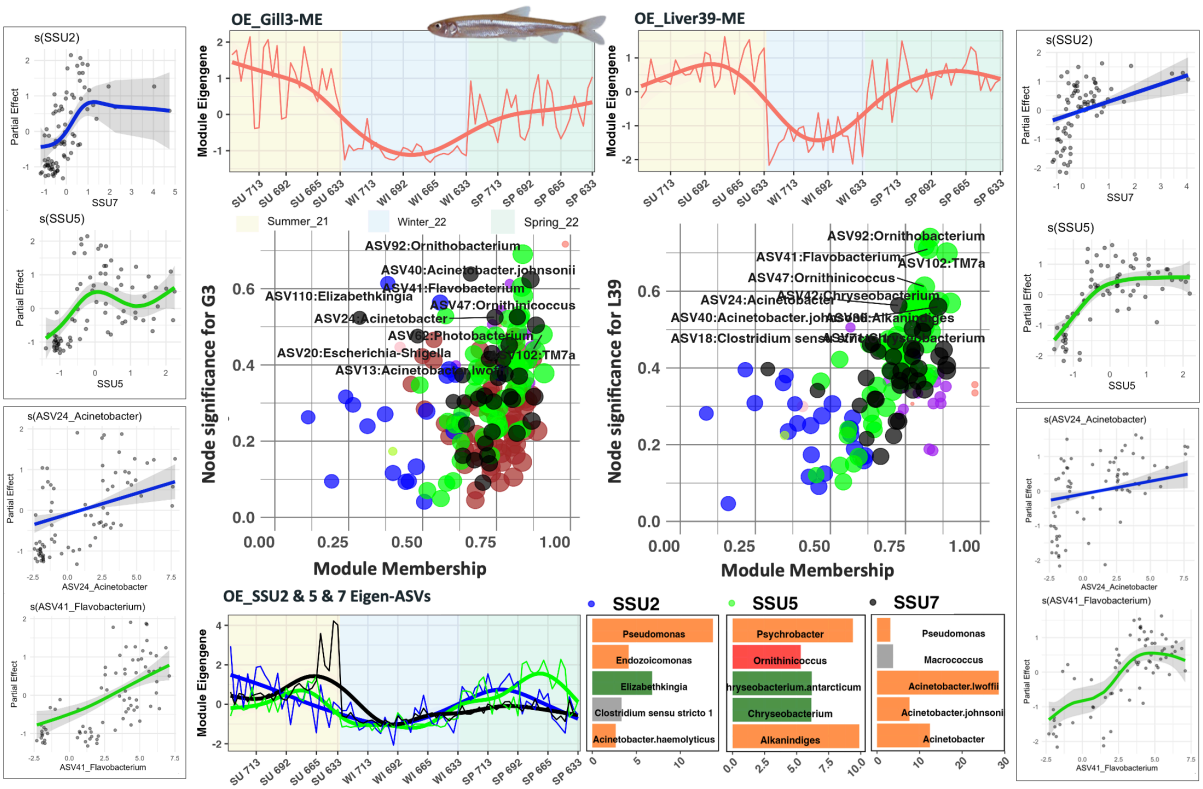


Fig. 7 Cellular stress response and potentially opportunistic pathogens. Line plots depict the Eigengenes (ME) of a gill and liver modules enriched in components of cellular stress response (CSR) (GC_G3, GC_L39) as well as those of the bacterial modules that show highest module correlation (OE_SSU2, 5 & 7) along the course of the estuary (left = downstream, right = upstream) and over seasons. Central dot plots depict the correlation of individual bacterial taxa with CSR MEs (y-axis) as well as the individual intramodular correlation (x-axis & point-size). Barplots for SSU2 -7 show dominant taxa in the modules by abundance. The outermost plots visualize the partial effects of the bacterial module eigen-ASVs (upper) and the dominant taxa by abundance (lowest) on CSR MEs (gill = left side, liver = right side) determined by generalized additive models. Model results in section **Tab.1**, model validation in **Fig.S9**.

For OE we observed similar patterns as described above indicating a strong, almost linear increase in cellular stress responses in both tissues with increasing EigenASVs explaining 61% & 68.5% variance, or individual abundancies of the dominant taxa ASV41_Flavobacterium & ASV24_Acinetobacter explaining 53.2% & 63.2% in gill and liver, respectively.

Tab. 1 Generalized additive models for adaptive immune and cellular stress response module Eigengenes in both fish species. See supplement **Fig. S.8– 13** for model diagnostics and complete reports.

Response	Predictor #1	edf	F	p	Predictor #2	edf	F	p	R-sq.adj, Deviance
GC_G3	O_2	3.0	20.156	< 0.001	NH_4	4.094	4.432	0.00306	0.61, 64%
GC_L37	O_2	2.177	30.398	< 0.001	NH_4	2.313	2.754	0.0431	0.51, 53%
GC_G24	O_2	5.418	6.383	< 0.001	NH_4	6.129	7.997	< 0.001	0.75, 78%
	GC_SSU2	1	5.909	< 0.001	GC_SSU5	3.4	8.63	0.0173	0.41, 45%
	ASV24 Acinetobacter	3.068	24.59	< 0.001	ASV41 Flavobacterium	2.299	1.884	0.148	0.58, 61%
GC_L26	O_2	5.869	35.856	< 0.001	NH_4	4.883	5.371	< 0.001	0.87, 88%
	GC_SSU2	1.377	13.607	< 0.001	GC_SSU5	3.05	15.243	< 0.001	0.55, 58%
	ASV24 Acinetobacter	1	41.12	< 0.001	ASV41 Flavobacterium	1	33.6	< 0.001	0.62, 63%
OE_G5	O_2	7.593	69.421	< 0.001	Salinity	2.52	3.299	0.0247	0.9, 92%
OE_L18	O_2	3.145	22.521	< 0.001	Salinity	2.969	3.592	0.105	0.58, 61%
OE_G3			No fit						
	OE_SSU5	3.942	4.705	< 0.001	OE_SSU7	3.729	4.624	< 0.001	0.61, 65%
	ASV24 Acinetobacter	1	10.88	0.0015	ASV41 Flavobacterium	1.625	13.62	< 0.001	0.53, 55%
OE_L39	O_2	3.213	55.66	< 0.001					0.75, 76%

OE_SSU7	1	15.883	< 0.001	OE_SSU5	3.273	18.224	< 0.001	0.69, 71%
ASV24 Acinetobacter	1	5.899	0.0178	ASV41 Flavobacterium	3.899	13.239	< 0.001	0.63, 66%

5.2. II Cold response

Cold adaptation in GC gill tissue comprised specifically autumn enriched OXPHOS, proteasome, spliceosome & ribosome pathways coordinated by *CIRBP* (cold-inducible RNA-binding protein B-like) (GC_G8, GC_G16) and amino acid metabolism in liver (GC_L35, 36).

Global low temperature adaptations then comprise ubiquitin mediated proteolysis (GC_L6, GC_G1, GC_G54), ER protein procession (GC_L2), ribosome and spliceosome regulation (GC_L3, GC_L18, GC_G5) in both tissues. Also, metabolic pathways (TCA, Glucagon & Fat degradation together with AMPK) in gill (GC_G11) and autophagy in liver (GC_L7). Further there is a slight enrichment in bacterial invasion and T-helper cell differentiation during the cold seasons nested within the large ECM receptor interaction and focal adhesion pathways.

Low temperature response in OE was likewise related to proteome adaptation via protein processing in ER coordinated by cold-inducible RNA-binding protein B (OE_L4), transcription & translation regulation (OE_L6), ribosome biogenesis & spliceosome (OE_L3, OE_Gill6), polycomb repressive complex (OE_L7, OE_L17), DNA integrity (OE_L38, OE_Gill13), as well as several pathways related to energy metabolism comprising proteasome, TCA, (OE_L5, OE_Gill20), autophagy and mitophagy (OE_Gill1, OE_L19, OE_L58), OXPHOS, TCA, Glycolysis and amino acid metabolism (OE_L48, OE_L49). Specific to the gill tissue were immune responses during low temperatures comprising leucocyte migration and neutrophil trap (*NCF2, CYBB, CYBA, MMP9*) (OE_G37, OE_G47), and a sub-section of the anti-viral response (*TLR3, RIDI, ADAR*) (OE_G44). The immune responses in both species gill tissues were correlated with the abundances of various cold-enriched *Flavobacteria* strains, in OE comprising the *F. psychrophilum* causative agent of cold-water disease.

5.3. III Antiviral responses

The network approach revealed highly overlapping cross tissue anti-viral response patterns in both fish species (GC_G21, GC_L13 & OE_G28, OE_L40) including recognition of viral RNA in cytoplasm (*IFIH1, DHX58*), type I interferon expression (IFN-α, IFN-β) (*IRF3, IRF7*), activation of interferon-stimulated genes (ISGs) (igldcp), direct inhibition of viral replication (*MX1, EIF2AK2*), counteractors of ISGylation (*ISG15, USB18*), ubiquitination for proteasomal degradation (*RNF135, TRIM39, RNF213*, and *HERC3*) as well as apoptosis inducer (*CASP8*). A multitude of TRIMs, known for their key role in viral signaling (Langevin et al., 2019) are comprised in the responses, especially TRIM21 known for coordinating the antibody-dependent intracellular neutralization (ADIN) of viruses (McEwan et al., 2011) forms a whole module in GC gill response (GC_G53). While unmatched by global abiotic or bacterial patterns, the viral responses are highly interrelated with constitutively expressed immune pathways. In GC, the anti-viral responses from a correlated cluster with cross-tissue expression of MHC and specific Immunoproteasome genes (*TAP1* & *2, psmb13a, 8a, 12, 10*) in two identical gill and liver modules (GC_L28, GC_G37) as well as hundreds of NLR subfamily genes (GC_L29, GC_L46, GC_G28). A similar correlation to antigen-processing (*TAP1, UBA1, RNF31, NLRC5, PSME2, PSMB10*) (OE_G42) exists in OE. In smelt the pattern recognition receptors *RIGI* and *TLR3* are highly correlated within liver but captured in another anti-viral module (OE_Gill44) with deviating, strongly negatively correlation to temperature (r -0.72).

5.4. IV Salinity

In GC, exposed gill tissue responses were captured in one large module summarizing autophagic processes and ABC transporters with hub genes directly involved in cell survival (*RICTOR,*

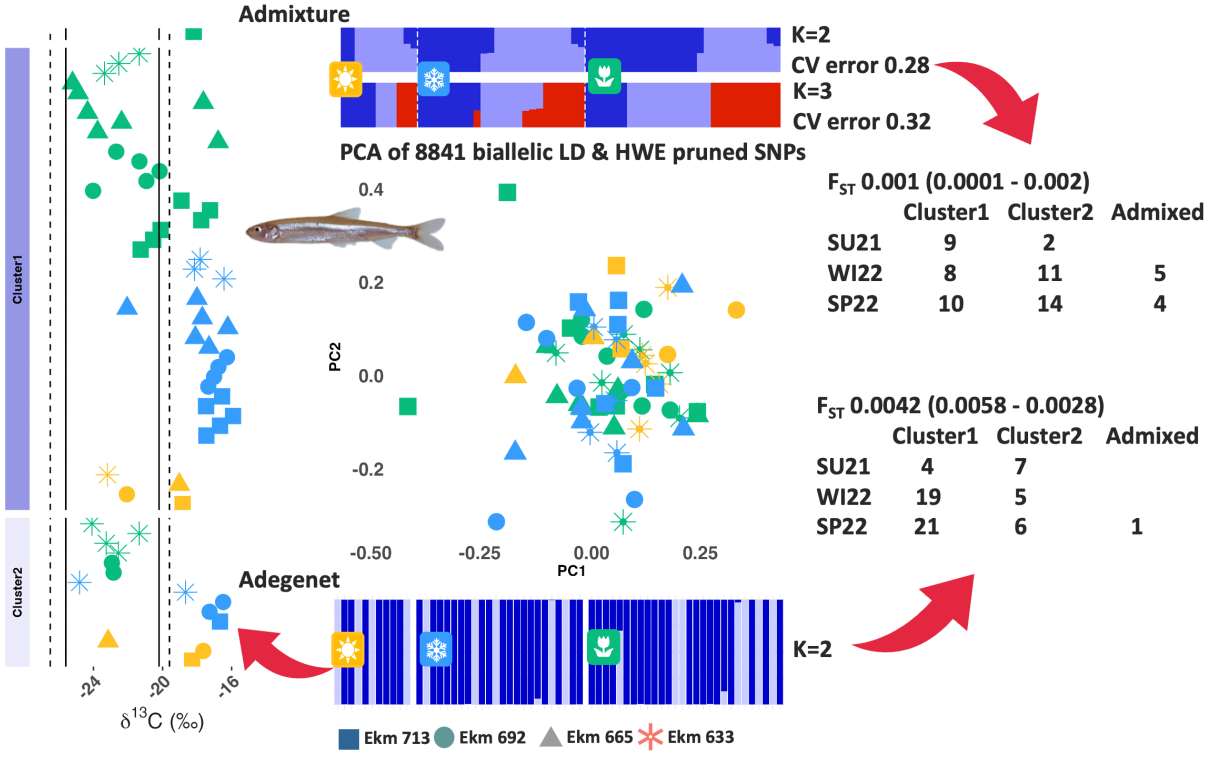
HERC1, AMBRA1, WNK1, MADD, EP400, PIK3R4), ion balance (*CLCN4*), proteostasis (UBR5) as well as long-chain fatty acid transport (*ABCA5, ABCD1*) (GC_Gill2) and Wnt signaling (GC_32).

Liver specific mainly innate immune system components, Nod- & toll-like receptor (TLR) signaling cascades (*IRAK4, IRAK3, TLRP5*), NFκB signaling (GC_L14) and complement components (various CFH and C3 related genes) (GC_L45). Interestingly a module comprising Bifunctional hemolysin/adenylate cyclase & Iron-regulated Protein FrpC mainly associated with bacterial pathogens used lyse blood cells, modulate immune responses and acquire iron was co-regulated (GC_L50). These responses are all aligned with higher fish age and length and unrelated to seasonal fluctuations in temperature as well a bacterial modules enriched in higher salinity: *Dinguibacter, Caulobacteraceae, Sediminibacterium, Afipia* (GC_SSU10) and *Illumatobacter, Halioglobus, Persicirhabdus, Luteolibacter* (GC_SSU7).

In anadromous OE, distributed along the higher spectrum of salinity levels, there was a low response to salinity levels in network modules as in mantel tests. Besides hemoglobin assembly, some metabolic adaptation and proteostasis pathways were found elevated in the liver encompassing enriched in glycerophospholipid metabolism, autophagy (OE_L22), amino acid and cholesterol metabolism (OE_L41), and OXPHOS (OE_L37) in low correlation to salinity levels. These responses were aligned with the appearance of high salinity indicator taxon (*Persicirhabdus*) in OE_SSU3.

5.5. Population differentiation

The exploratory approaches identified that all samples in the individual fish species most likely belonged to a single population, respectively. For OE cross validation between K 1-5 identified 1 with the lowest error value (0.22) followed by K = 2 (0.28). We assigned individuals with admixture Q-values > 0.85 to either of two clusters of equal size (n = 27). The data suggest a weak (F_{ST} = 0.0012) population structure between a cluster 1 present at all seasons along the estuary and a cluster 2 more likely to be present in spring and winter (**Fig. 8**). Similar, adegenet identified a single cluster by smallest BIC followed by two that showed similar patterns as in admixture: Most animals in cluster 1 were present in spring and winter and a smaller cluster more present in summer and upper estuary in winter and spring.



F_{ST} 0.001 (0.0001 - 0.002)

	Cluster1	Cluster2	Admixed
SU21	9	2	
WI22	8	11	5
SP22	10	14	4

F_{ST} 0.0042 (0.0058 - 0.0028)

	Cluster1	Cluster2	Admixed
SU21	4	7	
WI22	19	5	
SP22	21	6	1

Fig.8 Admixture and adegenet runs exploring population structure in the OE gill datasets. The analysis comprised 63 Individuals in 8,841 biallelic SNPs filtered for Hardy–Weinberg equilibrium, presence of 95% of the datasets and pruned for linkage disequilibrium putatively representing neutral SNPs clustered in a PCA in the middle plot. Both analyses supported the presence of only one population most, two populations was the next likely possibility. Weir and Cockerham's pairwise FST and confidence intervals with 1,000 bootstrapped iterations were calculated between the non-admixed individuals assigned to one cluster with Q-values > 0.85 by admixture or adegenet find clusters (upper and lower plot and results in the tables on the right side). Population assignments from adegenet matched with stable isotope signal for δ13 C from fish muscle tissue indicate in which region inside and outside the estuary the individual animals fed over the last weeks to month. The limits of the estuary are −20.2 ± 0.6 (lower reaches) and −25.6 ± 0.9 (upper reaches). Values above this come from the marine, below this from the freshwater reaches upstream. Four summer fish miss SIA data.

For GC similarly both approaches predicted K = 1 as the most likely outcome and PCA presentation indicated little seasonal and mixed spatial clustering with most animals from the lower section clustering part from the middle section on PC2 (**Fig. 9**). The second likely scenario of two clusters resulted in a similar pattern in both exploratory approaches with one cluster present all along the estuary and one especially abundant in the middle section. Stable isotope signals match exactly the location of capture in this species but gives no further insight into the population structure.

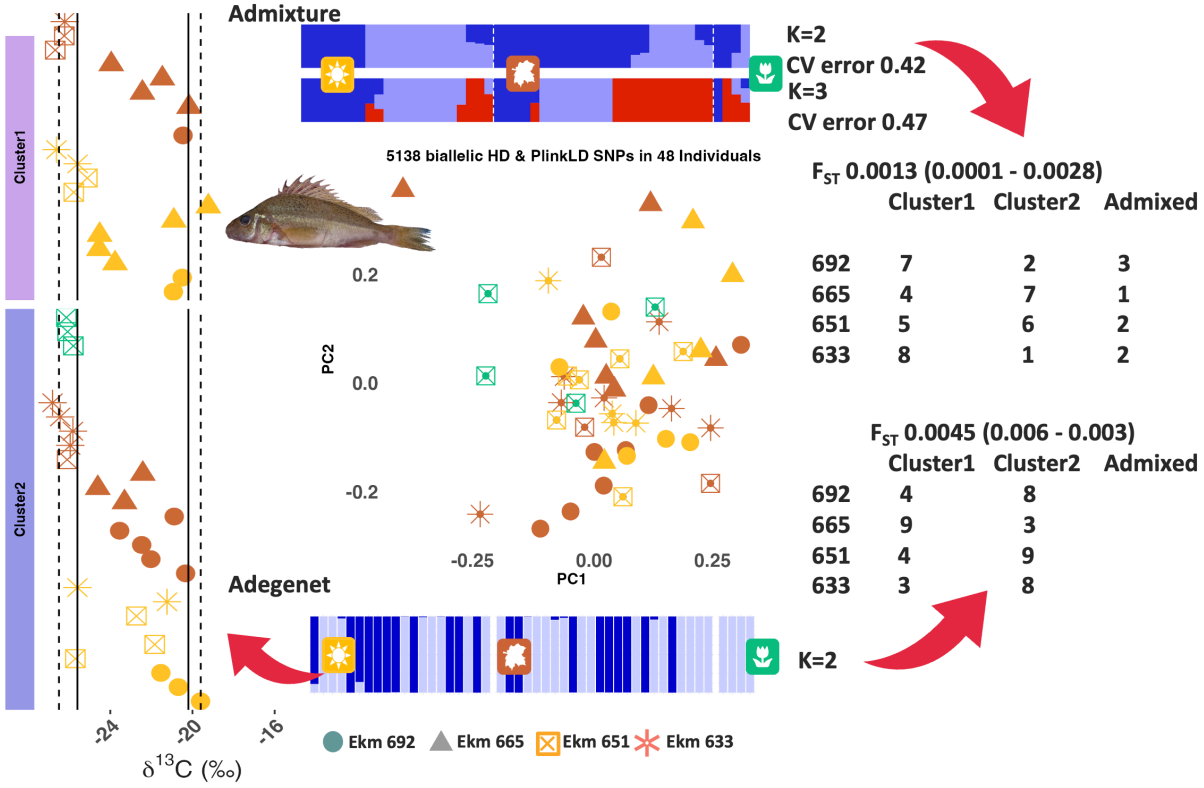


Fig.9 Admixture and adegenet runs exploring population structure in the GC gill datasets. The analysis comprised 48 Individuals in 5,128 biallelic SNPs filtered for Hardy–Weinberg equilibrium, presence of 90% of the datasets and pruned for linkage disequilibrium putatively representing neutral SNPs clustered in a PCA in the middle plot. Both analyses supported the presence of only one population. Two populations was the next likely possibility. Weir and Cockerham's pairwise F_{ST} and confidence intervals with 1,000 bootstrapped iterations were calculated between the non-admixed individuals assigned to one cluster with Q-values > 0.85 by admixture or adegenet find clusters (upper and lower plot and results in the tables on the right side). Population assignments from adegenet matched with stable isotope signal for δ13 C from fish muscle tissue indicate in which region inside and outside the estuary the individual animals fed over the last weeks to month. The limits of the estuary are −20.2 ± 0.6 (lower reaches) and −25.6 ± 0.9 (upper reaches). Values above this come from the marine, below this from the freshwater reaches upstream.

5.6. Signatures of selection

The pcadapt analysis of GC gill on a total of 93,492 SNPs identified 6,218 outliers and 1305 gene clusters carrying more than one SNP of which 74% could be mapped to the network modules. High and low temperatures had similar effects as 18 and 23% of SNPs were bundled in high and low temperature correlated modules (-0.5 < r > 0.5), genes in relation to salinity fluctuation made up only 2.8%. The PCA rather showed spatial patterns than seasonal effects, PCs 1 and 2 slightly split a larger portion of upstream animals apart from those caught in downstream direction while on PC3 and 4 there were no patterns related to location and season of sampling (**Fig.10**). About 18% of SNPs were caught in the uncorrelated gene module 0, followed by 9% in the broad gill immune module (GC_G3) (*IFI44L, HLA-DRA, HLA-DQB1, AIF1L, MPEG1, TRIM25*), apoptosis and DNA damage (*DNASE1L3, STK17A, MOB1A*), oxygen binding (*MB*) and Ion regulation (*VDAC, CA4*) (**Tab. 2**). Further related to high temperatures and hypoxia 3,7% in GC_G17 (*HIF1A, CBS*), paletet activation (*PRGIS, F2R*), Immune (*BTN1A1, NFKBIA, SOCS3*), and 3,4 in CSR (GC_G24) in ROS defense (*GPX1*), proteostasis (*CALR*) and genes related to cycle progression (*MOB3A, HDAC2, PPP1CA*). Related to cold temperatures on the other hand 8,6% in GC_G1 in genes related to immune responses (*PTGER2, IFRD1, IRF6*), lysosomal and autophagosomal degradation (*FUCA1, MVB12A, VMP1*) and 8% in GC_G11 in genes involved in energy metabolism (*ATP1A1, NDUFS1, GOT1, PFHA1, MPC1, ACO2, CYP46A1*).

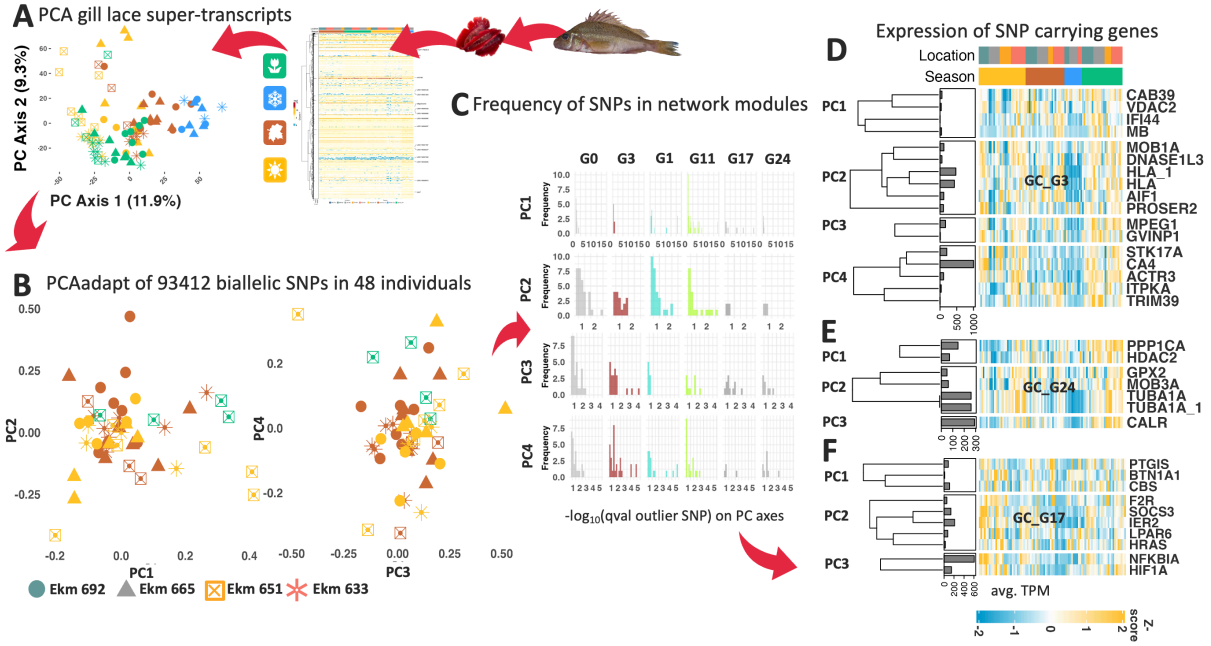


Fig. 10 Signatures of selection in GC. Super-transcripts clustered from *de Novo* assembly (**A**) used for SNP calling. Principal component implementation from pcadapt with colors showing seasonal sampling and symbols locations along the course of the estuary (**B**). 93,412 biallelic SNPs were called in 48 animals and filtered for quality, minor allele frequency > 0.05 and presence in at least 80% of the samples. This set was used for outlier analysis without filtering for Hardy–Weinberg equilibrium or pruned for linkage disequilibrium, thus more likely indicating patterns of adaptive variation in the system. Principal components (PC 1 - 4) represent slight seasonal patterns. Transcripts were filtered for containing at least two outlier SNPs before mapping to the network modules (**C**). The six modules containing most SNP carrying genes are shown in histograms indicating significance of outlier SNPs and frequencies in network modules. The heatmaps (**D - F**) depict z-score of TPM-expression genes carrying at least two SNPs for Modules GC_G3, 11, 24 indicating seasonal and spatial expression patterns. Barplots on the right side of the heatmaps show the average TPM value of each gene filtered for TPM > 5.

Tab. 2 Genes carrying multiple SNPs in GC gill data. Column 1 shows the seven network modules with most SNP carrying genes, the Eigengene correlation with oxygen saturation and temperature as well as the module KEGG enrichment. Column 2 contains the pcadapt principal component and column 3 individual genes with ≥ 2 outlier SNPs significant at Benjamini–Hochberg adjusted p-value.

GC_G0	PC1	*TLR7, SERINC1, PRKCD, SLCO3A1, GADD45A, TRIM16, STX10, JAG1, PAF1, TCP11L2, SORT1*
0.6 O2Sat, 0.77 Temp	PC2	*ERVFRD-1, TMEM165, VWA1, IFITM3, SERINC1, PPL, PRKCD, TLR7, EVPL, MVP, BCL11A, MBP, VGLL4, CREB3L3, TMEM127, GOLPH3, PSG1, SLCO3A1, DCAF4, DNAJC7, DDX5, CR2, NRXN2, ARF1, TAAR5, SERPINA10, ALDOA*
	PC3	*NRXN2, GSK3B, SORT1, TSPO, CR2, CREB3L3, CLDN19, PPL, SLC2A1, TMEM165, NANS, VWA1, ERVFRD-1, JAG1, TMEM127, HK1, SLCO3A1, STX10, VGLL4, SERPINA10, EVPL, PSG1, GOLPH3, IFITM3, PAF1*
	PC4	*ERVFRD-1, TAAR5, MTDH, TRIM16, CR2, BCL11A, HK1, DDX5, TLR7, DCAF4, SERPINA10, GSK3B, MBP, NRXN2, ARF1, MVP, ALDOA*
GC_G3	PC1	*IFI44L, ACTR3, RAB25, HLA-DRA, CAB39, VDAC2, MB*
-0.44 O2Sat, 0.47 Temp	PC2	*AIF1L, HLA-DQB1, MOB1A, HLA-DRA, PROSER2, RAB25, CA4, TRIM25, MPEG1, DNASE1L3, ITPKA*
Immunodeficiency, Th1, 2, 17, NK, NFkB	PC3	*TRIM25, AIF1L, MPEG1, STK17A, IFI44L, MOB1A, PROSER2, RAB25, HLA-DQB1, CAB39*
	PC4	*CA4, TRIM25, ACTR3, STK17A, MPEG1, HLA-DQB1, HLA-DRA, ITPKA, PROSER2, DNASE1L3*
GC_G1	PC1	*PTGER2, MVB12A, IRF6, SMAP1, SCCPDH, MAPK1, SRSF5, VMP1, TUBA1C*
0.78 O2Sat, -0.98 Temp	PC2	*PPP2CA, SMAP1, CTSK, ETNK1, WAC, FUCA1, MTHFD1, MAPK1, TUBA1C, DDX19B, IFRD1, MVB12A, SCCPDH*
Focal adhesion, ECM, Protein digestion	PC3	*ETNK1, SCCPDH, DDX19B, FUCA1, SEC61A1*
	PC4	*TUBA1C, IRF6, WAC, VMP1, SEC61A1, ETNK1, DDX19B, MVB12A, CTSK, SRSF5, PPP2CA, IFRD1*
GC_G11	PC1	*ATP1A1, NDUFS1, GOT1, NLRP12, PDHA1, ABCA1, MPC2, RAB8A, CLDN4*
0.41 O2Sat, -0.57 Temp	PC2	*ACO2, ABCA1, CALM1, ATP1A1, NLRP12, CLDN4, ZP4, MPC2, GNS, NDUFS1*
Glucagon signaling, TCA, Fat degradation AMPK	PC3	*CALM1, ZP4, NLRP12, CYP46A1*
	PC4	*CALM1, GOT1, SAT1, ABCA1, PDHA1, CYP46A1, CLDN4, NDUFS1, GNS*
GC_G8	PC1	*RAP1A, COX4I1, EMC4*
OXPHOS, Proteasome, Spliceosome	PC2	*PFDN2, RBM4B, CDC42, EMC4, DDX39A, RHOA, PGK1, RAP1A, TLE5*
	PC3	*CSNK2B, PGK1, PFDN2, TLE5, RHOA, EMC4, SET, PSMD4, ARF6, BRI3, ARPC4*
	PC4	*CDC42, DDX39A, SET, BRI3, ARPC4, COX4I1, PSMD4*
GC_G17	PC1	*CBS, PTGIS, BTN1A1, NFKBIA*
-0.33 O2Sat, 0.44 Temp	PC2	*BTN1A1, NFKBIA, HIF1A, PTGIS*
MAPK	PC3	*F2R, HRAS, SOCS3, LPAR6, IER2, HIF1A*
	PC4	*NFKBIA, HIF1A, BTN1A1, IER2*
GC_G24	PC1	*TUBA1A, CALR, MOB3A, HDAC2*
-0.45 O2Sat, 0.45 Temp	PC2	*HDAC2, GPX1, PPP1CA, CALR*
CSR, antigen processing	PC3	*TUBA1A, GPX1, CALR, TUBA1B, MOB3A*
	PC4	*CALR, TUBA1A, GPX1, PPP1CA*

In OE, there was a slight seasonal pattern between summer and spring animals clustering apart from winter caught fish within the first four PCs from the pcadapt analysis (**Fig.11**). From a total of 127,000 biallelic SNPs, 2414 appeared as outliers (q < 0.05) on the first four PCs with 474 gene clusters containing more than one SNP that were used in the further analysis. 75% of the gene clusters carrying more than one SNP could be matched against the OE genome, 46 % of which matched with network modules related to high (r > 0.5) temperature while only 13% were related to those with low temperature (r < -0.5) and none with salinity. Overall, 13% were related to module zero, containing un-correlated genes within the network analysis, while a very high amount (13% and 10%) where contained in the CSR and immune modules OE_G3 and OE_G5 followed by 8% in OE_G2 (protein digestion and focal adhesion). Several mutations within the cellular stress response module (OE_G3) fall within the enriched signaling pathways MAPK pathway (*DUSP1*), TNF signaling (*TNFRSF10B, TRAF2, TNFAIP8L2*), general immune response (*PTGFRN, TIMP3, MYD88, CD44*) and heat shock & hypoxia (*HIF1A, HSPA8, HASP90AB1, RNF19B, TCP1*) (**Tab. 3**). SNPs in adaptive immune module OE_G5 genes comprise the major histocompatibility complex I (*MR1*) and II genes (*HLA-DPA1, HLA-DPB1*) as well as further immune related responses (*MPEG1, IFI30, FCGBP, CCR6, NLRP12, ALOX5*) but also the cross-tissue cross-species adaptive immune hub gene *INPP5D*.

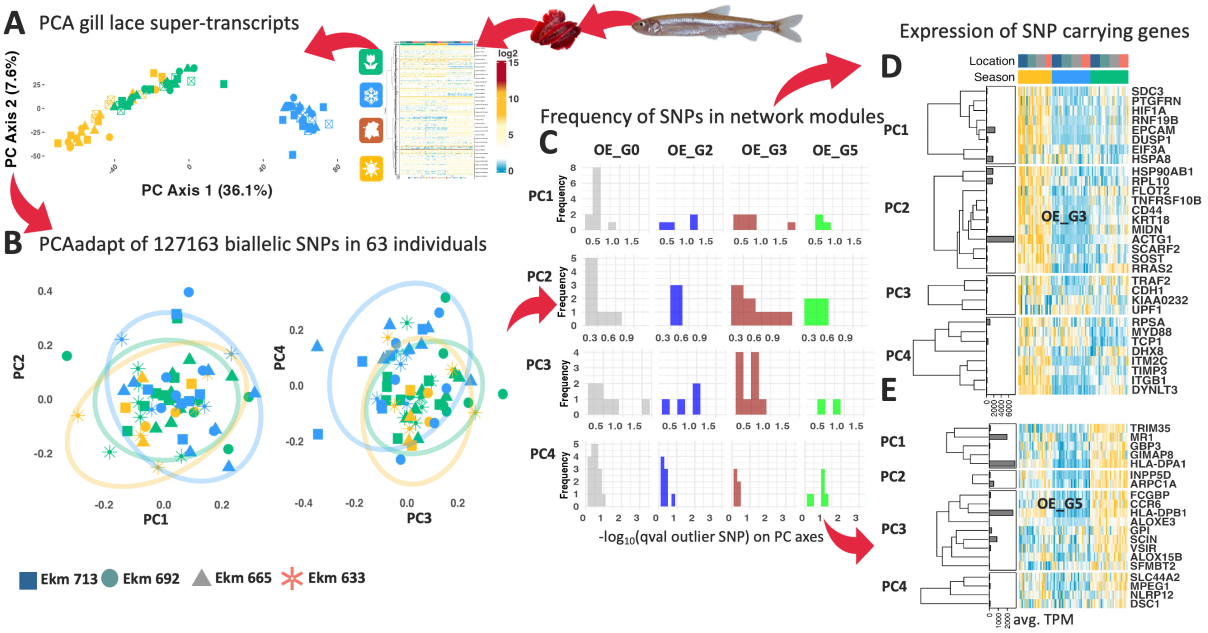


Fig. 11 Signatures of selection in OE. Super-transcripts clustered from *de Novo* assembly (**A**) used for SNP calling. Principal component implementation from pcadapt with colors showing seasonal sampling and symbols locations along the course of the estuary (**B**). 127,163 biallelic SNPs were called in 63 animals and filtered for quality, minor allele frequency > 0.05 and presence in at least 80% of the samples. This set was used for outlier analysis without filtering for Hardy–Weinberg equilibrium or pruned for linkage disequilibrium, thus more likely indicating patterns of adaptive variation in the system. Principal components (PC 1- 4) represent slight seasonal patterns. Transcripts were filtered for containing at least two outlier SNPs before mapping to the network modules (**C**). The four modules containing most SNP carrying genes are shown in histograms indicating significance of outlier SNPs and frequencies in network modules. The heatmaps (**D** & **E**) depict z-score of TPM-expression genes carrying at least two SNPS for Modules OE_G3 and 5 indicating seasonal and spatial expression patterns. Barplots on the right side of the heatmaps show the average TPM value of each gene filtered for TPM > 5.

Tab. 3 Genes carrying multiple SNPs in OE gill data. Column 1 shows the four network modules with most SNP carrying genes, the Eigengene correlation with oxygen saturation and temperature as well as the module KEGG enrichment. Column 2 contains the pcadapt principal component and column 3 individual genes with ≥ 2 outlier SNPs significant at Benjamini–Hochberg adjusted p-value.

OE_G0 -0.52 O2Sat, 0.95 Temp	PC1	*ITM2B, FAM83C, HEXB, FRMD6, BTNL2, LMNB2, PNN, URGCP, PSMB8, BHLHE40, KRT17, PSAP, PAWR, AKAP9, GUK1, DAAM1, N4BP2L1, CLDN4, LPCAT3, RALGAPB, ZDHHC23, TYMP*

	PC2	*DCTD, Cluster-51770.20637, ATP5F1A, GOLGB1, PSAP, LPCAT3, URGCP, ALAD, C1GALT1, CLDN4, GIMAP4, TAPBP, KDM5A, FAM83C*
	PC3	*CTSB, DAAM1, PAWR, RALGAPB, TYMP, ATP5F1A, ZDHHC23, GOLGB1, TAPBP, KRT17, AKAP9, PNN, ALAD, Cluster-51770.20637, CAVIN2*
	PC4	*PGRMC2, ALAD, GIMAP4, CLDN4, URGCP, ATP5F1A, ANXA1, KDM5A, LPCAT3, GUK1, LMNB2, CTSB, ITM2B, CAVIN2*
OE_G3	PC1	*SDC3, EPCAM, HSPA8, PTGFRN, ITM2C, RNF19B, MIDN, HIF1A, TNFRSF10B, DUSP1, TIMP3, DHX8, RPSA, RRAS2, MYD88, RPL10, KIAA0232, CDH1, TRAF2, SOST, EIF3A*
-0.45 O2Sat, 0.78 Temp		
MAPK, bacterial-viral infection,		
Apoptosis, Rap1, TNF, PI3K-akt, IL-17	PC2	*SDC3, FLOT2, RRAS2, TNFRSF10B, MIDN, CDH1, ACTG1, CD44, SOST, RPL10, KRT18, SCARF2, TCP1, HSP90AB1, ITGB1*
	PC3	*KIAA0232, UPF1, CDH1, RPSA, RNF19B, PTGFRN, TIMP3, ITM2C, EPCAM, CD44, TRAF2, DUSP1, DYNLT3, HSP90AB1*
	PC4	*RPSA, DHX8, KIAA0232, UPF1, ITM2C, TIMP3, MYD88, TCP1, DYNLT3, ITGB1, KRT18, ACTG1, CD44*
OE_G5	PC1	*HLA-DPA1, MR1, HLA-DQB1, MPEG1, GIMAP4, TRIM35, GBP2, INPP5D, VSIR*
-0.47 O2Sat, 0.88 Temp	PC2	*INPP5D, ARPC1A, GSN*
NK-cell cytotoxicity, B-, T-cell	PC3	*HLA-DQB1, FCGBP, HLA-DPA1, ALOXE3, DSC1, CCR6, ALOX5, SFMBT2, MR1, GSN, GIMAP4, GPI, SLC44A2, VSIR, NLRP12*
receptor signaling, B cell, Th1, 2, 21, Chemokine	PC4	*HLA-DPA1, MPEG1, NLRP12, DSC1, FCGBP, SFMBT2, SLC44A2, ALOXE3, HLA-DQB1, GSN*
OE_G2	PC1	*ADH5, RBM39, ATP1A2, FN1, EEF2, TLN1, MR1, CLK1, PTPRF*
-0.54 O2Sat, -0.95 Temp	PC2	*CLK1, FN1, EEF2, MBP, SGCG, TLN1, GSN, FBN1*
Protein digestion, ECM, Focal-Adhesion	PC3	*ATP1A2, CLK4, FN1, CCN1, MR1, CLK1*
	PC4	*SGCG, ATP1A2, ADH5, CLK4, COL6A1, COL6A3, GSN, CCN1, FBN1, MBP*

6. Discussion

6.1. Stress response strategies in the Elbe estuarine fish

The interaction of multiple stressors can narrow the capacity for compensation in fish (Petitjean et al., 2019) and might lead to a switch from stress compensation to energy conservation strategy (Sokolova, 2013; Sokolova et al., 2012). Reactive oxygen species (ROS) are one universal aspect of stress exposure, arising from disruptions in the mitochondrial electron transport chain during to oxygen deficiency, increased metabolism from rising temperatures, immune system activation to kill pathogens, or xenobiotic-induced protein malfunctions, either individually or cumulatively during these interactions (Birnie-Gauvin et al., 2017). Scavenging enzymes (SODs, CAT, GPXs, PRXs) and antioxidants (Sulmon et al., 2015) belonging to CSR category I anti-oxidative systems were universally coordinated with increasing temperatures in both estuarine fish. With ROS production exceeding the defenses, probably especially when accumulating during multiple-additive/synergistic stressor interaction, macromolecules get damaged (Lushchak, 2011). High damage levels lead to genotoxicity (Birnie-Gauvin et al., 2017) with potentially cascading effects on the survival of the individual up to population dynamics (Cheng et al., 2018; Malev et al., 2010). Indicative cellular stress responses of categories II (cell cycle control) and IV (cell death) were determined especially related to prolonged hypoxia in summer in the upper part of the estuary in both fish species. Species-specific difference appeared in the capacity of GC to maintain large parts of the immune functions in parallel while in OE global immune responses drop with the steep increase in CSR categories. The global results indicate **I.** that both species at the prevailing levels of multiple-abiotic stressors mount appropriate compensatory responses instead of conserving energy, **II.** trade-off between cellular stress responses and immune components takes place in both species at differing levels, **III**. benthopelagic ruffe appear to have a higher capacity to tolerate prevailing situations.

6.2. Estuarine stressors

6.2.1. Salinity gradient and fluctuations

Environmental salinity influences a wide range of physiological responses in fish (reviewed in Evans & Kültz, 2020) and can strongly affect the energy budget (Bœuf & Payan, 2001). Responses differ greatly between direction of the osmotic pressure, acute or chronic exposure of fluctuations (Foskett et al., 1981; Sangiao-Alvarellos et al., 2003; Tseng & Hwang, 2008). In both fish, stable isotope and bacterial patterns indicate little short-term migration along the estuarine salinity gradient (0.5 – 15 psu), but rather exposure to lower tidal salinity fluctuations (± 0.5 psu lower section, ± 0.05 psu upper section). The lack of expression changes related to salinity fluctuations in OE gill indicated that no tissue remodeling took place and capacity for ion transport with and against the osmotic gradient are likely kept in reserve in the anadromous species (Evans & Kültz, 2020). Weak increases in liver energy metabolism are further in line with responses in other euryhaline fish indicating slightly increased energy demand (Aragão et al., 2010; Tseng & Hwang, 2008; Xu et al., 2015a, 2015b). In the potamodromous GC on contrary increased autophagy, Wnt-pathway activation and long-chain ABC transporters expression, indicated rearrangements in the epithelial tissues exposed to osmotic gradients (Evans & Kültz, 2020; Jeffries et al., 2019; Kültz, 2005; Shivkamat & Roy, 2005). Unexpected was a strong, seasonally independent innate immune pathway upregulation in the liver of GC especially in NFκB-, TNF-, NOD-like signaling and complement factors, that are mainly synthesized in this tissue (Wang et al., 2015; Wang & Secombes, 2003). Non-specific modulation of the innate immune response in fish under fluctuating salinity regimes are commonly observed, especially in the liver tissue playing a decisive role in the cross-tissue comparison (El-Leithy et al., 2019; Maryoung et al., 2015; Xu et al., 2015b; Y. Zhang et al., 2022). In downstream estuarine sections, GC reach their salinity tolerance limits around 12 psu (Lind, 1977), where only larger, perennial animals were caught. We cannot rule out parasitic or other pathogenic infections favoring the liver specific innate immune activation. This response is however presumably energetically costly and may be associated with resource allocation trade-offs that impair mounting adaptive immune responses when necessary, shown to render exposed fish vulnerable for infection (Birrer et al., 2012). Over the last two decades, a trend towards an increase in salinity has already been detected in the estuarine area (Scholle & Schuchardt, 2020), and with progressing climate change saltwater intrusions are expected to increase especially due to lowered river discharge in the summer month (Lee et al., 2024). These processes lower the distribution ranges of GC, change the holobiont response, might introduce new parasites (Lee et al., 2022) and could already be related to the major population decline (95.3%) observed over the last decades.

6.2.2. Immunosuppression under high temperature, hypoxia and eutrophication

Continuous exposure to stressors involves continuous allocation of energy towards stress responses. Mechanisms necessitating continuous energy availability by the immune system (antibody production, complement system, differentiation of leukocytes) can be subjected to a lack of resources, decreasing efficiency and leading to immunosuppression (Tort, 2011). Several factors in our data suggest multi-stress related immunosuppression in both fish species, particularly associated with prolonged lowered oxygen levels. Nearly all immune patterns increase strongly with increasing temperatures and decreasing oxygen levels in upstream direction in spring. In GC, large adaptive patterns around T- and B-cell signaling are maintained with decreasing oxygen levels, but immunoglobins such as IGM associated with the B cell receptor in fish gills (Salinas et al., 2011) and pattern recognition factors (NLRs, C-type lectins) decrease under prolonged hypoxia in the harbor area in summer. In OE, we see a pronounced tissue-wide decline in overall adaptive and especially in liver also innate immune responses starting when oxygen levels fall below 7 mg/L. Since adaptive immune functions such as immunoglobins enhance antigen-specific opsonization and phagocytosis allowing more efficient immune responses to repeated pathogen exposure compared to innate immunity (Mashoof & Criscitiello, 2016), immune responses in summer in both fish species can be expected to be less efficient. This is in line with recent studies from laboratory or aquaculture experiments where especially adaptive immune components are often impaired by prolonged hypoxia such as reduced

antibody titer following vaccination (Gallage et al., 2017; Wang et al., 2018), impaired lymphocyte migration (Schäfer et al., 2021) or even complete prevention in raise in immunoglobin (IgM) levels upon bacterial infection (Leeuwis et al., 2024). Also innate humoral responses like complement factors or lysozyme production can be impaired (Abdel-Tawwab et al., 2015; Douxfils et al., 2012) rendering affected fish overall immunocompromised.

During our sampling campaign, almost 80% (800 individuals/mio.m^3) of the total catches of GC occurred in summer in the harbor area. The largest abundance of adult smelt stayed in the lower reaches in summer and spring (38% between Ekm 712 - 691) avoiding areas with oxygen levels below 5 mg/L (Möller & Scholz, 1991). However, this does not apply to juveniles with restricted movement capacities whose main abundance were caught at Ekm 633. Based on stable isotope data and bacterial patterns indicating high site fidelity, we assume that fish caught during our sampling remained exposed to local conditions for extended periods, with limited ability to escape low DO areas. Hypoxic areas between spring and summer may act as migration barriers (Roberts et al., 2009; Thiel et al., 1995). Thus, our results represent a significant portion of the prevailing populations, with annual summer mass mortalities being only direct, visible effects of ongoing ecosystem degradation.

Aside from lowered oxygen, ammonia and nitrite itself accumulate in summer and spring in the harbor area (Ingeniero et al., 2024) and can reach values > 0.3 mg/L respectively over periods of several weeks (Sanders et al., 2018). Environmental nitrite changes hemoglobin to non-oxygen carrying methemoglobin (Bodansky, 1951) causing anoxia in fish (Lewis & Morris, 1986). The concentrations in fish blood plasma can amplify 60 times compared to surrounding water (Fontenot et al., 1999) and concentrations of only 0.3 mg/L nitrite as a single stressor in laboratory experiments promoted protein catabolism and inflict histological damage and hamper growth after 60 days in a percid species (Zhang et al., 2021). Both sublethal hypoxia and nitrite exposures affect the interplay between host and its associated microbiome (Yu et al., 2021). Irrespective of changes in the bacterial composition in the water columns, sublethal nitrite change the microbiome (Ortiz et al., 2023) and render them prone to infectious diseases (Carballo et al., 1995).

6.3. Holobiont interaction

6.3.1. Bacterial abundances

With increasing temperatures in spring and summer highly similar and correlated clusters of Flavobacteriaceae (GC_SSU5, OE_SSU5) accounting for 7% in overall average abundance in gill mucus in both fish species increased in upstream direction. These are almost entirely composed of genera ranging among the top contributors to disease-associated losses in fish farming (e.g., *Flavobacterium, Chryseobacterium, Elizabethkingia, Ornithobacterium*) (Loch & Faisal, 2015). Causing commonly known infections like columnaris, bacterial gill and bacterial cold water disease (BCWD) (*F. psychrophilum*), and various further ones related to by *F. spp.* and *Chryseobacterium spp.* (Loch & Faisal, 2015).

Flavobacteria and *Chryseobacterium* have an almost universal strong proteolytic activity, their virulence is achieved and dependent on secretion of proteases, the efficiency of iron uptake, the bacterial biofilm production and nutrient availability (Wahli & Madsen, 2018). In infected fish they induce strong regulation of lectins, interferon-inducible signaling, but commonly also cause immunosuppression (downregulation of NFκB pathways, MHC, IGs, TLRs, chemokine genes), immune evasion and gill tissue necroses and high mortality (Declercq et al., 2015; Sun et al., 2012; Xu et al., 2024). Increasing in abundances in strains from estuarine fish gill mucus aligned with strong upregulation of adaptive immunity components: B- & T -cell signaling, T-helper cell differentiation, NFκB and chemokine signaling in both species and tissues. This indicates a coordinated response in

the hosts in an attempt to manage homeostasis in its microbiota with increasing temperatures and decreasing DO levels.

An interesting pattern is the emergence of another highly overlapping bacterial cluster in both fish species (GC_SSU2, OE_SSU7) composed of strains of *Acinetobacter* (A. *spp., iwofii, johnsonii*), *Macrococcus*, *Shewanella* (S. *baltica* & *putrefaciens*), *Chryseobacterium*, *Aeromonas* & *Pseudomonas* only in summer under prolonged hypoxia and high loads of nitrogen compounds coupled with a strong decline in otherwise spatially and seasonally stable core microbiota composition (Koll et al., 2024). Especially *Acinetobacter* strains considered as conditional pathogens causing serious infections in humans and fish with high persistence against environmental pressures (Sheldon & Skaar, 2020) showed highest correlation to cellular stress and immune responses in both fish. All of *A. lwoffii* (Cao et al., 2018)*, A. johnsonii* (Bi et al., 2023), *A. junii* (Malick et al., 2020)*, A. pittii* (Wang et al., 2020) and *A. baumannii* (Xia et al., 2008) were described opportunistic pathogens infecting various marine and freshwater fish.

Their emergence coincides on one side with the signs of immunosuppression, but on the other with increased cellular stress responses and cell cycle control patterns. These host responses were strongly determined by oxygen levels in GAMs. But also showed an almost linear increase with the *Acinetobacter*-module or abundance of most abundant individual *Acinetobacter* strain whereas the *Flavobacteriaceae* impact was shown plateauing in OE or in GC even insignificant. Interpretation leads to a multi-stressor (Temperature, hypoxia, NH_4, and opportunistic bacteria) level driven immunosuppression and cellular damage in gill and liver tissues of both estuarine fish species. Monitoring the bacterial composition on the fish gills had a high explanatory potential for cellular stress responses (50 – 70%) and could therefore add a valuable monitoring tool as shown in other recent estuarine studies (Suzzi et al., 2022; Suzzi et al., 2023).

6.3.2. Antiviral responses

Viruses are highly abundant in the aquatic biomes strongly affecting and determining survival of bacterial and eukaryotic hosts (Q.-Y. Zhang et al., 2022). In Elbe estuarine OE, virally induced skin papillomatous lesions caused by herpesvirus HVOE1 (Jakob et al., 2010) are described during spawning period (Anders, 1985; Möller, 1984). These are related immunosuppression caused by lowered temperature (Bly & Clem, 1992) and related to spawning related stress (Getchell, 1998). However, here we detected distinct cross-tissue antiviral responses around RIG-I pathways activation in both fish species, seemingly randomly increased in about 10% of all samples at all seasons and sampling locations. Hub gene to the response patterns in gill and liver tissues was an immunoglobulin (Ig)-like domain-containing protein (igldcp), described as interferon-stimulated gene (ISGs) highly induced by different fish viruses, capable of decreasing viral infection and leading to high mortality in in vivo knockout (Lama et al., 2022; Martínez-López et al., 2025). Further, in both species the proteasome machinery was correlated with these responses, including specific immunoproteasome subunits induced by interferon secretion upon viral infection. Due to altered cleavage specificity those facilitate the creation peptides for MHC I improving immune responses (Rivett & Hearn, 2004; Shin et al., 2006). Involved genes, especially in GC co-regulated also with hypoxia stress (psmb13a, psmb8a, psmb10, psmb12, psmb9, psmb9a) have been described regulated during viral infection and tissue regeneration in fish (Hou et al., 2020; krishnan et al., 2021; McConnell et al., 2016). The specificity of the responses indicates spawning-unrelated infections with further viruses throughout the year and highly conserved response mechanism in both fish species.

6.4. Population differentiation and signatures of selection

From both fish species in this study, populations with morphological and life-history differences are described in proximity to the Elbe estuary. Local populations of anadromous OE in

North Sea, in the Baltic Sea as well as resident lacustrine ones are described (Shpilev et al., 2005; Thiel & Thiel, 2015). While the majority of adult smelt leaves the estuary following reproduction in spring, individuals can be caught all year along the entire tidally influenced range and further above into the freshwater system (Thiel & Thiel, 2015). For GC, freshwater populations inhabit the tributaries around the estuarine area, but recent stable isotope data indicate that the brackish caught specimen from the Elbe complete their entire life-cycle within the brackish area with high site fidelity. Known adaptations in ruffe from brackish populations comprise the production of larger eggs and a higher tolerance of embryos to salinity (Albert et al., 2006; Svirgsden et al., 2015). Divergent environmental pressures such as the described temperature, hypoxia, accumulation of nitrogen compounds and other xenobiotic loads, especially in the upper reaches in summer, accompanied by fluctuations in salinity affect populations within the estuary.

Sequencing of mRNA has been proven useful to provide insides into fish population structure (Jeffries et al., 2019; Thorstensen et al., 2020). From *de novo* assembled data we determined a weak population structure in both fish species, related to slight seasonal pattern in OE and spatial pattern in GC, indicating constant gene flow between fish caught over different seasons of the year and along the course of the estuary. Biological differentiation with high connectivity and weak population structure is however common in marine (Aykanat et al., 2015; Knutsen et al., 2011) and freshwater fishes (Chen et al., 2020; Thorstensen et al., 2020).

Functional analyses determined gene clusters with multiple SNPs specifically enriched in network modules related to immune- and cellular stress response in both fish species that were most related to elevated temperatures and lowered oxygen levels. Genetic diversity at functional gene loci might be important for the adaptive potential of populations in the wild and low levels of immune gene diversity could increase pathogen susceptibility (Magid et al., 2022; Spielman et al., 2004). The major histocompatibility complex with its key function in antigen-specific adaptive immune functions is known as the region including the largest number of SNPs per size in human genomes (Monos & Winchester, 2019). In accordance we found a large number of SNPs in related genes but also within a broader range of immune related genes. A highly conserved response to lowered oxygen levels in metazoa is coordinated by the HIF transcription factor that activates expression of target genes involved in energy metabolism, growth-, angiogenic- and apoptosis signaling (Mandic et al., 2021). Mutations in HIF-pathway-related genes are involved in hypoxia tolerance and might provide adaptive potential in wild populations. Mutations in hif2ab and hif1an went along with increased erythrocyte numbers, glutathione, SOD and CAT expression during acute hypoxia exposure in fish (Yang et al., 2020; Zhao et al., 2023). On the opposite, low nucleotide diversity in HIF-related genes in a marine pleuronectiform might relate to the loss of hypoxia tolerance (Xiao & Wang, 2024). Both estuarine fish species had multiple outlier SNPs in teleost specific *hif1aa* variants which are O_2 sensitive regulator during acute hypoxic response (Rytkönen et al., 2013) found especially expressed in summer and the upper estuary in both species gill tissue. These were accompanied by a range of mutations in cellular stress response genes like heat shock proteins in OE highly expressed in summer or GPX2 highly expressed under prolonged hypoxia in GC.

The results indicate a functional diversity in genes important for high temperature and hypoxia tolerance and the high loads of opportunistic bacteria present in the estuarine system and imply adaptive divergence despite weak population differentiation. With the recent decline in abundance over the last decade (92% in OE, 95 % in GC) the populations are likely undergoing a genetic bottleneck, reducing overall genetic diversity. Following (Kardos et al., 2021) the effects of prolonged genetic drift from this bottleneck could further diminish the genetic variation present in important hypoxia response and immune system genes. Significant loss of crucial nucleotide diversity would weaken the populations' adaptive capacities to response to future environmental challenges expected with climate change. This genetic erosion can exacerbate the populations' decline by reducing it resilience to further stressors, leading to increased vulnerability and risk of extinction. The effects of

such bottlenecks are however influenced by their durations, with longer bottlenecks increasing risks by extending the amount of time that genetic drift acts. Therefore, fast action in ecosystem restoration can mitigate the loss of adaptive capacity induced by genetic bottlenecks

6.5. Omics in estuarine fish monitoring

Conventional methods in fish health monitoring often involve laborious assessments (necropsies, measurements of energy metabolites, immune cell counts (Adams et al., 1993; Adams & Ham, 2011)) that often require lethal sampling and fail to identify important pathogens and holobiont interactions (Chapman et al., 2021; Semeniuk et al., 2022). The integrative holistic omics approach used in this study instead overcomes many these shortcomings. Different from highly variable physiological endpoint measurement omics data showed high accordance along sampling-points and times requiring less animals to be analyzed. Further, miniature gill tissue dissections and gill mucus samples can be taken non-lethally, and have been shown to not affect individual survival (Jeffries et al., 2021). The rapid turn-around times, affordability of holistic mRNA or DNA sequencing, availability of bioinformatic pipelines for high throughput processing massively decrease worktime in the field and lab. Most important however, they allow identification of complex response patterns to known stressors and identification of unknown ones. The integration of different omics approaches further allows approximation of the holobiont interaction and in parallel cross-validate individual findings (e.g. immune responses and abundance of opportunistic bacteria). Finally, determination of population structure and genetic variability can inform about adaptive processes and forces.

7. Data availability

The sequence data are deposited in the ENA Sequence Read Archive under the study XXX. The complete analysis is available in stepwise R markdown files, including metadata, ready to run supplementary lists as well as step-by-step visualizations as HTMLs at DOIXXX

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9. Permits

Sampling procedures were according to the standards described in the German Animal Welfare Act (§4 TierSchG). After being brought on board, the fish were stunned with a blow to the head, before being killed with a heart stab. The implementation of the stow-net fishing for scientific purposes is approved by the Authority for the Environment Climate, Energy and Agarwirtschaft, by the State Fisheries Office Bremerhaven and by the State Office for Agriculture, Environment and Rural Areas of Schleswig-Holstein. Exemptions to the ordinances on nature reserves Mühlenberger Loch/Neßsand as well as a nature conservation permit to conduct research fishing in protected areas

in the NSG "Rhinplate und Elbufer südlich Glückstadt"/FHH area DE 2393-393 from the Office of Environmental Protection were obtained.

10. Author contributions

RTK: Conceptualization, Methodology, Investigation, Formal analysis, Data curation, Validation, Writing- original draft, Visualization, Project administration. EH: Investigation, Visualization, Formal analysis, Data curation, Review & Editing. JT: Investigation, Permit acquisition, Review & Editing. CB: Data curation, Resources, Review & Editing. MJT & KMJ: Methodology, Resources, Review & Editing, RT & CM: Resources, Review & Editing. JNW: Supervision, Validation, Review & Editing. AF: Funding acquisition, Conceptualization, Supervision, Validation, Review & Editing, Project administration.

11. Acknowledgements

We would like to thank Claus & Harald Zeeck and Dirk Stumpe for their efforts in collecting the samples and the hundreds of hours spent together on their fishing vessel. We thank Prof. Dr. Kathrin Dausmann for mentoring the project.

12. Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work RK used DeepL/translate/write in order to improve language of the manuscript. After using this tool, RK reviewed and edited the content as needed and takes full responsibility for the content of the publication.

13. Appendix A. Supplementary data

All metadata, intermediate data and full analyses outcomes are provided at https://github.com/vollkorrn/EstuarineFishOMICS

13.1. Global patterns in gene expression

First, we visualized the tissue specific transcriptomic responses to seasonal and spatial gradients and in relation to physiological measurements by computing average Euclidean distance-based redundancy analyses (dbRDA) (Legendre & Anderson, 1999) based on variance stabilized expression data via vegan *capscale* function. Variables were selected by stepwise model building for constrained ordination (*ordistep*) and assessed for multicollinearity by measuring variance inflation factors (VIF) keeping *ordistep* selected parameters with VIF < 10 as explicative variables. Association strength between explicative variables and gene expression was then measured by fitting environmental vectors on the reduced model ordination using vegan *envfit*. In addition, unsupervised individual Mantel tests were used to explore global associations between tissue specific gene expression, bacterial gill mucus assemblages and environmental and physiological measures (Fig. S1). This analysis was intended to track global trends, justify and validate the following specific analyses.

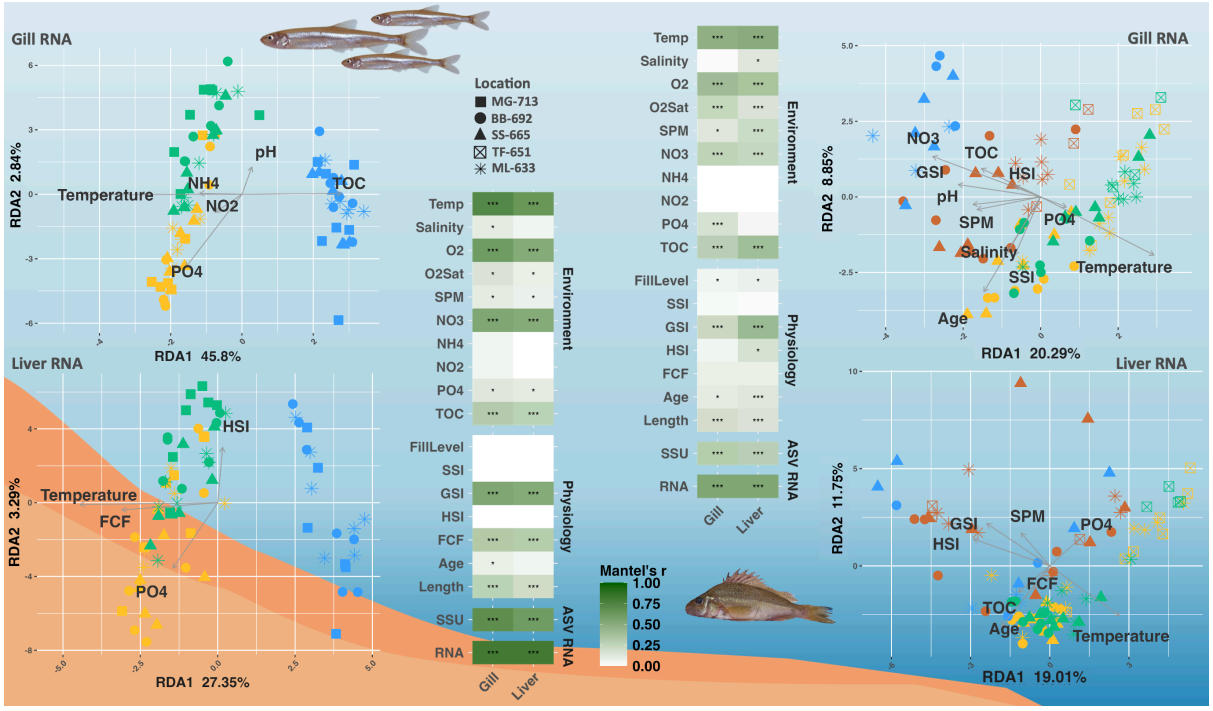


Fig S1. Environmental and physiological drivers in tissue specific gene expression. Euclidean distance-based redundancy analyses (dbRDA) and global matrix correlation (Mantel tests) on variance stabilized RNAseq data in two host species *O. eperlanus OE* (left) and *G. cernua GC* (right). Datapoints within every dbRDA plot indicate gill or liver gene expression patterns colored for season of sampling and shaped for sampling location along the course of the estuary. Physiological and environmental variables were selected by stepwise model building for constrained ordination (ordistep) and VIF < 10. TOC stands for total organic carbon (mg/L), SPM (suspended particular matter), O2Sat (Dissolved oxygen saturation levels), FCF (Fulton's condition factor), HSI (hepato-somatic index), SSI (spleno-somatic index), GSI (gonado-somatic index). Oxygen levels were removed from dbRDA for multicollinearity with temperature and nutrient loads. Global matrix correlation between tissue specific gene expression (gill & liver), gill mucus microbiota (SSU/ASV) and environmental and biometric data tested by Mantel test are shown for both species in the middle of the plot. Significance levels are depicted as < 0.05 (*), < 0.01 (**) and < 0.001 (***).

Non-parametric distance-matrix correlation identified temperature (r 0.5) and DO levels (r 0.4) as dominant cross-tissue driver in GC gene expression. Surprisingly, oxygen saturation levels, salinity levels and SPM all had small global correlation (r 0.1 – 0.2) with gene expression in both tissues. Similar patterns are shown by dbRDA summarizing 29,1% and 30,7% on the first two axes on both tissues with envfit indicating a slightly more pronounced effect of fish age in gill (R2 0.6, p 0.001) and liver (R2 0.4, p 0.001) and a slight but significant effect of salinity in gill tissues (R2 0.2 p 0.002). Spring and summer samples from the upper estuary form a prominent cluster in both tissues.

For OE, there is an outstandingly strong distance-matrix correlation (gill r 0.7 gill, liver r 0.64), as well as dbRDA clustering along the first axis summarizing 45.8% and 27.35% of variance with an R2 of 0.9 to temperature values. Interestingly DO is similarity highly correlated (r 0.5) but oxygen saturation levels are way less influential (r 0.2 in gill and 0.1 in liver). Salinity levels show very low correlation in both tissues. The correlation to physiological parameter is strongest in both tissues to GSI (r 0.5) and FCF (r 0.3). In both fish species, there is strong distance-matrix correlation between tissue specific gene expression as well as bacterial composition on the fish gill. However, these were more pronounced in the anadromous OE with (r 0.8) between tissues as well as bacterial composition (r 0.6 – 0.7), while in GC these range between 0.6 between tissues and only 0.3 to microbiota.

13.2. Complete network visualization

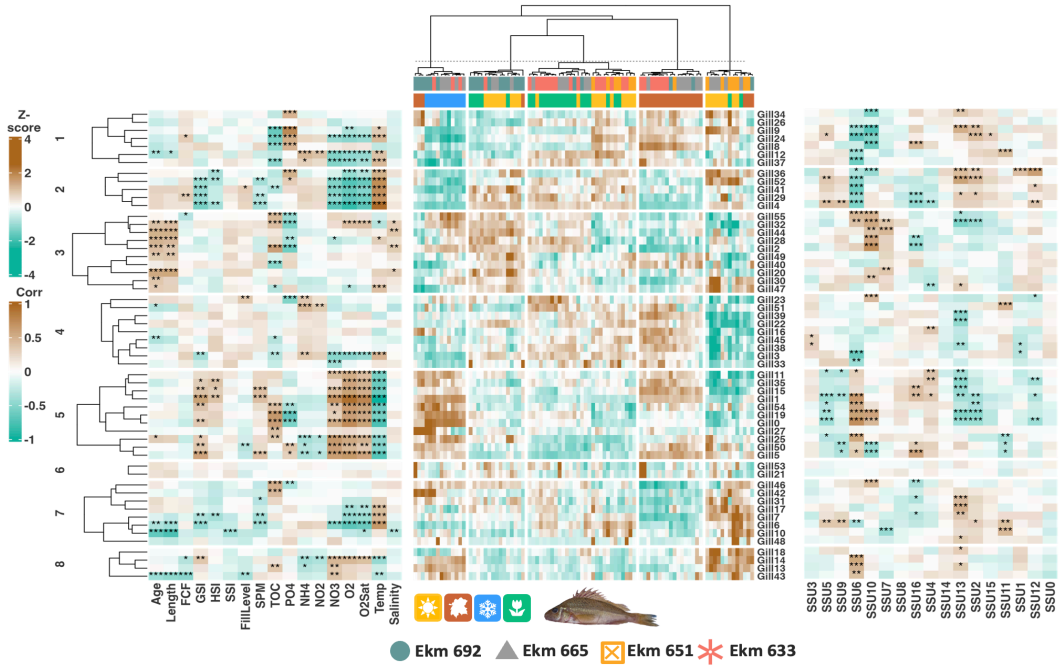


Fig. S2. GC Gill network trans-kingdom heatmap. Relationship between gene expression, bacterial composition, biometric fish data and abiotic conditions for Elbe estuarine ruffe (GC). Integrated heatmap of gill network analysis (right panel) showing correlation between host gene expression module eigengenes (middle panel), physiological traits (left panel: FCF, FillLevel, HSI (hepatosomatic index)) and relevant abiotic factors (SPM – Salinity) (left panel). The middle panel shows Z-score of eigengene values per module as rows for individual fish ordered for sampling location in upstream direction as columns. The left panel shows Pearson correlation strength between eigengene and host/external traits, the right panel shows Pearson correlation to SSU eigenASVs (brown representing positive, zero white and green negative correlation) and statistical significance indicated by stars: * $p < 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

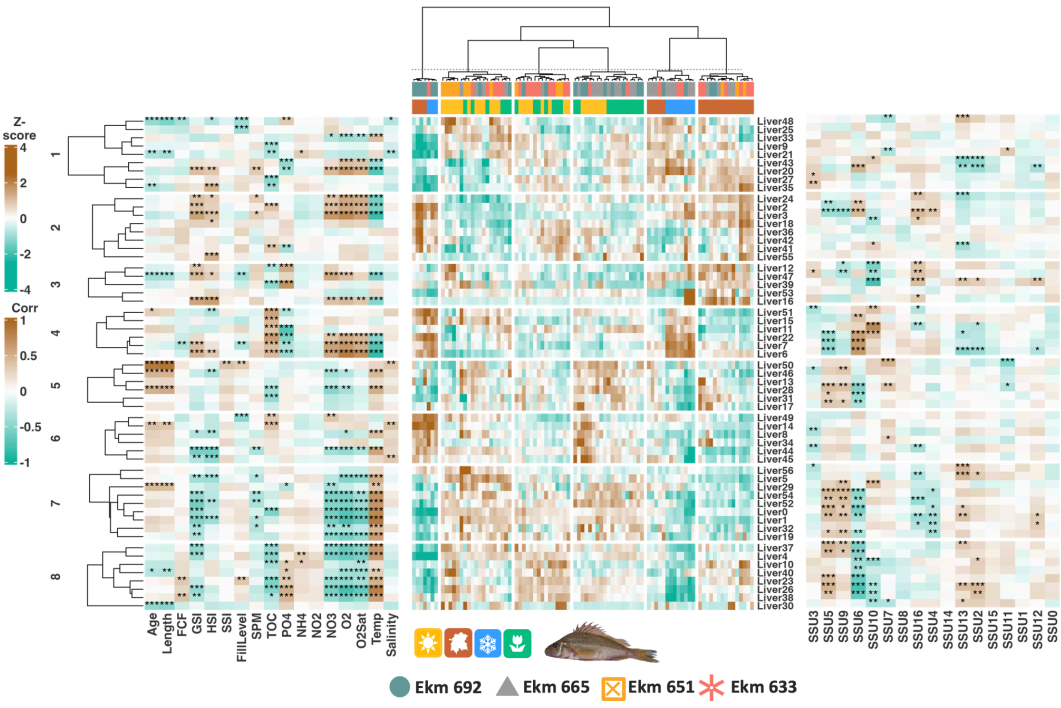


Fig. S3. GC Liver network trans-kingdom heatmap. Relationship between gene expression, bacterial composition, biometric fish data and abiotic conditions for Elbe estuarine ruffe (GC). Integrated heatmap of liver network analysis (right panel) showing correlation between host gene expression module eigengenes (middle panel), physiological traits (left panel: FCF, FillLevel, HSI (hepatosomatic index)) and relevant abiotic factors (SPM – Salinity) (left panel). The middle panel shows Z-score of eigengene values per module as rows for individual fish ordered for sampling location in upstream direction as columns.

The left panel shows Pearson correlation strength between eigengene and host/external traits, the right panel shows Pearson correlation to SSU eigenASVs (brown representing positive, zero white and green negative correlation) and statistical significance indicated by stars: * p < 0.05, ** p ≤ 0.01, *** p ≤ 0.001.

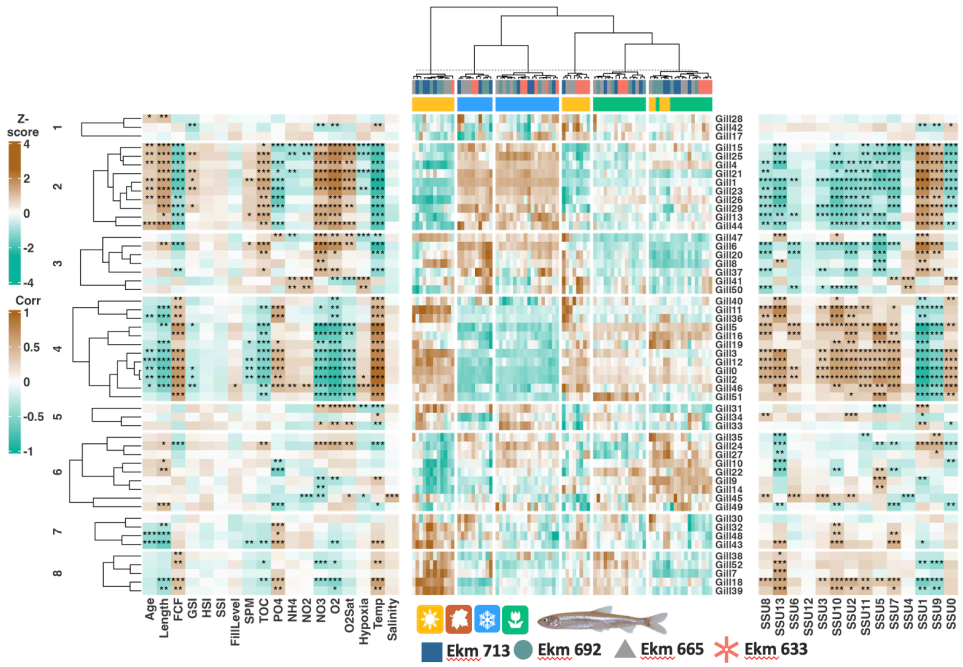


Fig. S4. OE Gill network trans-kingdom heatmap. Relationship between gene expression, bacterial composition, biometric fish data and abiotic conditions for Elbe estuarine smelt (OE). Integrated heatmap of gill network analysis (right panel) showing correlation between host gene expression module eigengenes (middle panel), physiological traits (left panel: FCF, FillLevel, HSI (hepatosomatic index)) and relevant abiotic factors (SPM – Salinity) (left panel). The middle panel shows Z-score of eigengene values per module as rows for individual fish ordered for sampling location in upstream direction as columns. The left panel shows Pearson correlation strength between eigengene and host/external traits, the right panel shows Pearson correlation to SSU eigenASVs (brown representing positive, zero white and green negative correlation) and statistical significance indicated by stars: * p < 0.05, ** p ≤ 0.01, *** p ≤ 0.001.

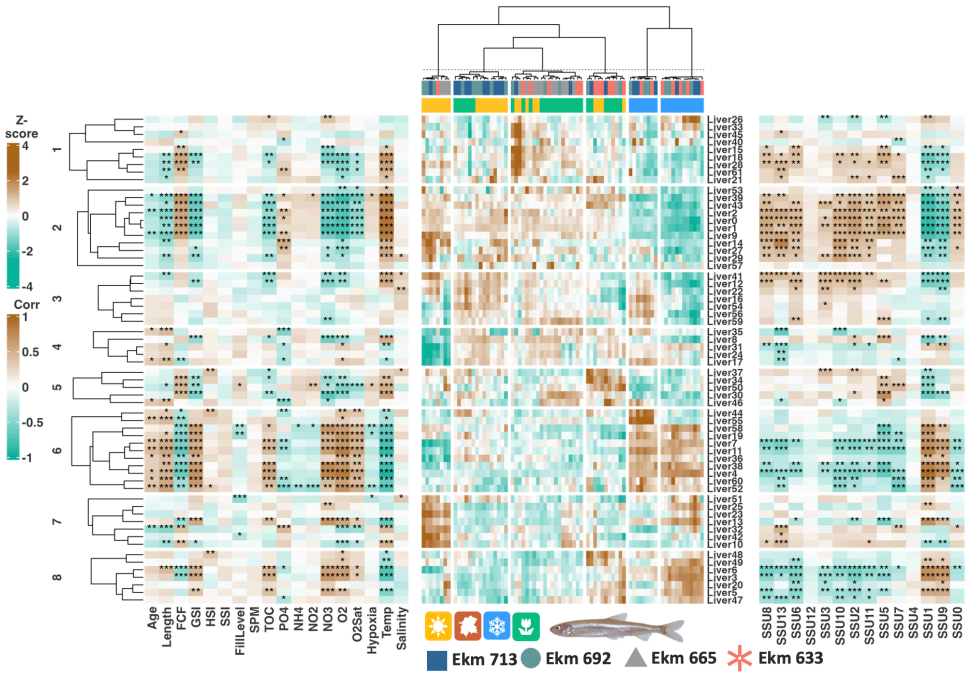


Fig. S5 OE network trans-kingdom heatmap. Relationship between gene expression, bacterial composition, biometric fish data and abiotic conditions for Elbe estuarine smelt (OE). Integrated heatmap of liver network analysis (right panel) showing correlation between host gene expression module eigengenes (middle panel), physiological traits (left panel: FCF, FillLevel, HSI (hepatosomatic index)) and relevant abiotic factors (SPM – Salinity) (left panel). The middle panel shows Z-score of eigengene values per module as rows for individual fish ordered for sampling location in upstream direction as columns. The left panel shows Pearson correlation strength between eigengene and host/external traits, the right panel shows Pearson correlation to SSU eigenASVs (brown representing positive, zero white and green negative correlation) and statistical significance indicated by stars: * $p < 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

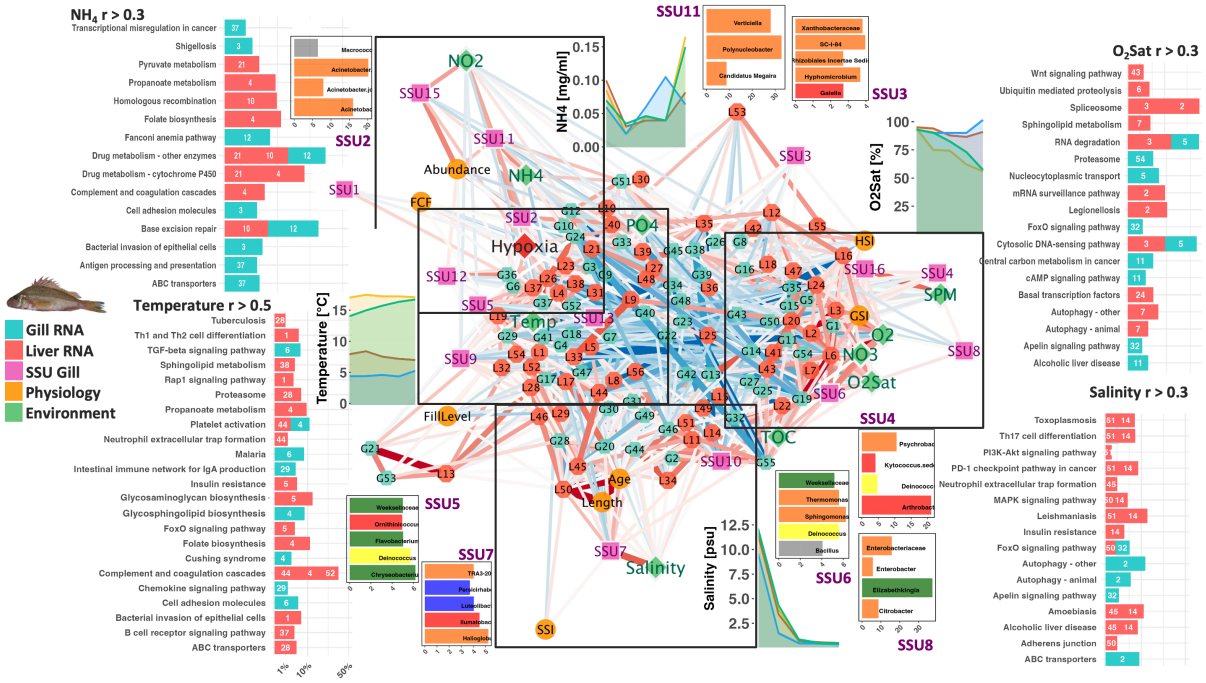


Fig. S6. Full GC cross-tissue network visualization. Edge-weight Spring-embedded network visualization of Pearson correlations between GC tissue specific (gill, liver) and bacterial (gill mucus) WGCNA eigen-genes/ASVs and physiological and abiotic measurements. Hypoxia is coded as binary variable for DO levels < 6 mg/L. Continuous line colors and width represent the Pearson correlation coefficients (red, thick = 1, blue, thick = -1, white, thin ± 0.3). Only significant adjusted correlations r > ± 0.3 are depicted. Line graphs show selected abiotic conditions over seasons and along the course of the estuary (left = estuary mouth, right = freshwater region close Hamburg). Stacked barplots represent tissue-specific pathway enrichments of modules correlated to abiotic conditions. Specific module numbers are shown in white within the barplots. Dominating bacterial taxa within selected modules are shown in barplots SSU2-8 & 11. SSU8 represents the gill core microbiome of this species with ca. 50% abundance over all samples.

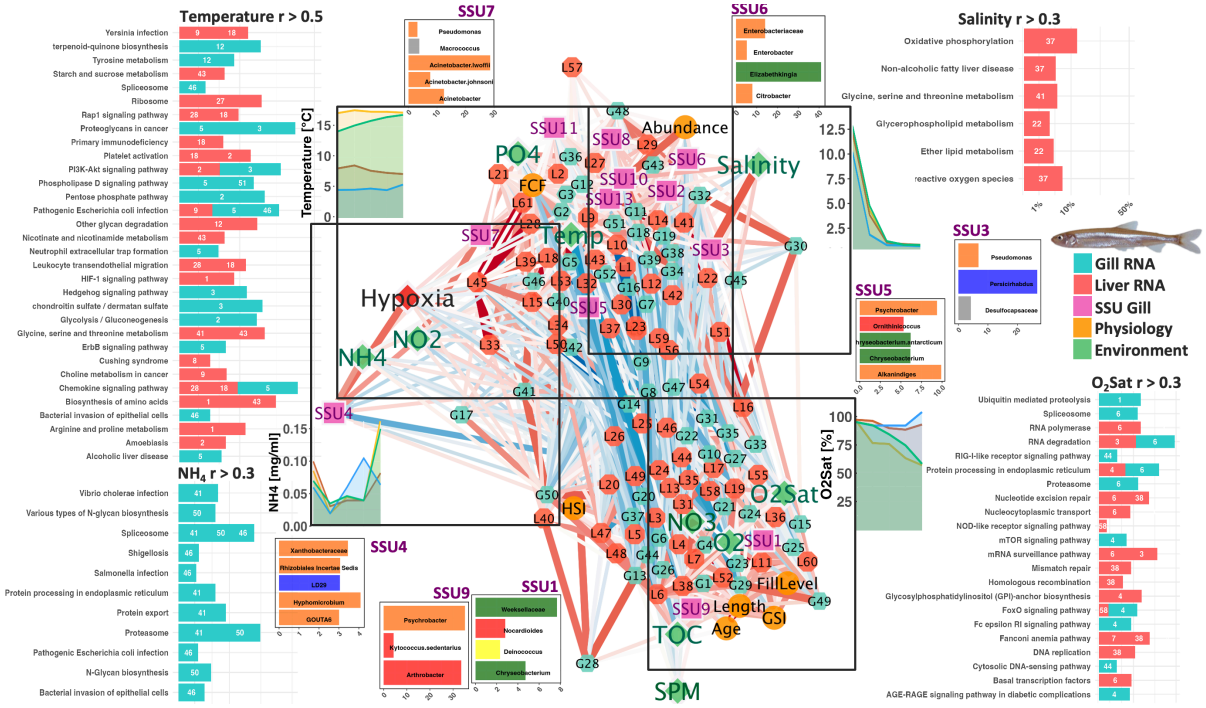


Fig. S7. Full OE cross-tissue network visualization. Edge-weight Spring-embedded network visualization of Pearson correlations between OE tissue specific (gill, liver) and bacterial (gill mucus) WGCNA eigen-genes/ASVs and physiological and abiotic measurements. Hypoxia is coded as binary variable for DO levels < 6 mg/L. Continuous line colors and width represent the Pearson correlation coefficients (red, thick = 1, blue, thick = -1, white, thin ± 0.3). Only significant adjusted correlations r > ± 0.3 are depicted. Line graphs show selected abiotic conditions over seasons and along the course of the estuary (left = estuary mouth, right = freshwater region close Hamburg). Stacked barplots represent tissue-specific pathway enrichments of modules correlated to abiotic conditions. Specific module numbers are shown in white within the barplots. Dominating bacterial taxa within selected modules are shown in barplots SSU2-8 & 11. SSU6 represents the gill core microbiome of this species with ca. 40% abundance over all samples

13.3. GAM models evaluation

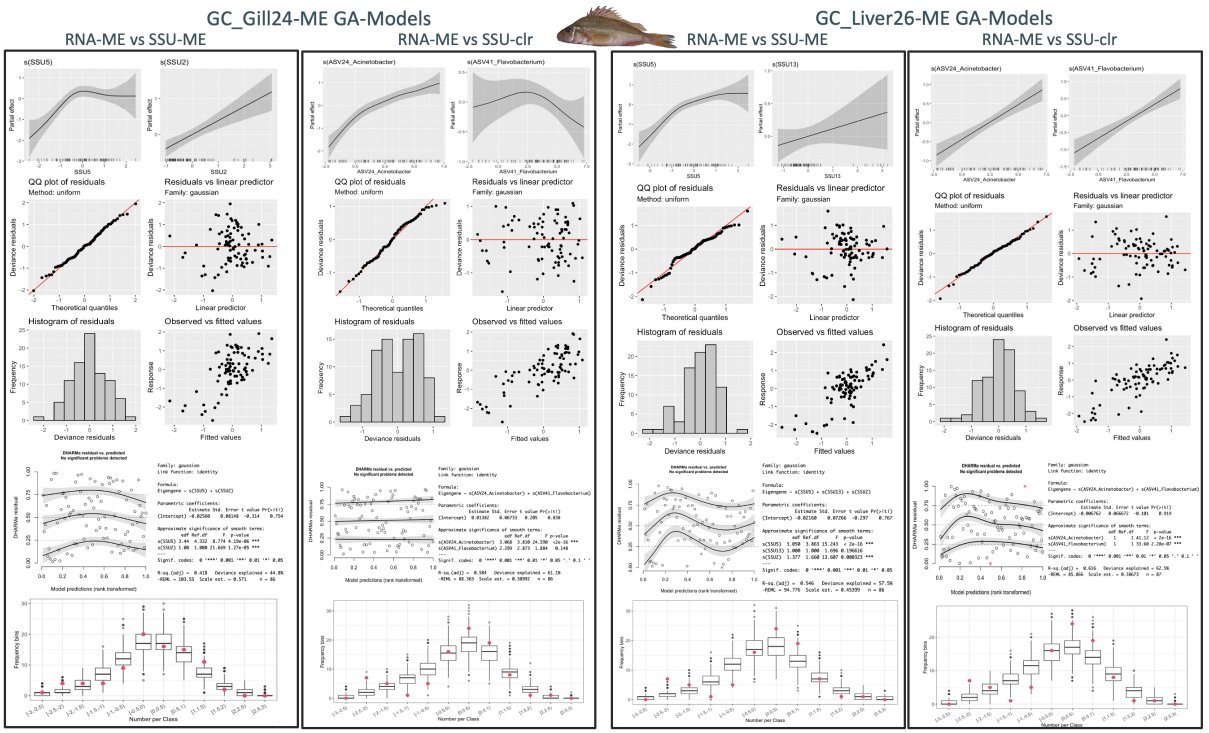


Fig. S8. GC GAM model metrics: Gill & Liver CSR vs bacteria. Shown are the smooth terms on top, followed by gratia and DHARMA model validation. Finally model statistics and a model validation via binned predictions (boxplots) vs. measures values (red dots).

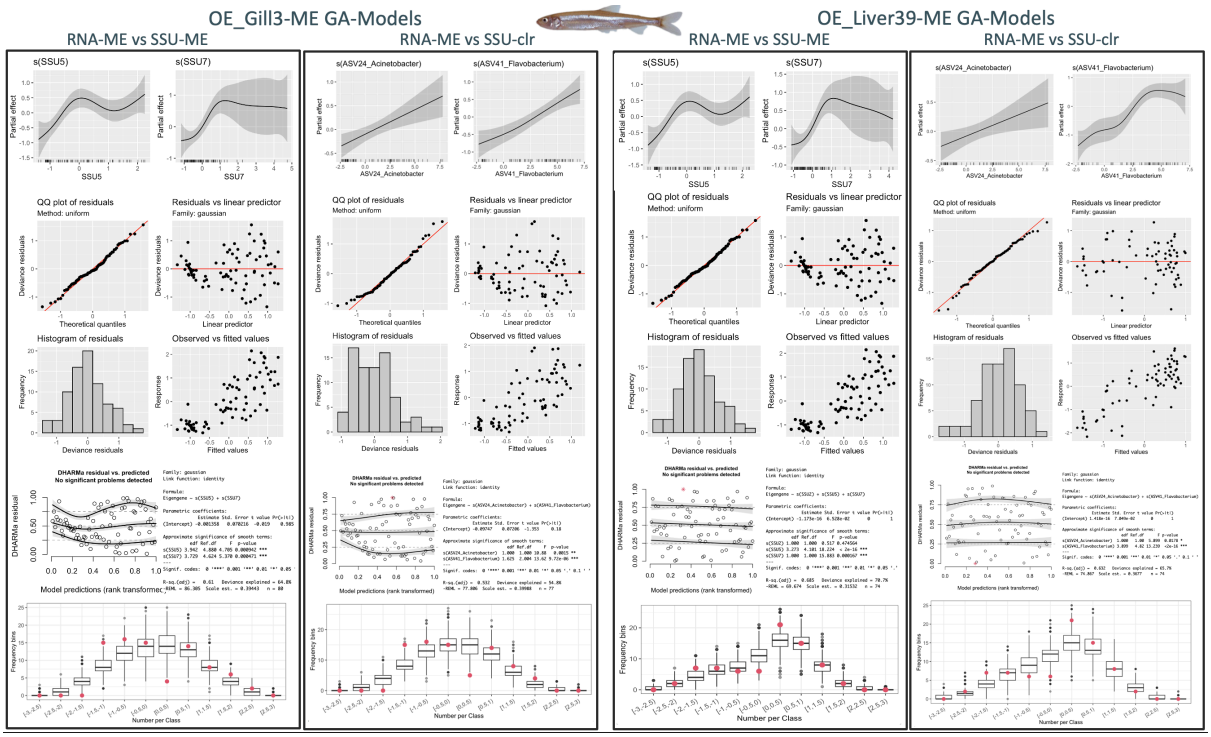


Fig. S9. OE GAM model metrics: Gill & Liver CSR vs bacteria. Shown are the smooth terms on top, followed by gratia and DHARMA model validation. Finally model statistics and a model validation via binned predictions (boxplots) vs. measures values (red dots).

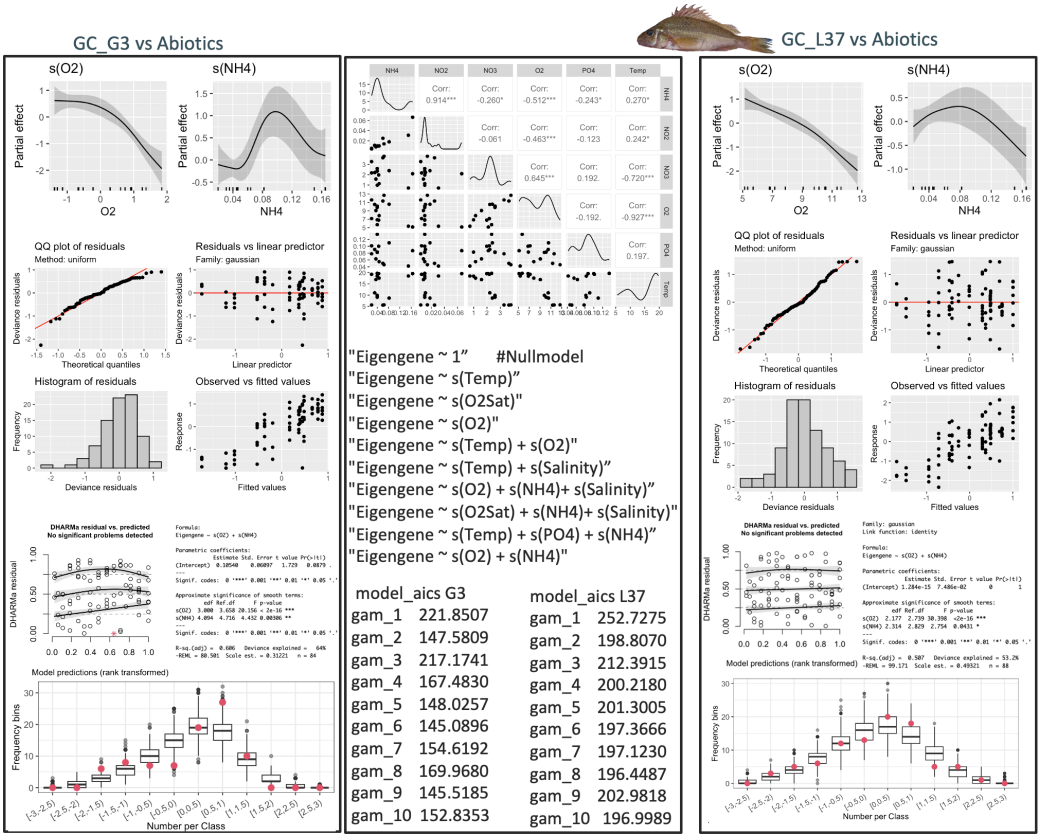


Fig. S10. GC GAM model metrics: Gill & Liver Immune vs abiotics. Shown are the smooth terms on top, followed by gratia and DHARMA model validation. Finally model statistics and a model validation via binned predictions (boxplots) vs. measures values (red dots). The middle panel shows global correlation of predictors and GAM-models stated in model theoretical approach and compared by AIC. Models containing O_2 were chosen over Temperature as both variables are highly collinear but O_2 as predictor has higher biological relevance.

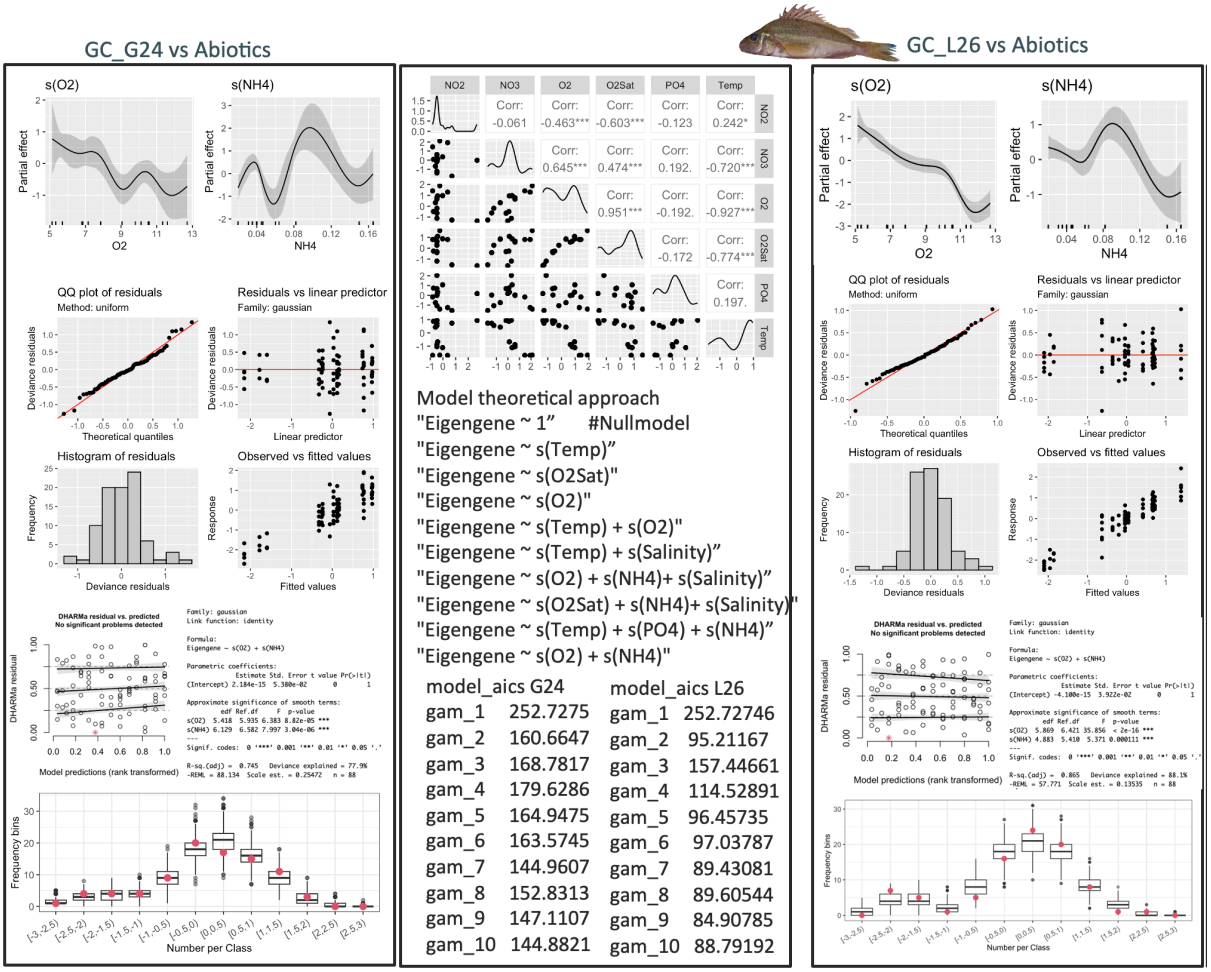


Fig. S11. GC GAM model metrics: Gill & Liver CSR vs abiotics. Shown are the smooth terms on top, followed by gratia and DHARMA model validation. Finally model statistics and a model validation via binned predictions (boxplots) vs. measures values (red dots). The middle panel shows global correlation of predictors and GAM-models stated in model theoretical approach and compared by AIC. Models containing O_2 were chosen over Temperature as both variables are highly collinear but O_2 as predictor has higher biological relevance.

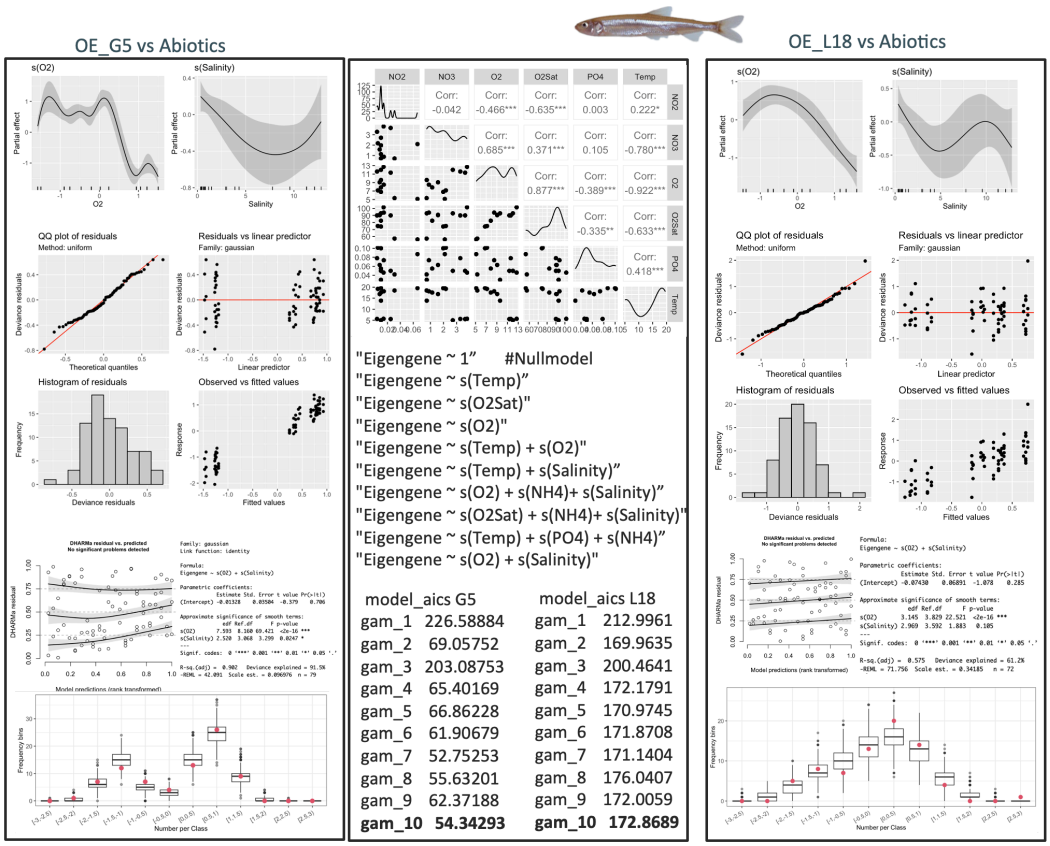


Fig. S12. OE GAM model metrics: Gill & Liver adaptive Immune vs abiotics. Shown are the smooth terms on top, followed by gratia and DHARMA model validation. Finally model statistics and a model validation via binned predictions (boxplots) vs. measures values (red dots). The middle panel shows global correlation of predictors and GAM-models stated in model theoretical approach and compared by AIC. Models containing O_2 were chosen over Temperature as both variables are highly collinear but O_2 as predictor has higher biological relevance.

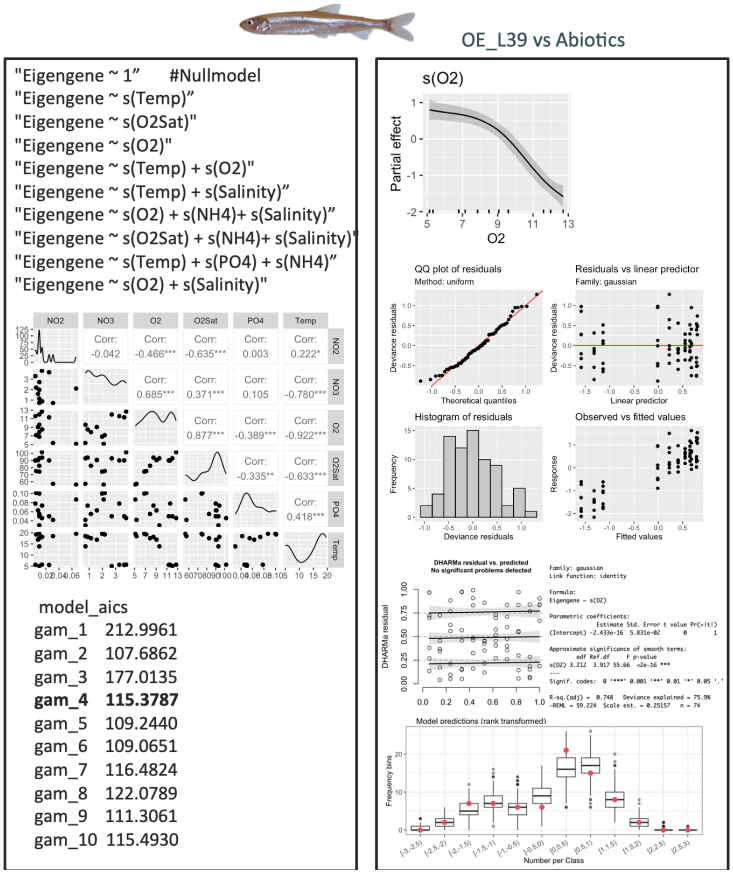


Fig. S13. OE GAM model metrics: Gill & Liver CSR vs abiotics. Shown are the smooth terms on top, followed by gratia and DHARMA model validation. Finally model statistics and a model validation via binned predictions (boxplots) vs. measures values (red dots). The middle panel shows global correlation of predictors and GAM-models stated in model theoretical approach and compared by AIC. Models containing O_2 were chosen over Temperature as both variables are highly collinear but O_2 as predictor has higher biological relevance.

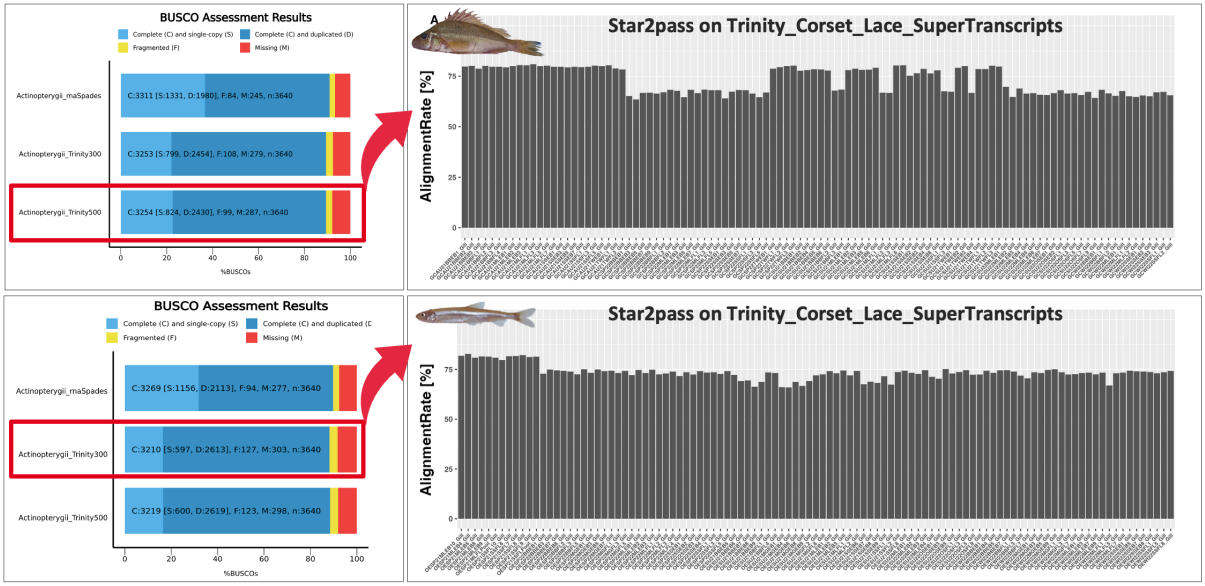


Fig S3. Three individual de novo assemblies run with Trinity and rnaSpades with different settings, compared by BUSCO scores. Further Assembly evaluation measures are contained in the code-files on Zenodo DOI:XXX. The right side shoed Star2pass backmapping rates after Lace super transcript creation of all samples.

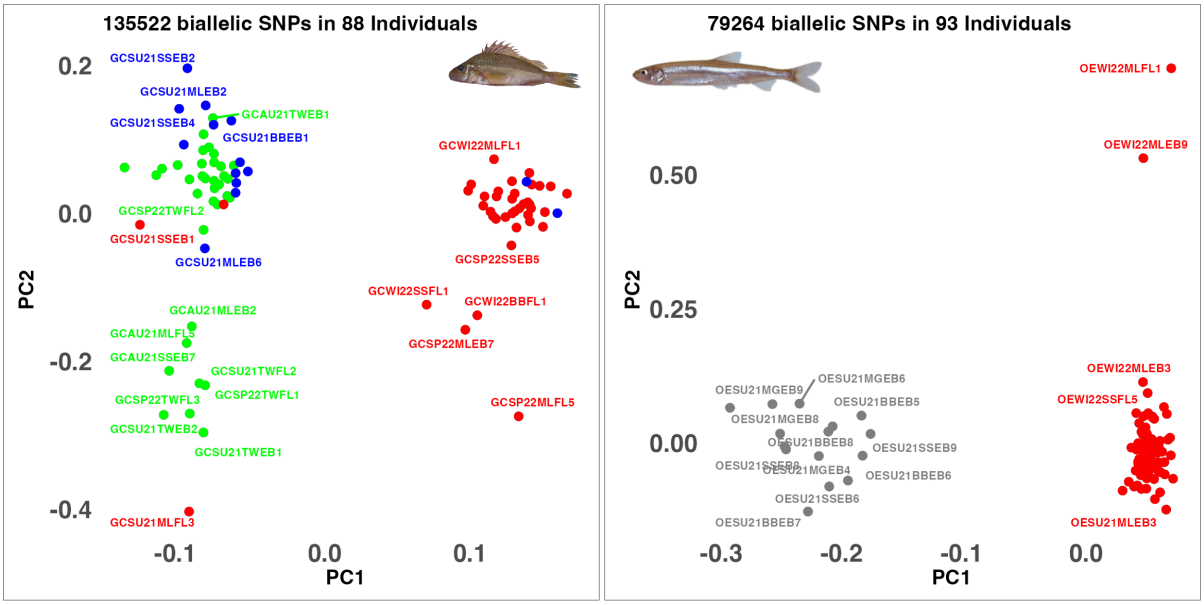
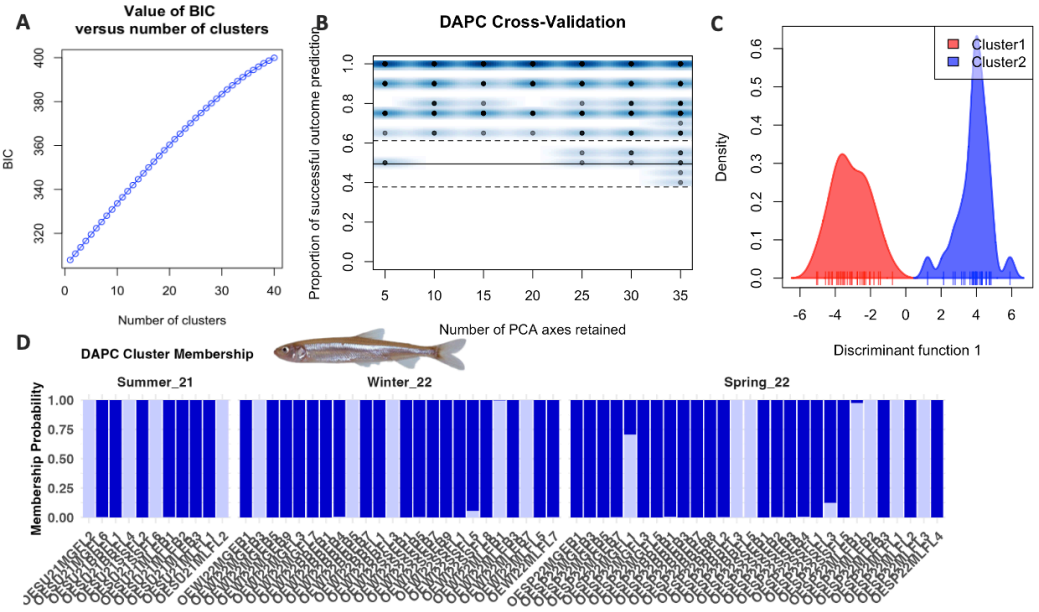
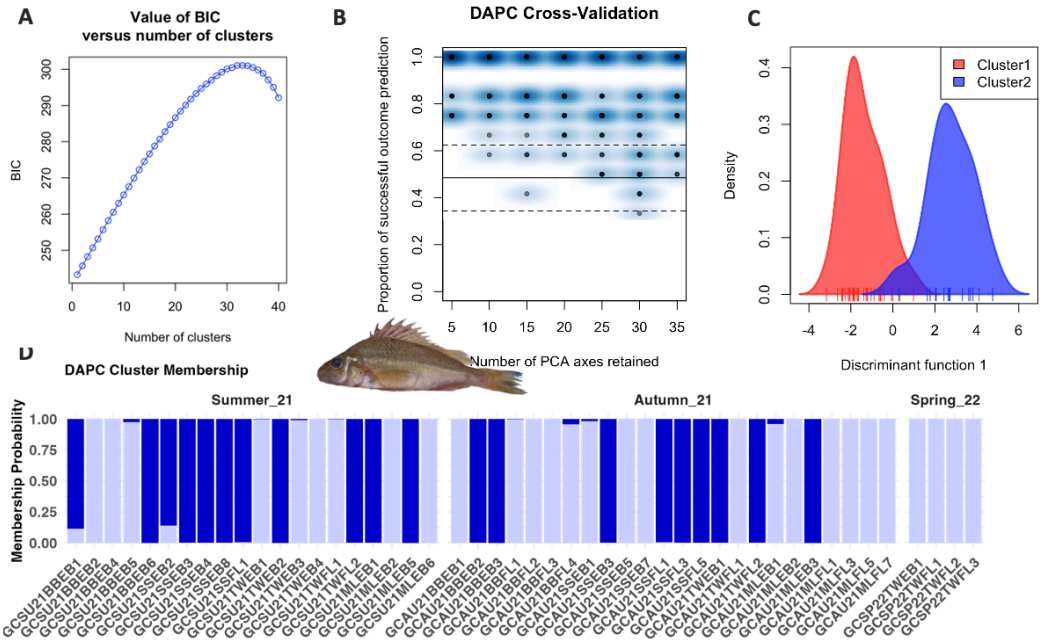


Fig S4. Effect of different sequencing centers on SNP calling. Colors depict different sequencing facilities. For GC only green and blue have been depicted resulting from the same center but different flow cells, for OE the red ones have been chosen all from one sequencing run.



Supplementary Figure XX. Bayesian Information Criterion (BIC) values versus number of clusters found when using Adegenet to explore population reassignment in all 63 smelt (*Osmerus eperlanus*) collected over summer, winter and spring 2021 and 2022, over 4 sites representing a latitudinal gradient in along the Elbe estuary (A). These values were found using 8,841 Hardy-Weinberg Equilibrium filtered and linkage disequilibrium pruned, putatively neutral single nucleotide polymorphisms. DAPC cross validation determining the number of PCs to retain for the analysis and (B) and the Discriminant function density curves (B) determining the final clusters shown as individual barplots (Cluster 1 = Blue, Cluster 2 = light blue) in the data (D)



Supplementary Figure XX. Bayesian Information Criterion (BIC) values versus number of clusters found when using Adegenet to explore population reassignment in all 48 ruffe (*Gymnocephalus cernua*) collected over summer, winter and spring 2021 and 2022, over 4 sites representing a latitudinal gradient in along the Elbe estuary (A). These values were found using 8,841 Hardy-Weinberg Equilibrium filtered and linkage disequilibrium pruned, putatively neutral single nucleotide polymorphisms. DAPC cross validation determining the number of PCs to retain for the analysis and (B) and the Discriminant function density curves (B) determining the final clusters shown as individual barplots (Cluster 1 = Blue, Cluster 2 = light blue) in the data (D)

13.4. Xenobiotic pattern in GC

The Elbe estuary is a highly anthropogenically engineered waterway, a site of various industrial plants with the largest German harbor residing in its freshwater part. It also has a long history of organic compound and heavy metal pollution with toxicity values expected to affect fish communities especially in upper reaches (Wetzel et al., 2013). Besides large xenobiotic and ROS detoxification patterns in both species' liver tissues highly related to temperature and hypoxia, another liver specific xenobiotic response pattern with seasonally independent freshwater, ammonia and age correlation was determined in GC liver (**Fig.X.**).

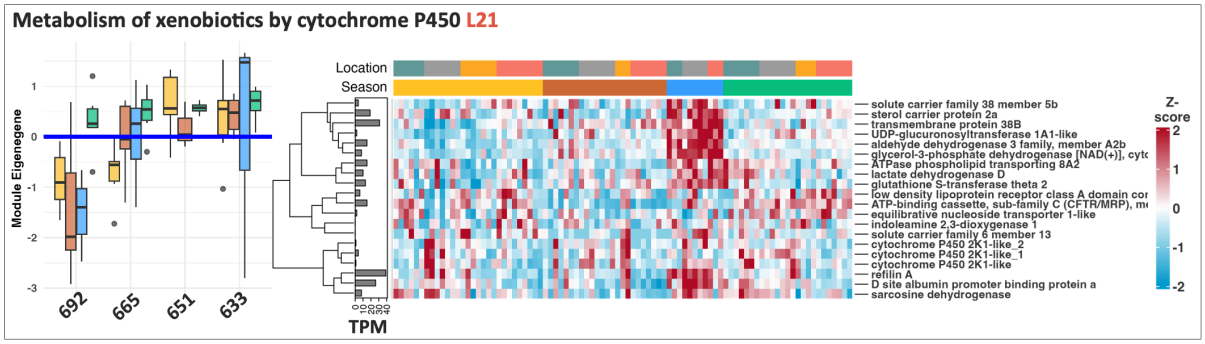


Fig S2. Xenobiotic response in GC liver. Boxplots depict module eigengenes over time and space while the heatmap show the z-score of TPM values for the twently genes with highest intramodular correlation. Average TPM expression over all samples is shown in bar plots.

Essential components of the two-phase biotransformation process of xenobiotic substances, primarily taking place in the liver, comprise the chemical modification to increase water solubility, enzymes involved include cytochrome P450 enzyme family (4 variants of CYP2K1-like, CYP4V2), as well

as aldehyde oxidizers (ALDH3A2, ALDH6A1) (Hassan et al., 2015). In the second step, toxicity is reduced (GSTT2, UGT1A1) and intermediates are transported across cell membranes (SLC6A13, SLC29A1, SLC38A5b). SARDH and IDO1 can also contribute to the processing of nitrogen compounds. The final coordinated excretion of compounds involved xenobiotic ATP-binding cassette (ABC) family transporters (ABCC2) (Christine Paetzold et al., 2009).

Cytochrome P450 enzymes can indicate the presence of various contaminants (Van Der Oost et al., 2003), CYP2K1 has been shown increased upon oil contamination in tropical fishes (Zhu et al., 2006). In principle, the expression patterns match with the location of highest contamination from Wetzel et al., 2013, and the detection in GC with a more benthic lifestyle.

Especially high values were detected in younger fish in winter, indicating an immediate response rather than an accumulation. However, specific studies are needed to determine which xenobiotic substances are involved and to what extent they might influence fish health.

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Chapter 6 Fish fauna time series

Environmental factors shaping fish fauna structure in a temperate mesotidal estuary: periodic insights from the Elbe estuary across four decades

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Keywords

Estuary; fish assemblage; guild composition; fish stocks; environmental influences

Highlights

- Changing environmental factors over a 40-year time series have affected life cycle guilds in fish stocks in the Elbe estuary
- Eutrophication and deoxygenation caused decline in fish stocks in the 1980s that majorly recovered and peaked until 2010 by effective pollution management
- Hydromorphological perturbations causing high suspended particle loads co-working with hypoxia and reduced river runoff led to recent decline in fish density

- Environmental factors and fish stocks were most dominantly affected in freshwater nursery areas

Abbreviations

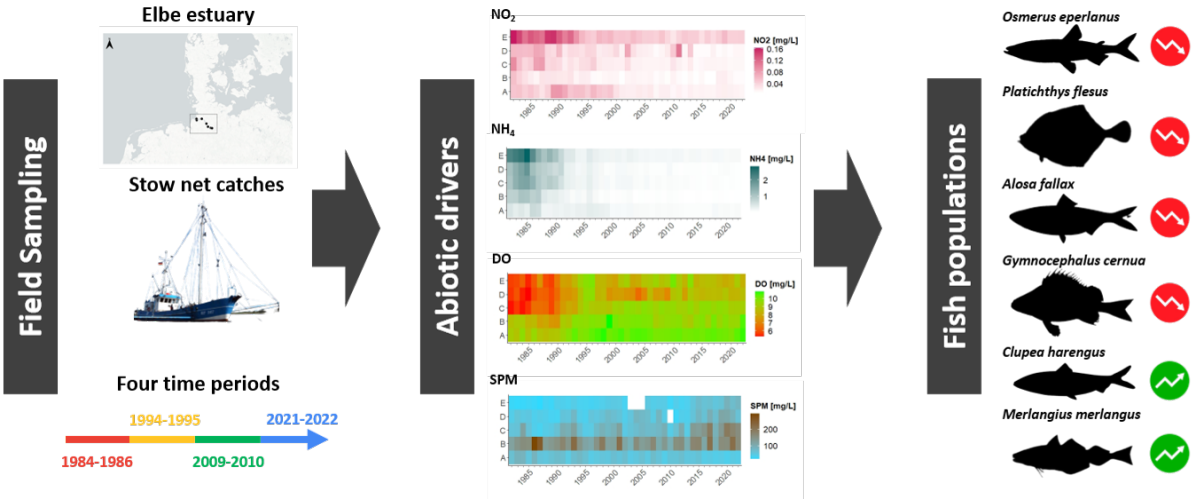
A	anadromous
ANOSIM	analysis of similarities
B	estuarine-marine
Biog.	biogeographic affinity
DO	dissolved oxygen
E	solely estuarine
EC	electric conductivity
Ekm	Elbe river kilometre
envfit	environmental fit
F	freshwater
FNU	Formazin Nephelometric Units
FGG	Flussgebietsgemeinschaft (river drainage basin community)
C	catadromous
LCG	life cycle guild
M	marine straggler
MTZ	maximum turbidity zone
NH_4	ammonium
NMDS	non-metric multidimensional scaling
NO_2	nitrite
NO_3	nitrate
NTU	Nephelometric Turbidity Unit
O	marine estuarine-opportunist
PERMANOVA	permutational multivariate analysis of variance
pH	potential of hydrogen
PO_4	phosphate
SIMPER	similarity percentages
SPM	suspended particular matter
TOC	total organic carbon
wTemp	water temperature

1. Abstract

Anthropogenic perturbations paired with increasing climatic changes, affect the biota composition and ecosystem services provided by highly productive estuarine transitional ecosystems worldwide. To determine driving forces affecting fish stocks in an estuarine habitat, we created a periodic time series over the last four decades (1984 – 2022) combining fish species compositions and densities with environmental conditions along the course of the temperate mesotidal Elbe estuary. We detected major changes in the species composition alongside with changes in life cycle guilds composition. With a relative increase of marine-estuarine opportunists and reduction of diadromous species, the fish fauna of the Elbe estuary has become more similar in guild structure compared to macro tidal estuaries in Europe. Improvements in water quality in the 1990s were accompanied by increased fish densities, specially smelt (*Osmerus eperlanus*), until 2010. Anthropogenic hydromorphological interventions, however, could have led to an increase in suspended particular matter until 2022, which combined with reduced river runoff and poor oxygen concentrations in

summer months acted as poor environmental conditions for fishes in the estuary. Mean fish densities dropped by over 91 % compared to 2010 to an all-time low in the data. This reduction was primarily a result of a decline of the key species smelt in the system along with declines of twaite shad (*Alosa fallax*), flounder (*Platichthys flesus*), ruffe (*Gymnocephalus cernua*), common bream (*Abramis brama*) and other species. On the contrary, marine species herring (*Clupea harengus*) and whiting (*Merlangius merlangus*) densities increased. Overall, the time-series provides insight into the strong impact of human intervention that are however expected to lead to further stressors.

2. *Graphical Abstract*



3. *Introduction*

Estuaries constitute only 5.8 % of the coastal area (Wetzel et al. 2014), but are among the most important environments of the coastal zone providing habitats for numerous plant and animal species (Barbier et al. 2011). While the faunal composition varies due to spatial and temporal dynamics, estuaries are often characterized by high productivity (e.g. Thiel et al. 1995, Ducrotoy et al. 2019). However, environmental conditions of many estuaries worldwide are heavily influenced by humans (Ahlhorn 2009) through impacts from industrial sewage, dyke construction, deepening of navigation channels and dumping of sediments to name a few (e.g. Barbier et al. 2011, Drabble 2012, Wetzel et al. 2014). Today, estuaries are among the most heavily anthropogenically modified aquatic habitats on earth (e.g. Haedrich 1983, Whitfield 1996, Pein et al. 2023). Exogenous pressure is further exerted by climate change in particular (van Beusekom et al. 2018). Differences in the trends of climate change and human activities result in highly variable long-term developments (Ducrotoy et al. 2019), with anthropogenic factors generally influencing species richness, biomass, genetic structure and food webs in estuaries (Gilarranz et al. 2015).

The Elbe estuary, as one of the largest in Europe, shared a common fate of the above-mentioned interventions with estuaries worldwide (Pein et al. 2023), which has been shaped by a long history of human alterations (Bergemann et al. 1996, Schroeder 1997, Kerner 2007, Amann 2012, Pein et al. 2021). Since the mid nineteenth century the Elbe estuary has been under anthropogenic influences, which includes channelization for shipping, increasing inlet of industrial, agricultural and communal sewage as well as abstraction of water for power plant cooling (Thiel et al. 2003, Kerner 2007, Illing 2009, Eick & Thiel 2014). Hydromorphological alterations of the main channel had strong influences on the river runoff dynamics, tidal range and current velocities. The tidal range at the port of Hamburg (100 km upstream of the river mouth) doubled from 1.9 m to 3.8 m over the last century (Hein et al. 2021). As a further consequence of channelization, the sub- and intertidal shallow water areas, especially in the freshwater regions, were reduced. At the same time, oxygen deficiency

situations in the Hamburg port region increase during summer months (Riedel-Lorjé & Gaumert 1982, Thiel 2011, Eick & Thiel 2014, Scholle & Schuchard 2020). However, the Elbe estuary still harbours internationally important ecological populations and habitats (Thiel & Thiel 2015), like a number of other temperate estuaries worldwide (Ducrotoy et al. 2019).

The Elbe estuary serves as an important nursery habitat for several fish species. Especially the southern marginal areas of the estuary are characterized by very high fish production with over 200kg/ha per annum (Thiel 2001). These marginal areas provide shelter from predation by larger fishes due to shallower waters and reduced flow velocities and provide abundant food resources such as copepods (*Eurytemora affinis*) and mysids (*Neomysis integer*) (Sepúlveda et al. 1994). These southern marginal areas were found directly downstream of the Hamburg harbour at Mühlenberger Loch (Ekm 633-636), Hanhöfer Nebenelbe (Ekm 636-644) and Lühesander Süderelbe (Ekm 647-652). Larval retention and accumulation of smelt was described by Sepúlveda et al. (1994), by active vertical migration, migrating to the surface layers during flood tides and to the bottom during ebb tides. Active vertical migration, upstream transport and accumulation in the southern marginal areas of the Elbe estuary was also described for flounder larvae (Bos 2000). Besides providing essential feeding, spawning grounds and migration routes for a number of fish species (Nellen & Thiel 1994, Thiel et al. 1996, Thiel et al. 2003) the Elbe estuary with around 80 fish species is the most species-rich estuary in Europe (Thiel 2011).

There is a lack of quantitative long-term studies on the influence of environmental and anthropogenic factors on the fish fauna composition in a number of European estuaries (e.g. Thiel 2011) and estuaries worldwide (Pasquaud et al. 2015). A consistent and continuous dataset covering the entire period from the 1980s to the present day is still lacking, since evaluation of fish species composition in large estuaries is cost-intensive. Even when these data exist, there can be difficulties obtaining the data, since the data at the authorities/data portals are not always up to date. Monitoring data of fish composition and densities available from state funded monitoring used varying fishing methods rendering catch data from before 2000 and recent data not useable for this study. With the implementation of the Water Framework Directive (WFD) in 2000, fish surveys are conducted twice a year, however, these data are insufficient to study trends in fish communities since fish populations exhibit significant seasonal variations. Therefore, only data from stow net catches at five consecutive station were used, to insure best comparability.

To fill the knowledge gaps shown above, the aim of our study was to analyse historical and present compositions of the ichthyofauna in the Elbe estuary using a dataset of comparable traditional fishing methods incorporating seasonal sampling since the 1980s (Möller 1988, Thiel et al. 1995, Thiel & Potter 2001, Thiel et al. 2003, Thiel 2011, Eick & Thiel 2014). The aims of the study include (1) the assessment of changes in the life cycle guilds, (2) the quantification of changes in the densities of the fish species and (3) the identification of long-term shifts of environmental factors and their influence on the fish species densities. In this regard, the Elbe estuary can be seen as a case study for other temperate mesotidal European estuaries, as it is one of the few estuaries in Europe for which quantitative data for fish and relevant environmental factors are available over longer time series.

4. Material and Methods

4.1. Study area and stations

The study was conducted in the Elbe estuary, which is located in north-west Germany and ranging from a weir at the city of Geesthacht downstream to its mouth into the southern North Sea, close to the city of Cuxhaven. The strongly tidally influenced Elbe estuary is classified as mesotidal, coastal-plain estuary and has no marked halocline (Thiel & Potter 2001, Eick & Thiel 2014, Pein et al. 2023). In past studies, the Elbe estuary was categorized as a well-mixed estuary (Muylaert & Sabbe

1996, Amann et al. 2012, Eick & Thiel 2014), but recent findings from Pein et al. (2021), argue that hydrodynamic modelling approaches showed buoyancy driven density gradients, that induce periodic stratification, especially during summer. In extreme cases water temperature reaches above 25° C during summer months and down to -1° C in extreme winters (FGG-Elbe 2024). The freshwater region (salinity < 0.5 PSU) extends from the Geesthacht weir (Ekm 586; upper end of tidal influence) to Stade (Ekm 654), the brackish oligohaline area (salinity 0.5 – 5 PSU) reaches down to Brokdorf (Ekm 684) and the brackish mesohaline area (salinity 5 – 18 PSU) continues downstream to the city of Cuxhaven (Ekm 725) (ARGE Elbe 2007). Turbidity in the Elbe estuary is high, and has increased in recent years, which is mainly driven by marine sediments brought upstream by increased tidal pumping and resuspension by extensive dredging for channel maintenance (Kerner 2007, Schulz et al. 2023). Kappenberg & Grabemann (2001) described a variability of suspended particular matter (SPM) in the Elbe estuary, with a maximum turbidity zone (MTZ) located between Ekms 670 and 690 before the year 2000. Due to seasonal variation of river runoff, the SPM concentration varies throughout the year.

The hydromorphological changes by channel deepening were accompanied by increased tidal pumping and therefore increased sediment transport towards the Hamburg port (Pein 2021). Scholle & Schuchard (2020) showed that the shallow water areas in the Mühlenberger Lock were reduced from 210.000 m^3 in the year 2000 to 113.000 m^3 in the year 2016 by construction and siltation processes (Scholle & Schuchard 2020), which could have influenced the estuary's nursery function. The filling of a portion of the Mühlenberger Loch (2001-2004) led to a direct loss of shallow water areas in the southern marginal areas.

We selected five stations along the salinity gradient at regularly spaced distances within the Elbe estuary (Fig. 1): mesohaline (river mouth section): Medem Reede/Medemgrund at Elbe-kilometre (Ekm) 713/716 (A'/A), oligo-mesohaline (lower section): Brunsbüttel at Ekm 692 (B), oligohaline (middle section): Schwarztonnensand at Ekm 665 (C), freshwater-oligohaline (upper section): Twielenfleth at Ekm 651 (D) and freshwater (upper section): Neßsand/Mühlenberger Loch at Ekm 633/639 (E'/E). Out of these five stations, three stations (B, C, D) were sampled at the exact same location throughout entire investigation period, while stations A and E had to be slightly relocated (approx. 6 km difference) due to changed nautical circumstances and regulations.

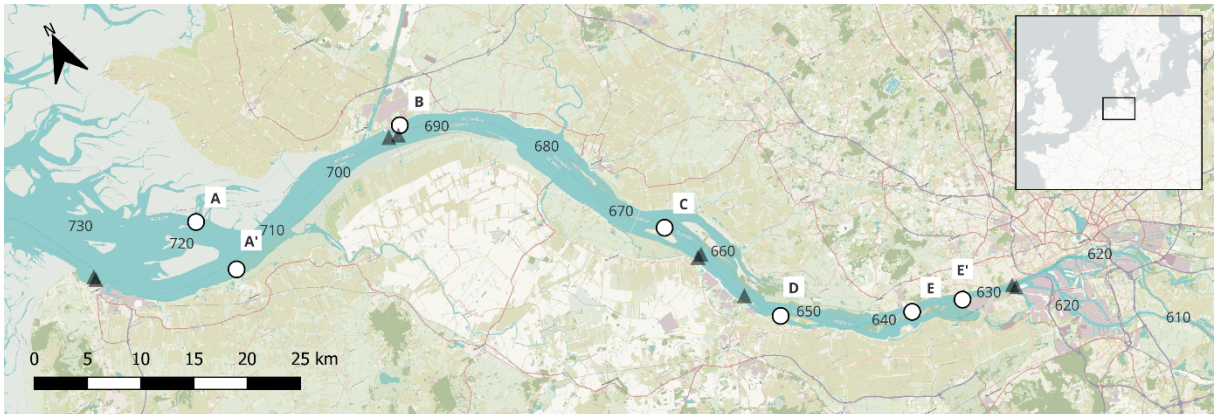


Fig. 1: Elbe estuary with the sampled stow net stations (white circles): Medem Reede/Medemgrund at Ekm 713/716 (A'/A), Brunsbüttel at Ekm 692 (B), Schwarztonnensand at Ekm 665 (C), Twielenfleth at Ekm 651 (D) and Neßsand/Mühlenberger Loch at Ekm 633/639 (E'/E). Stations from which environmental data were obtained from the FGG-Elbe data portal were indicated with triangles (Map sources: NextGIS, CartoDB).

4.2. Field sampling strategy

Sampling was conducted during four time periods (period I: 1984-1986, period II: 1994-1995, period III: 2009-2010, period IV: 2021-2022) covering all seasons (spring, summer, autumn, winter) in each period. Fishes were caught (day and night) using commercial stow net vessels having mesh sizes

between 5 and 10 mm at the cod end (Fig. A1). The vessel was anchored at each sampling site and the net immersed, so that the net opening faced the direction of the water flow (Thiel et al. 1995, Thiel & Potter 2001, Thiel et al. 2003, Eick & Thiel 2014). Species identification of the caught fish was conducted on board immediately. Fishes were counted, weighted (wet weight, 1 g accuracy) and total lengths were measured. Specimens that were not directly identifiable on board were either frozen using dry ice, freezers (-20 °C) or were preserved in formaldehyde solution (5 %) for later taxonomical identification in the laboratory following Whitehead et al. (1985), Muus & Nielsen (1999), Kottelat & Freyhof (2007), Knebelsberger & Thiel (2014) and Froese & Pauly (2022).

4.3. Harmonization of fish density data

In the initial time period I from 1984-1986, used stow nets had a net opening area of 80 m^2 and 95 m^2. One tidal phase (A at flood tide; B, C, D and E at ebb tide) was sampled per station (Möller 1988), which resulted in one haul per sampling station at each sampling campaign. For comparison purposes, all catches in period I have already been converted to a net opening of 80 m^2 by Möller (1988). In time period II from 1994-1995 a stow net with a net opening of 90 m^2 was used (Thiel et al. 1995, Thiel et al. 2003). During period II one stow net haul was performed during both tidal phases (flood an ebb tide) at each station resulting in two hauls per sampling station at each sampling campaign. In period III from 2009-2010 a stow net with a net opening of 135 m^2 was used (Eick & Thiel 2014). During period III, during each tidal phase two hauls were conducted. For comparability these two hauls were summarized into one haul per each tidal phase. In period IV from 2021 to 2022 the stow net had a net opening of 135 m^2. The mean haul duration per period was 4 h for period I (Möller 1988), 4 h during period II (Thiel et al. 2003), 3 h (combined) during period III (Eick & Thiel 2014) and 3 h during period IV.

Fish densities were calculated based on the filtered water volume of each haul and the number of individuals of each species caught. The filtered water volume was determined by using the net opening area and water flow, which was measured using mechanical flow meters (Hydro-Bios® and/or General Oceanics®) during period II, II and IV. From the filtered water volume and the number of individuals caught was expressed as density, which was standardized to a fixed water volume (individuals per 1 million m^3) for each haul by proportional rule. This standardization ensures consistency and comparability across hauls, regardless of the variations on the filtered water volumes. Water flow in period I was not measured directly but was approximated by using the haul durations of period I and comparable flow velocities from period II. In assumption that between period I and period II no major changes in the hydromorphology by channel deepening (Gaumert & Bergemann 2007) of the Elbe estuary occurred, approximation of the water volume based on these two periods was regarded as sufficiently accurate. During period IV only the five sampling sites A-E were sampled and only one sampling campaign during each season was performed, whereas during the periods I (Möller 1988), II (Thiel et al. 1995, Thiel et al. 2003) and III (Eick & Thiel 2014) a higher spatial and temporal resolution of the sampling data were available. In order to ensure the best possible comparability of the density data from the four different periods, the more detailed data records from periods I to III were reduced and adjusted to the resolution of period IV by utilizing records from the very same months.

4.4. Life cycle guilds and geographical distribution categories

Fish species in the Elbe estuary were grouped into life cycle guilds (LCG) following Thiel & Potter (2001), Thiel et al. (2003), Elliott et al. (2007), Franco et al. (2008), Thiel et al. (2011) and Eick & Thiel (2014): S: marine stragglers (e.g. lesser sand eel, *Ammodytes tobianus*), O: marine estuarine-opportunists (e.g. herring), E: solely estuarine (e.g. common goby, *Pomatoschistus microps*), C: catadromous (e.g. European eel, *Anguilla anguilla*), A: anadromous (e.g. smelt), F: freshwater (e.g. zander, *Sander lucioperca*). These definitions were based on the most recent work by Potter et al.

(2013), who classified life cycle guilds of estuarine fish species worldwide. To ensure exact comparison with previous data (Thiel et al. 1995, Eick & Thiel 2014), a classification with six LCG was chosen.

Freshwater fish species in the Elbe estuary were further grouped into two geographical distribution categories (Leroy et al. 2019): Palearctic fish species that have European, Siberian and East-Asian distributions and Nearctic fish species that are introduced from North America. Spatial and temporal variation in the fish fauna composition of the Elbe estuary can be described by their guild composition. A longitudinal change in the fish community in upstream direction corresponds with a decrease of densities of marine species and an increase of densities of freshwater species (Thiel 2011, Eick & Thiel 2014). In the past century, the anadromous smelt has been dominant fish species in the Elbe estuary since it occurs in the entire estuary with high densities and biomasses (Ehrenbaum 1894, Lillelund 1961, Möller 1988, Thiel et al. 1995, Eick & Thiel 2014). The invasive round goby (*Neogobius melanostomus*) was assigned to the freshwater LCG.

4.5. Collection of environmental data

Environmental data originated from long-term routine monitoring programs by the local authorities and were downloaded from the FIS Elbe data portal (FGG-Elbe 2024). The variables electric conductivity (EC µS/cm: DIN EN 27888 C8), water temperature (wTemp °C: DIN 38404-4 C4), dissolved oxygen (DO mg/L and %: DIN ISO 17289 G25 and DIN 38408-23 G23), pH (DIN EN ISO 10523 C5), suspended particular matter (SPM mg/L: DIN EN 872 H33), ammonium (NH_4 mg/L: DIN EN ISO 11732:2005-05), nitrite (NO_2 mg/L: DIN EN ISO 13395 D28), nitrate (NO_3 mg/L: DIN EN ISO 10304-1 D20), phosphate (PO_4 mg/L: DIN EN ISO 15681-2 D46) and total organic carbon (TOC mg/L: DIN EN 1484 H3) were downloaded between the years 1982 and 2022. Monitored stations were chosen in closest proximity possible to the fishing sampling stations (see Appendix Tab. A.1).

The monitoring program of the local authorities included measurements from monthly helicopter flights (longitudinal profile) from 1982 to 1986, eight times a year from 1987 to 1993 (February, April, May, June, July, August, September, November) and six times per year from 1994 to 2022 (February, May, June, July, August, November). The water samples were extracted from the middle of the channel at a depth between 0.5 and 1 m during low tide (ARGE Elbe 1999, FGG-Elbe 2021). From the stations Brunsbüttelkoog (1982-2022), Grauerort (1982-2022) and Seemannshöft continuous measurements with usually one to four measurements per month were available. Monthly and annual means were calculated based on stationary continuous measurements and helicopter flights measurement.

4.6. Ordination and statistical analyses

Ordinations were computed using the R-package ‘vegan’ (Oksanen et al. 2022). Principal component analyses (PCA) was performed on standardized environmental variables (as percentage of maximum value recorded for the specific variable). Detrended correspondence analysis (DCA) was carried out on log10 transformed species density data was carried out for further data exploration (see Appendix Fig. A12). With the fist axis of the DCA expressing a gradient length > 4, the data were suitable for unimodal analyses (ter Braak & Smilauer 2012). The relationship between fish species composition and environmental gradients were determined using unimodal canonical correspondence analysis (CCA). Statistical significance of the relationships between each environmental variable and the species composition were tested using the post-hoc permutation test anova.cca (999 permutations) of the ‘vegan’ R-package. Similarity percentage (SIMPER) analysis was carried out in order to identify species contribution to ordination. Analysis of similarities (ANOSIM) was used to determine differences of the fish fauna between periods, while using pairwise ANOSIM with Bonferroni correction to address differences between individual periods. Over the entire time series, fish density data from stow net hauls was assigned to environmental data by matching sampling

month. Monthly median values of not normally distributed data were calculated for the ordination matrix of environmental factors. Hauls were removed from the dataset when no environmental data could be assigned. From originally n = 365 stow net hauls (period I n = 85, period II n = 72, period III n = 159, period IV n = 49) a total of n = 310 hauls was used for ordination computation (period I n = 79, period II n = 63, period III n = 126, period IV n = 42).

5. Results

5.1. Fish species composition

A total of 69 fish taxa were recorded across four time periods wherein period I (1984-1986) had 47 species, declining to 38 in period II (1994-1995), during period III (2009-2010) there was an increase to 53 species, that recently declined again to 44 species in period IV (2021-2022). During the entire investigation period, the 15 most abundant fish species were: smelt, herring, twaite shad, three-spined stickleback (*Gasterosteus aculeatus*), ruffe, flounder, sprat (*Sprattus, sprattus*), silver bream (*Blicca bjoerkna*), common bream, lesser pipefish (*Syngnathus rostellatus*), whiting, zander, common/sand goby (*Pomatoschistus* spp.), common seasnail (*Liparis liparis*) and European eel (Fig. 2, Tab. A.2). While smelt was the most abundant fish species in the Elbe estuary during all four time periods the ranks of the remaining species were different between the investigated time periods. Twaite shad was the second most abundant species during the 1980s, while in the 1990s twaite shad was on the 7th rank and during 2009-2010 it was on the 3rd rank and during recent survey (2021-2022) it was on the 11th rank. Herring displayed the third most abundant species during the 1980s and 1990s, the fourth most abundant species in 2009-2010 and the second most abundant species during the 2020s. Other abundant species were ruffe, which was the second most abundant species during 2009-2010 (1980s: 15th, 1990s: 5th, 2020s: 7th) and three-spined stickleback which was the second most abundant species during the 1990s (1980s: 4th, 2009-2010: 11th, 2020s: 9th). While whiting was the third most abundant species in the 2020s, the ranks of *whiting* were lower in the 1980s (42nd), 1990s (10th), 2009-2010 (26th).

The mean density per haul of several species exhibited varying patterns over time (Fig. 2, Tab A.2). Specifically, there was an increase in density observed from the 1980s through the 1990s, followed by a peak during the 2009-2010 period. However, our subsequent survey conducted in the 2020s indicated a decline in mean density of fish species such as smelt, zander, ruffe, river lamprey (*Lampetra fluviatilis*), silver bream, common bream and lesser pipefish in comparison with the previous period. In the following we report the approximate number of fishes as individuals per 1 million m^3 water. Especially the mean density per haul of smelt reached from ca. 12,000 during the 1980s to 29,000 in the 1990s to 168,000 during 2009-2010 and was reduced with the most recent period (2020s) to 13,000. European eel expressed a gradual decrease over time, displaying the highest density during the 1980s and the lowest during 2020s. The density of twaite shad decreased from the 1980s from 1,700 to 52 in the 1990s. With the period 2009-2010 density of twaite shad increased to 1,000 and decreased with the latest period (2021-2022) to 12. Mean density of flounder increased in the 1990s up to 600, but exceeding to the time periods 2009-2010 (240) and 2021-2022 (33) the density was reduced. Common/sand goby had the highest density during the 1980s (165), which decreased to 15 in the 1990s and 0.7 during 2009-2010, while the density of common/sand goby increased in the time period 2021-2022 (26). Additionally, during 2021-2022 herring (1,500) and whiting (314) displayed their highest mean densities in the analysed time frame.



Fig. 2: Fish taxa found in in the Elbe estuary at the distinctive time periods 1984-1986, 1994-1995, 2009-2010 and 2021-2022. Changes of mean densities per haul (Log transformation) indicated by heatmap. Log-transformation of density data for enhanced visualization (large ranges of densities between species), untransformed data see Tab. A2.

5.2. Spatiotemporal density variability of dominant fish species

Differences in the distribution of species density from the 1980s to the 2020s aim to illustrate variations in species dynamics, focusing on spatial disparities across five locations along the salinity gradient in the Elbe estuary. Boxplots for of fish species that were among the most abundant species (smelt, herring, twaite shad, three-spined stickleback, ruffe, flounder) as well as important predatory fish species (zander, whiting), river lamprey as endangered anadromous species and European eel as endangered catadromous species across various stations in the Elbe estuary were chosen to indicate their variance across spatiotemporal scales (Fig. 3).

Densities of smelt, changed most substantially in the upstream regions being completely absent at several occasions during the 1980s ((Jun./Jul./ Aug./ Oct. 1985, Jun./Jul. 1986), D (May/Jun./Oct. 1985, Jun. 1986) and C (May 1986). In the most recent survey (2021-2022) the highest smelt densities were observed the Hamburg region. At E/E' with a median density of 25,000 during period IV, the population was reduced by nearly 85 %, compared to period III with a median density

of 162,000. In the same time period the median density at D was reduced from 76,000 to 5,000 while at C the density was reduced from 33,000 to 2,200.

Generally, herring exhibited its highest median densities in the downstream areas, specifically at stations A/A' (1,690 at period IV) and B (651 at period IV) near Cuxhaven and Brunsbüttel. Throughout all four time periods, herring was found up to the mid estuarine section (C). Density of herring was notably lower during the 1980s compared to surveys conducted in the 2020s, where the density reached its peak in that region. Only during the periods of 2009-2010 and 2021-2022 the presence of herring extended till the upper estuary (D), with higher density observed in the 2020s.

The highest median density (27) of twaite shad was recorded at A/A' during the 1980s, which had decreased to the 1990s (4.5), increased to 2009-2010 (26) and again decreased after the 2020 (4). During the 2020s, the highest maximum densities of *twaite shad* were observed at E/E' with 40 (spawners) and B with 262 (juveniles).

Three-spined stickleback density was highest during the 1990s period, especially at station C, where the median density of three-spined stickleback was17. During the most recent period (2021-2022) densities of three-spined stickleback were considerably lower at all five stations compared to period III (2009-2010).

During the 1980s, ruffe was absent in the downstream regions at A/A' and B, while in the 1990s, 2009-2010 and the 2020s ruffe occurred occasionally in low densities. However, during the 1990s and the 2009-2010 period, the density of ruffe was highest in the freshwater region, particularly at D and E/E'.

Flounder was found at its highest density during the 1990s with A/A' (median density: 3,000). Lower median densities of flounder during the 1990s were found upstream at B (451), at C (43), at D (44) and at E/E' (23). Decrease of flounder over was most prominent in the downstream areas A/A' and B from period II to period III.

The highest densities of zander were particularly found close to the Hamburg area at E/E', during the 2009-2010 period. Zander were also most abundantly found at E/E' during the 2021-2022 survey. Between those two periods the median density of zander increased from 7.5 to 17 at E/E'. At the more downstream areas zander densities were lower during all four time periods.

Whiting was found exceptionally in the downstream regions in the river mouth at A/A' and B, where the higher densities were found at the outermost station A/A', especially during the 2021-2022 survey. During period IV at A/A' the median density of whiting reached 263, while the median density at the other periods was 0, since whiting occurred only sporadically during those time periods.

Generally, the density of river lamprey decreased with our recent sampling in the 2020s. The highest median densities of river lamprey were found in the river mouth are, especially during the 2009-2010 period at A/A' (2.1). At B the median densities were lower: 0.7 (1980s) and 0.8 (1990s). During period III at D the maximum density of river lamprey recorded was 177, followed by 89 at C and 82 at D. The maximum density of river lamprey in the 2021-2022 period was found at B with 11, which had also decreased.

Densities of European eel decreased consistently at all five stations across the Elbe estuary over the four observed time periods.

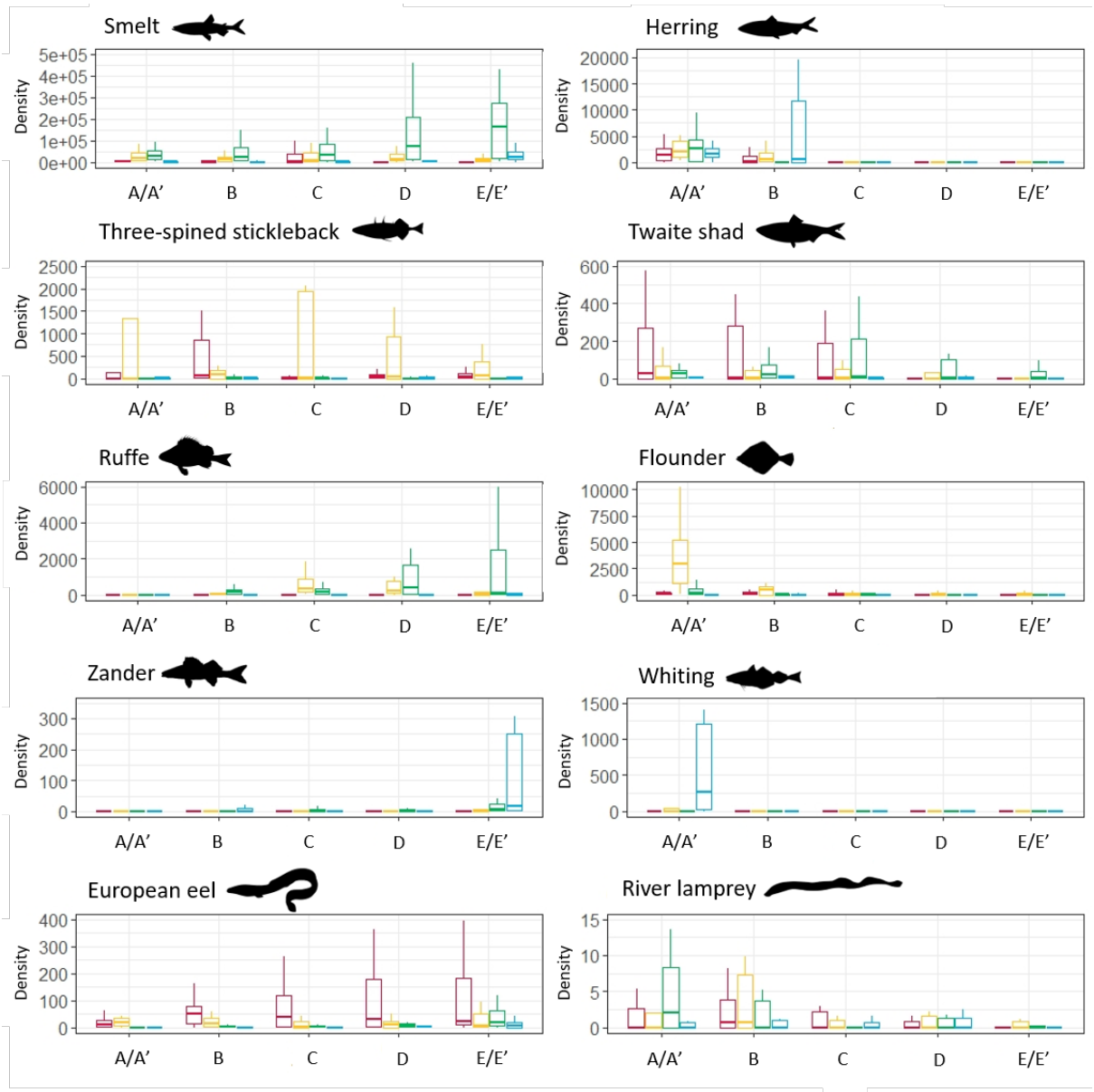


Fig. 3: Densities of ten of the key fish species along the longitudinal course of the Elbe estuary. A line at zero indicating complete absence of the species at the specific station during the referring time period. Medem Reede/Medemgrund at Ekm 713/716 (A/A'), Brunsbüttel at Ekm 692 (B), Schwarztonnensand at Ekm 665 (C), Twielenfleth at Ekm 651 (D) and Neßsand/Mühlenberger Loch at Ekm 633/639 (E/E'). Colours indicating the respective sampling periods: red (1984-1986), yellow (1994-1995), green (2009-2010) and blue (2021-2022). Outliers (> 1.5 x interquartile range) removed for enhanced visualization of quartiles.

5.3. Spatiotemporal variations of life cycle guilds

Marine stragglers were found only in the downstream areas (A/A' and B), with relatively low and consistent densities, with less than 0.1 % (Fig. 4, Tab. A.3). The highest density of marine stragglers, with a maximum of 1.6, was recorded during period IV (2020s) sampling at the downmost region (A/A').

At A/A' the marine estuarine opportunists increased from 178 (1980s) to 597 (1990s) to 517 (2009-2010). From 2009-2010 to the 2020s at, the number of marine-estuarine opportunists decreased from 517 to 255. In contrast, the relative proportions between the 1980s and 2009-2010, remained nearly constant (8.6 %, 10.9 %, 7.6 %). However, compared to the most recent sampling

relative proportion of marine estuarine opportunists increased from 7.6 % to 18.5 % at A/A'. At B, the number of marine estuarine opportunists from 1980s to the 1990s remained constant with densities of 220 and 191 (6.9 %, 5.1%), while from 2009-2010 to 2021-2022 marine estuarine opportunists increased from 116 to 464 at station B, which was a relative increase from 1.4 % to 36.9 %. In the middle section of the estuary the density of marine-estuarine opportunists devolved from 14.8 (1980s) over to 9.3 (1990s) to 12.9 (2009-2010) and 17.0 (2020s). Therefore, the relative proportion increased from 0.1 % to 0.3 % (1980s until 2009-2010) to 3.9 % during the 2020s. In the upper sections the densities of the estuarine-opportunists LCG were lower.

In the river mouth section (A/A') the density of the estuarine LCG decreased from 660 (1980s) to 99 (1990s), to 567 (2009-2010) and 12 (2020s), while in the lower section at B, densities decreased from 173 during the 1980s to 21 in the 1990s, 1.0 in 2009-2010 and 2.0 during the 2020s. Their numbers remained nearly constant at the freshwater to upper oligohaline areas with densities around 0.5 (< 0.1 %).

The number of catadromous species consistently decreased throughout the entire estuary from the 1980s to the 2020s. The mean density of anadromous species increased from the 1980s to the 1990s and further to 2009-2010, but then drastically decreased in the 2020s. In the downstream areas, changes in anadromous LCG densities were less pronounced compared to the upstream areas. From 2009-2010 period to 2021-2022 period the density of freshwater species declined notable in the freshwater regions of E/E' and D.

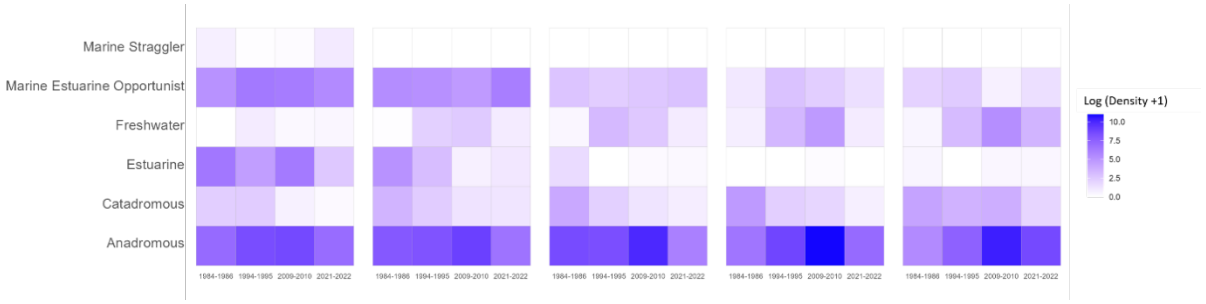


Fig. 4: Spatiotemporal variation of mean densities of LCG at four distinct time periods along the salinity gradient of the Elbe estuary: Medem Reede/Medemgrund at Ekm 713/716 (A/A'), Brunsbüttel at Ekm 692 (B), Schwarztonnensand at Ekm 665 (C), Twielenfleth at Ekm 651 (D) and Neßsand/Mühlenberger Loch at Ekm 633/639 (E/E'). Log-transformation of density data for enhanced visualization (large ranges of densities between guilds), untransformed data see Tab A.3.

5.4. Long term shifts of environmental factors

Over the course of the past 40 years, the Elbe estuary underwent changes in environmental conditions (Fig. 5). EC in the upstream regions (D, E/E') decreased from 1980s until the early 2000s, but increased from there slightly. The pH was lowest during the 1980s and early 1990s in the upstream regions (C- E). TOC had decreased in all five stations since the 1980s. At region C the total organic carbon concentration increased again. Phosphate concentration was highest in the upstream regions (D & E) during the 1980s and gradually decreased across the time scale. Nitrate concentration at all five regions decreased from 1980s until 2022. Maximum nitrate values were around 8 mg/L at region B during the early 1980s and early 1990s. Nitrite concentration was highest at region E/E', especially during the entire 1980s decade, shown by highest annual means. At region E/E' and B nitrite peaks above 0.8 mg/L. On multiple occasions nitrite concentrations above 0.5 mg/L were found at stations E/E', D and C. Generally, the nitrite concentration decreased over time. The highest annual ammonium concentrations were found in the upper and middle sections E/E', D, and C. The maximum concentrations in the upper and middle sections E/E', D and C ranged between 4 mg/L and 5 mg/L during the 1980s. From the beginning of the 1990s the ammonium concentration decreased in the entire estuary.

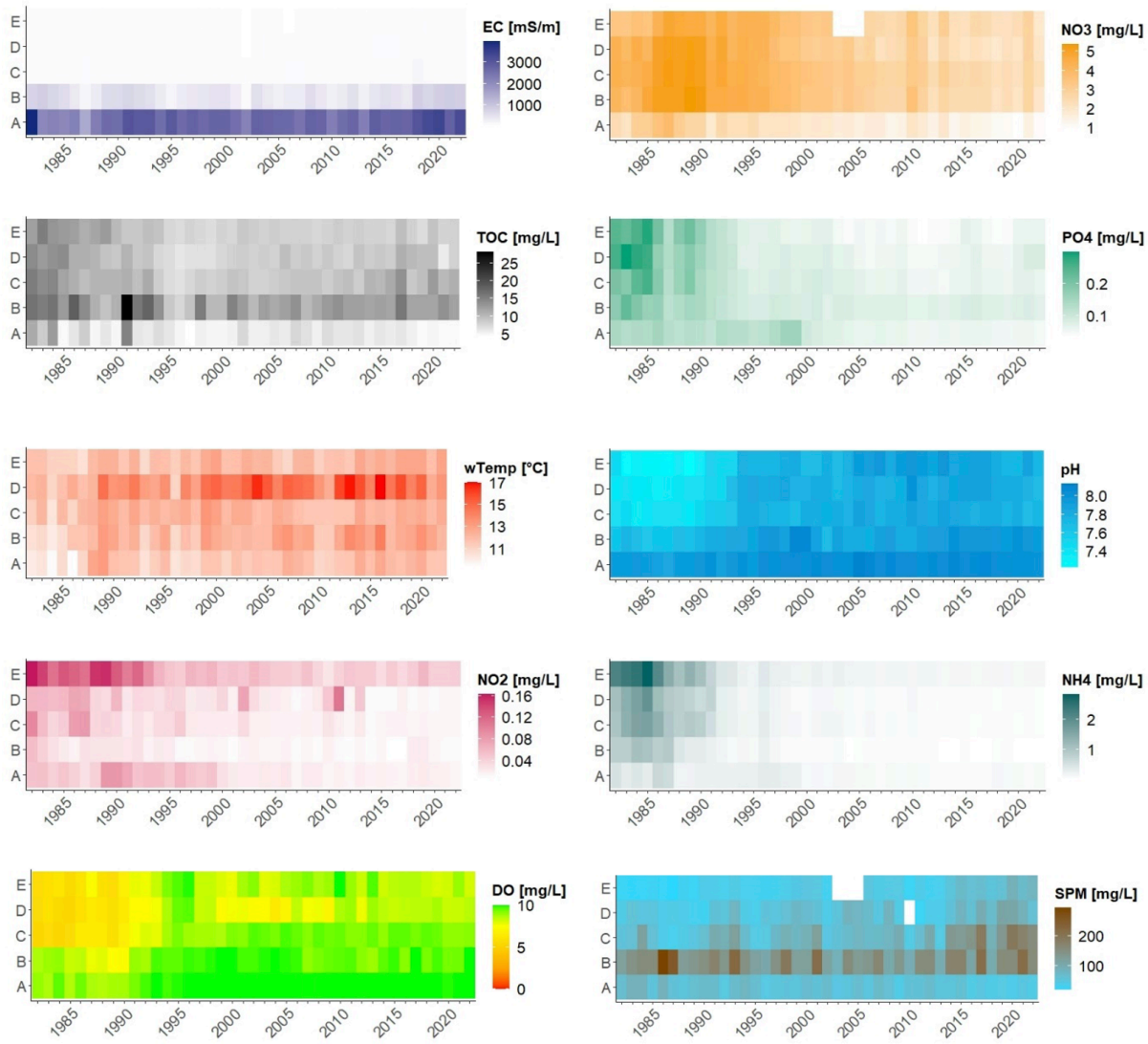


Fig. 5: Environmental parameter electric conductivity (EC), nitrate (NO_3), nitrite (NO_2), ammonium (NH_4), phosphate (PO_4), total organic carbon (TOC), suspended particular matter (SPM), water temperature (wTemp), dissolved oxygen (DO) and pH as heatmap (annual mean) from 1982 to 2022. A = Medem Reede/Medemgrund (Ekm 713), B = Brunsbüttel (Ekm 692), C = Schwarztonnensand (Ekm 665), D = Twielenfleth (Ekm 651), E = Neßsand/Mühlenberger Loch (Ekm 633).

The oxygen concentration in the Elbe estuary was particularly lowest during the 1980s in the upstream region E/E', D and C, shown by the annual mean oxygen levels. In the river mouth at station A/A', no critical oxygen values were measured at any time. At region B, especially during the 1980, critical oxygen levels were measured. In this region, on multiple occasions the oxygen concentration fell below 4 mg/L, with incidences below 2 mg/L in the early 1980s. Going further upstream lower oxygen concentrations were more frequently measured, which resulted in lower annual mean DO. At regions C and D oxygen concentrations below 2 mg/L occurred most frequently during the 1980s and oxygen situation improved from the 1990s on. At region E/E', located in the Hamburg port area, oxygen concentrations close to zero were measured during the 1980s. Although the oxygen situation had improved from the 1990s onward, low oxygen levels were still regularly found in that region until the year 2022. During hypoxic events in that time period oxygen concentrations below 4 mg/L and 2 mg/L were measured, especially during summer months.

At the station at A/A', located at the river mouth, the annual mean of SPM ranged from 38.1 mg/L to 89.7 mg/L. In the lower section at station B, the SPM measurements reached the maximum, with annual means ranging from 90.5 mg/L (2003) to 293.9 mg/L (1986). At station C in the time frame

from 1982 to 2010 (a 28-year period), the annual mean of SPM exceeded 100 mg/L on four occasions: 1984, 1991, 1993, and 2001. However, in a shorter 12-year period from 2010 to 2022, 100 mg/L was surpassed nine times, peaking at 193.8 mg/L in 2020. In region D, the highest annual means of SPM were recorded in 2019 (111.3 mg/L), 2020 (107.7 mg/L), and 2017 (97.2 mg/L). In the region of E/E' the annual mean of SPM concentration had more than tripled from 1982 to 2022. From 1982 to 2013 the SPM annual mean at station E/E' exceeded a concentration of 50 mg/L only once in 2006 (50.3 mg/L), however since 2014, the annual mean concentration of SPM has consistently surpassed 50 mg/L each year. During the 1980s, concentrations ranged between 18.6 mg/L and 30.7 mg/L. Subsequently, in the 1990s, they increased to a range of 30.5 mg/L to 44.6 mg/L. In the 2000s, concentrations continued to rise, reaching concentrations between 31.8 mg/L and 50.6 mg/L. In the 2010s concentrations were varying from 26.3 mg/L to 72.5 mg/L, and in the 2020s, SPM peaked between 71.1 mg/L and 92.1 mg/L.

The PCA (Fig. 6) shows spatial and periodical separation on both axis by the environmental variables. The axis PC1 accounted for 30.4 % while PC2 accounted for 21.7 % of the variation. The samples show a spatial gradient with station A as the station in the river mouth mostly ordinated at -2.5, while freshwater station (C, D, E) were mostly ordinated in positive ordination space extending to above +7 on PC1. Samples from period I are clearly separated from the other periods due to changed environomental conditions.

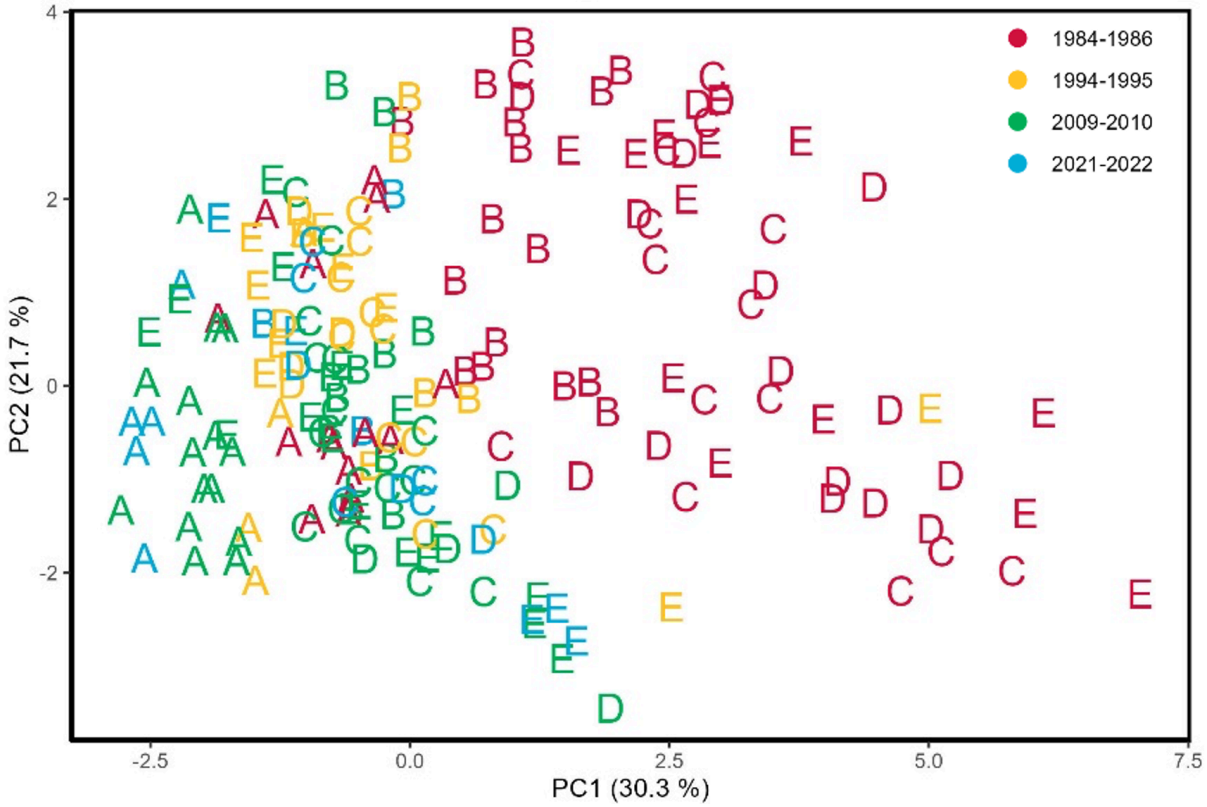


Fig. 6: Principle component analysis (PCA) classified by solely environomental variables measured in the Elbe estuary between 1984 and 2022. A = Medem Reede/Medemgrund (Ekm 713), B = Brunsbüttel (Ekm 692), C = Schwarztonnensand (Ekm 665), D = Twielenfleth (Ekm 651), E = Neßsand/Mühlenberger Loch (Ekm 633).

5.5. Influence of environmental factors on the fish fauna

The CCA ordination CCA1 (eigenvalue = 0.35) and CCA2 (eigenvalue = 0.12) accounted for 67.3 % of the variation (Fig. 7). The ordination showed the spatial distribution of the samples with stations in the river mouth (A, B) orientated in the positive ordination space along axis CCA1, while freshwater

samples (D, E) were oriented in the negative ordination space. The fish species were oriented in a similar pattern: while marine stragglers e. g. hooknose (*Agonus cataphractus*) or tub gurnard (*Chelidonichthys lucerna*) and marine estuarine opportunists (e.g. herring, whiting) were oriented in the positive ordination space along CCA1 axis, freshwater species were orientated in the negative space. Diadromous fish species (e.g. smelt, twaite shad, three-spined stickleback) were ordinated centralized in the ordination space. This is also reflected by the salinity gradient as expressed by the vector EC in which the EC acts as a proxy for the salinity. Samples with higher water temperature are ordinated in the negative ordination space along CCA2 axis. The vectors for water quality parameters nitrate, nitrite, ammonium and TOC showed that samples from the 1980s mainly in the freshwater region (D, E) and the middle section (C) had higher concentrations compared to samples from the other periods.

SIMPER analysis (Tab. 1) revealed that the largest influence on the ordination is caused by variation of density from smelt (21.5 %). The species that had the second most influence on the dissimilarity matrix was herring (8.9 %) followed by three-spined stickleback (7.9 %), ruffe (7.4 %), twaite shad (7.2 %), sprat (5.7 %), flounder (5.0 %), European eel (4.4 %), zander (3.5 %) and common bream (3.0 %).

Tab. 1: Cumulative percentages of the ten fish species responsible for the most variation (75 %) in the Elbe estuary (SIMPER-Analysis).

Species	Cumulative contribution [%]
Smelt	21.5
Herring	30.4
Three-spined stickleback	38.3
Ruffe	45.7
Twaite shad	52.9
Sprat	58.6
Flounder	63.6
European eel	68.0
Zander	71.5
Common bream	74.7

The four time periods 1984-1986, 1994-1995, 2009-2010 and 2021-2022 were statistically distinguishable by ANOSIM analysis ($R^2 = 0.219$, $p < 0.001$). Post-hoc pairwise ANOSIM with Bonferroni correction (adjusted alpha level reduced to $1/6^{th}$ of 5 %) showed that dissimilarities between period I and IV were not significant ($R^2 = -0.01$, $p = 0.606$) as well as dissimilarities between period II and III ($R^2 = 0.05$, $p = 0.034$). Dissimilarities between period I and III were highest ($R^2 = 0.29$, $p = 0.001$), while dissimilarities between period III and IV ($R^2 = 0.17$, $p = 0.001$) or between period II and IV ($R^2 = 0.11$, $p = 0.002$) were also significant.

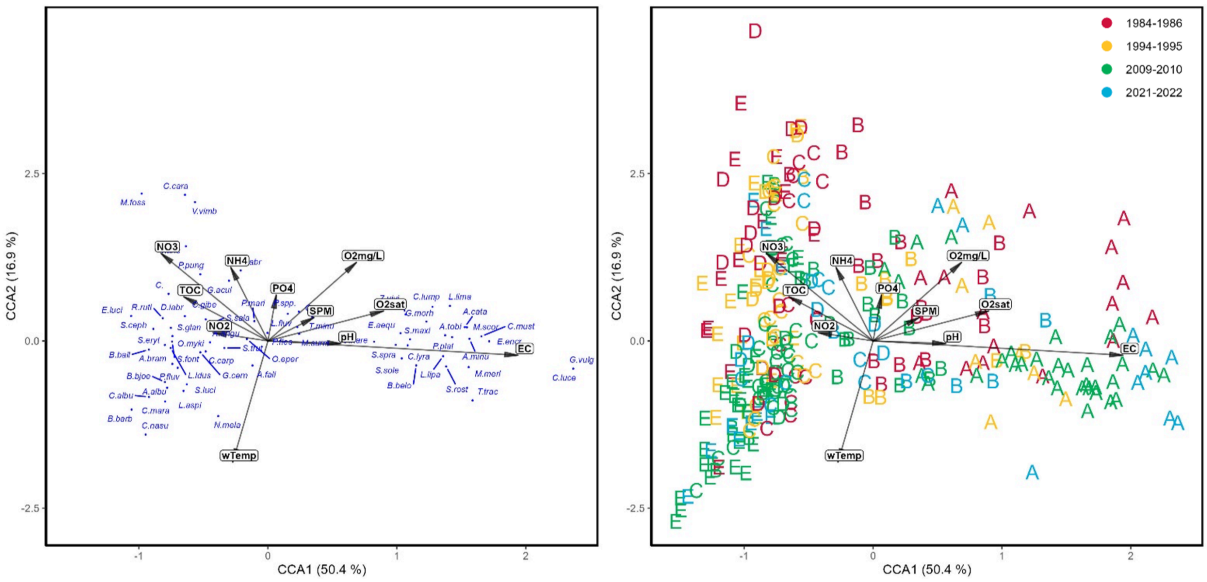


Fig. 7: Canonical correspondence analysis, separated for visualization by top: species as abbreviations; and bottom: environmental variables as vectors: EC (electric conductivity), SPM (suspended particular matter), NO_2 (nitrite), NO_3 (nitrate), NH_4 (ammonium), PO_4 (phosphate), TOC (total organic carbon), Wtemp (water temperature), O_2 (oxygen saturation in % and concentration in mg/L) and pH. ANOSIM ($R^2 = 0.219$, $p < 0.001$). A = Medem Reede/Medemgrund (Ekm 713), B = Brunsbüttel (Ekm 692), C = Schwarztonnensand (Ekm 665), D = Twielenfleth (Ekm 651), E = Neßsand/Mühlenberger Loch (Ekm 633). Species abbreviations: *Abramis brama (A.bram), Alburnus alburnus (A.albu), Alosa fallax (A.fall), Ammodytes tobianus (A.tobi), Anguilla anguilla (A.angu), Ballerus ballerus (B.ball), Carassius carassius (C.cara), Carassius gibelio (C.gibe), Chelon labrosus (C.labr), Chelon ramada (C.rama), Clupea harengus (C.hare), Coregonus albula (C.albu), Coregonus maraena (C.mara), Cyprinus carpio (C.carp), Dicentrarchus labrax (D.labr), Gadus morhua (G.morh), Gasterosteus aculeatus (G.acul), Gymnocephalus cernua (G.cern), Lampetra fluviatilis (L.fluv), Leuciscus aspius (L.aspi), Limanda limanda (L.lima), Merlangius merlangus (M.merl), Myoxocephalus scorpius (M.scor), Neogobius melanostomus (N.mela), Oncorhynchus mykiss (O.myk), Osmerus eperlanus (O.eper), Perca fluviatilis (P.flvi), Petromyzon marinus (P.mari), Platichthys flesus (P.fles), Pleuronectes platessa (P.plat), Pomatoschistus spp. (P.spp.), Pungitius pungitius (P.pung), Rutilus rutilus (R.ruti), Salmo salar (S.sala), Salmo trutta (S.trut), Salvelinus fontinalis (S.font), Sander lucioperca (S.luci), Scardinius erythrophthalmus (S.eryt), Scophthalmus maximus (S.maxi), Silurus glanis (S.glan), Solea solea (S.sole), Sprattus sprattus (S.sprat), Squalius cephalus (S.ceph), Tinca tinca (T.tinc), Trisopterus minutus (T.minu), Vimba vimba (V.vimb), Zoarces viviparus (Z.vivi).*

The post-hoc permutation test for constrained correspondence analysis showed that all factors had significant influence on the species composition (Tab. 2). Strongest influence was expressed by EC (F = 41.91), which acts as proxy for the salinity, since presence of marine species is highly depended on the salinity. Second strongest environomental variable was oxygen concentration (F = 8.44), since oxygen minimum zones had affected the species composition. Third and fourth strongest influences were expressed by nitrogen compounds nitrate (8.42) and ammonium (F = 5.79). SPM (F = 5.33) was identified as fifth most influential environomental variable affecting the fish species composition. Although being of minor importance, variables such as pH (F = 1.92) or TOC (F = 2.01) still had significant influence on the compositing of the fish fauna.

Tab. 2: Influences of environomental variables on the fish species composition by post-hoc permutation test. Higher F-value indicating more influence on the species composition.

Environomental variable	Chi-square	F-value	Pr(>F)
EC	0.33608	41.91	0.001***
O2mg/L	0.06767	8.44	0.001***
NO3	0.06754	8.42	0.001***
NH4	0.04646	5.79	0.001***
SPM	0.04275	5.33	0.001***
PO4	0.03470	4.33	0.001***

NO2	0.03325	4.15	0.001***
wTemp	0.02016	2.51	0.001***
O2sat%	0.02010	2.51	0.003**
TOC	0.01615	2.01	0.008**
pH	0.01541	1.92	0.021**

6. Discussion

6.1. Changes of life cycle guilds

In this study we aimed to describe changes in the density and the species composition in a temperate mesotidal estuary across four distinct periods extending over four decades and determine the driving environmental factors for fish occurrences.

As being among the most abundant species, smelt and twaite shad account for the largest portion of the anadromous LCG. Since the Elbe estuary provides suitable nursery areas for both species, we found juvenile life stages in high numbers. However, as both species have faced a reduction in their population sizes, the anadromous LCG decreased within recent years. From period III to period IV the mean density of the anadromous LCG was reduced from 20,000 to 1,500 individuals per 1 million m^3 resulting a decline of over 92 %. This LCG was mainly represented by smelt, twaite shad and three-spined stickleback.

The recent decline in the density of anadromous (juvenile) fish species in the Elbe estuary can be linked to a combination of factors contributing to the overall degradation of habitat quality. Key stressors include the recently increased oxygen deficiencies and SPM loads. Additionally, the reduction of shallow water areas within the freshwater region further increased the stress on growing larval and juvenile fish (Scholle & Schuchard 2020, Illing et al. 2024). While tested environomental factors play significant roles, it is important to note that this decline is most likely a result of multiple interacting stressors. Further investigation with mesocosm experiments could further address these issues, in order to quantify the effects of multiple environomental stressors.

The catadromous LCG was mainly represented by European eel, whose populations decreased throughout Europe and therefore listed as ‘critically endangered’ on the IUCN Red List (Pike et al. 2020). The factors resulting in the collapse of European eel populations are versatile: migration barriers, silver eel mortality by hydropower plants, parasites e.g. swim bladder nematode *Anguillicola crassus*, disease spreading of anguillid herpesvirus by stocking, and fisheries (especially glass eel) (Behrmann-Godel & Eckmann 2003, Briand et al. 2007, Dekker 2016, Kullmann et al 2017). These problems are not specifically related to factors found exclusively in the Elbe, but are found in other European water bodies as well.

The increase of marine species in the Elbe estuary can be explained by an increase of marine influence by the flood stream paired with reduced river runoff. The river runoff between 2014 and 2020 was 32 % lower than the long-term 1960-2020 mean (Rewrie et al. 2023). Due to enlarged channel diameter, larger quantities of marine waters can enter the Elbe estuary during high tides (Zhu et al. 2014, Ralston & Geyer 2019), which favors the inland prevalence of marine fish species.

With the relative increase of marine-estuarine opportunists and the reduction of diadromous species, the Elbe estuary has become somewhat more similar in its guild structure to the macrotidal estuaries found in the UK (i.e. Forth, Tyne, Humber). In these estuaries the ecological guilds of estuarine residents, marine juvenile migrant and marine adventitious species displayed the largest portion of the fish fauna, while diadromous fish species were of less importance (Elliott & Dewailly

1995). These alterations suggest an ongoing transition of the fish guild composition towards those found in a macrotidal estuary.

6.2. Environmental factors structuring the fish fauna

Over the entire time scale, certain environmental factors had a major influence on structuring the fish species composition in the Elbe estuary. The strongest influence was attributed to EC, which acts as a proxy for salinity. Stations with higher salinities at the river mouth and lower sections (A and B, see Fig. 1) had higher densities of marine stragglers and marine-estuarine opportunists, which confirms the findings of the spatiotemporal structure found in previous studies (e.g. Thiel & Potter 2001, Eick & Thiel 2014). With increasing sea level rise and reduced river runoff due to climate change, these marine influences are likely to increase even more in the future (Little et al. 2022, Pein et al. 2023).

Seasonal changes of the fish fauna composition in relation to water temperature were also described by Thiel & Potter (2001). With diadromous species such as smelt, twaite shad, three-spined stickleback in the fish community, species composition changes during migration periods and juvenile nursery time. Smelt densities peaked between early June and late August, when the population consists predominantly of juvenile young-of-the-year specimens. Densities of three spined-stickleback tended to be highest in colder temperatures during winter and early spring samples. In the freshwater sections common bream densities tended to be higher at warmer temperatures during summer months. Upstream migration of anadromous fish can be induced by factors such as rising water temperature (Aprahamian et al. 2003) and increased river runoff (Lillelund 1961). The seasonal variation of the fish species composition is characterized by spawning and nursing activities, with high densities of juveniles occurring in the estuary during spring and summer. As water temperature follows seasonal patterns, the significant influence on the species composition expressed in Fig. 6, reflects these seasonal changes rather than long-term shifts by increased temperature.

In the 1980s water quality in the Elbe estuary was poor due to waste water sewage (ARGE Elbe 1984, Möller & Scholz 1991), which was expressed by high concentrations of nitrogen compounds (ammonium, nitrite, nitrate), TOC and phosphates. The concentrations of those compounds were highest in the freshwater and upper oligohaline areas of the estuary (Fig. 2). Nitrite is particularly toxic to fish by reducing oxygen uptake ultimately causing death (Russo & Thurston 1977) by suffocation which is even more unfavourable during events with low oxygen concentration. Schäperclaus (1979) described concentrations of nitrite of 1 mg/L as non-toxic (with regard to lethal exposure). However more recent studies investigating long-term effects of sublethal nitrite exposure of 0.3 mg/L for 60 days indicated significantly lower food intake and growth performance in juvenile percids (Zhang et al. 2021). With values regularly exceeding 0.3 mg/L in the freshwater regions during the 1980s these values have been exceeded for long times in the Elbe estuary classified as highly polluted (LAWA 1998). Adding to this, ammonium values that, depending on temperature and pH becomes fish toxic ammonia, reached values of 2.4 mg/L during multiple years rendering the estuarine waters as severely polluted by classification of LAWA (1998).

The water quality described in the 1980s was accompanied by recurring low oxygen concentrations, occasionally even anoxic conditions (Möller & Scholz 1991). During anoxic events in the years 1985 and 1986, *O. eperlanus* was completely absent from E/E' (seven samples), D (four samples) and C (one sample) between May and August. Entire smelt generations can be destroyed by an early onset of oxygen depletion in their life cycle (Möller & Scholz 1991, Thiel et al. 1995). During 1985 highest catches of juvenile smelt were in areas with more than 5 mg/L DO (Möller & Scholz 1991). In the 1980s the oxygen minimum reached downstream to B, where values below 2 mg/L were measured. After the 1990s at C and D no DO concentrations below 2 mg/L were measured, but occasionally values below 4 mg/L were reached. Generally, the oxygen situation improved compared to the 1980s, especially at B, C and D. Although the oxygen situation had improved after 1990, at E/E'

concentrations below 4 mg/L and 2 mg/L were still regularly measured during summer, meaning the oxygen situation at E/E' remained relatively unchanged after the late 1990s. With regard to fish larvae that use the region around the Mühlenberger Loch (E/E') as feeding area, Sepúlveda (1994) showed that DO concentrations below 4.5 mg/L already reduced the growth performance of smelt larvae.

6.3. Anthropogenic influences on fish densities

Historically, nutrient loads and eutrophication have severely impacted fish populations, leading to significant oxygen depletion and causing destruction of entire cohorts (Möller & Scholz 1991). As water quality improved, fish stocks had recovered between the mid-1990s and early 2000s. However, compared to the time period 2009-2010, our most recent survey showed that several of the dominant fish species in the Elbe estuary declined in their density.

In a scenario of intensified river dredging, the effects of tidal pumping are amplified, resulting in several stress factors for the fish fauna in the Elbe estuary. Bilkovic (2011) found that recently dredged tidal creeks provide less suitability nursery habitats for fish, while Yang et al. (2019) concluded that suspended sediments can have direct impacts on the survival of fish eggs and larvae. Model calculations showed that channel deepening appeared to be the main enhancing factor for an upstream transport of sediments (van Maren et al. 2015). Sexton et al. (2024) concluded that ship traffic in freshwater waterways is related with declines in biodiversity for fish and macroinvertebrates. They concluded that channelization and riparian degradation have a detrimental effect on these ecosystems. Findings from Thiel & Pezenburg (2001) showed that elevated SPM concentration in the Mühlenberger Loch region can induce alterations in the gill epithelial tissue (increase of mucocytes) of juvenile smelt and therefore could cause raised mortality rates and stunted growth.

Hecht & van der Lingen (1991) found that turbidity affects feeding behaviour in fish. Under different turbidity conditions, changes in feeding strategies were described, whereby visual predators are more affected by turbidity than fish that feed on macroinvertebrates. Higher concentrations of SPM were shown to affect lower trophic levels, resulting in less phytoplankton production by light limitation (de Jonge et al. 2014, Steidle & Vennell 2024) leading to lower densities of zooplankton and benthic invertebrates (Shoup & Wahl 2009) and therefore restrict food availability for fish. This is supported by findings from Bernát et al. (1994), who found that increased SPM negatively affects the filtration rate of calanoid copepod *Eurytemora affinis* in nauplii stages and adults, thus affecting copepod densities in recent years and disrupting the entire food web of the estuary. The increased turbidity in this scenario can affect the fishes in the Elbe estuary on various levels: firstly, the suspended solids could have caused irritation in the gill tissue, secondly, foraging behaviour could have been impaired and finally, overall food availability could have been reduced.

Increasing SPM concentration at C (Schwarztonnensand) showed that the reach of the maximum turbidity zone has migrated upstream. In the description of Lang (1990), in a mesotidal estuary the highest concentrations of SPM are found in the gradient zone, while in a macrotidal estuary higher concentrations of SPM can be found in the freshwater areas. With the increased tidal amplitude and the upstream migration of the MTZ, the Elbe estuary is driven towards a macrotidal estuary. Upstream migrations of the MTZ have already been observed in other heavily modified estuaries such as the Ems estuary (de Jonge et al. 2014). Especially the side channels of southern marginal area in the upper section of the estuary (between D and E/E') Hahnöfer Nebenelbe, Lühesander Süderelbe and the shallow bay Mühlenberger Loch were described as locations where the annual production of fish was the highest (more than 200 kg/ha per year) in the entire estuary (Thiel 2001). In the shallow water, the ratio between surface and water volume is in favour for the fish larvae, since diffusion of atmospheric oxygen and penetration through the shallow water column mitigates low oxygen situations, which occur in the main channel during summer. Copepod densities are highest in the Mühlenberger Loch area around E/E' (Fiedler 1991, Kafemann et al. 1996, Thiel 2001), making this area the favoured nursery habitat for fish larvae, as prey organisms of the optimal size are

available here. The upstream sediment transport from tidal pumping also carries organic compounds. These particles include organic carbon and active microorganisms that consume and break down the organic compounds (Tobias-Hunefeldt et al. 2024), leading to higher oxygen demands and oxygen deficiencies during summer. These oxygen deficiencies, especially in the freshwater regions of the estuary, which are the nurseries for several fish species lead to a reduction of survival of fish larvae and juveniles.

Another factor that can affect the autochthonous fish fauna are invasive species. In 2008 the first individual of round goby (*Neogobius melanostomus*) was caught by commercial fisherman in the Elbe estuary (Hempel & Thiel 2013). The high numbers of records and juveniles by the year 2012 and 2013 suggested the establishment of the species in the Elbe estuary. Round goby can negatively impact local fish populations by competition for habitats and food resources or direct predation of eggs and juveniles. But they have also incorporated in the food web by being part of the diet composition of predatory fish such as zander (Hempel et al. 2016). While adult predatory fish benefit from round goby as an easily available prey throughout the year, they may suffer from them at earlier life stages, since percid fish eggs were found in the diet of round goby in the Elbe at Hamburg and the adjacent Kiel Kanal (Giesler 2015, Hempel 2017).

While several fish species in the Elbe estuary suffered from negative impacts, the marine-estuarine opportunist LCG (e.g. herring and whiting) did benefit from changing environmental factors. The upstream shift of the upper brackish water boarder allowed them to access a larger area of the estuary more easily. Whiting is a lusitanian fish species and according to Thiel et al. (2025) densities of whiting have recently increased in the German North Sea areas. Although high densities of juvenile whiting are not an event that occur every year, with the increasing temperature in the North Sea and the Elbe estuary due to climate change, the appearance of large juvenile whiting schools in the Elbe river mouth is likely to occur more regularly.

7. Conclusion

Our long-term case study in the Elbe estuary demonstrates the significant influence of environmental factors, many altered by human impacts, on fish fauna composition in European estuaries. Despite improvements in water quality and oxygen conditions boosting fish densities in the 1990s and early 2000s, recent years have seen a dramatic decline. Increased SPM concentrations, upstream shifts in turbidity, reduced river runoff, and hypoxic summer conditions have severely affected fish stocks, with mean fish densities plummeting by over 91 % from 2009-2010 to 2021-2022, primarily impacting smelt populations. These findings highlight the critical importance of protecting estuarine nurseries to maintain ecosystem services, which are currently undermined by ongoing ecological degradation. For key species like smelt, the lack of refuge areas due to blocked upstream migration exacerbates their plight which is likely to be intensified by climate change effects.

8. Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work the author JT used DeepL for translation and for improvement of language and readability of the manuscript. After using this tool, JT reviewed and edited the content as needed and takes full responsibility for the content of the publication.

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10. Author contributions

JT: conceptualization, investigation, field work (methodology), project administration, data curation, formal analyses, writing – original draft; **VS:** data curation, formal analyses, writing – review & editing; **EH:** field work (methodology), investigation, writing – review & editing; **RK:** field work (methodology), investigation, review and editing; **CM, AF:** conceptualization, supervision, funding acquisition, writing – review & editing; **RT:** funding acquisition, conceptualization, supervision, formal analyses, writing – review & editing, project administration.

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Chapter 7 General Discussion

1. General Discussion

Within the climate change debate, in particular the large capacity for carbon storage in estuarine habitats is recognized (Abrantes et al., 2015; Bulmer et al., 2020; Krauss et al., 2018). However, this, like many other services delivered by estuarine systems, is heavily dependent on an intact ecosystem. Disturbances in the systems, such as the disappearance of large predatory fish, affects the carbon cycle and storage capacity (Atwood et al., 2015).

Many fish species use estuarine habitats as essential parts of their life cycle (Whitfield et al., 2022): As nurseries, feeding grounds, migration routes, refuge or overwintering areas (Elliott & Hemingway, 2008). This requires special adaptations towards salinity, temperature, oxygen levels and suspended particulate matter (SPM) tolerance (Able, 2005; Lam et al., 2005). For example, adaptations in the gill morphology including the permeability for Ions, metabolic differences, regulation of the heat shock response such as the abundance of inducible or constitutively regulated heat shock proteins (HSPs), potentially also variations in coding sequences, even an adaptation to human induced toxins by induction of different variants of detoxification related genes like CYP1A (Schulte, 2007; Scott et al., 2004).

However, over the last decades, habitat degradation by direct human intervention and climate change related impacts amplified the pressure on estuarine communities (Cloern et al., 2016; Colombano et al., 2021; Lauchlan & Nagelkerken, 2020).

Among others, the following are of particular importance and will be increasingly so:

I. Hypoxia, resulting from a combination of nutrient input and temperature increase far surpassing the impact of all other factors in estuarine biota (Breitburg et al., 2018; Cottingham et al., 2018; Diaz & Rosenberg, 2008; Sampaio et al., 2021)

II. Temperature rise and occurrence of extreme events like heatwaves. Here global models on ocean warming seem not to be applicable to estuaries as these were shown to increase in temperature at a magnitude higher speed (Scanes et al., 2020; Tassone et al., 2022)

III. Sea level rise and altered patterns in river runoff will globally increase saltwater incursion into tidally influenced oligohaline and freshwater areas (Lee et al., 2024; Little et al., 2017). Natural upstream relocation of these habitats is prevented by human constructions such as dams, like the weir upstream of Hamburg, and thus a squeezing of these low salt areas will appear (Little et al., 2022; Pein et al., 2023).

Only 0.1% of all climate change research focusses on observational changes in estuaries with a lack of long-term studies (Biguino et al., 2023). The integration of molecular methods into habitat and fish monitoring, enabling an evaluation of responses and stress factors over an extended range of levels of biological organization and adaptive trajectories, is largely missing in estuaries like the tidal Elbe. Such studies have only recently been conducted in French estuaries on pooled fish responses (Laurent et al., 2022; Laurent et al., 2023) or in Japanese (Wei et al., 2018) and Australian ones focussing on fish-gut associated microbiota (Suzzi et al., 2023) but neither in combined approaches aimed at describing holobiont responses nor over spatio-temporal scales.

The aim of this thesis was to gain a holistic overview of stress responses in estuarine fish from the very low level of biological organization (molecular) and interaction (holobiont) till high level population scale long-term dynamics. Previous studies on molecular level in fish stress response from the Elbe study system evaluated punctuated responses from single time points and selected stressors giving first glances at the additive/ synergistic nature of stressors in the wild compared to mimicked

responses in the lab (Tiedke et al., 2015; Tiedke et al., 2014). The continuous drop in costs for Omics methods such as transcriptomes via RNAseq or the recording of bacterial compositions via 16S sequencing over the last decade, however, has opened up a completely new field of possibilities for *in situ* application (within the wild habitat) and the integration into wildlife monitoring and conservation efforts (Connon et al., 2018; Miranda et al., 2023).

1.1. Key findings

1.1.1. Network based integration of omics

The integration of holistic molecular data from gill tissue (highly involved in immune responses and gas exchange), liver tissue (similarly involved in the immune system but key site for metabolic stress acclimatization), gill mucus bacterial composition, physiological and abiotic measurements in a network approach allowed us to determine stress patterns with high resolution: Fish from the maximum turbidity zone showed signs of starvation via low body condition and upregulated autophagy and fat degradation pathways in the liver. Fish from the upper estuary living under high temperatures and nutrient load as well as low dissolved oxygen showed adaptive and innate immune responses correlated with abundances of specific opportunistic bacterial taxa. A large number of stress and immune response mechanisms had not been described for the species before. Overall, the study proved the concept of how individual molecular data can be recorded in the field, and used for estuarine fish health monitoring (**Fig. 7.1**).

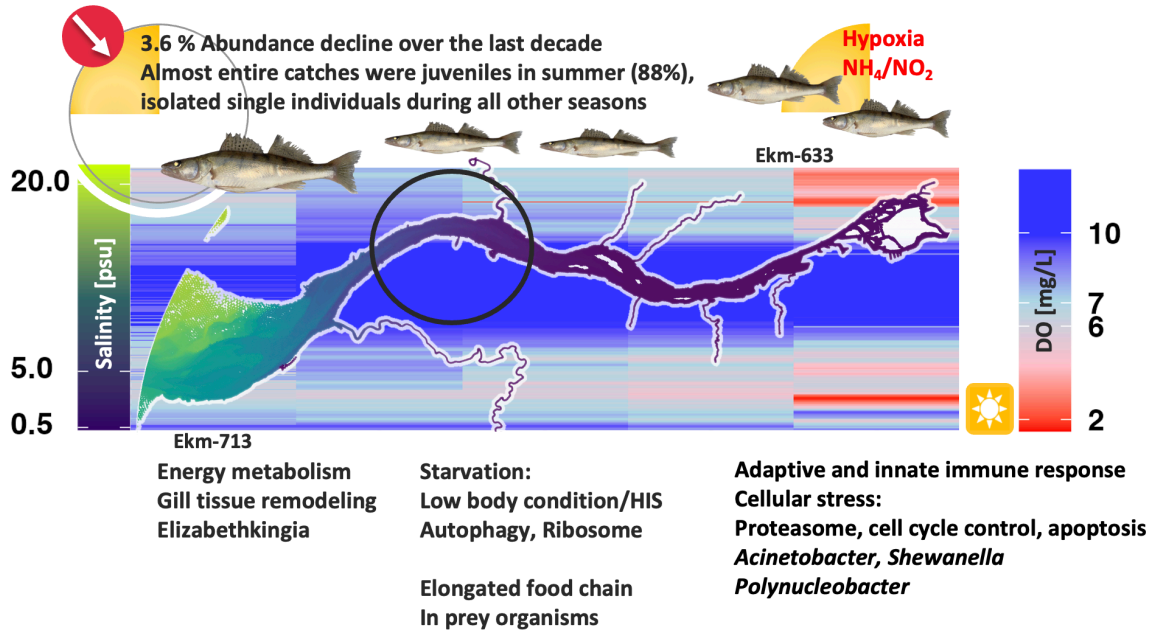


Fig. 7.1 Key findings in integrated molecular, physiological and abundance study on *Sander lucioperca* (SL): Only summer animals could be studied in detail due to disappearance during other sampling campaigns (88% caught in summer, 1% in autumn, 11% in spring and 0.07% in winter).

1.1.2. Tracking life history of estuarine fishes

Via stable isotope analyses we tracked the habitat exploitation and annual migration patterns of potamodromous ruffe (GC) and facultative catadromous flounder (PF). GC showed overall high site fidelity, with low signs of migration between locations along the estuary reflected by a small isotopic niche. The data suggested that the individuals caught also did not leave the study area over the course of the year, promoting a hypothesis of a locally adapted brackish population granting genetic confirmation. PF on the other side reached the estuary as juveniles in summer to autumn and migrated upstream especially in winter, showing a significantly larger isotopic niche. Deviating isotope patterns combined with delayed arrival times of juvenile flounder indicated the presence of two flounder populations exploiting the estuary as a nursery area in accordance with the literature.

1.1.3. Spatio-temporal plasticity of gill microbiota in estuarine fish

The interaction between host species and their associated microbiota is of tremendous importance for the host species health. Fish gills are multifunctional tissues involved in gas exchange, osmoregulation and waste secretion but in permanent, immediate exchange with environmental conditions. Patterns in bacterial compositions in the gill mucus could therefore provide insights into factors immediately affecting wild fish populations that could be gained minimal invasively, fast and at cheap costs. We determined a similar and highly abundant core microbiota composition of around 30 genera in GC and OE gill mucus indicating a small number of generalist species capable of coping with the estuarine conditions. The combination with stable isotope data allowed us to determine patterns of gradual adaptation in the microbiota composition in OE migrating from the sea upstream for reproduction. In both species prolonged hypoxia and high loads of nutrients in the system were found associated with signs of dysbiosis, a sudden decline in core taxa abundance going along with a takeover by opportunistic taxa (*Acinetobacter, Shewanella, Aeromonas*).

1.1.4. Advancing molecular estuarine wild fish monitoring

In this large-scale approach, spatio-temporal holobiont dynamics in the dominant estuarine fish (OE, GC) were explored over 340 tissue specific transcriptome datasets integrated with bacterial, stable isotope, fish abundance, physiological and abiotic data. The molecular responses towards dominant abiotic factors in the Elbe estuary, salinity, temperature and hypoxia, were explored and in parts co-working or interactive effects could be disentangled. We found compensatory strategies applied by both estuarine species towards prevailing conditions. Models exploring the impact of abiotic conditions or bacterial abundances on cellular stress and adaptive immune responses in the host indicated strong relations: The abundances of opportunistic taxa and hypoxia had strong impact on cellular stress and damage indicators in gill and liver. In OE even signs of immunosuppression appeared with oxygen levels falling below 7 mg/L. Making use of the qualitative nature of transcriptome sequencing data, we further explored the population structure in both species in the Elbe estuary for the first time, showing high connectivity between the caught fish in each species respectively, but signatures of selection and genetic variability in key genes responsible for hypoxia and immune tolerance indicating driving forces on the Elbe fish stocks.

1.1.5. Environmental factors shaping Elbe estuarine fish fauna across four decades

Analyzing a 40-year time series of periodic catch data combined with environmental factors from four sampling periods in the Elbe estuary indicated major changes in species composition and life cycle guilds. With the improvement of water quality in the 1990s fish densities increased. Climate related changes paired with direct anthropogenic interventions however increased suspended particulate matter (SPM) within the last decade, which coupled with hypoxic situations and reduced river runoff, was related to a 91% decline in overall fish density. The effect was pronounced in the

decline of diadromous and freshwater key species (OE, GC, SL, PF, *Abramis brama* and *Alosa fallax*). Increased saline water inflow, however, was related to an increase in marine-estuarine-opportunists (*Clupea harengus*, *Merlangius merlangus*).

1.2. Estuarine sections and abiotic gradients

1.2.1. Salinity Gradient: Subtle and Species-Specific Molecular Responses

The estuarine mouth is the entrance for marine species to exploit the estuarine habitat (Elliott et al., 2007), with entrance width and tidal range going along with higher biodiversity (Teichert et al., 2017). In many European estuaries, marine fish dominate in number and abundance (Eick, 2015; Teichert et al., 2017; Thiel & Potter, 2001). In the Elbe estuary they account for the highest biodiversity, but only a small proportion of the overall fish abundance (Eick & Thiel, 2014). Our long-term study however showed a recent increase in the abundance of marine species (**Chapter 6**) driven by increased inflow of saltwater due to channelization and reduced river runoff (Ralston & Geyer, 2019; Rewrie et al., 2023; Scholle & Schuchardt, 2020). The occurrence and abundance of the marine species fluctuates spatially and seasonally, with whiting juveniles, for example, reaching locations up to Ekm 693 in summer and spring, herring even up to 651. On the contrary the system experienced declines in anadromous species (OE, *Alosa fallax*), relying on freshwater areas for reproduction, and freshwater fish species (GC, SL).

The success of all species exploiting the estuary is largely dependent on the respective ability to osmoregulate (Thiel & Potter, 2001). Environmental salinity influences a wide range of physiological responses in fish (Evans & Kültz, 2020) and acclimatization to fluctuating salinity levels can be among the most energy consuming processes (Bœuf & Payan, 2001; Hasenbein et al., 2013). Responses differ greatly between direction of the osmotic pressure, acute or chronic exposure of fluctuations. Large amounts of energy metabolites can be released acutely to fuel ion exchange in salinity-adapted fish (Sangiao-Alvarellos et al., 2003; Tseng & Hwang, 2008), prolonged exposure however leads to remodelling of osmoregulatory epithelia within a few days in euryhaline fish (Foskett et al., 1981). From analysis on disease signs in fish from Elbe estuary it was concluded that large tidal fluctuation of salinity were the major stressors triggering disease in fish (Möller, 1984) as most diseases (virally caused lymphocystis, fin rot, skin ulceration, pharyngeal granuloma) in OE, but also bleaching syndrome in PF and cauliflower disease in *Anguilla anguilla* were found in estuarine mouth up till Ekm 693.

On the molecular level, we therefore expected high responses directed towards energy metabolism, tissue remodelling and immune system activation with changing levels of environmental salinity and salinity fluctuations in the estuarine fish, and pronounced differences between freshwater (SL, GC) and anadromous (OE) species.

The salinity gradient within the study area ranged from 0.5 – 15 psu, while local fluctuations within the upper section were neglectable (min. 0.28 – max. 0,48 psu ± 0.05 psu between seasons and ± 0.026 between tides at Ekm 633), they were higher in the lower sections (min. 9.0 – 15.2 ± 2.6 psu between seasons and ± 0.5 psu between tides at Ekm 713).

Global distance matrix correlation of tissue specific gene expression however indicated low or absent global liver and gill responses in all species independent of life cycle guild. Also, spatial 1:1 comparisons of SL gill and liver between estuarine mouth (15 psu) and freshwater (0.4 psu) showed no pathway enrichment at a gene expression log fold change threshold of 1 (genes with double expression strength).

The bacterial composition in all fish species (SL, GC, OE) on the contrary showed spatial patterns indicatory for the sampling location. Especially the larger datasets of GC and OE allowed for

the identification of bacterial taxa indicatory for high salinity residence of the host (*Persicirhabdus*, *Ilumatobacter, Halioglobus*). In combination with the stable isotope patterns in GC and OE (**Chapter 3**), the conclusion leads to overall low short-term migration along the salinity gradients. An exception was the spawning migration of OE in fall and winter, where bacterial composition and stable isotopes showed clear divergent patterns. Therefore, all fish species might mostly be affected by the comparably lower tidal salinity fluctuations on a daily basis instead of the strong spatial gradient. These bi-directional osmotic fluctuations do not appear to require as high fold-changes in gene expression in the highly adapted estuarine fishes.

The more fine-tuned network approach, however, indicated global shifts in gene expression indicating several tissue specific patterns and contrasting acclimatizational processes in the different life-history guilds.

Upregulation of MAPK and PI3K-Akr pathways, cell-cell/ECM interactions in SL (**Chapter 2**) and autophagy, long-chain ABC transporters, Wnt pathway regulation in GC gill tissues (**Chapter 5**) are all processes that have been described as essential for tissue remodelling in fish exposed to salinity fluctuations (Evans & Kültz, 2020; Jeffries et al., 2019; Lam et al., 2014; Zhou et al., 2022). The anadromous species on contrary showed no gill specific regulations towards the whole salinity gradient indicating a high capacity for osmoregulation, suggesting that capacity for ion transport with and against the osmotic gradient is likely kept in reserve (Evans & Kültz, 2020).

OE as an anadromous species and also SL survive high salinity levels and fluctuations, the latter even full marine conditions for up to several hours (Brown et al., 2001), while the limit for GC might be lower at around 12 psu (Lind, 1977).

In overlap between OE and SL, both indicated a slight increase in energy metabolism, lipid metabolism in SL and lipid & amino-acid metabolism and autophagy in OE in liver tissues. These are usual responses of euryhaline fish coping with fluctuating salinity levels necessitating energy investment (Aragão et al., 2010; Tseng & Hwang, 2008; Xu et al., 2015a, 2015b). Stable isotope and stomach analyses of OE indicated the richest food supply in the estuarine mouth (Hauten, 2024). Also, large body size, body condition and hepatosomatic indices in SL indicated that the metabolic demands related to salinity could be compensated by the respective life-stages (adult OE, juvenile SL). On molecular level, there was no association between immune system processes and salinity levels in both fishes.

In contrast, GC liver showed a comparably strong (r 0.4) correlation of essential components in the innate immune response (interleukin signalling, NOD-like receptors, complement system) with salinity levels. Similar unspecific responses are often recorded in euryhaline fish under salinity stress (El-Leithy et al., 2019; Xu et al., 2015a; Zhang et al., 2022) (**Chapter 5**). While there was no major increase in known opportunistic gill bacteria detected, there was a salinity related change in overall microbiota composition (**Chapter 4**). We cannot rule out parasitic or other pathogenic infections favouring the liver specific innate immune activation. This response is however presumably energetically costly and may be associated with resource allocation trade-offs that impair mounting adaptive immune responses when necessary, shown to render exposed fish vulnerable to infection (Birrer et al., 2012).

Over the last two decades, a trend towards an increase in salinity has already been detected in the estuarine area (Scholle & Schuchardt, 2020), and with progressing climate change saltwater intrusions are expected to increase especially due to lowered river discharge in the summer month (Lee et al., 2024; Pein et al., 2023).

These processes presumably lower the distribution ranges of GC in the estuary. They also change the holobiont response and might introduce new parasites (Lee et al., 2022) and could already

be related to the major population decline of 95.3% in this species observed over the last decade (**Chapter 6**).

1.2.2. Turbidity in the MTZ: Influencing Foraging Success in Juveniles

The maximum turbidity zone (MTZ) defines the interface of marine and freshwater where organic matter and sediments accumulate and turbidity increases by flocculation of suspended matter (Fockedey & Mees, 1999). This is also the area with the highest salinity fluctuation (discussed above) (Taupp & Wetzel, 2014) and usually also location of highest nitrite turnover (Telesh et al., 2013), which, however, has shifted in the Elbe estuary due to the deepening of the harbour (Sanders et al., 2018).

High turbidity decreases photosynthetic primary production due to light limitation (Kundu et al., 2021). In the Elbe estuary mixotrophic phytoplankton species increase (Martens et al., 2024) and zooplankton species switch in feeding preferences in the MTZ. This led to an elongated food chain displayed by enriched δ15N values in consumers, which were also measured in juvenile OE but not in adults (Hauten, 2024). Food intake was recently shown to be affected by turbidity levels in juvenile smelt (Illing et al., 2024). Adults instead might avoid areas with low food supply. Juvenile SL as visual predators however seem strongly affected by the local conditions, individuals caught here showed signs of starvation on molecular (autophagy, mitophagy, ribosomal pathways, fat degradation) and individual physiological levels (lowered HSI, FCF) (**Chapter 2**). For GC and adult OE, we did not determine an obvious impact on growth parameters and no signs of starvation on molecular level in this region. However, the sample sizes used during this study (**Chapter 5**) did also not allow for the identification of variation on physiological end point parameters like body condition. GC are also described effective predators under dim light conditions due to modified eyes, extensive canal system in the head region and well-developed lateral system (Disler & Smirnov, 1977; Hölker & Thiel, 1998), which might explain lower impact on their foraging success.

Turbidity levels and in particular SPM, beneath affecting foraging success (Liljendahl-Nurminen et al., 2008), might damage the exposed gill structures (Costa et al., 2007; Costello et al., 2007; Hess et al., 2015; Maitland, 2000) and causes shifts in microbial mucus communities (Hess et al., 2015). Human interventions in the hydro-system (waterway maintenance, dredging, shipping traffic) increased turbidity levels (Illing et al., 2024; Kerner, 2007; Scholle & Schuchardt, 2020; Weilbeer et al., 2021) matching with our surveys showing three-fold increases in SPM from 19 – 31 mg/L in 1980s to 71 – 92 mg/L in 2021 (**Chapter 6**). These effects underlie, however, strong spatial and temporal variability and fluctuations affecting the whole study area. Therefore, larger, time-series data in molecular and bacterial analyses would be required to sufficiently capture the organismal and holobiont response.

1.2.3. Hypoxia and eutrophication in the freshwater area: A Cross-Species Stressor Hotspot

The upper estuarine area between Ekm 632 and 635 is dominated by freshwater species and also the nursery area for different anadromous species (Kafemann, 1992; Thiel, 2011). It hosts the highest annual fish production in the Elbe estuary (Thiel & Potter, 2001) and was also the region where the largest numbers of the three study species were caught (**Chapter 6**). Low flow velocity and temporarily flooded tidal flats retain planktonic organisms longer than the main channel (Steidle & Vennell, 2024) which is associated with highest densities of zooplankton species (Kafemann et al., 1996; Thiel, 2001). Despite its enormous ecological value, 37% of these highly productive shallow areas have been destroyed by construction measures within the last two decades (Scholle & Schuchardt, 2020). The 40-year time series analyses of fish populations showed particular declines in species using these regions for nursery (**Chapter 6**) but also in freshwater species that have highest abundances here (SL, GC).

The major stressor in this area of the estuary was the reappearing, prolonged situations of severe hypoxia. Deepening of the harbour and main channels led to intensified stratification of the water column in summer month (Pein et al., 2021). Different from other estuaries where the maximum turbidity zone at the marine transition constitutes the hotspot for nitrogen turnover (Telesh et al., 2013), the deepened port region in Hamburg leads to algae die-off in the freshwaters zone favouring heterotrophic conditions and nitrification (Amann et al., 2012; Sanders et al., 2018; Schöl et al., 2015). Measurements of ammonium and nitrite show seasonal fluctuations and accumulation in summer and spring in the harbour area (Ingeniero et al., 2024) where they can reach values > 0.3 mg/L, respectively, over periods of several weeks (Sanders et al., 2018). Nitrification alone has been estimated to contribute up to 30% to oxygen depletion (Schöl et al., 2015), coinciding with critical oxygen concentrations < 4 mg/L for weeks to months every year.

Hypoxia has substantial effects on fish physiology, the immune system and behaviour. Fish like OE display avoidance reactions towards areas with lowered oxygen levels (Möller & Scholz, 1991) with DO levels below 7.5 mg/L and temperatures higher than 15°C shown to act as migration barriers for fish movement in estuaries (Pomfret et al., 1991). At the physiological level, the efficiency of oxygen transport is impaired, leading to disruptions in energy production, the development of oxidative stress resulting in cell damage, energy redistribution and impaired immune system function, as well as impaired survival, which can have a cascading effect on populations (Abdel-Tawwab et al., 2019). In essence, exposure to environmental nitrite has similar effects, changing haemoglobin to non-oxygen-carrying methemoglobin (Bodansky, 1951) causing anoxia in fish (Lewis & Morris, 1986) leading to the above-described responses. Concentrations of NO_2 in fish blood plasma have been shown amplifying compared to surrounding water (Fontenot et al., 1999), and concentrations as low as 0.3 mg/L NO_2 as a single stressor in laboratory experiments already promoted protein catabolism and inflicted histological damage in percide fish over weeks of exposure (Zhang et al., 2021).

In direct response to lowered oxygen levels, fish increase their capacity for oxygen transport, for example by increasing red blood cell formation (Jensen et al., 1993). Consistent with previous studies on gills of GC exposed to hypoxia (Tiedke et al., 2015), we determined genes directly linked to erythropoiesis (haemoglobin subunit beta-2-lile *hbb2* in SL) and a large variety of haemoglobin subunits (HBA1-like, *hbaa1, hbb2, hbb1, hbad* in GC & OE gill and liver tissues) regulated. While this response is among the most aligned ones with oxygen saturation in GC and SL, it is especially correlated with salinity levels and high temperatures in summer in OE. Composition in haemoglobin chains in fish is more variable than in mammals resulting from selection pressures from changing oxygen availability compared between water and air (Souza & Bonilla-Rodriguez, 2007). The results indicate similar cross-species and cross-tissue acclimatory processes in response to changed oxygen availability. The co-correlation with salinity levels in OE could result in different requirements for oxygen transport in fish from different life history guilds as environmental salinity requires modification in gill structures (Evans & Kültz, 2020).

In all three species under study, we found strong signs for cellular stress response activation in all stages (**I.** prevention and repair of damage, **II.** control of the cell cycle, **III.** reallocation of energy and finally **IV.** cell death) most enriched in association with the prevailing situations during summer (low DO, high temperature and NO_2/NH_4) in the upper reaches. Categories **II.** and **IV.** are especially related to tissue damages, which were pronounced in all fish species in the Ekm 633 region in summer. SL showed upregulating in cell cycle control and arrest (*PPP5C*) and DNA repair pathways (**Chapter 2**). In GC heat shock response (*HSBP1, HSPE1, CDC37*), detoxification (*GSTZ1, GASTA, SUMO2a, SUMO3a*), apoptosis (*PYCHARD, BCL2L10, CASP3, CASP8*) were most pronounced. In OE chaperones (*FKBP1A*), ROS protection (*PRDX2, COX8B*), DNA repair (*XRCC6, OGG1*), apoptosis and cell cycle control (*BTG1, CCNG2, MCL1, MNT, DIABLO*) as well as HIF-1a responses (**Chapter 5**) were regulated.

Especially in the freshwater species, we found a strong involvement and modifications in the proteasomal degradation of proteins as part of the temperature/hypoxia and immune system

activation that were less pronounced in the anadromous OE. Organisms owe various pathways to prevent cytotoxicity from denatured or damaged proteins resulting from oxidative- or heat-stress gathered around the ubiquitin-proteasomal machinery (Bard et al., 2018; Hwang & Qi, 2018). An adaptive modification of this system under oxidative stress and inflammatory conditions involves the integration of differentiating beta-type subunits (PSMB8, PSMB9, PSMB10) with altered cleavage specificity into the 20S core. This process speed up and in parts enables ubiquitin-independent degradation of damaged proteins and peptides preventing aggregation-associated cell death under oxidative stress conditions (Pickering et al., 2010) and the creation of major histocompatibility complex I antigen-peptides for presentation to T cells boosting immune responses (Rivett & Hearn, 2004; Shin et al., 2006). So far, there is relatively little research focusing in depth on proteasomal gene expression in fish. The homologs of the human PSMB8, 9 & 10 encode subunits of the immunoproteasome in fish. PSMB proteins are highly expressed during anti-bacterial and anti-viral responses and used as discriminative markers in infection monitoring in sea bream and salmon (Kasthuri et al., 2014; Miller et al., 2017). Individual versions e.g. PSMB8 were found important regulators of NFkB responses during viral infection in fish (krishnan et al., 2021). Further, less studied variants without human homologs (*psmb13a, psmb12* and *psma6l*) were suggested to function in tissue regeneration in fish (Hou et al., 2020; McConnell et al., 2016). In both freshwater species (GC, SL) various B-type subunits were cross-tissue hub genes (*psmb13a, psmb8a, psmb10, psmb12, psmb9, psmb9a, psma6*) taking part in temperature and hypoxia stress response, and further highly co-regulated in immune (anti-viral/anti-bacterial) responses. The high intra-modular correlation within the respective sub-networks in both species indicated functional importance (Langfelder et al., 2013) of these special subunits, which was not the case for the anadromous OE and might be related to its lower coping capacity under prevailing multi-stress conditions (**next chapter**).

The specific conditions during summer in the upper estuarine area were the most impactful for holobiont interaction in all three fish species. The composition of host-associated bacterial taxa can generally be seen as a biomarker for environmental pressures and health situation in the host (Sehnal et al., 2021). Especially, the analysis of microbial frequencies over time and space allows for the determination of a stable core community which is proposed to be important for the health in the host (Zaneveld et al., 2017). In all three species we determined an about 30 genera comprising highly abundant (30 – 60% overall bacterial abundance) estuarine gill-mucus core composition dominated by *Elizabethkingia* and Enterobacteriaceae taxa (**Chapter 2 & 5**). The small number of bacterial taxa and similarity between fish species of different life-history guilds and trophic levels was indicative for either strong host selection or harsh estuarine physiochemical conditions shaping the composition (Luan et al., 2023; Sharpton et al., 2021). Specifically important was that fluctuations in abundances served as indicator for strong reorganization and dysbiosis in all fish species under prolonged hypoxia. The abundance of core taxa diminished to 18 - 28% in all species accompanied by increase in opportunistic bacterial taxa with high disease potential in fish (*Acinetobacter, Aeromonas, Shewanella*) that were highly correlated with cross-tissue immune system activation.

The integration of different omics methods shows a strong mutual validation by molecular stress and immune responses going along with the presence of opportunistic bacteria in all fish species. The variations in the bacterial composition of the gill mucus enabled a precise, rapid and cost-effective indication of fish movement patterns and increased host stress responses applicable for continuous monitoring programs, when selectively underpinned with host gene expression data.

1.3. Spatio-temporal responses in individual fish species

1.3.1. Smelt

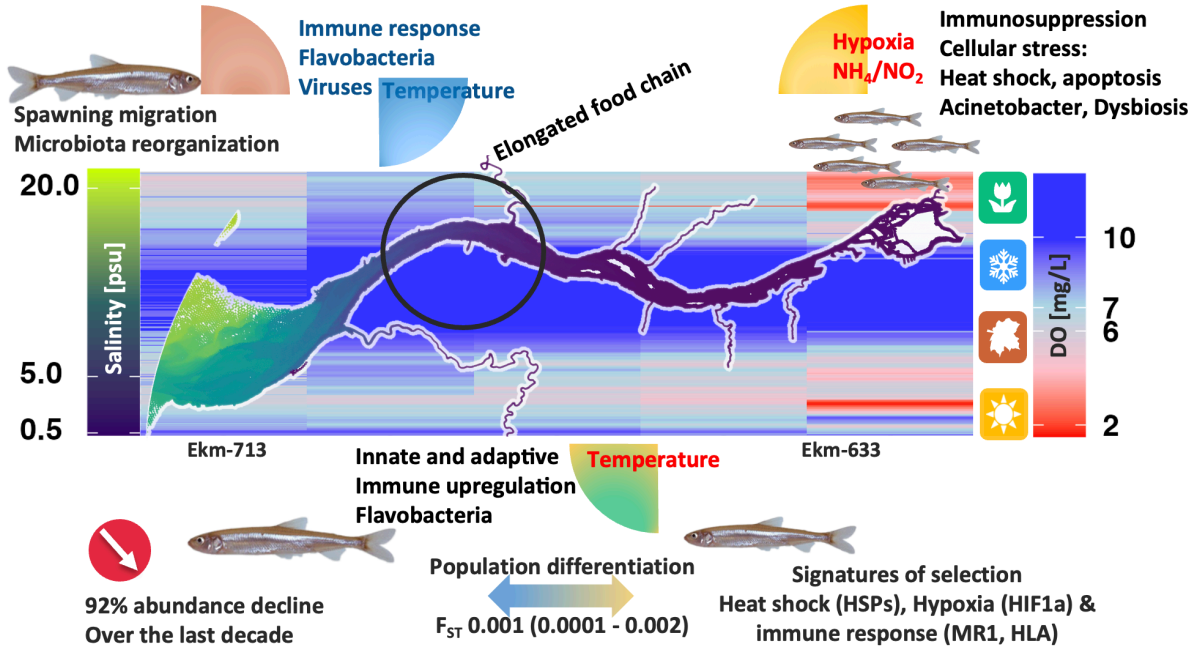


Fig. 7.2. Key findings in integrated molecular, physiological, stable isotope and abundance study on *Osmerus eperlanus* (OE).

Anadromous species are the most successful life history guild (98% abundance) in the Elbe estuary according to previous studies, with OE accounting for 96% of fish abundance in the system alone (Eick & Thiel, 2014). However, during our sampling campaign, the overall fish density has dropped by 91%, especially driven by the decline in anadromous OE and *Allosa fallax* (92%) (**Chapter 6**). These effects have been mostly related to the facets of habitat degradation explained above: reappearing hypoxia, increased suspended particular matter, reduction in shallow areas in freshwater (Illing et al., 2024; Scholle & Schuchardt, 2020).

Main reasons for the success of OE in the habitat were that the spawning takes place upstream the main channel and harbour and that eggs can temporary attach to substrate (Thiel & Potter, 2001). They were therefor less heavily affected by dredging works (Sepúlveda et al., 1993) as SPM directly affects fish eggs and larvae (Yang et al., 2019). Following, the larvae drift with tides to the nursery areas downstream of the harbour (Thiel & Potter, 2001) where they rely primarily on local food supply and remain in one place for an extended period of time which increases vulnerability with regard to environmental conditions.

While adult smelt are described to avoid areas with DO levels below 5 mg/L (Möller & Scholz, 1991), juveniles, larvae and embryos have restricted movement capacities. From DO levels falling below 4.5 mg/L growth depression is recorded in smelt larvae (Sepulveda, 1994), and with values < 3 mg/L hardly any smelt (neither adults nor juveniles) have been caught anymore in the wild habitat (Thiel et al., 1995).

Our molecular analyses have shown that in adult OE with decreasing oxygen levels, immunosuppression occurs in conjunction with increased cellular stress responses. In parallel bacterial taxa with high opportunistic or pathogenic potential were associated with dysbiosis and a takeover of the microbiota composition (**Chapter 4 & 5**, most important findings in **Fig.7.2**). In particular, immunosuppression becomes evident across tissues from oxygen levels below 7 mg/L,

above the 5 mg/L at which avoidance reactions are described for OE but within the range that can represent migration barriers in estuarine fish (Pomfret et al., 1991).

The effect of hypoxia is exceeding the impact of other stressors on aquatic organisms related to climate change (Rosenfeld & Lee, 2022; Sampaio et al., 2021). Definitions for what is seen as hypoxia however vary between a drop to 50% of the ecosystem mean (Breitburg, 2002; Hughes et al., 2015), 50% of maximum saturation (Breitburg, 2002) or DO values between 2 - 2.5 mg/L (Gray et al., 2002). Recent meta-analyses of freshwater and marine fish species found a critical non-lethal impact on growth and nutrient uptake between 4.5 - 6 mg/L DO (Hrycik et al., 2017; Rosenfeld & Lee, 2022). Matching with recent observations of hypoxia related immunosuppression from the lab and aquaculture studies (Abdel-Tawwab et al., 2019; Leeuwis et al., 2024), we add further evidence for the recognition of higher thresholds as impactful for fish and the holobiont interaction in the wild that might guide conservation efforts.

The effects on immune pathways were likely the result of an energy reallocation trade-off directed towards essential cellular stress response stages (increased heat shock response, cell cycle control, apoptosis). While this indicated a compensatory strategy in stress coping used by the species (Sokolova et al., 2012), it might have serious effects on the organism. Unknown are long-term effects with intensifying pressure under climate change scenarios and the interactive effects of stressors: Metabolic rates increased by temperature are accompanied by increased foraging for compensatory purposes during single stressor exposure in fish (Bartolini et al., 2015; Johansen & Jones, 2011; Nowicki et al., 2012). On the contrary, immune challenge generally reduces activity saving energy for the immune response (Bonneaud et al., 2003; Johnson, 2002; Kirsten et al., 2018; Volkoff & Peter, 2004). Multi-stress experiments have shown such antagonistic effects with potential for detrimental consequences for populations (Petitjean et al., 2020). Likewise, avoidance reactions could be impaired by bacterial infections favoured by hypoxia driven immunosuppression.

At the molecular level the tissue specific transcriptome data from gills and livers did not align with described increased visual illness observed during the spawning season and in association with the salinity gradients (Möller, 1984). High prevalence in disease signs in OE (12.7%) increasing with age, most (spawning papillomatosis, skin ulceration, fin rot), especially during the spawning season (February to May) were reported. Further, most diseases (virally caused lymphocystis, fin rot, skin ulceration, pharyngeal granuloma) in OE, were found in estuarine mouth up till Ekm 693. It was concluded that not pollution or hypoxia but large tidal fluctuations of salinity were major stressors triggering visible diseases in OE (Möller, 1984). Comparison with our long-term study (**Chapter 6**) showed however, that OE had lowest abundance during these times (12,000 Individuals/m^3 water 1980s compared to 170,000 in 2010 and 13,000 in 2020). Water quality within the estuary was poor in 1980s due to waste water sewage (Möller & Scholz, 1991) (high nitrogen compounds, heavy metal loads, lowest oxygen levels in upper estuary during whole observational timeframe) killing entire populations of OE (Möller & Scholz, 1991; Thiel et al., 1995). Eutrophication has improved over the last 40 years, but re-emerging hypoxia is still a problem, and turbidity levels tripled by the 2020s. Because we did not conduct visual observations of signs of disease and sampling only occurred at four time points that did not cover the entire spawning season, patterns of visual disease and activation of molecular immune responses are difficult to compare in the context of changing environmental conditions. Further, the restriction to externally visible disease signs might have overlooked inner infections.

Our data indicated a clear pattern of temperature driven enhancement in cross-tissue adaptive and innate immune response in spring and in summer until the upper reaches, found elevated and increasing in upstream direction. These were highly correlated with increased abundances of opportunistic bacterial taxa belonging to the Flavobacteriaceae (*Flavobacterium, Chryseobacterium, Elizabethkingia, Ornithobacterium*), the top contributors for disease-associated losses in farmed fish (Loch & Faisal, 2015) (**Chapter 5**).

Further, highly correlated cross-tissue (and cross-species) indications for anti-viral response (upregulation of RIG-1 pathways and marker genes like *igldcp*, *MX1*, type I interferon expression) were increased in animals during all seasons and over all locations indicating a spawning migration independent viral burden.

1.3.2. Ruffe

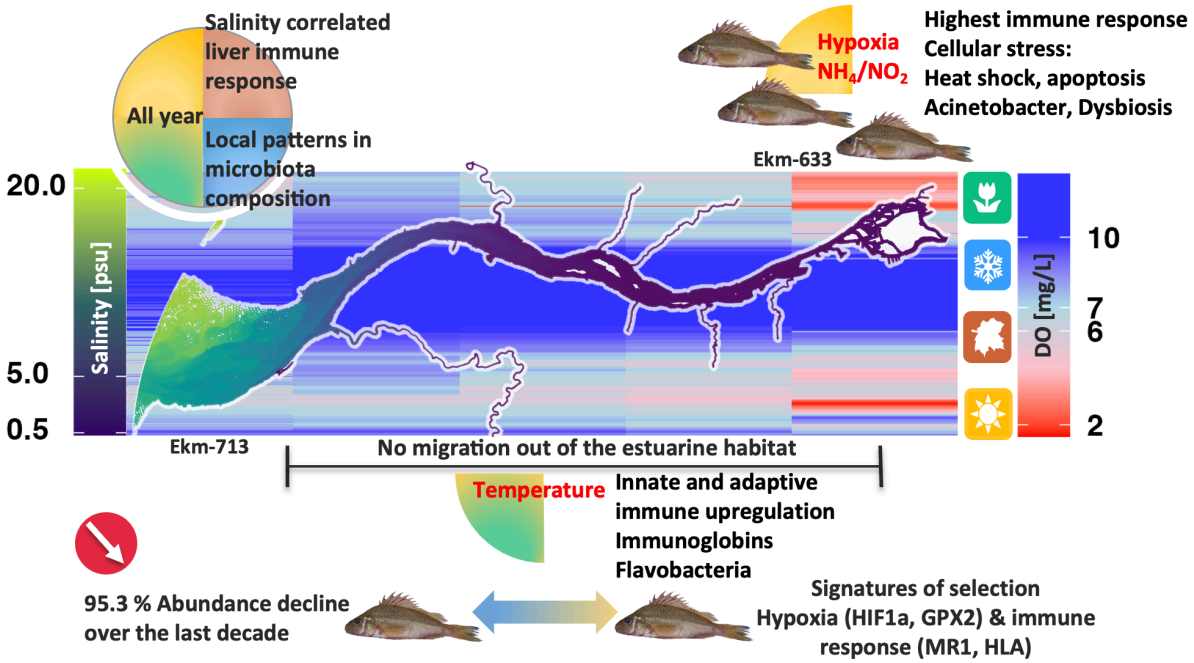


Fig. 7.3 Key findings in integrated molecular, physiological, stable isotope and abundance study on *Gymnocephalus cernua* (GC).

GC, as potamodromous species, complete their entire life cycle in freshwater and undertake migrations for feeding, overwintering and spawning habitats (Gutsch & Hoffman, 2016; Morais & Daverat, 2016). While they are overall described as robust, tolerating a wide range of conditions and are successful invaders in North American lakes (Gutsch & Hoffman, 2016; Svirgsden et al., 2015), we found a 95.3% decline in abundance over the last decade in the Elbe estuarine population (**Chapter 6**).

Tissue specific molecular analyses, in overlap with anadromous OE, indicated temperature as a major driver in GC gene expression (most important findings in **Fig. 7.3**). Almost identical, immune responses were strongly upregulated in spring aligned with abundances of Flavobacteriaceae in the gill microbiota. GC showed a large number of specific patterns such as highly correlated NOD-like receptor genes, specific C-type lectins, and a central organization of different immune components around immunoproteasomes.

Matching with its described robustness, GC was able to maintain most immune responses during prolonged hypoxia and eutrophication in the wild habitat. However, immunoglobins, of major importance for host-microbiota cross-talk in the gill associated lymphoid tissue (Yu et al., 2021) and the major constituents in pathogen defence (Uribe et al., 2011) were lowered in summer compared to spring despite an increase in opportunistic bacteria. The combination of presumably energetically costly salinity triggered innate immune system component upregulation in the lower estuary (**Chapter 7.2.1**), potentially hypoxia compromised adaptive responses (Leeuwis et al., 2024) and energetically costly cellular stress and xenobiotic responses could relate to the recently recorded population declines. Especially, as stable isotope analyses (**Chapter 3**) of Elbe estuarine GC indicated high site

fidelity and resident behaviour throughout the year with highest abundances caught between Ekm 633 till 651 most exposed to hypoxic conditions and xenobiotic loads (Wetzel et al., 2013).

The specimens caught hardly exceeded isotopic limits of sampling sites within the estuary, therefor their migration for spawning between March and May as well as for overwintering in deeper water (Gutsch & Hoffman, 2016) probably takes place within the tidally influenced habitat in side and main channels (Thiel et al., 1995). This led to the hypothesis of a brackish population that might be isolated from pure freshwater GC occurring in adjacent rivers or upstream (**Chapter 2**). Local adaptations in several GC populations have been recorded showing the production of larger eggs and salinity tolerant juveniles (Albert et al., 2006; Svirgsden et al., 2015). To evaluate this hypothesis, we analysed the population structure in GC with an exploratory approach based on single nucleotide polymorphisms (SNPs) (**next chapter**).

1.4. Population structure and signatures of selection

Consistent with studies on other estuarine habitats (McLean et al., 2019; Nicolas et al., 2010; Pasquaud et al., 2015; Teichert et al., 2017), local human disturbances and climate change related impacts affected the Elbe estuarine fish community over the last 40 years study period (**Chapter 6**). Different from other European estuaries, where marine fish species dominate, the Elbe estuary is dominated by anadromous OE (Eick, 2015; Maes et al., 1998; Thiel & Potter, 2001), but has recently shown tendencies towards an alignment of the fish community with that of other macro-tidal systems. In overlap with recent studies on marine fish dominated estuarine systems along the coast of France (McLean et al., 2019), species with r-selected traits/opportunistic-periodic traits like OE and GC declined most in the Elbe estuarine system. These traits are vulnerable to short-term fluctuations in abiotic conditions (Perry et al., 2005; Schweiger et al., 2008; Simpson et al., 2011), but are expected to have high capacity for evolutionary adaptation given short generation times (Rijnsdorp et al., 2009). Making use of the qualitative information gained from holistic mRNA sequencing, we aimed to explore not only respective tissue specific response mechanisms in the dominant fish taxa, but further to gather inside on signatures of adaptational processes and overall population structure by evaluation of SNPs. Intra-specific variation could have a high impact on the stress coping (Petitjean et al., 2021), but has not been explored in the Elbe estuarine fish stocks to the best of my knowledge.

The data showed overall weak indication for population differentiation in OE and GC which was to be expected within such a small, connected habitat. Significant biological differentiation was however shown in fish with high connectivity and weak population structure (Aykanat et al., 2015; Chen et al., 2020; Knutsen et al., 2011; Thorstensen et al., 2020). As no GC caught outside the estuarine system were included in the analysis, the hypothesis of a differentiated estuarine population remains to be investigated.

However, outlier SNPs in important genes for hypoxia response (i.e., HIF1a, and related pathway genes) and immune system (i.e., major histocompatibility complex) indicate potential adaptive divergence despite weak overall population differentiation, consistent with anthropogenically-influenced stressors over the past two decades in this estuarine system. With the current massive decline in fish abundance in the system, a genetic bottleneck is expected to reduce genetic diversity and will be exacerbated by genetic drift the longer it persists (Kardos et al., 2021), which will decrease the capacity for populations to adapt to future climate change (e.g., increasing hypoxia, temperature). The short generation times of these fish present a two-sided coin: on the one hand, they can rebound with rapid conservation action and the genetic effects of a decreased population can be mitigated, but on the other hand, they are likely to lose genetic variation quickly as genetic drift predominates at lower population sizes. Because mutation rates build up genetic variation much more slowly than drift removes variation at low population sizes (Lynch, 2010), future adaptation to climate change will be influenced by population dynamics in the present, and whether the populations can be fostered back to high abundances in the near future.

1.5. Advantages of omics techniques in estuarine monitoring

In face of environmental change, adjusting the abundance of messenger RNA (mRNA) is a primary mechanism underlying physiological responses (Connon et al., 2018; Faught et al., 2016). Sequencing of mRNA followed by mapping to reference genomes or *de novo* transcriptome assembly allows for the determination of expression changes as well as examination of genomic sequence differences (SNPs) relevant for determination of physiological responses and can be used to guide conservation efforts (Connon et al., 2018; Miranda et al., 2023; Oomen & Hutchings, 2017).

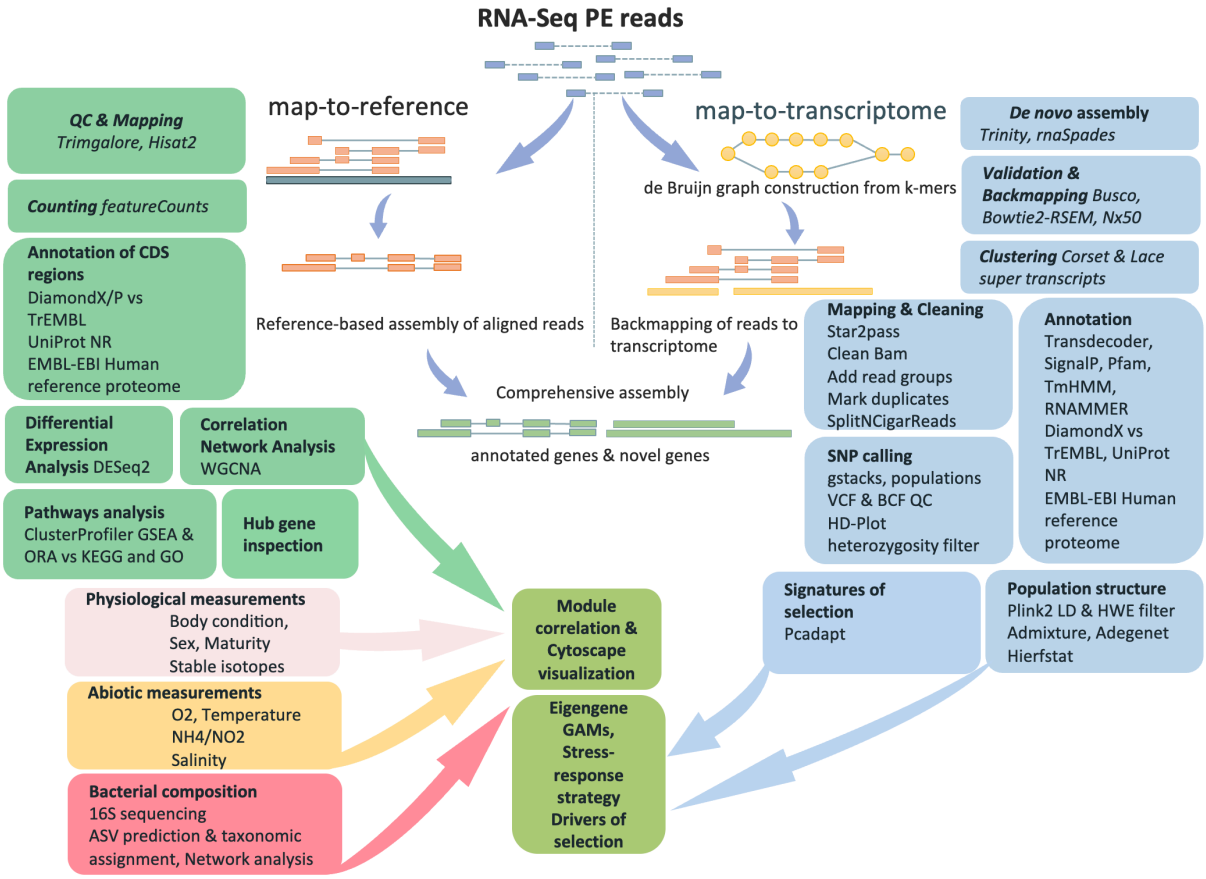


Fig.7.4 Bioinformatic pipeline applied and developed for data procession and integration to evaluate molecular stress response patterns in estuarine fish. All workflows are available as ready-to-run Linux and R-scripts published with the respective publications.

As recently reviewed in (Semeniuk et al., 2022) conventional methods in fish health monitoring involve laborious assessments via necropsies (Adams et al., 1993) or physiological measurements (energy metabolites, stress hormones, immune cell counts (Adams & Ham, 2011) which usually require lethal sampling and tend to fail to test important pathogens (Chapman et al., 2021) or holobiont interactions. Omics methods instead overcome many of these shortcomings (Leese et al., 2016), especially in an integrated approach used in this thesis:

I. Low amounts of tissue or mucus are needed that can be sampled non lethally by swapping and dissection of miniature tissue pieces that in case of gill do not affect the survival of the individual (Jeffries et al., 2021).

II. The higher precision of molecular methods allows for lower sampling sizes compared to often highly fluctuating and imprecise physiological end-point measurements (**Chapter 1,3,5**)

III. Processing times in the field and laboratory decrease, lowering large cost drivers in monitoring procedures. On the contrary, affordability of holistic mRNA or DNA sequencing, rapid turnaround times (Qian et al., 2014) and availability of bioinformatics pipelines allow high-throughput and broad-scale application in monitoring (**Fig.7.4**).

IV. Complex response patterns to known stressors can be disentangled and unknown or unexpected stressors can be identified from gene expression patterns.

V. Qualitative information of genomic sequencies allow to determine population structure, genetic variability, signatures of adaptation and driving forces.
Sequencing of mRNA has been proven useful to provide these insights into fish population structure (Jeffries et al., 2019; Thorstensen et al., 2020) although it comes with several limitations: SNP discovery might be biased towards highly expressed genes and mutations in mRNA are widely under selection (Chamary & Hurst, 2005). Propper linkage pruning would require chromosome level genome assemblies, of which only for OE one was very recently published out of the data-procession range of this study.

VI. Integration of different omics approaches in parallel validates the individual findings (e.g. immune response and abundance of opportunistic bacteria) and enables the determination of movement patterns via bacterial patterns.

VII. Biodiversity and complex biotic interactions can be described e.g. in holobionts which improves the overall understanding of ecosystem functioning and adaptive potential.

VIII. Dominant stressors, stressor hotspots and genetic structure in population inform ecosystem conservation and restoration efforts.

The time passing between capturing of fish and tissue preservation influences gene expression patterns, especially stress-inducible genes (Jeffries et al., 2021). While immediate early genes show fast responses peaking after 30 to 60 minutes of stress exposure (Bahrami & Drabløs, 2016; Cullinan et al., 1995), a steady state in global gene expression under hypoxia stress can be reached after two hours (Bendjilali et al., 2017). Innate immune responses after stimulation show fast responding patterns with maximum regulation after two hours, genotoxic stress genes like p53 show maximum in upregulation after 6 hours, large amounts of genes even after 9 hours and return to baseline after 2 – 5 days (Schlamp et al., 2021).

These effects can either be overcome by using individual biomarker genes that reflect slow responding processes (Jeffrey et al., 2020). Alternatively, highly standardized workflows in capture and immediate processing in samples and reasonable amounts of biological replicates and replicated captures (like distribution of animals from repeated low-tide and high-tide catches, random selection of animals from the catch) can mitigate these effects. Also, the interpretation of the results must be cautious and consider the temporal patterns of expression of the determined genes.

Comparing different ways to integrate molecular data into monitoring, there is a trade of between cost and ease of interpretability given the use of biomarker genes as single measurements or via gene panels comprising 30 – 100 genes (Semeniuk et al., 2022), but a loss of information when compared to the analysis of holistic mRNA that delivers a high-resolution snapshot of gene expression profiles allowing the detection of subtle patterns and potentially unknown insights. This becomes visible in the expression differences at different fold changes in gene expression (**Fig.7.5**). Biomarker genes would not determine a useful pattern as expression differences are subtle, however as the analysis on lower fold changes or via network showed, there are large scale adjustments that allow the fish to cope with strong variable stressor mosaics.

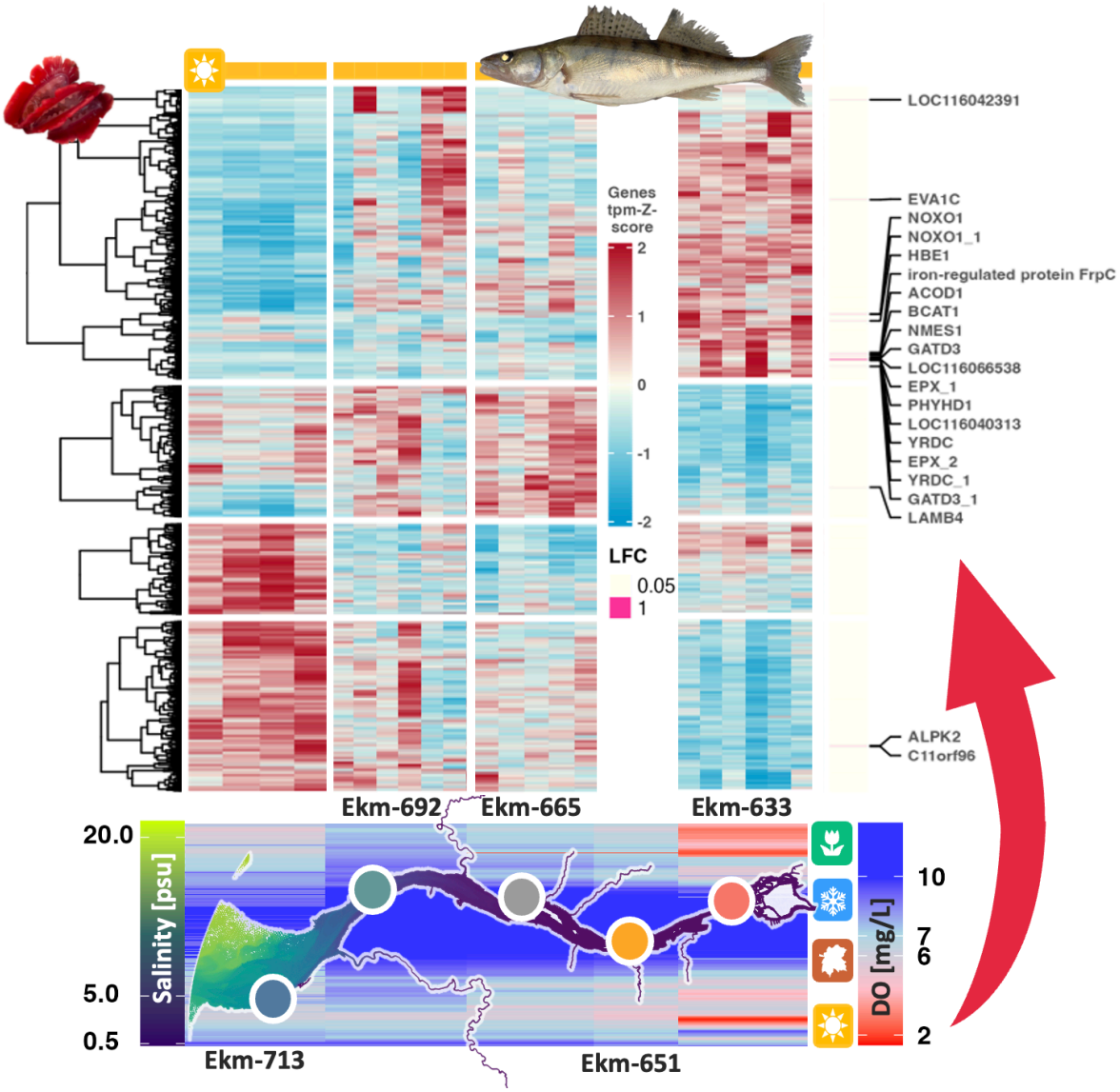


Fig.7.5 Differential gene expression between gill datasets from SL caught in summer 2021 along the course of the estuary. The Heatmap depicts 4519 cumulative DEGs between pairwise sampling groups at log foldchange (LFC) 0.05 significant at FDR corrected qvalue < 0.05, but only 21 genes significant at LFC 1. Results for liver tissues are similar with 2775 DEGs at LFC 0.05 and 21 at LFC 1.

Further integrative systems level approaches, as used in this study, combining holistic transcriptome data with and an approximation of the holobiont interaction via bacterial 16S metabarcoding, allows for cross-validation of individual results.

Network analyses via WGCNA (Langfelder & Horvath, 2008) appeared be particularly useful in all studies. Networks naturally describe interactions, as for example bacterial taxa exist as complex networks, co-occurrence analyses represent an especially suited tool to study interaction in complex bacterial communities (Boutin et al., 2013; Freilich et al., 2010; Röling et al., 2010). They further enable a strong reduction in complexity, integration of different kinds of omics datasets and robust predictions of putative interactions between them (Strand et al., 2021). Different to discriminative analyses they do not rely on pre-defined comparison groups and appear robust against variance potentially arising from different movement patterns in fish.

Summarizing, this Phd-project shows that holistic integrated omics are powerful tools to determine genetic mechanisms promoting acclimatization and holobiont interaction in escalating

conditions in aquatic multi-stress habitats and analyses of genomic differences. They can further provide context for genetic differentiation and adaptive processes and should be integrated into ecosystem monitoring efforts.

1.6. Outlook



Juliane Schulte, 2024

1.6.1. Life-stage vulnerability

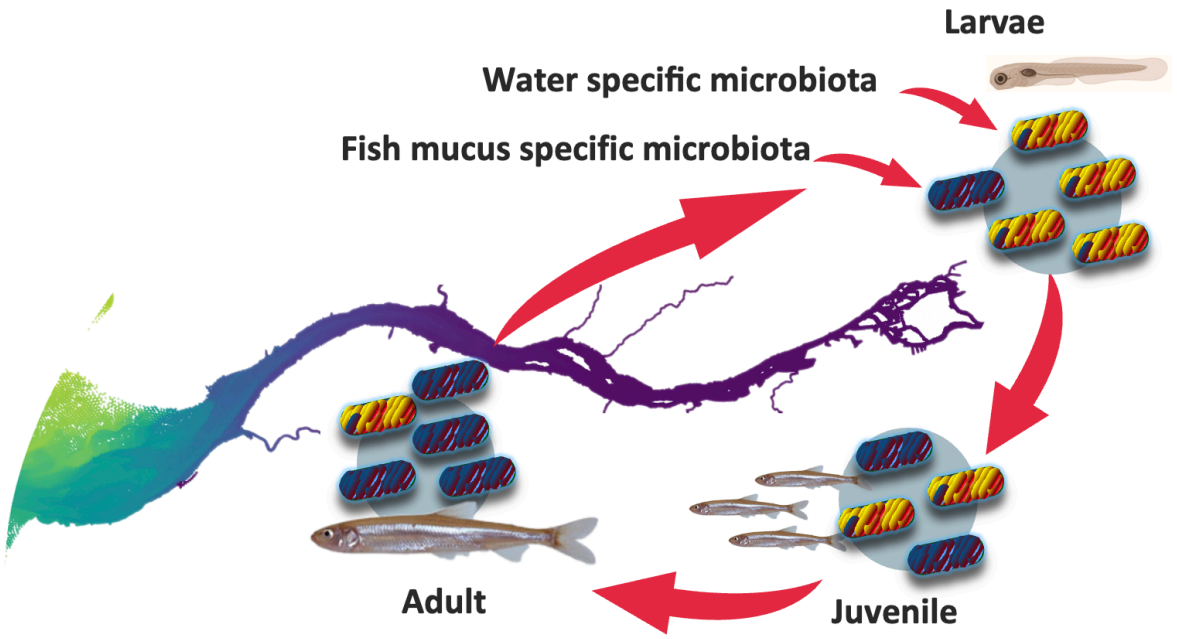


Fig.7.6 Expected effect of the water microbiome on OE mucus microbiota composition over different developmental stages. Adapted from (Morshed & Lee, 2023; Stephens et al., 2016). Nursery areas are located where most opportunistic bacterial strains were identified in correlation with immune and cellular stress responses in all analysed fish species.

In fish, in contrast to terrestrial animals, the sterile egg chorion is first colonized by microbiota from the surrounding water, the initial host-microbe interaction also shapes the immune development in the host (Giatsis et al., 2015). While the microbiota composition diverges from the surrounding water during adulthood (Stephens et al., 2016) (**Fig. 7.6**), early environmental effects can still be observed in adult fish (Minich et al., 2020).

The co-occurrence of opportunistic pathogenic bacteria in relation to the decline of core microbiota abundance identified the eutrophied and hypoxic areas of the estuarine system to cause dysbiosis in the gill bacterial composition going along with increased immune and cellular stress responses in all three fish species analysed in adult (OE, GC) and juvenile (SL, GC) life-stages.

Research on life-stage vulnerability towards temperature increase, based on mortality, tissue pathology and the capacity to employ cellular stress responses, indicated different environmental niches of different life stages with larvae and adults more severely affected than juveniles. Juveniles showing adaptive phenotypic plasticity in coastal and estuarine water coped well with elevated temperature by rapid induction of cytoprotective proteins protecting integrity of vital organs. Larvae lacked acclimation capacity resulting in tissue damage and elevated mortality and adults displayed lower plasticity showing tissue damage in brain, liver and muscle (Madeira et al., 2020). Data from almost 700 marine and freshwater fish species indicated highest vulnerability of embryos and breeding adults towards climate warming constituting potential bottlenecks in the life cycle of fish (Dahlke et al., 2020).

Lacking at the moment are in-depth *in-situ* (within the wild habitat) studies holistically analysing the holobiont interaction across life-stages. Recent studies have shown that stress factors during development affect the external bacterial mucus community. Overlapping with our data, *Acinetobacter* and *Aeromonas*, in particular, play a role and even subtle non-lethal stressors affect the microbiome persistently (Uren Webster et al., 2021).

Considering that estuaries are rearing areas, especially juveniles of species like OE and GC might largely be affected by the high loads of opportunistic bacteria in the upstream areas. Studies on the pathogenicity of bacterial strains and the physiological effects on fish health and possible recovery are lacking at the moment. Lowered movement capacities in juveniles, eutrophication and hypoxia within the nursery areas might however affect the expectedly higher tolerances of juveniles towards stressors like temperature gathered from laboratory and meta-analyses due to intensified biotic interaction with opportunistic taxa in the real-world. Multi-stress experiments (temperature, hypoxia, pathogen loads) using integrative omics tools over different life-stages paralleled with *in-situ* monitoring could elucidate interactive effects of stressors on the holobiont.

1.6.2. Functional trait network

Surveillance programs like the Water Framework Directive in Europe require the establishment of biotic indicators for ecological status of water bodies including fish communities because of their sensitivity to environmental disturbances (Birk et al., 2012; Hering et al., 2010; Reyjol et al., 2014) and roles in ecosystem functioning (Holmlund & Hammer, 1999). Functional approaches describing assemblage composition and functional guilds (ecological, trophic) and functional structure (functional diversity, evenness and redundancy) are described useful to detect the impact of disturbances (anthropogenic, climate change) on fish assemblages (Baptista et al., 2014; Sagouis et al., 2017; Teresa & Casatti, 2017). Based on the work of (Teichert et al., 2018) and (McLean et al., 2019) for French estuarine ecosystems, a functional trait network for the Elbe estuary can be established and integrated with (Eick & Thiel, 2014; Thiel, 2011), the data summarized in **Chapter 6** and upcoming species composition data gathered by the RTG2530.

1.7 References: General Discussion

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Affidavit

Affidavit:

I hereby declare and affirm that this doctoral dissertation is my own work and that I have not used any aids and sources other than those indicated. If electronic resources based on generative artificial intelligence (gAI) were used in the course of writing this dissertation, I confirm that my own work was the main and value-adding contribution and that complete documentation of all resources used is available in accordance with good scientific practice. I am responsible for any erroneous or distorted content, incorrect references, violations of data protection and copyright law or plagiarism that may have been generated by the gAI.

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Hamburg, den 15.02.2025



Chapter/Publications and Contribution of Authors

Presented and discussed within this thesis

Chapter 2: Network-based integration of omics, physiological and environmental data in real-world Elbe estuarine Zander

Authors: **Raphael Timotheus Koll**, Jesse Theilen, Elena Hauten, Jason Nicholas Woodhouse, Ralf Thiel, Christian Möllmann, Andrej Fabrizius

This chapter is a manuscript published in Science of the Total Environment (09/2024). https://doi.org/10.1016/j.scitotenv.2024.173656

Raphael Timotheus Koll (RTK) conceptualized the study together with Andrej Fabrizius (AF). RTK, Elena Hauten (EH) & Jesse Theilen (JT) were involved in the sampling. RTK conducted sample preparation, bioinformatics work (data processing, coding), implementation of data integration approaches, formal analysis, data curation, validation, visualization and writing of the original draft. RTK is the corresponding author. Work by others involved in this study: JT provided permit acquisition. Funding for the study was acquired by AF, Ralf Thiel (RT) & Christian Möllmann (CM). AF, CM & Jason Nicholas Woodhouse (JNW) supervised the study. JNW evaluated the statistical methods used. All co-authors critically reviewed and edited the original draft written by RTK.

Chapter 3: Tracking life cycle strategies of flounder and ruffe in the Elbe estuary

Authors: Elena Hauten, Johanna Biederbick, Steffen Funk, **Raphael T. Koll**, Jesse Theilen, Andrej Fabrizius, Ralf Thiel, Peter Grønkjær and Christian Möllmann

This chapter is a draft currently being prepared for publication in a scientific journal.

Raphael Timotheus Koll (RTK) was involved in sampling the fish in the field, conducted the sample preparation on board and was involved in the critical review and editing of the original draft written by Elena Hauten (EH). The contribution of the authors is as follows: Elena Hauten (EH) and Christian Möllmann (CM) were responsible for the conceptual design of this study. EH, Raphael T. Koll (RTK) and Jesse Theilen (JT) collected fish in the field. Sample preparation in the laboratory was conducted by EH. EH, CM and Steffen Funk (SF) developed the data analysis concept, which was implemented and visualized by EH. Ralf Thiel (RT) and CM generated the funding for this study. Peter Grønkjær (PT) and Johanna Biederbick (JB) improved the writing process with their expertise, CM supervised the study. The original draft was written by EH and edited and reviewed by all coauthors.

Chapter 4*:* Spatio-temporal plasticity of gill microbiota in estuarine fish

Authors: **Raphael T. Koll**, Elena Hauten, Jesse Theilen, Corinna Bang, Michelle Bouchard, Ralf Thiel, Christian Möllmann, Jason Nicholas Woodhouse, Andrej Fabrizius

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Raphael Timotheus Koll (RTK) and Andrej Fabrizius (AF) were responsible for the conceptual planning and administration of the study design. RTK collected the samples during field research, conducted laboratory work and was supported by Masters student Michelle Bouchard (MB). Data curation was implemented by RTK, MB, EH and Corinna Bang (CB). RTK conducted the data analysis under supervision of Jason Nicholas Woodhouse (JNW) and AF. Data processing, formal analysis and visualization was conducted by RTK. The original draft of the manuscript was written by RTK with the supervision of AF. RTK is the corresponding author. The contribution of the authors is as follows: Elena Hauten (EH) and Jesse Theilen (JT) participated in the collection samples during field research. EH provided stable isotope data (δ13C) of ruffe and smelt samples, visualized the isotope data and created parts of the map describing the study area. Funding acquisition was coordinated by AF, Ralf Thiel (RT) and Christian Möllmann (CM), additionally funding for sequencing was acquired by a combined sequencing application at the DFG with the work of AF, RTK and others from the RTG2530. JT provided permit acquisition. All coauthors reviewed and edited the manuscript.

Confirmation of correctness

Hamburg, 15.02.2025 A. Fabrizius

Andrej Fabrizius

Chapter 5: Advancing molecular wild fish monitoring: Spatio-temporal holobiont dynamics in an estuarine system

Authors: **Raphael T. Koll**, Elena Hauten, Jesse Theilen, Corinna Bang, Ralf Thiel, Christian Möllmann, Jason N. Woodhouse, Matt J. Thorstensen, Ken M. Jeffries, Andrej Fabrizius

This chapter is a draft currently being prepared for publication in a scientific journal.

Raphael Timotheus Koll (RTK) conceptualized the study together with Andrej Fabrizius (AF). RTK, Elena Hauten (EH) & Jesse Theilen (JT) were involved in the sampling. Sample preparation, bioinformatics work (data processing, coding), implementation of data integration approaches, formal analysis, data curation, validation, visualization, writing of the original draft was done by RTK. Ken M. Jeffries (KMJ) hosted RTK for a three-month internship at the University of Manitoba, Canada, Matt J. Thorstensen (MJT) & KMJ trained RTK in population genetic analyses, and conceptualized the respective analyses. AF, CM & Jason Nicholas Woodhouse (JNW) supervised the study. JNW evaluated statistical methods used. JT provided permits for the study. Funding for the study was acquired by AF, Ralf Thiel (RT) & Christian Möllmann (CM), additionally funding for sequencing was acquired by a combined sequencing application at the DFG with the work of AF, RTK and others from the RTG2530. All co-authors critically reviewed and edited the original draft written by RTK.

Chapter 6*:* Environmental factors shaping fish fauna structure in a temperate mesotidal estuary: periodic insights from the Elbe estuary across four decades

Authors: Jesse Theilen, Victoria Sarrazin, Elena Hauten, **Raphael T. Koll**, Christian Möllmann, Andrej Fabrizius & Ralf Thiel

This chapter is a draft submitted to Estuarine, Coastal and Shelf Science (08/2024) and passed the first revision.

Raphael Timotheus Koll (RTK) was involved in collecting fish and implementation of data acquisition in the field and involved in the evaluation of statistical methods used in the publication. Jesse Theilen (JT) and Ralf Thiel (RT) designed the objectives and administration of this study. The concept of this study was developed by JT, RT, Andrej Fabrizius (AF) and Christian Möllmann (CM). JT, Elena Hauten (EH) and RTK collected data in the field. Funding acquisition and supervision were implemented by AF, CM and RT. The curation of the long-term data was coordinated by Victoria Sarrazin (VS) and JT. JT analyzed and visualized the data under the advice of RT and VS. All co-authors critically reviewed and edited the original draft written by JT.

Additional participation in projects and manuscripts

Title: Characterizing intraspecific habitat exploitation of anadromous key species Osmerus eperlanus along the salinity gradient of a large European estuary

Authors: Elena Hauten, Johanna Biederbick, Steffen Funk, **Raphael Timotheus Koll**, Jesse Theilen, Andrej Fabrizius, Ralf Thiel, Kai Jensen, Peter Grønkjær, Christian Möllmann

This is a chapter in (Hauten, 2024) and a draft currently being prepared for publication in a scientific journal. Authors contributions are as follows: EH: Conceptualization, data curation, formal analysis, investigation, methodology, project administration, visualization, writing – original draft preparation, JB: Writing – review & editing; SF: Methodology, writing – review & editing, **RTK: Methodology, investigation, writing – review & editing;** JT: Data curation, methodology, investigation, project administration, writing - review & editing; AF: Funding acquisition, writing - review & editing; RT: Funding acquisition, project administration, writing - review & editing; KJ: Funding acquisition, writing - review & editing; PG: conceptualization, methodology, supervision, writing - review & editing; CM: conceptualization, funding acquisition, supervision, writing - review & editing

Title: Seasonal and spatial variations in microbial communities in the Elbe estuary

Authors: Vanessa Russnak, **Raphael Timotheus Koll**, Jason Woodhouse, Sabine Keuter, Kirstin Dähnke

This is a manuscript draft currently being prepared for publication in a scientific journal. Authors contributions are as follows: VR: Conceptualization, data curation, formal analysis, investigation, methodology, project administration, visualization, writing – original draft preparation, **RTK: Was involved in the bioinformatics analysis (raw data processing, evaluation, visualization). All co-authors reviewed and edited the manuscript.**

Confirmation of correctness

Hamburg, 15.02.2025 A. Fabrizius

Andrej Fabrizius