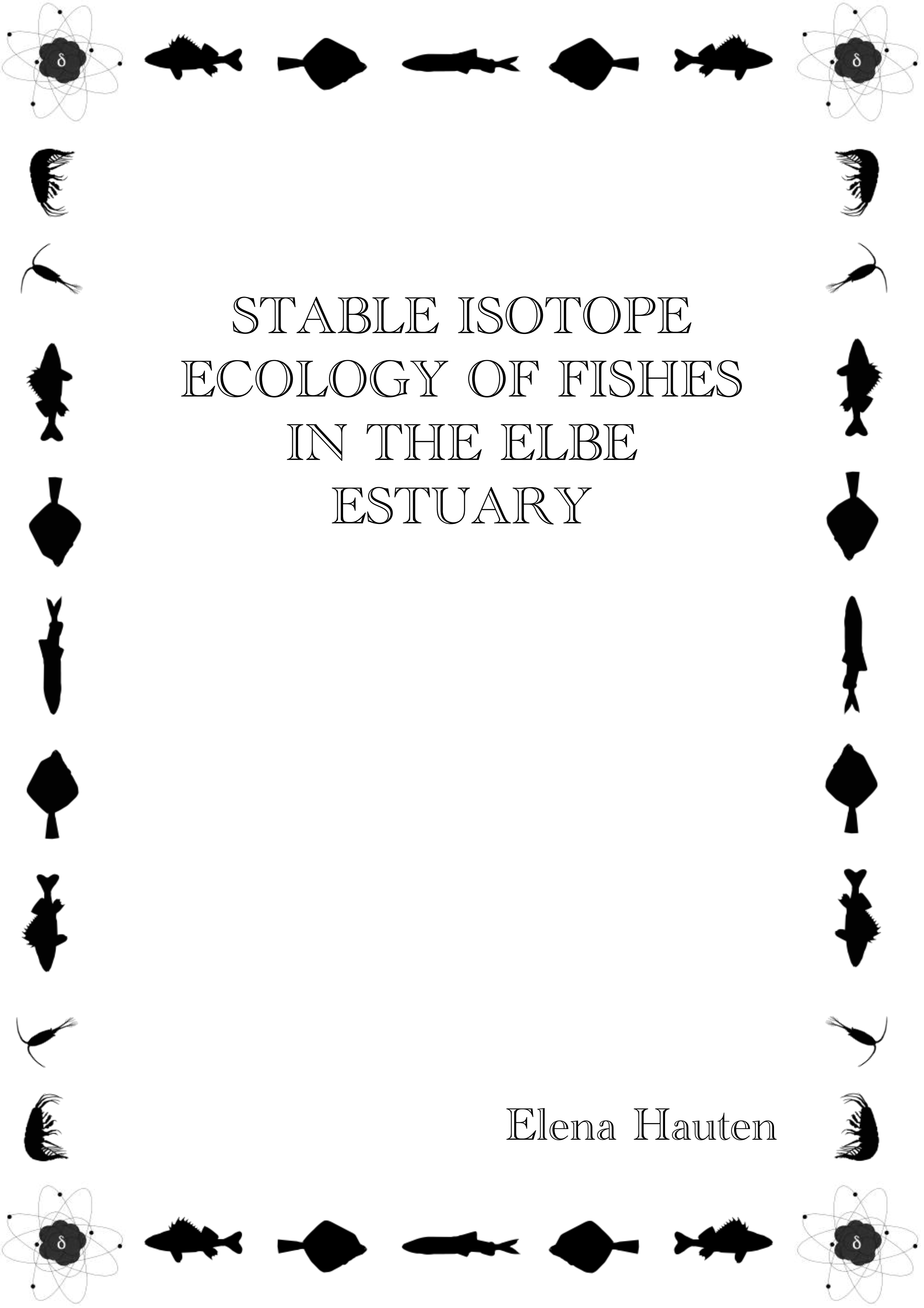

STABLE ISOTOPE ECOLOGY OF FISHES IN THE ELBE ESTUARY

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Stable isotope ecology of fishes in the Elbe estuary

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Summary

Stable isotope analysis of $\delta^{13}C$ and $\delta^{15}N$ evolved into a favored tool for ecologists enabling them, among others, to identify aquatic food web structures and nutritional pathways from lower to higher trophic levels. Especially the ecological role of aquatic consumers, such as fishes, can be uncovered as stable isotope analysis provides insights into their feeding strategies, migrations, foraging habits and dietary niche overlaps. Estuaries link marine and freshwater ecosystems, forming isotopic imprints with distinct stable isotope signatures along the salinity gradient and enabling them to be excellent study sites that can be analyzed using stable isotopes (chapter 1).

In the Elbe estuary European smelt *Osmerus eperlanus* (Linnaeus, 1758) is the dominating fish species that contributes up to 96% of the overall fish abundance, fulfilling a key function. However, the smelt population has declined dramatically in recent years, leading to a necessity to further improve our knowledge on its habitat exploitation in the estuary (chapter 2). Due to smelt´s anadromous life cycle juveniles and adults share the same areas and resources during springtime. In our study we conducted stomach content and stable isotope analysis of both life stages, providing information on long-term and short-term feeding preferences and isotopic niche overlaps along the salinity gradient. The dietary preferences changed from smaller to larger prey during ontogeny with a cannibalistic feeding habit, whereby feeding presumably depended on food availability. As we observed both overlapped isotopic niches and prey items (mainly mysids and gammarids), the risk of intraspecific competition in smelt can be discussed. However, as the species richness is overall low in the estuary, but biomass rates are high, we assume minor competition pressure within the smelt population. Along the salinity gradient we observed different isotopic niche widths and overlaps, indicating spatial differences in food web structures. Enriched $\delta^{15}N$ values in the maximum turbidity zone of the Elbe estuary revealed an extended food chain in this area that mainly affected juvenile smelt. Based on our findings we conclude that juveniles are exposed to the prevailing food supply and conditions, while adults can leave areas when the environmental circumstances are unfavorable.

Various fishes developed different life cycle strategies to exploit estuarine resources, whereby abiotic induced stressors, such as osmoregulation, may hamper their distribution ranges. Ruffe *Gymnocephalus cernua* (Linnaeus, 1758) and European flounder *Platichthys flesus* (Linnaeus, 1758) are eponymous for lower regions in temperate European rivers (*ruffe-flounder-region*). We used stable isotope analysis of $\delta^{13}C$ and $\delta^{15}N$ of fish data sampled seasonally from spring 2021-2022 to track movements, habitat exploitation, spatial connectivity and potential diet shifts during ontogeny (chapter 3). Our study revealed new insights on fish migrations and habitat use in estuaries. Facultative catadromous flounder use the Elbe estuary especially during early life stages, with juveniles occurring in summer and autumn and migrating upstream of our sampling area. We found two isotopic patterns of flounder throughout the year that indicate two North Sea populations exploiting this area

for nursery. The isotope data of ruffe, in contrast, showed residency in the estuary for its entire life cycle, as hardly any marine and riverine isotope signatures were found in the individuals examined. These findings might indicate a distinct brackish water population, while a freshwater population exists further upstream. However, deeper knowledge is needed to confirm our assumption.

To uncover trophic interactions on lower trophic levels on a seasonal scale along the salinity gradient of the Elbe estuary, we investigated $\delta^{13}C$ and $\delta^{15}N$ of particulate organic matter (POM) and dominant meso- and microzooplankton species (chapter 4). High quality carbon (C) was derived from freshwater sources upstream of the city of Hamburg, whereby C sources further downstream were of lower quality ($C:N > 8$). Overall, the POM $\delta^{13}C$ values reflected a mixture of riverine and coastal C that was used by most taxa studied. Calanoid copepods *Eurytemora affinis* contained a broader range in $\delta^{13}C$ than POM, suggesting that this species fed selectively on POM components. Brackish mysid shrimps of *Mesopodopsis slabberi* showed the least variation in its isotopic values which did not overlap with dietary niches of other taxa. During winter and autumn zooplankton increased carnivorous foraging (enriched $\delta^{15}N$ values), especially in the middle section, due to high suspended matter loads and the limitation of high-quality autochthonous phytoplankton. Zooplankton of the Elbe can therefore deal with rapid changing environmental dynamics through their trophic plasticity (e.g., feeding niches partioning, habitat shifting).

Some aquatic organisms bind biogenic carbon, e.g., in their shells, bones or exoskeletons, that reflect inorganic carbon from the surrounding environment rather than assimilated carbon derived from their diet, which can be problematic while conducting stable isotope analyses. However, the removal of inorganic carbon (IC) is often neglected in aquatic food web studies. Therefore, we analyzed the effects of acidification versus no acid treatment on both $\delta^{13}C$ and $\delta^{15}N$ ratios of four common estuarine macroinvertebrates (chapter 5). Our results showed that the necessity of acid treatment prior to stable isotope analysis depends on the species-specific carbonate content. As carbonate is especially high in *Gammarus* spec. and *Palaemon longirostris*, we here recommend a removal of IC either by acid or mechanical procedures. In contrast, mysid shrimps showed no strong effect in stable isotope values. We further discussed other options to remove IC based on previous studies as a general protocol on how to proceed is missing.

As estuaries are often affected by anthropogenic threats, environmental conditions may change over time, thus shaping fish community compositions, life cycle guilds and abundances. In our 40-year long-term study we compared four seasonal fish monitoring periods from 1980s-2020s (chapter 6). Generally, we observed changes in fish life cycle guilds, especially in marine-estuarine opportunists, anadromous and catadromous species. Overall low fish abundances in the 1980s were potentially induced by poor water quality, due to high concentration of nitrogen compounds, such as NO_2 and NH_4, that negatively affected fish health and recruitment success. As water quality improved during the 1990s, fish abundances started to increase until 2010. Recently, we observed declining trends in fish stocks again, especially in smelt, flounder, twaite shad and ruffe. Marine-estuarine opportunists, such as herring and whiting, on the other hand increased as they potentially benefit from widening and deepening of the main channel leading to an amplified inflow of saline water. Recent declines in fish abundances were mainly triggered by increased suspended organic matter (SPM) concentrations, reduced river runoff and low oxygen concentrations. We found most significant changes of abiotic parameters and fish stocks in the freshwater and oligohaline areas of the Elbe estuary, where important nursery areas of certain fish species are located.

Movements between habitats can lead to certain stress responses in fish and can further affect fish health (chapter 7). Fish gills are in direct connection with the surrounding water, so the microbial composition of gill mucus reflect environmental conditions and represent a major entry for pathogens. We compared gill microbiota using prokaryotic sequencing of resident ruffe and anadromous smelt in space and time to further establish fish health indicators. To estimate migratory behavior, we integrated a stable isotope approach based on $\delta^{13}C$. Ruffe microbiota composition was mainly influenced by spatial drivers with bacterial indicator taxa *Verticiella, Polynucleobacter* and *Candidatur Megaira*, reflecting freshwater residency. Anadromous smelt arriving from the adjacent North Sea in autumn showed a stepwise adaptation of the gill microbiome, whereby *Luteolibacter* became dominant and hampered bacterial dysbiosis in the host. We found an overall small core biome containing ~30 genera that can cope with estuarine conditions. Hypoxia and high nutrient loads lead to dysbiosis states, largely driven by takeover of opportunistic pathogens (*Acinetobacter, Shewanella, Aeromonas*).

In conclusion, we revealed new insights into trophic relationships and habitat exploitation of aquatic consumers in the Elbe estuary on a spatial and temporal scale (chapter 8). Based on our study we were able to prove the efficiency of stable isotope analysis in an estuarine ecosystem to track migration patterns and population dynamics of the most important fish species that has not been investigated before. We found four areas along the salinity gradient that are of crucial relevance in understanding the food web of the Elbe estuary. Especially shallow habitats are important for early fish life stages, such as smelt, flounder and ruffe. Therefore, we conclude that our findings could contribute to future conservation strategies in the Elbe estuary.

Zusammenfassung

Die stabile Isotopenanalyse von $\delta^{13}C$ and $\delta^{15}N$ hat sich zu einem beliebten Instrument für Ökologen entwickelt, das es ihnen u.a. ermöglicht, die Strukturen des aquatischen Nahrungsnetzes und die Ernährungswege von der unteren zur oberen trophischen Ebene zu ermitteln. Insbesondere die ökologische Rolle aquatischer Konsumenten, wie z. B. von Fischen, kann so untersucht werden, da die Analyse Einblicke in ihre Ernährungsstrategien, Wanderungen, Nahrungsgewohnheiten und Überschneidungen von Nahrungsnischen gewährt. Ästuare verbinden Meeres- und Süßwasser-Ökosysteme und formen *Isotopensignaturen* entlang des Salinitätsgradienten, wodurch sie sich hervorragend als Untersuchungsgebiete eignen, die mit dieser Methode analysiert werden können (**Kapitel 1**).

Im Elbeästuar ist der Stint *Osmerus eperlanus* (Linnaeus, 1758) die dominierende Fischart, die bis zu 96 % des Gesamtfischbestandes ausmacht und damit eine Schlüsselfunktion erfüllt. Da die Stintpopulation in den letzten Jahren dramatisch zurückgegangen ist, bedarf es einer weiteren Verbesserung unserer Kenntnisse über die Nutzung der Lebensräume im Flusseinzugsgebiet (**Kapitel 2**). Aufgrund seines anadromen Lebenszyklus, teilen sich Jungfische und Adulte im Frühjahr dieselben Gebiete und Ressourcen im Ästuar. In unserer Studie führten wir Mageninhalts- und stabile Isotopenanalysen beider Lebensstadien durch, die Informationen über lang- und kurzfristige Ernährungspräferenzen und isotopische Nischenüberschneidungen entlang des Salzgradienten liefern. Die Nahrungspräferenzen änderten sich im Laufe der Ontogenese von kleineren zu größeren Beutetieren mit steigender kannibalistischer Ernährung. Die Wahl der Beuteorganismen hing dabei primär von der Nahrungsverfügbarkeit ab. Da wir sowohl überlappende Isotopennischen als auch Beutetiere (hauptsächlich Mysiden und Gammariden) finden konnten, kann über eine mögliche intraspezifische Konkurrenz diskutiert werden. Durch den geringen Artenreichtum im Ästuar und der hohen Biomasse von Beuteorganismen gehen wir jedoch von einem geringen Konkurrenzdruck innerhalb der Stintpopulation aus. Entlang des Salinitätsgradienten beobachteten wir unterschiedliche isotopische Nischenbreiten und Überlappungen, was auf räumliche Unterschiede in den Strukturen des Nahrungsnetzes hinweist. Angereicherte $\delta^{15}N$ -Werte in der maximalen Trübungszone des Elbeästuars zeigten eine verlängerte Nahrungskette in diesem Gebiet, die hauptsächlich juvenile Stinte betraf. Aus unseren Ergebnissen schließen wir, dass Jungfische dem vorherrschenden Nahrungsangebot und Bedingungen ausgesetzt sind, während ausgewachsene Stinte Gebiete mit schlechten Umweltbedingungen aktiv verlassen können.

Verschiedene Fische haben unterschiedliche Lebenszyklusstrategien entwickelt, um die Ressourcen eines Ästuars zu nutzen, wobei abiotisch bedingte Stressfaktoren, wie die Osmoregulation, ihre Verbreitungsgebiete behindern können. Der Kaulbarsch *Gymnocephalus cernua* (Linnaeus, 1758) und die Flunder *Platichthys flesus* (Linnaeus, 1758)

sind namensgebend für untere Regionen in gemäßigten europäischen Flüssen (*Kaulbarsch-Flunder-Region*). Um Bewegungen, Habitatnutzung, räumliche Konnektivität und potenzielle Ernährungsumstellungen während der Ontogenese zu verfolgen, nutzten wir stabile Isotopenanalysen von $\delta^{13}C$ and $\delta^{15}N$ von Individuen, die innerhalb eines Jahres vom Frühjahr 2021-2022 entnommen wurden (**Kapitel 3**). Die fakultativ katadrome Flunder nutzt das Elbeästuar vor allem während der frühen Lebensstadien, wobei Jungfische primär im Sommer und Herbst vorkamen und die Überwinterungsgebiete weiter stromaufwärts von unserem Probenahmegebiet lagen. Wir fanden zwei Isotopenmuster der Flunder im Jahresverlauf, die auf zwei Nordseepopulationen hinweisen, die dieses Gebiet als Aufwuchsgebiet nutzen. Die Isotopendaten des Kaulbarsches hingegen zeigen, dass dieser sich während seines gesamten Lebenszyklus im Ästuar aufhält, da bei den untersuchten Individuen kaum marine und fluviale Isotopensignaturen gefunden wurden. Diese Ergebnisse könnten auf eine ausgeprägte Brackwasserpopulation hinweisen, während weiter flussaufwärts eine Süßwasserpopulation existiert. Um diese Annahme zu bestätigen, sind jedoch weitere Erkenntnisse erforderlich. Unsere Ergebnisse haben gezeigt, dass die Bewegungsmuster von Fischen in Flussmündungen komplexer sind als zunächst vermutet.

Um trophische Wechselwirkungen auf niedrigeren trophischen Ebenen auf einer saisonalen Skala entlang des Salinitätsgradienten des Elbeästuars aufzudecken, untersuchten wir $\delta^{13}C$ and $\delta^{15}N$ von partikulärem organischem Material (POM) und dominanten Meso- und Mikrozooplanktonarten (**Kapitel 4**). Hochwertiger Kohlenstoff (C) stammte aus Süßwasserquellen flussaufwärts der Stadt Hamburg, während C-Quellen weiter flussabwärts von geringerer Qualität waren (C:N > 8). Insgesamt spiegeln die $\delta^{13}C$-Werte der POM eine Mischung aus Fluss- und Küstenkohlenstoff wider, der von den meisten untersuchten Taxa genutzt wurde. Die calanoide Copepoden *Eurytemora affinis* wiesen eine größere $\delta^{13}C$-Spanne auf als POM, was darauf hindeutet, dass sich diese Art selektiv von POM-Komponenten ernährte. Brackwasser-Mysidenkrebse der Art *Mesopodopsis slabberi* zeigten die geringste Variation in ihren Isotopenwerten, die sich nicht mit den Nahrungsnischen anderer Taxa überschnitten. Während des Winters und im Herbst nahm das Zooplankton aufgrund der hohen Schwebstoffbelastung und der Begrenzung des hochwertigen autochthonen Phytoplanktons vor allem im mittleren Abschnitt verstärkt karnivoren Nahrung auf (angereicherte $\delta^{15}N$ -Werte). Das Zooplankton der Elbe kann also durch seine trophische Plastizität (z.B. Aufteilung von Nahrungsnischen, Verlagerung von Lebensräumen) mit einer sich schnell ändernden Umweltdynamik an die gegebenen Umstände anpassen.

Einige aquatische Organismen binden biogenen Kohlenstoff, z.B. in ihren Schalen, Knochen oder Exoskeletten, der eher anorganischen Kohlenstoff aus der Umgebung als assimilierten Kohlenstoff aus ihrer Nahrung widerspiegelt, was bei der Durchführung von stabilen Isotopenanalysen problematisch sein kann. Der Abbau von anorganischem Kohlenstoff (AC) wird jedoch in Studien bezüglich des aquatischen Nahrungsnetzes oft vernachlässigt. Daher haben wir die Auswirkungen einer Ansäuerung im Vergleich zu einer

Behandlung ohne Säure auf die δ^{13}C- und δ^{15}N-Verhältnisse von vier häufigen Makroinvertebraten im Ästuar untersucht (**Kapitel 5**). Unsere Ergebnisse zeigten, dass die Notwendigkeit einer Säurebehandlung vor der Analyse stabiler Isotope vom artspezifischen Carbonatgehalt abhängt. Da der Carbonatgehalt bei *Gammarus* spec. und *Palaemon longirostris* besonders hoch ist, empfehlen wir hier eine Entfernung von AC entweder durch Säure oder mechanische Verfahren. Im Gegensatz dazu fanden wir kaum Auswirkungen auf die stabilen Isotopenwerte von Mysiden. Auf der Grundlage früherer Studien haben wir weitere Möglichkeiten zur Entfernung von AC erörtert, da ein allgemeines Protokoll für die Vorgehensweise fehlt.

Da Ästuare häufig von anthropogenen Bedrohungen betroffen sind, können sich die Umweltbedingungen im Laufe der Zeit ändern und so die Zusammensetzung der Fischgemeinschaften, die Lebenszyklusgilden und die Abundanzen beeinflussen. In unserer 40-jährigen Langzeitstudie haben wir vier saisonale Fischmonitoring-Perioden aus den 1980er bis 2020er Jahren verglichen (**Kapitel 6**). Wir beobachteten Veränderungen bei den Lebenszyklusgilden der Fische, insbesondere bei den marin-ästuarinen Opportunisten, anadromen und katadromen Arten. Die insgesamt geringen Fischvorkommen in den 1980er Jahren sind vermutlich auf die schlechte Wasserqualität zurückzuführen, die durch hohe Konzentrationen von Stickstoffverbindungen wie NO_2 und NH_4 verursacht wurde und sich negativ auf die Gesundheit der Fische und deren Rekrutierungserfolg auswirkte. Mit steigender Wasserqualität im Laufe der 1990er Jahre erholten sich die Fischbestände und stiegen bis 2010 an. In jüngster Zeit wurden wieder rückläufige Trends bei den Fischbeständen beobachtet, insbesondere bei Stint, Flunder, Finte und Kaulbarsch. Die Bestände von marinen und ästuarinen Opportunisten wie Hering und Wittling nahmen dagegen zu, da sie möglicherweise von den durch den Menschen verursachten Veränderungen des Hauptkanals profitieren, die zu einem erhöhten Einstrom von salzhaltigem Wasser führen. Der Rückgang der Fischbestände in jüngster Zeit wurde hauptsächlich durch erhöhte Konzentrationen organischer Schwebstoffe (SPM), einen geringeren Abfluss und niedrige Sauerstoffkonzentrationen verursacht. Die deutlichsten Veränderungen der abiotischen Parameter und der Fischbestände fanden wir in den Süßwasser- und oligohalinen Bereichen des Elbeästuars, wo sich wichtige Aufwuchsgebiete von zahlreichen Fischarten befinden.

Der Wechsel zwischen verschiedenen Lebensräumen kann zu bestimmten Stressreaktionen bei Fischen führen und deren Gesundheit beeinträchtigen (**Kapitel 7**). Die Kiemen von Fischen stehen in direkter Verbindung mit dem umgebenden Wasser, sodass die mikrobielle Zusammensetzung des Kiemenschleims die Umweltbedingungen widerspiegelt und eine wichtige Eintrittspforte für Krankheitserreger darstellt. Wir verglichen die Kiemenmikrobiota mittels prokaryotischer Sequenzierung von ortsansässigen Kaulbarschen und anadromen Stinten in Raum und Zeit, um daraus Indikatoren für die Fischgesundheit zu ermitteln. Um das Wanderverhalten abzuschätzen, integrierten wir δ^{13}C Daten der jeweiligen Fischarten. Die Zusammensetzung der

Kaulbarsch-Mikrobiota wurde hauptsächlich durch räumliche Faktoren beeinflusst, wobei die bakteriellen Indikatortaxa *Verticiella*, *Polynucleobacter* und *Candidatur Megaira* den Aufenthalt im Süßwasser widerspiegeln. Anadrome Stinte, die im Herbst aus der angrenzenden Nordsee eintrafen, zeigten eine schrittweise Anpassung des Kiemenmikrobioms, wobei *Luteolibacter* dominant wurde und die bakterielle Dysbiose im Wirt behinderte. Insgesamt fanden wir ein kleines Kernbiom mit ~30 Gattungen, die im Ästuar überleben können. Hypoxie und hohe Nährstoffbelastungen führen zu Dysbiosezuständen, die weitgehend durch die Übernahme von opportunistischen Pathogenen (*Acinetobacter*, *Shewanella*, *Aeromonas*) verursacht werden.

Diese Arbeit ermöglichte uns neue Einblicke in die trophischen Beziehungen und Habitatnutzung im Elbe-Ästuar auf räumlicher und zeitlicher Ebene (**Kapitel 8**). Auf der Grundlage unserer Studie konnten wir die Effizienz der Analyse stabiler Isotope in einem Ästuar-Ökosystem nachweisen, um Migrationsmuster und Populationsdynamiken der wichtigsten Fischarten zu verfolgen, die bisher nicht untersucht wurden. Wir fanden vier Bereiche entlang des Salinitätsgradienten, die für das Verständnis des Nahrungsnetzes des Elbeästuars von entscheidender Bedeutung sind. Besonders flache Gebiete sind für frühe Fischlebensstadien von u.a. Stint, Flunder und Kaulbarsch wichtig. Wir kommen daher zu dem Schluss, dass unsere Ergebnisse einen Beitrag zu künftigen Schutzstrategien im Elbästuar leisten könnten.

Chapter 1

General introduction

“Elbe, stadtstinkende kaiklatschende schilfschaukelnde sandsabbelnde möwenmützige graugrüne große gute Elbe! Links Hamburg, rechts die Nordsee, vorn Finkenwerder und hinten bald Dänemark. Um uns Blankenese. Über uns der Himmel. Unter uns die Elbe. Und wir: Mittendrin!“

Wolfgang Borchert - Die Elbe - Blick von Blankenese

Old Harbor Tidal Creek (2016)
© Wolfgang Hauten; used with permission

Chapter 1 - Introduction

1.1 Estuaries

1.1.1 Importance and threats

Estuaries provide a multiple collection of ecosystems, offering various habitats for estuarine flora and fauna but also great ecosystem services for humans. The network of different habitats (e.g., salt marshes, intertidal flats, seagrass meadows) and topographic structures (e.g., mud flats, rocky substrates) connect marine, freshwater and terrestrial worlds and form special intermediate and complex environments (Bulmer et al. 2020, Colombano et al. 2021b, Lal et al. 2023).

The term "estuary" dates back to the 16th century and originates from the Latin word *aestuarium*, which stands for "marsh" or "channel" (Collins 1979, Elliot and McLusky 2002). Although "estuary" is a common term in science, there is no coherent definition of the exact terminology. Faibridge (1980) claims that "*an estuary is an inlet of the sea reaching into a river valley as far as the upper limit of tidal rise, normally being divisible into three sectors: (a) a marine or lower estuary, in free connection with the open sea; (b) a middle estuary subject to strong salt and freshwater mixing; and (c) an upper or fluvial estuary, characterized by freshwater but subject to strong tidal action. The limits between these sectors are variable and subject to constant changes in the river discharges*". Hence, estuaries are commonly transitional regions where marine and riverine waters are mixed up, which is usually reflected by strong biogeochemical gradients such as salinity (Bauer et al. 2013). Besides the controversial discussion on how to define estuaries, it is known that these aquatic transition zones transport large amounts of energy and carbon from allochthonous and autochthonous sources within and outside the system on a longitudinal and lateral scale (Ricart et al. 2020). Due to the connection of different carbon pools, shaped by prevailing natural processes (e.g., photosynthetic pathways, temperature and salinity fluctuations) (Michener and Kaufman 2007), ecologists can track carbon transport isotopically as $\delta^{13}C$ values change over the course of the estuary with decreasing isotope values from marine to riverine waters (Fry 2002) (Fig. 1.1, see section 1.3 for further information). The high productivity of estuaries is mainly driven by (1) year-round primary production units such as marsh grass, benthic algae, and phytoplankton, (2) the continuously water movement due to tidal fluctuations, (3) high nutrient supply and (4) the effective recycling of nutrients by microorganisms and filter feeders (Schelske and Odum 1962, Day et al. 2013).

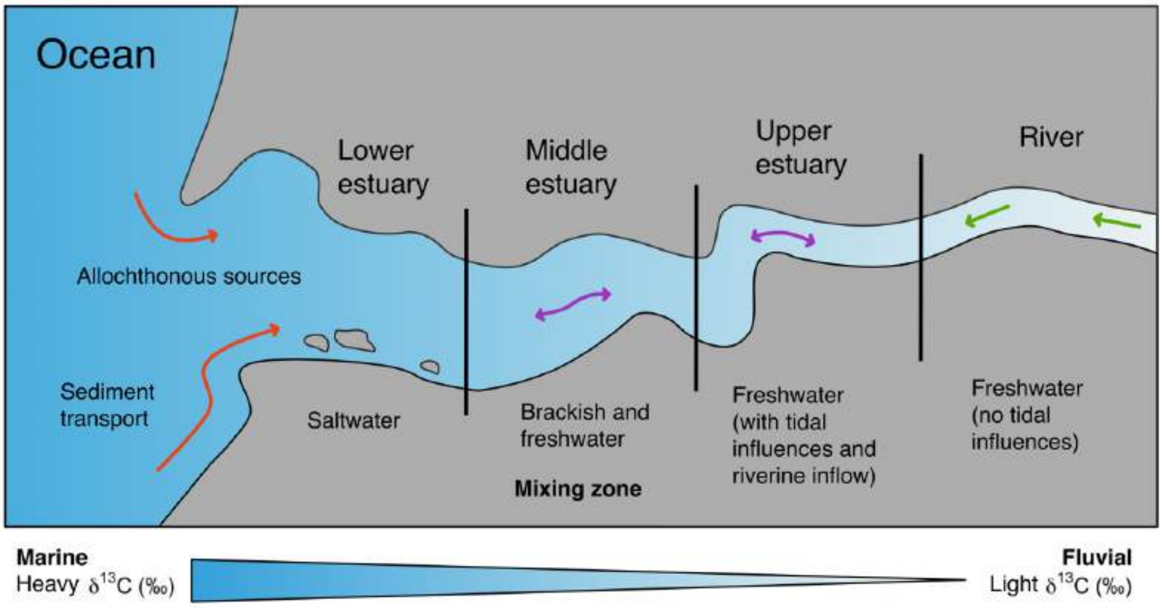


Fig. 1.1 General characteristics of an estuary with lower, middle, and upper sections as well as river area which are mainly shaped by a salinity gradient (blue shaded arrow), water mixing and distinct $\delta^{13}C$ ratios based on individual C pools (modified from Fairbridge 1980, RTG2530 2021).

Estuaries are globally important as they fulfil multiple ecosystem services (e.g., carbon storage, fish nursery), facilitate international trade and economic competitiveness through inland ports, and serve as cooling water reservoirs for power plants (Wetzel et al. 2013). During the industrialization process, people started modifying estuaries for economic purposes, such as dike constructions as well as straightening and deepening of main channels to optimize commercial ship traffic (Wetzel et al. 2013). Agricultural use of adjacent landmarks fuel nutrient load in estuaries that is assumed to increase in the future, enhancing eutrophication and oxygen minimum zones (Statham 2011, Altieri and Gedan 2015). The impacts of climate change will lead further to changes in nutrient loads due to rapid changes in temperature, wind patterns and sea level rise, among others, that force changes in community structures of biota (Statham 2011). The spread of hypoxic dead zones, which have been shown to double every decade since the 1950s in coastal ecosystems, also intensifies the pressure on estuarine communities, leading to an associated reduction in native biodiversity and biomass as well as their functionality (Diaz and Rosenberg 2008, Altieri and Gedan 2015, Francescangeli et al. 2021). It can therefore be assumed that the consequences of increasing anthropogenic impacts will further exacerbate the pressure on estuarine communities in the future.

1.1.2 Fishes in estuaries

As estuaries are known to be enormous productive ecosystems with high food availability, they offer ideal conditions for fish and crustaceans supporting large populations (Haedrich, 1983, Elliot and Hemingway 2002). Various fish species exploit estuaries in multiple ways: as nursery and feeding areas as well as migratory routes (Whitfield et al. 2022). However, they also represent a challenging habitat for aquatic consumers due to constantly fluctuating natural conditions and other stresses that affect the aquatic community structure supporting an overall low species richness (Whitfield and Harrison 2020, Mosman et al. 2023). That, in turn, leads to higher importance of individual species, a common generalist feeding pattern and competitive pressure among species (Whitfield and Harrison 2020). Fishes and other organisms that are well-adapted to this variability and can cope with estuarine-induced stresses without harmful effects are referred to as *Environmental Homeostasis* (Elliot and Quintino 2006). Varying spatial environmental conditions and rapid temporary fluctuations (e.g., in salinity, temperature) cause marine fish species to dominate in the transitional zone of the estuary and decrease markedly towards the inland. Limnic species, on the other hand, use the habitat further upstream, with their abundance declining downstream (Thiel and Potter 2011). For both marine and limnic organisms, the estuary is an exceptionally stressful environment, though, the resident species take the benefits of the habitat and incur extra physiological and energetic costs to achieve them (Elliot and Quintino 2006, Whitfield 2021).

The estuarine fish community structure is shaped by various types of life strategies. Overall, fish migration can be divided into: oceanodromy, potamodromy and diadromy (Myers 1949, Magath 2013). Oceanodromous and potamodromous fishes spend their lives exclusively in the ocean respectively in freshwater (Morais and Daverat 2016). Diadromy describes the migration by fish between freshwater and the sea and is further subdivided into anadromy, catadromy and amphidromy (Morais and Daverat 2016). While anadromous and catadromous species migrate between freshwater and saltwater for spawning (see section below for a more detailed description), amphidromous species move between these habitats, but not with the aim of reproducing (McDowall 1992). In general, the movement radius of fish underlies individual variability, which is referred to as partial migration (Chapman et al. 2012, Morais and Daverat 2016).

The further classification of fishes into life cycle guilds based on their distinct migration patterns enhances the understanding of habitat exploitation at specific life-stages and further illustrates spatial and temporal distributions of fishes in estuaries (Elliot et al. 2007). Elliot et al. (2007) defined 11 life cycle guilds (or categories) of estuarine fish: marine straggler, freshwater straggler, marine migrant, catadromous species, anadromous species, semi-catadromous species, semi-anadromous species, amphidromous species, freshwater migrant, estuarine resident and estuarine migrant. A selection of the six most important guilds (Theilen et al. *unpubl.*) occurring in the Elbe estuary is shown in Fig. 1.2 and can be described according to Thiel (2003, 2011) as:

a) **Marine stragglers** (e.g., Atlantic mackerel *Scomber scombrus*) are marine fish species that occur irregularly and in low densities in the river mouth where salinity is highest.
b) **Marine-estuarine-opportunists** (e.g., herring *Clupea harengus*) are marine species that occur more frequently and in higher densities, especially in the juvenile life-stage.
c) **Solely estuarine species/estuarine residents** (e.g., common goby *Pomatoschistus microps*) spend their entire life cycle in the estuary.
d) **Catadromous species** (e.g., European eel *Anguilla anguilla*) reproduce in the sea, where the larvae and juveniles are washed by ocean currents into adjacent rivers, where they remain until they reach sexual maturity (Morais and Daverat 2016).
e) **Anadromous species** (e.g., salmon *Salmo trutta*) live predominantly in the ocean and migrate to limnic waters to spawn, where they either leave the habitat (iteropar) or die immediately after reproduction (semelpar) (Magath 2013, Morais and Daverat 2016).
f) **Freshwater species/freshwater migrants** (e.g., zander *Sander lucioperca*) are species that are usually restricted to limnic habitats, but also occur in high densities at lower salinities.

Most of these life cycle guild classifications further reflect the ability of fishes to travel great distances based on their life cycle strategy and as an adaptive response to spatial differences in ecosystem resource availability leading to the connection of different habitats and aquatic food webs (Baker 1978, Mather et al. 2013).

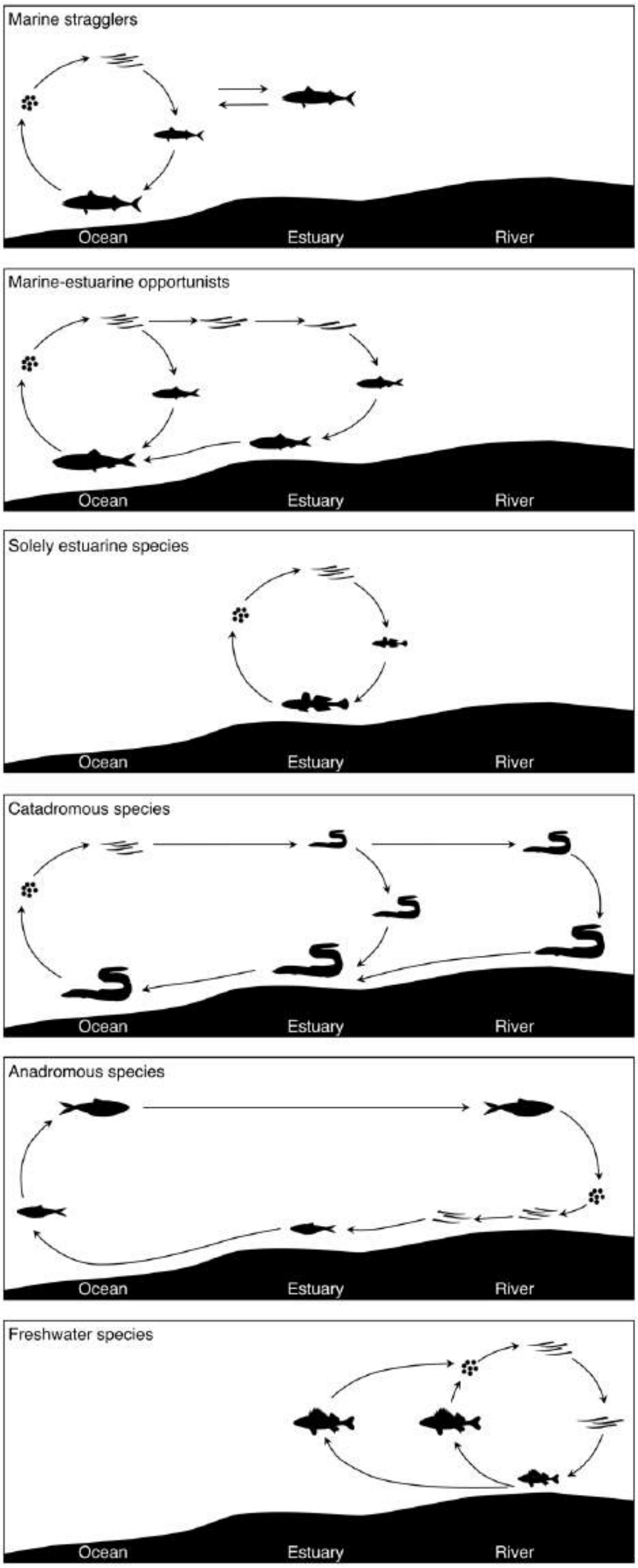


Fig 1.2 Most important life history guilds covering the migration patterns and habitat utilization during life of fish in the Elbe estuary (modified from Thiel 2011, Whitfield et al. 2022).

1.2 The Elbe estuary

The Elbe River has its source in the Czech Republic. It is one of the largest rivers in Europe having a length of 1,094 km and a catchment area of 148,268 km^2 (Francescangeli et al. 2021, van Neer et al. 2023). The transitional part between the Wadden Sea of the southern North Sea and the Elbe River can be termed as tidal Elbe, Lower Elbe or Elbe estuary and extends about 140 km from the weir of Geesthacht to the seaward border of Cuxhaven (Carstens et al. 2004, Francescangeli et al. 2021). At Elbe km (Ekm) 609, the river splits into the Norder- and Süderelbe forming an inland delta, which merge again at Ekm 636.6 and contain the Port of Hamburg, Germany´s largest seaport (Riedel-Lorjé and Gaumert 1982, Schwentner et al. 2021). Due to its access to the North Sea, the Elbe estuary is exposed to tides with high current rates of up to 1.8 m s^{-1} and a tidal range of 3.6 m in the harbor area (Schwentner et al. 2021).

1.2.1 Human interventions: past and present

The tidal Elbe is a river shaped by human activities and has been repeatedly modified in former centuries, decades, and years. Historically, the first artificial interventions in the form of dike construction works were documented around the year 1000 (Riedel-Lorjé and Gaumert 1982). This was followed by further dike buildings to prevent damage from storm surges and river engineering measures to improve navigability. The first major harbor project was planned by Hubbe and Lindley in 1845 and completed in 1866 in the so-called Sandtorhafen, an artificial harbor (Riedel-Lorjé and Gaumert 1982). Since then, the river has undergone continuous change. Before German reunification in 1989, the Elbe was considered one of the most polluted rivers in Europe in terms of organic enrichment, nutrients, and chemical contamination (Krysanova et al. 2006, Francescangeli et al. 2021) that led to an overall fish decline and the absence of indicator species, such as twaite shad *Alosa fallax* (Thiel et al. 2008, Thiel 2011). In the 1990s, the water quality improved significantly, which also contributed to the recovery of the native flora and fauna (Thiel et al. 1995, Maitland 2003, Eick 2016). However, the river has been constantly modified due to human activities, such as channelization, straightening, pollution, and agriculture, which have led to a transformation of topographical (e.g., high sedimentation rates) and abiotic conditions (e.g., lower dissolved oxygen level, increased turbidity values, reduction of the surface/volume ratio) in the river (Wetzel et al. 2013, Schwentner et al. 2021). Especially the frequent ship traffic (with 8,700 commercial vessels passing the Hamburg harbor annually) as well as immense dredging activities influence the estuarine environment around the main channel (van Neer et al. 2023). Lastly, 3-9 million m^3 of sediment was removed to deepen the channel in 2021 and 2022, resulting in an additional annual dredging of 12 million m^3 to maintain the intended depth (van Neer et al. 2023).

These intensive human activities led to the decline of biodiversity and -biomass (Diaz and Rosenberg 2008, Francescangeli et al. 2021) and further may result in disturbances of ecosystem functioning and changing food web structures at different trophic levels (Wetzel et al. 2013). In the Elbe estuary the overall fish abundance has been decreasing rapidly in recent years, presumably as a result of various anthropogenic disturbances that cause habitat loss, low food availability and expanded oxygen minimum zones during summer (Spiekermann et al. 2022).

1.2.2 Fish community

While the fish communities in many estuaries located in the Northern hemisphere are characterized by high abundances of marine species (e.g., Hamerlynck and Hostens 1994, Maes et al. 1998, Thomas 1998, Thiel and Potter 2001), the Elbe estuary is dominated by anadromous species, especially smelt *Osmerus eperlanus* and twaite shad *Alosa fallax* (Eick and Thiel 2014). Besides these two species, the ten most important fishes that occur here are: ruffe *Gymnocephalus cernua*, bream *Abramis brama*, white bream *Blicca bjoerkna*, herring *Clupea harengus*, European flounder *Platichthys flesus*, sprat *Sprattus sprattus*, zander *Sander lucioperca* and three-spined stickleback *Gasterosteus aculeatus* (Eick and Thiel 2014). However, the fish community is subject to environmental factors, which can affect their diversity and abundance spatially and temporally. For example, salinity primarily influences species composition and fish biomass on a spatial scale, while temperature is a good predictor of fish abundance throughout the year (Thiel et al. 1995). The Elbe mouth represents the area with the highest species richness with predominantly marine-estuarine opportunists, such as herring and sprat (Thiel and Potter 2001, Eick and Thiel 2014). Freshwater species (e.g., zander, ruffe) occur mainly in the least saline areas upstream, with a decreasing trend towards the river mouth (Möller 1984, Thiel 2011). However, both marine and freshwater species make up only a small proportion of the total abundance, as anadromous smelt dominate here with up to 96 % and thus play a key role in the Elbe estuary (Eick and Thiel 2014).

Due to the stressful biogeochemical conditions for the aquatic biota, these estuaries are often characterized by low biodiversity compared to adjacent marine habitats (Whitfield and Harrison 2021, Mosman et al. 2023). Smelt is particularly successful in establishing itself in this habitat, as it has adapted its life cycle best to the given stressors in order to derive maximum benefit from them. Thiel and Potter (2001) mentioned as an advantage the location of the spawning areas above the city of Hamburg, which were largely unaffected by channel alterations, but also the so-called *umbrella structure* of the smelt eggs, with which they attach to the substrate until the hatched larvae are carried downstream to their actual nursery area. Due to its high occurrence, smelt serves further as a link of upper and lower trophic levels (Hennig et al. 2016) and has been noticed being an indicator species for water quality (McCarthy et al. 2019). However, the smelt population has declined dramatically in recent years due to e.g., increased hypoxia events and loss of shallow water areas as a result of anthropogenic impacts (Illing et al. 2014). A continued decline in the population might have drastic consequences in the future, as the smelt further connects spatially separated food chains via feeding and excretion, thus promoting food web stability (Quevedo et al. 2009).

In the last 10 years, there has been hardly any research on the fish fauna in the Elbe estuary, in particular interactions with lower trophic levels and migration patterns to fulfill life cycle strategies of fish have not been studied. The last comprehensive study on the fish community in this region was carried out in 2009/2010 by Eick and Thiel (2014). Since then, however, several anthropogenic interventions, such as the deepening of the main channel to 16.1 m completed in January 2022 (Hafen Hamburg Marketing e. V. 2024), have been conducted, potentially affecting the dynamics of the food web and fish production. The fish community therefore particularly needs to be reassessed.

1.3 Stable isotope analysis

The world of stable isotopes enables researchers to entangle food web structures (Hansson et al. 1997), evaluating food chain lengths (Layman et al. 2007), estimating ecological niches (Newsome et al. 2007), calculating trophic levels (Post 2002) and tracking animal migrations (Hobson 1999, Cunjak et al. 2005, Durbec et al. 2010). Isotopes can be found everywhere in the biosphere from sediment compounds to living organisms, so that everything (and everyone) can be tracked isotopically in an ecosystem and beyond (Fry 2006). Stable isotope analysis offers therefore a toolbox for ecologists that can be used in multiple ways and in various habitats.

Isotopes are atoms of an element that contain a different number of neutrons but the same number of protons and electrons (Michener and Lajtha 2008). If the number of neutrons and protons is close, then the isotope is energetically stable and referred to as a stable isotope (e.g., ^{2}H, ^{13}C, ^{15}N, ^{32}S). Unstable isotopes, though, radiate and are radioactive. One of the best-known radioactive isotopes is ^{14}C, which is used for the radiocarbon method for age determination over a long period of time (up to 55,000 years) based on its decay rate in palaeontological research (Hajdas 2008).
Using a high precision IRMS (isotope-ratio mass spectrometry) isotopic compositions from organic samples (e.g., fish tissue, zooplankton, sediment) can be measured quantitatively (Morais and Daverat 2016). The δ notation is a common term while conducting stable isotope analysis and expresses parts per thousand (‰) differences from a standard using the formula: $\delta X = [(R_{sample}/R_{standard})-1] \cdot 10^3$(Peterson and Fry 1987). The IAEA (International Atomic Energy Agency) provides these standards that are used to calibrate the IRMS for the analysis (West et al. 2006, IAEA 2024). The δX value displays the proportion of heavy and light isotopes in a sample, whereby an increase indicates a higher amount of heavy isotope compounds (Peterson and Fry 1987).

Carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) are most frequently used in aquatic food web studies (Pasquaud et al. 2008). In the biosphere, ^{12}C makes up 98.89 % of the carbon atoms present, with only 1.11 % occurring as ^{13}C isotopes (Fry 2006). In nitrogen, this proportion is even lower, with ^{15}N making up 0.36 %, while the remaining N atoms from ^{14}N account for 99.64 % (Fry 2006). The difference between these two isotopes (^{12}C:^{13}C and ^{14}N:^{15}N) results in the delta values $\delta^{13}C$ and δ ^{15}N (Peterson and Fry 1987).
The isotopic composition of $\delta^{13}C$ in an animal´s tissue reflects that of its diet and is on average 0.8 – 1.1 ‰ enriched, following the principle "You are what you eat (plus a few ‰)" (DeNiro and Epstein 1976, DeNiro and Epstein 1978). $\delta^{15}N$ enriches stepwise per trophic level (2-4 ‰), enabling the calculation of the trophic position of a species in a food web (McCutchan et al. 2003, Nielsen et al. 2018, Quezada-Romegialli et al. 2018). This enrichment, or trophic discrimination, can vary between organisms based on the rate of assimilation and their position in the food chain, whereby lighter stable isotopes are excreted more frequently leading to heavier stable isotope ratios in the consumer (Nielsen et al. 2018).

To uncover the origin of diet in the consumer´s tissue, mixing models has been frequently applied in food web studies. The composition of stable isotopes in the consumer reflects the composition of its assimilated diet (Phillips 2012). This characteristic can be used to draw conclusions about food preferences and predator-prey relationships (Fry 2006, Layman et al. 2012, Smith et al. 2013), taking into account the trophic enrichment factor, turnover rate and variation of the prey stable isotope signatures (Cabana and Rasmussen 1996, Boecklen et al. 2011, Layman et al. 2012). Basic mixing models contain mathematical equations that "*explain the observed consumer isotopic composition as a simple mixture of the isotopic composition of its (assimilated) diet based on isotopic mass balance*" (Phillips 2012). However, these simple models cannot deal with missing information (e.g., a missing dietary source), hence the *point-in-polygon* approach is often used, which requires the isotopic data of the consumers to appear within the dietary signatures (Phillips and Gregg 2003, Benstead et al. 2006, Smith et al. 2013). The growing interest on stable isotopes in food web studies has also expanded the analytical toolbox of mixing models, especially Bayesian mixing models, which resulted in an increased provision of R packages (e.g., SIAR, MixSIAR, SIMMR) to make these methods available to a wide range of scientists (e.g., Jackson et al. 2011, Layman et al. 2012).

The characterization of trophic niche occupations of certain species is relevant in ecological studies. According to Hutchinson (1957), the trophic niche is defined as an "n-dimensional hypervolume" of all resources a population exploits. This hypervolume of the trophic niche is impossible to quantify (Syväranta et al. 2013), hence the niche width is often calculated using dietary information gathered from stomach content data or stable isotope approaches (Bearhop et al. 2004).

Chapter 1 - Introduction

In recent years, the term "isotopic niche" has been established in stable isotope research, being represented in a two-dimensional bi-plot of $\delta^{13}C$ and $\delta^{15}N$, and thus reflecting the isotopic dietary niche of multiple individuals in a population (Syväranta et al. 2013). The isotopic variability reflects, among others, the consumer´s feeding strategy (specialists vs. generalists) and further depends on available prey organisms and their composition (Bearhop et al. 2004). The isotopic niche may be considered part of the ecological niche, but it is not equivalent to it (Jackson et al. 2011). To calculate the isotopic niche width and to compare isotopic niches across communities, Jackson et al. (2011) established a Bayesian framework using standard ellipse areas (SEA) which is less dependent on sample size than the former used total area (TA) according to Layman et al. (2007).

When evaluating stable isotope data, researchers must consider that the assimilation process of a diet in the consumer's body requires a certain amount of time until an equilibrium is reached (Guelinckx et al. 2008, Durbec et al. 2010). Different tissues of the consumer "store" isotope signals for different durations based on their metabolic activity and growth rate that is known as the isotopic turnover rate (Ankjærø et al. 2012, Matich and Heithaus 2014). Thus, individual tissue types provide evidence of dietary habits over variable periods of time: in blood, the isotopic turnover rate amounts to a few days, in the liver a few weeks, in muscles several months and in bone structures (e.g., otoliths) years (Nielsen et al. 2018). The integrated utilization of these tissue types can thus significantly improve the temporal resolution of feeding behavior in ecological studies (Nielsen et al. 2018).

Furthermore, the combination of stable isotopes with other established approaches can be an effective way of overcoming the limitations of certain research methods. For instance, additional stomach analyses (e.g., Pasquaud et al. 2008, Davis et al. 2012, Leclerc et al. 2014), fatty acids (e.g., Petursdottis et al. 2012) and DNA metabarcoding analyses (e.g., Carreon-Martinez and Heath 2010, Compson et al. 2019) have proven to be particularly effective in the study of trophic interactions in food webs. Using these combined methods, ecologists can improve their understanding of feeding ecology in aquatic food webs at specific time frames (Nielsen et al. 2018), monitor changes in food chains due to invasive species (Rakauskas et al. 2013), decode trophic linkages (e.g., Petursdottis et al. 2012) and derive management strategies (e.g., Compson et al. 2019). Telemetry und mark-recapture integrated with stable isotope analysis have further been used in previous studies to track movement patterns in migrating fish species (e.g., Cunjak et al. 2005, Match and Heithaus 2014).

Stable isotopes can not only reveal information about what was eaten, but also where. Biogeochemical processes and nutrient inputs in an ecosystem create local *signatures* that helps to uncover spatial food preferences, migration patterns and food source origins (Peterson and Fry 1987). Marine and freshwater habitats, for instance, are fueled by different vegetations with distinct carbon pools that enable the tracking of migrating species via $\delta^{13}C$ (Morais and Daverat 2016). Plants with different photosynthetic pathways, for example, differ significantly in their isotopic values, with C_4 plants having significantly lower $\delta^{13}C$ ratios than C_3 plants (Smith and Epstein 1971, DeNiro and Epstein 1978). This is one of the processes that affect the local isotopic composition of organic matter (OM) in these habitats. Finley and Kendall (2007) illustrated that OM in river systems, which is ingested by consumers, derives mainly from allochthonous (terrestrial plant detritus, soils) and autochthonous sources (aquatic macrophytes, algae) as well as from aquatic heterotrophic bacteria and fungi. The proportion of these sources and their uptake (fractionation) of dissolved (e.g., NO_3^-, HCO_3^-) and gaseous compartments (e.g., CO_2, N_2) further influence the local isotopic composition of particulate organic matter (POM) and the primary producers that form the base of the aquatic food web (Finley and Kendall 2007). These biogeochemical processes result in carbon from river habitats generally having depleted $\delta^{13}C$ values (e.g., phytoplankton < -30 ‰), while the values in marine waters are more enriched (e.g., phytoplankton approx. -24 - 19 ‰) (Peterson and Fry 1987) (see Fig. 1.1). $\delta^{15}N$ ratios are also affected by these biogeochemical circumstances, leading to more depleted values in freshwater organisms than in marine specimens (Cloern et al. 2002, Morais and Daverat 2016). As estuaries connect fluvial and marine habitats, further enabling mobile consumers, such as fish to exploit them, they reveal excellent study areas with local distinct isotopic values to analyze fish migrations (Fry 2002).

1.4 Objectives

The Elbe estuary is subject to constant transformation and human intervention, causing the estuarine food webs to constantly be altering.
This doctoral thesis sheds the spotlight on the main fish species (chapter 2 and 3) and lower trophic levels (chapter 4) to gain a better understanding of the estuarine food web. The method of stable isotope analysis was carried out for the first time in the study area at this scale and therefore offers new insights into feeding habits, fish migrations, intraspecific interactions and habitat exploitation that have not been covered in previous studies. We also questioned our method and tested experimentally whether environmental conditions may influence the data quality (chapter 5). As continuous fish monitoring is necessary to record recent alterations in the estuary and, if necessary, to derive protective strategies from them, we analyzed how the fish community changed from the 1980s until today (chapter 6) and developed indicators for fish health monitoring using fish gill microbiota (chapter 7). In detail, this doctoral thesis is divided into the following chapters:

In chapter 2 we aimed to explore the feeding ecology and habitat exploitation of key species smelt *Osmerus eperlanus*. During springtime juveniles and adults utilize the same feeding grounds and resources. We used integrated stomach content and stable isotope analyses of

$\delta^{13}C$ and $\delta^{15}N$ to uncover short- and long-term feeding information on both ingested and assimilated prey along the salinity gradient. Further, we aimed to track migratory behavior of the life-stages, to determine local food web structures and to uncover important nursery and feeding areas for smelt in the Elbe estuary.

In chapter 3, we tracked annual habitat exploitation of two fish species with distinct life cycle strategies and movement patterns that occur regularly in the Elbe estuary (Eick and Thiel 2015). Facultative catadromous flounder *Platichthys flesus* rely on temperate estuaries as nursery habitats (Mendes et al. 2020), whereby larvae are washed into rivers shortly after spawning in adjacent marine waters (Morais and Daverat 2016). Potamodromous ruffe *Gymnocephalus cernua* on the other side is a resident species, occurring in both freshwater and brackish regions, but do not leave the estuary in the course of life. We used stable isotope ratios ($\delta^{13}C$ and $\delta^{15}N$) of both fish species caught at five stations along the salinity gradient during seasonal cruises from spring 2021 to spring 2022. Aim of this study was to explore annual fish migrations, spatial connectivity and potential ontogenetic shifts to gain further insights into population´s dynamics and habitat utilization on a spatial and temporal scale.

In chapter 4, we described the spatiotemporal zooplankton dynamics and carbon sources to uncover trophic interactions at the lower trophic levels and the origin of the carbon that fuels the estuary. We used stable isotope analyses ($\delta^{13}C$ and $\delta^{15}N$) of particulate organic matter (POM) and dominant meso- and macrozooplankton, including ichthyoplankton of *Osmerus eperlanus*. Sampling was conducted seasonally at five stations along the salinity gradient, including the freshwater stretch upstream of the port of Hamburg in 2022.

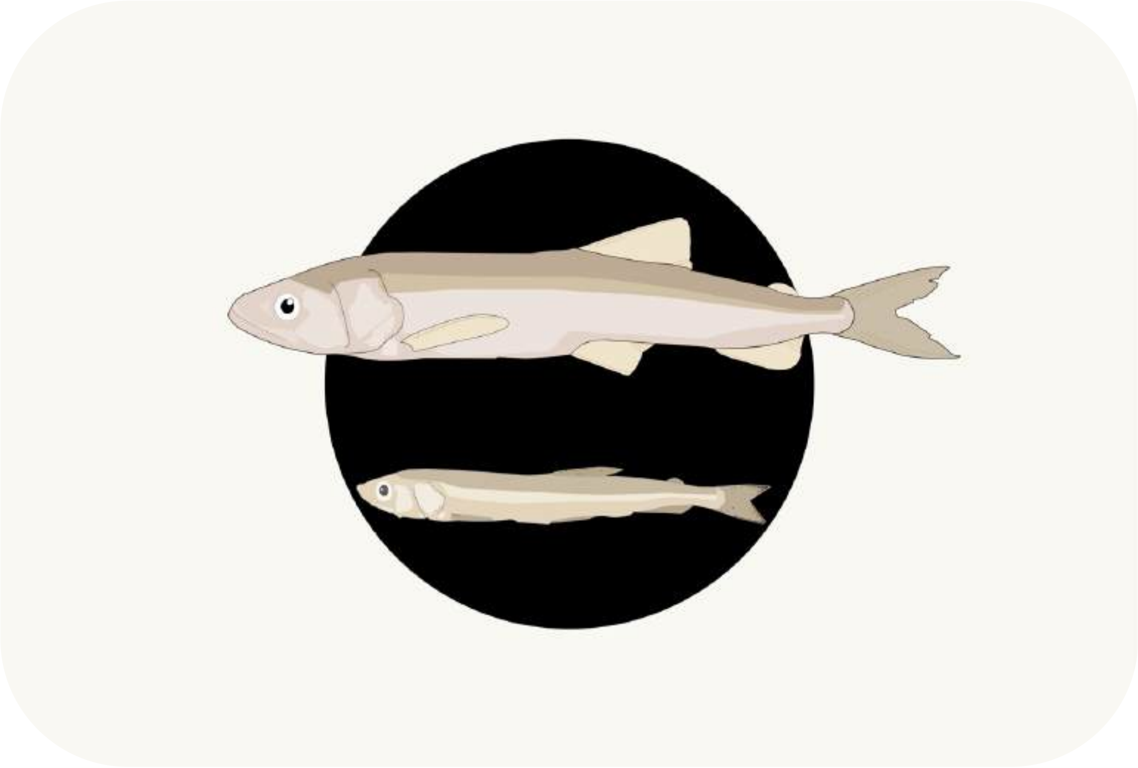
The impact of inorganic carbon signatures of calcified structures forming prey on stable isotope analysis is a common but often overseen problem in aquatic food web studies. However, calcified structures in organisms may rather reflect inorganic carbon signatures from surrounding water than $\delta^{13}C$ derived from diet and can therefore lead to misinterpretation of stable isotope data. Acidification is a known, but intricate method to remove calcified structures prior stable isotope analysis. In chapter 5 we analyzed acid treated and untreated samples of four macroinvertebrate species based on their $\delta^{13}C$ and $\delta^{15}N$ values. We further synthesize findings from previous studies to standardize how to proceed with the problem of inorganic carbon signatures in aquatic food web studies.

In chapter 6, we analyzed the effects of environmental factors on the fish fauna over the last 40 years using abundance data and abiotic measurements from four sampling periods. As the Elbe estuary has undergone drastic changes in recent years and decades, abiotic conditions and habitats have changed as well. In this study, we aimed to find out how the fish community has developed, and which main abiotic drivers generally influence the community in the Elbe estuary.

In chapter 7, we sequenced prokaryotic populations of fish gills of ruffe *Gymnocephalus cernua* and smelt *Osmerus eperlanus* in space and time as gill mucus provides information about the state of health in fish. Additional stable isotope analyses were used to generate information on site fidelity and seasonal migration behavior to compare with the abundance of microbiome marine and freshwater taxa. The aim of this study was to further develop this approach to characterize the health status of wild fish and to identify seasonal differences that may be related to migratory habits and environmental conditions.

Chapter 2

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



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

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Abstract

Estuaries are both highly productive and challenging habitats for aquatic organisms due to their rapid changing environmental conditions, with salinity acting as an important driver for species composition and richness. In the Elbe estuary, one of the largest estuaries in Europe, the anadromous smelt *Osmerus eperlanus* dominate the fish community, making it a key species. During spring, juveniles and adults occupy the same habitat and thus share the same feeding grounds and resources. However, essential aspects of the feeding ecology and habitat use of these life stages remain unknown. Using integrated stomach content and stable isotope analyses of $\delta^{13}C$ and $\delta^{15}N$ of smelt muscle tissue, we found distinct habitat exploitation and movements patterns of juveniles and adults. We observed a high overlap of shared resources with mysids and gammarids being the most important prey species. However, an ontogenetic shift was found, with isotopic overlaps between the life stages decreasing upstream. Adults mainly fed on prey from the mesohaline and oligohaline sections, but less on upstream freshwater areas. Enriched $\delta^{15}N$ values in the maximum turbidity zone indicated a locally extended food chain which primarily affected juveniles. Our results underline the importance of estuarine habitats serving as nursery and feeding areas for different life stages of migrating fishes. Our study contributes to a better understanding of habitat exploitation by the key species smelt along the estuarine salinity gradient. This knowledge further enhanced our expertise in smelt population dynamics, intraspecific interactions during ontogeny and the importance of estuarine services for migratory fishes.

Keywords: Estuary, food web, stable isotopes, key species, anadromous, nursery area

Chapter 2 -Smelt

Introduction

Estuaries are aquatic transition zones connecting marine and riverine habitats that are accessed by a variety of fish species as part of their life cycle to feed, seek refuge or spawn (Elliot and Hemingway 2002, Thiel 2011). Strong dynamic environmental processes cause high primary production rates (Schelske and Odum 1962, Day et al. 2013) and making them ideal for fish production (Haedrich 1983, Elliot and Hemingway 2002). However, the rapidly changing physico-chemical conditions, particularly the salinity gradient, present challenges for estuarine biota (Whitfield et al. 2022). These environmental conditions lead to generalist feeding strategies of consumers (Mosman et al. 2023), a lower species richness compared to adjacent freshwater and marine habitats (Whitfield and Harrison 2020, Whitfield et al. 2022) and the dominance of a few key species (Whitfield et al. 2022).

The European smelt, *Osmerus eperlanus* (Linnaeus, 1758), represent such a key species in the Elbe estuary (Illing et al. 2024), accounting for up to 96 % of the local fish community (Eick and Thiel 2014). As an anadromous fish, smelt spend their adult life primarily in marine waters and migrate annually to freshwater areas of estuaries to spawn (Kottelat and Freyhof 2007). The spawning migration takes place in late winter between February and March (Borchardt 1988, Thiel and Thiel 2015). Unlike other anadromous fishes, such as twaite shad (*Alosa fallax*) (Magath et al. 2013), mature smelt remain in the estuary after spawning to exploit the habitat´s benefits (e.g., high food supply, less predation) until summer (Borchardt 1988).

Smelt play a critical role as a trophic link between lower and higher trophic levels (Illing et al. 2024) and are also relevant for local fisheries (Eick and Thiel 2014). Due to its anadromous life cycle, smelt use the estuary as a spawning ground, nursery and feeding area (Elliot and Hemingway 2002), hence smelt exploit the estuary in various life stages throughout the year (Eick and Thiel 2014, Eick 2016). During spring, when the food supply peaks in the Elbe estuary (Borchardt 1988, Eick and Thiel 2014), juvenile and adult smelt utilize the same feeding areas. As the salinity gradient and other abiotic conditions (e.g., oxygen, turbidity) shape spatial estuarine community compositions (Henderson 1989, Thiel and Potter 2001, Breine et al. 2011), these local feeding areas differ in their quality and productivity throughout the river course (Selleslagh and Amara 2008).

Ontogenetic niche shifts and habitat exploitation in the smelt genus *Osmerus* have been investigated in landlocked (e.g., Vinni et al. 2004, Vinni et al. 2005, Salujõe et al. 2008, Hammar et al. 2018, Rosinski et al. 2020) and migrating populations (e.g., Franek 1988, Taal et al. 2014). However, these studies often overlooked the spatial characterization of feeding areas and intraspecific features such as movement patterns or dietary preferences. Understanding how smelt exploit resources within the estuary is essential to determine whether intraspecific competition occurs and if distinct strategies for resource use and migrations have developed. This knowledge is critical for understanding population dynamics and promoting the conservation of this key species, especially considering the recent population decline (Illing et al. 2024).

In this study, we applied a combination of stomach content and stable isotope analyses of white muscle tissue to investigate the feeding ecology of European smelt. Stomach content analyses provide direct observations of prey items and their quantities (Pasquaud et al. 2008), offering taxonomic resolution (Lin et al. 2007) and insights into predator-prey relationships, species-specific feeding strategies, and the main trophic pathways of a species (Leclerc et al. 2014, Poiesz et al. 2021). This method offers a snapshot of recently ingested prey (Klarian et al. 2022), complementing stable isotope approaches.

Stable isotope analysis has emerged as a powerful tool to elucidate the functioning of ecological networks and food web structures (Pasquaud et al. 2008). Nitrogen isotope ratios ($\delta^{15}N$) exhibit stepwise per trophic level and thus indicate the trophic position of an organism in an ecosystem (DeNiro and Epstein 1981). In contrast, carbon stable isotope ratios ($\delta^{13}C$) are only little enriched per trophic level but offer insights into the production base of the food web (Peterson and Fry 1987) and potential food source preferences of a species over a longer time frame (Kling et al. 1992, Harvey and Kitchell 2000).

In our study, we used both approaches, as the combination of these methods allows us to investigate whether the snapshots from the stomach contents are representative of the general diet over longer periods of time or whether they only reflect short-term fluctuations in prey availability and the smelt's food preferences (e.g., Nielsen et al. 2018). Goal was to (1) asses general feeding strategies and preferences of juvenile and adult smelt and to (2) examine these findings by analyzing ontogenetic niche shifts, isotopic niche widths, and isotopic niche overlaps along the salinity gradient of the Elbe estuary. In addition, we employed a Bayesian mixing model (3) based on $\delta^{13}C$ and $\delta^{15}N$ data to reveal the habitat use and movement patterns of both life stages.

Our findings contribute to a deeper understanding of habitat exploitation by the estuarine key fish species smelt along the estuarine salinity gradient. This knowledge enhances our understanding of smelt population dynamics, intraspecific interactions during ontogeny, and the importance of estuarine services for migratory fish species.

Material and methods

Sampling

We collected smelt at five fishing stations covering the Elbe estuary from the river mouth to the city of Hamburg (Fig. 2.1). For comparability with previous studies (e.g., Magath and Thiel 2013), we categorized the Elbe estuary into upper, middle, and lower sections, representing the salinity gradient in the area (Fig. 2.1a). Fishing was conducted with a commercial stow net vessel for 3-4 hours during high and low tide at each station from May 31st to June 4th, 2022. The stow net has an opening of 135 m^2 with a mesh size of 10 mm at the cod end. Additionally, a ring net with a mesh size of 1,000 µm, 94 cm diameter and a length of 2.8 m was used to collect potential prey organisms for stable isotope analysis. Standard measurements such as the total length of the fish (in cm) were recorded. Individuals were measured until normal distributions of the life stages were achieved to ensure a representative sample size (Fig 2.1c). Smelt were grouped into juveniles (age group 1: 5.6 -13.4 cm) and adults (age group 2+: length > 13.5 cm) (Fig. 1c) according to Lillelund (1961) (Supplement material Tab. S2.1). Further, we verified the life stages by irregularly examining the maturity stages of the fish.

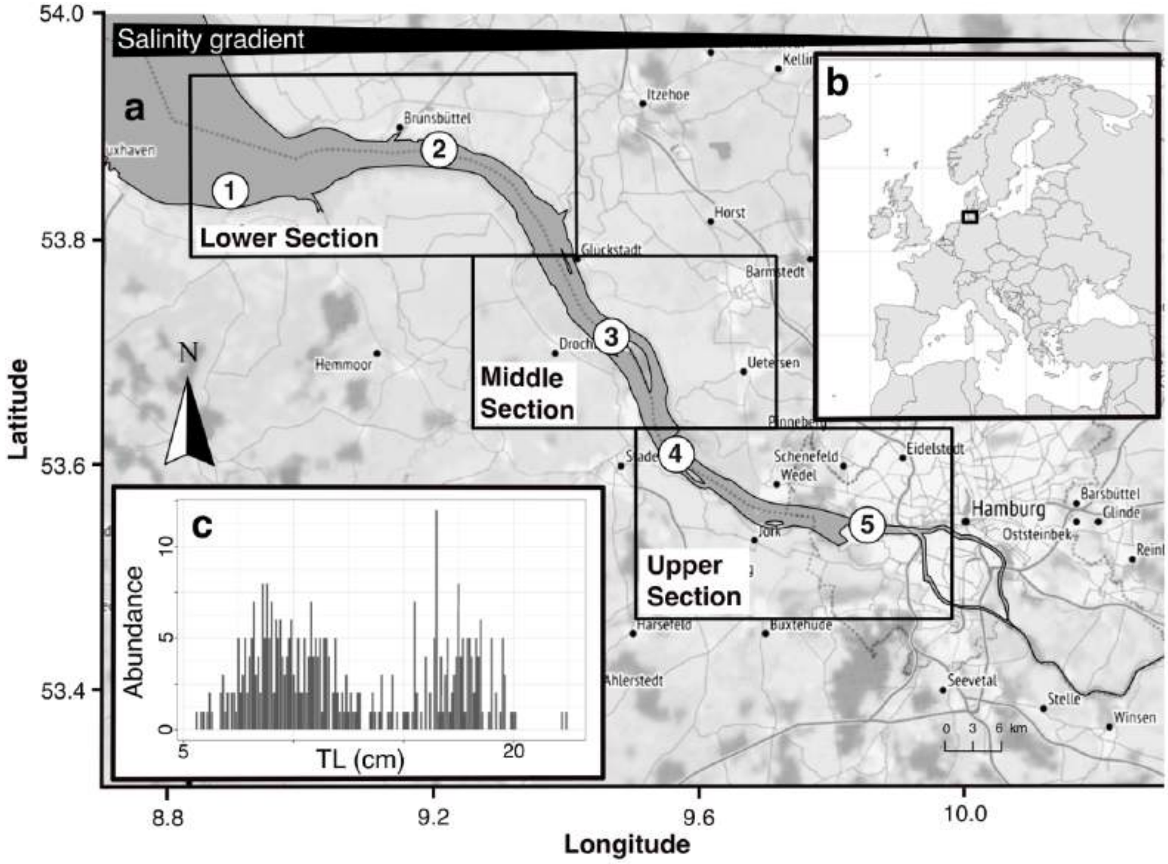

Fig. 2.1 (a) The Elbe estuary with sampling stations (1) Medemgrund, (2) Brunsbüttel, (3) Schwarztonnensand, (4) Twielenfleth and (5) Mühlenberger Loch along the salinity gradient. (b) The study area is located in Central Europe. (c) Barplot shows the abundance and TL = total length (cm) of sampled estuarine smelt *O. eperlanus* indicating the pattern of two occurring age groups.

Stomach content analysis

For stomach content analysis (n = 265) prey organisms were counted, measured, and determined to the lowest possible taxonomic level. Empty stomachs were excluded from the analysis. Highly digested fish that could not be identified properly were categorized as *Pisces*. Fragments of incomplete specimens were photographed and measured using the ImageJ software (Schneider et al. 2012) to reconstruct biomasses using linear length-weight regression models according to Pihl and Rosenberg (1982), Christiansen (1988), Mason (1986), Marsh et al. (1989), Oesmann (1994), Peitsch (1995), Debus and Winkler (1996), Wang and Zauke (2002) and Lindén et al. (2003) (see Supplement Tab. S2.2).

Numbers of fragmented organisms were estimated by counting heads or eyestalks and their biomasses were computed as mean values of intact individuals of the same species. Specimens that were found only very rarely, such as annelids, isopods or cladocerans were grouped into the category *other*.

Relative frequency of the biomass (%W) was calculated for all identified prey species per station and life-stage using the equation (eq.):

$$\%\mathrm{W} = \left(\frac{\sum \mathrm{B_i}}{\sum \mathrm{B_t}}\right) \cdot 100 \qquad \text{Eq. 2.1}$$

where B_i is the stomach content weight of prey $\mathbf{i}$ and B_t the total weight of the stomach content.

Prey-specific biomass (%P_i) is defined as the percentage of a prey taxon i averaged over all investigated fish stomachs in which prey i occurred and can be mathematically expressed as:

$$\%\mathrm{P_i} = \left(\frac{\sum \mathrm{S_i}}{\sum \mathrm{S_{t_i}}}\right) \cdot 100 \qquad \text{Eq. 2.2}$$

where $\sum \mathrm{S_i}$ is the sum of weights of prey item $\mathbf{i}$ and $\sum \mathrm{S_{t_i}}$ the sum of all biomasses in stomachs where prey item i occurred (Amundsen et al. 1996).

Frequency of occurrence (FO_i) was calculated by using the formula:

$$\mathrm{FO_i} = \frac{\mathrm{n_i}}{\mathrm{n}} \qquad \text{Eq. 2.3}$$

where $\mathbf{n_i}$ is the number of stomachs that contain prey $\mathbf{i}$ and $\mathbf{n}$ is the total number of investigated stomachs (Amundsen et al. 1996; Brown et al. 2012).

We visualized prey-specific abundances (P_i) and frequencies of occurrence (FO_i) using modified Costello plots to analyze feeding strategy, generalist-specialist dichotomy and niche width contribution based on stomach content data of juvenile and adult smelt (Amundsen et al. 1996). Although the prey composition changes at the species level due to changing salinity conditions along the estuary, these species can be categorized into superordinate prey taxa. To generalize results of feeding patterns of smelt life stages using modified Costello plots, we therefore grouped the prey species into higher taxonomic levels, i.e. Copepoda, Mysidae, Amphipoda, Caridea, Pisces and *other* taxa.

Stable isotope analysis

White muscle tissue was dissected from the dorsoventral bodyside of each smelt, rinsed with distilled water and subsequently stored at -80 °C on board. In the laboratory, all tissue samples were dried for 24 hours using a freeze dryer and homogenized using a tissue homogenizer and cell lyser. The powdered samples were weighed (0.8 – 1.2 mg) and filled into tin capsules. Stable isotope ratios of ^{13}C and ^{15}N in the samples were measured by the commercial laboratory of 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).

The final data is expressed relative to international standards VPDB (Vienna Pee Dee Belemnite) for carbon and atmospheric air for nitrogen using the delta notation:

$$\delta X = [\left(\frac{R_{Sample}}{R_{Standard}}\right) - 1] \qquad \text{Eq. 2.4}$$

where X is the stable isotope value of C or N in per mille (‰), and R the mass ratio of heavy and light stable isotope ratio ($^{13}C/^{12}C$ and $^{15}N/^{14}N$) for either the standard or the sample.

Statistical analysis

Normality and variance homogeneity of $\delta^{13}C$ and $\delta^{15}N$ ratios were statistically tested using Shapiro-Wilk-tests and variance tests in R. Overall differences in isotopic values between juvenile and adult smelt were determined afterwards using the non-parametric Mann-Whitney U test ($p < 0.05$). All statistical tests were performed using R studio (version 4.0.4, R Studio 2024).

The isotopic niche width among smelt life stages, overall and spatially separated via sections, were estimated using standard isotopic ellipses (‰^{2}) using the SIBER package (Jackson et al. 2011, version 2.1.6). Standard ellipse areas (SEA) were corrected for a small sampling size (SEA_c). Further, we estimated isotopic niche overlap by the standard ellipse function containing ~ 40% of the isotopic data for each smelt group (juvenile vs. adult). A Bayesian standard ellipse was estimated (SEA_b) to measure uncertainty by calculating credible intervals around the individual data (Jackson et al. 2011). Summary statistics of corrected SEA were used to determine confidence intervals (CI) of 95% and 40% for each group. To estimate the overall isotopic niche distribution of juvenile and adult smelt we calculated Layman metrics total area (TA), centroid (CD), nearest neighborhood distance NND, and standard deviation of NND (SDNND) of each investigated group (Layman et al. 2007).

Proportions of dietary isotope origins and spatial resource preferences of juvenile and adult smelt were determined using the SIMMR package (Parnell and Inger 2016, version 0.4.5) which is based on a Bayesian mixing model (JAGS – Just Another Gibbs Sampler, Plummer 2003). We used $\delta^{13}C$ and $\delta^{15}N$ data of smelt and their prey, concentrations of C (%) and N (%) of the selected prey organisms, including trophic enrichment factors (TEF) and smelt life stages to run the model. SIMMR applies Gaussian likelihood and fits the data to the model using Markov chain Monte Carlo (MCMC) (Parnell and Inger 2023). TEF were set to 0.7‰ for $\delta^{13}C$ and 3.0‰ for $\delta^{15}N$ per trophic level (Sweeting et al. 2007a, b, Ankjærø et al. 2012).

As SIMMR can only handle one model at a time, we analyzed each section of the estuary separately for smelt age groups and finally compared the estimated proportions of all life stages and sections using the *compare_sources* function. The more prey organisms are fed into the model, the more it leads to an overall depletion of the estimated proportions as the model is not able to distinguish between the sources. To avoid this effect, we chose the top three prey species per section that occurred most in terms of frequency and biomass based on stomach content and isotopic data. To generate isotopic information of zooplankton, we pooled mesozooplankton *Daphnia* spec. and calanoid copepod *Eurytemora affinis* from the upper section of the estuary, which are similar in their isotopic compositions and occurring simultaneously in this area (e.g., Riedel-Lorjé et al. 1998). For Clupeidae we used isotopic values from the literature of young-of-the-year *Sprattus sprattus* from the southern North Sea by Das et al. (2003). Finally, we summarized the proportions calculated by the model per section to determine the spatial isotopic source origin.

Results

Stomach content analysis

We initially used stomach content analysis to identify the recent feeding history of smelt in the Elbe estuary (Fig. 2.2). Modified Costello plots demonstrate an overall mixed feeding strategy with varying degrees of generalization and specialization on different prey groups (Fig. 2.2a). We found the diet of juvenile smelt to be dominated by Mysidae (*Neomysis integer* and *Mesopodopsis slabberi*) and to a lesser degree on Amphipoda (Gammaridae and *Corophium volutator*). Rare taxa in juvenile smelt diets were Caridea (*Crangon crangon* and *Palaemon longirostris*), Copepoda and *other* group. Fish was rarely identified in stomachs of juvenile smelts but occurred occasionally in high abundances pointing towards a high-between-individual-variability. Compared to juvenile smelt, adults consumed fish more frequently, being the dominant food item together with Amphipoda. In addition, the diet of adult smelt was observed to contain less Mysidae than that of juveniles and only rarely Copepoda or prey assigned to the other prey group. Comparing the diets of smelt along the salinity gradient revealed the dominance of Mysidae (*N. integer* and *M. slabberi*) and Amphipoda (Gammaridae and *C. volutator*) in stomachs of juveniles to exist at all stations but the “Mühlenberger Loch” (station 5 in the upper section, Fig. 2.2b). At this least saline sampling station fish was dominating the diet of both juvenile and adult smelt. In contrast to the juvenile conspecifics, fish was also dominating the diet of adult smelt in the lower section of the Elbe estuary where highest salinities prevail. Here, the fish diet constituted mostly of Clupeidae, Gobiidae and smelt itself (i.e. cannibalism). Caridea (*C. crangon* and *P. longirostris*) was particularly found in smelt´s stomachs from the lower and middle sections in both life stages.

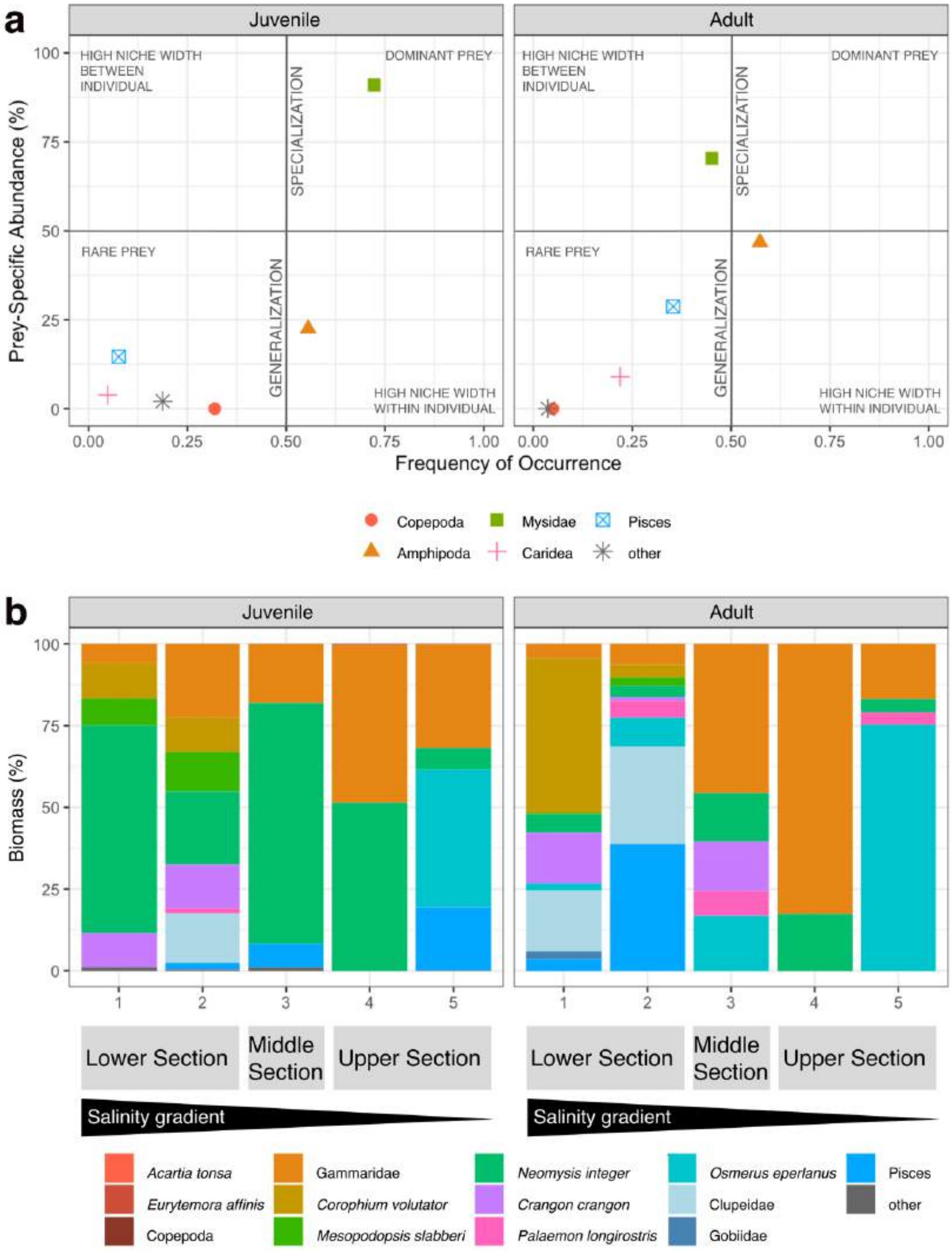

Fig. 2.2 Feeding preferences based on stomach contents of juvenile and adult smelt. (a) Modified Costello plots using prey taxa and averaged over sampling stations, and (b) percentage of biomass of prey species from smelt caught along the salinity gradient of the Elbe estuary.

Stable isotope analysis

Ontogenetic differences in isotopic niches

We first explored differences in stable isotope ratios between juvenile and adult smelt integrated over the entire sampling area in the Elbe estuary. We found juvenile smelts to have significantly lower $\delta^{13}C$ values compared to adults (Mann-Whitney U test: W = 7112.5, $p < 0.01$) (Fig. 2.3a, Tab. 2.1). The $\delta^{15}N$ values of juveniles were only slightly, but significantly lower (Mann-Whitney U test: W = 13418, $p < 0.01$) than of adults. Next, we analyzed the overlap in isotopic niches using standard ellipse areas (SEA_c) based on both isotope ratios together. We found a similar isotopic composition between both groups with an isotopic niche overlap of 16.4% (40% CI) of the SEA_c or 52.4% with 95% CI (Fig. 2.3b).

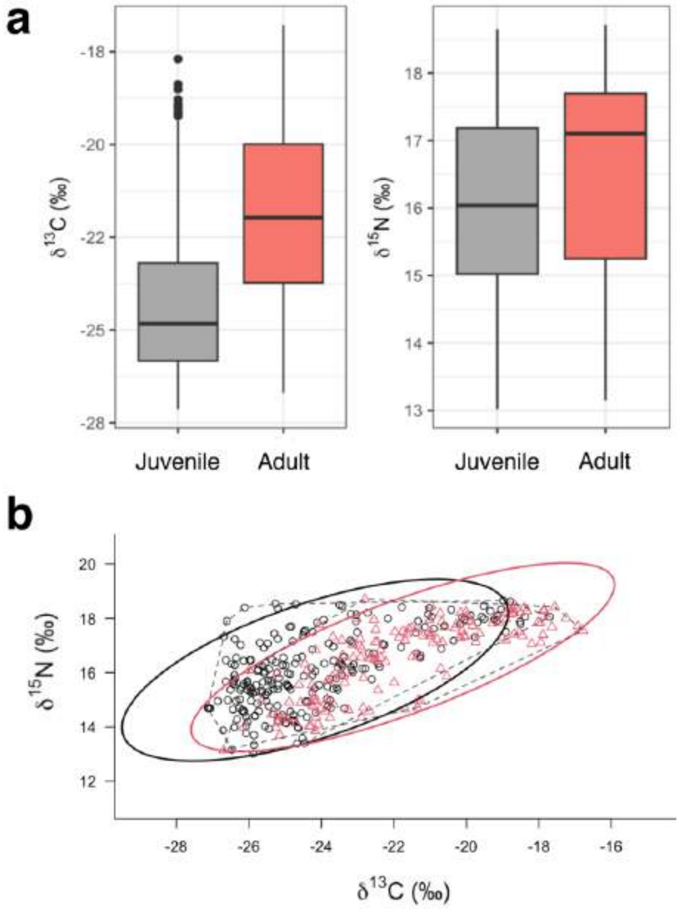

Fig. 2.3 Isotopic niches in juvenile and adult smelt aggregated over the Elbe estuary. (**a**) Boxplots with median as well as upper and lower quartiles of $\delta^{13}C$ and $\delta^{15}N$ of juveniles and adults. (**b**) Biplot of $\delta^{13}C$ and $\delta^{15}N$ ratios of juveniles (black circles) and adults (red triangles) with overall 95% CI ellipses (solid lines) and total area (TA) (dashed lines) of the isotopes.

In addition, we analyzed the spatial variation in isotopic niche overlap between juvenile and adult smelt along the salinity gradient of the Elbe estuary (Fig. 2.4). We found a pronounced decrease in isotopic niche overlap from mesohaline to freshwater conditions (Fig. 2.4a). In the lower, high saline section, juveniles showed a broader range of δ^{13}C values. Furthermore, adults had higher δ^{15}N values, except in the middle section. Standard ellipse areas (SEA_c) from adult smelt caught at the middle and lower section of the Elbe estuary showed overall highest variation in isotopic ratios and revealed a broader isotopic niche width compared to its juvenile conspecifics (Fig 2.4b). However, in the middle section the large SEA_c and total area (TA) were mainly affected by three individuals containing low δ^{13}C values, potentially derived from marine carbon pools (e.g., Peterson and Fry 1987). These outliers influenced markedly the isotopic niche width of adults from the middle section but had less effect on the isotopic niche overlap between the life stages (isotopic niche overlap with outliers: 9.0% vs. without outliers: 10.6%).

Low values of mean nearest neighbor distances (NND) and standard deviation of NND (SDNND) revealed similar trophic ecologies of the life stages and an even distribution of trophic niches. However, these metrics differed slightly between the life stages. Juveniles generally showed smaller mean nearest neighbor distances (NND) and standard deviation of NND (SDNND) than adults, except for the lower section. Highest NND was estimated for adults from the upper and middle section.
In the middle and upper sections of the estuary both juvenile and adult smelts are well-separated in both δ^{13}C and δ^{15}N values. Further analyses of SEA_c indicated that in the lower, high saline section of the estuary, both life stages have a similar isotopic niche width (Fig. 2.4b, Tab. 2.1). Towards the middle and upper sections adults have increasingly large ellipse areas than juveniles, indicating a broader isotopic niche.
Overall, our analysis revealed distinct isotopic niches for juveniles and adults along the salinity gradient, showing greater dietary and/or habitat diversity, especially in the lower region.

Tab. 2.1 Characterization of isotopic niche width of smelt life stages on a general (aggregated) and spatial scale (lower, middle and upper sections) using Layman metrics (TA = total area, NND = nearest neighborhood distance, SDNND = standard deviation of NND) and SIBER calculations of standard ellipse areas (SEA_c = standard ellipse area with correction) with n = sample size.

Section	Life-stage	n	δ^{13}C (‰)	δ^{15}N (‰)	δ^{13}C range	δ^{15}N range	TA	NND	SDNND	SEA_c (‰^{2})
Aggregated	Juvenile	227	-24.2 ± 2.2	16.1 ± 1.4	9.4	5.6	31.5	0.17	0.13	7.3
	Adult	147	-21.8 ± 2.4	16.6 ± 1.4	9.9	5.6	29.2	0.23	0.16	6.8
Lower	Juvenile	95	-22.8 ± 2.4	16.5 ± 1.3	8.9	4.9	21.6	0.26	0.19	5.3
	Adult	100	-20.9 ± 2.1	17.0 ± 1.2	8.2	5.0	22.8	0.25	0.19	5.7
Middle	Juvenile	67	-25.0 ± 1.4	16.2 ± 1.5	5.9	5.4	22.3	0.32	0.24	5.4
	Adult	26	-23.4 ± 2.5	15.6 ± 1.4	9.4	4.1	20.9	0.52	0.27	7.8
Upper	Juvenile	65	-25.4 ± 0.9	15.4 ± 1.1	3.6	5.4	13.6	0.25	0.21	3.2
	Adult	21	-23.7 ± 1.4	15.5 ± 1.2	5.4	4.8	12.1	0.61	0.54	4.0

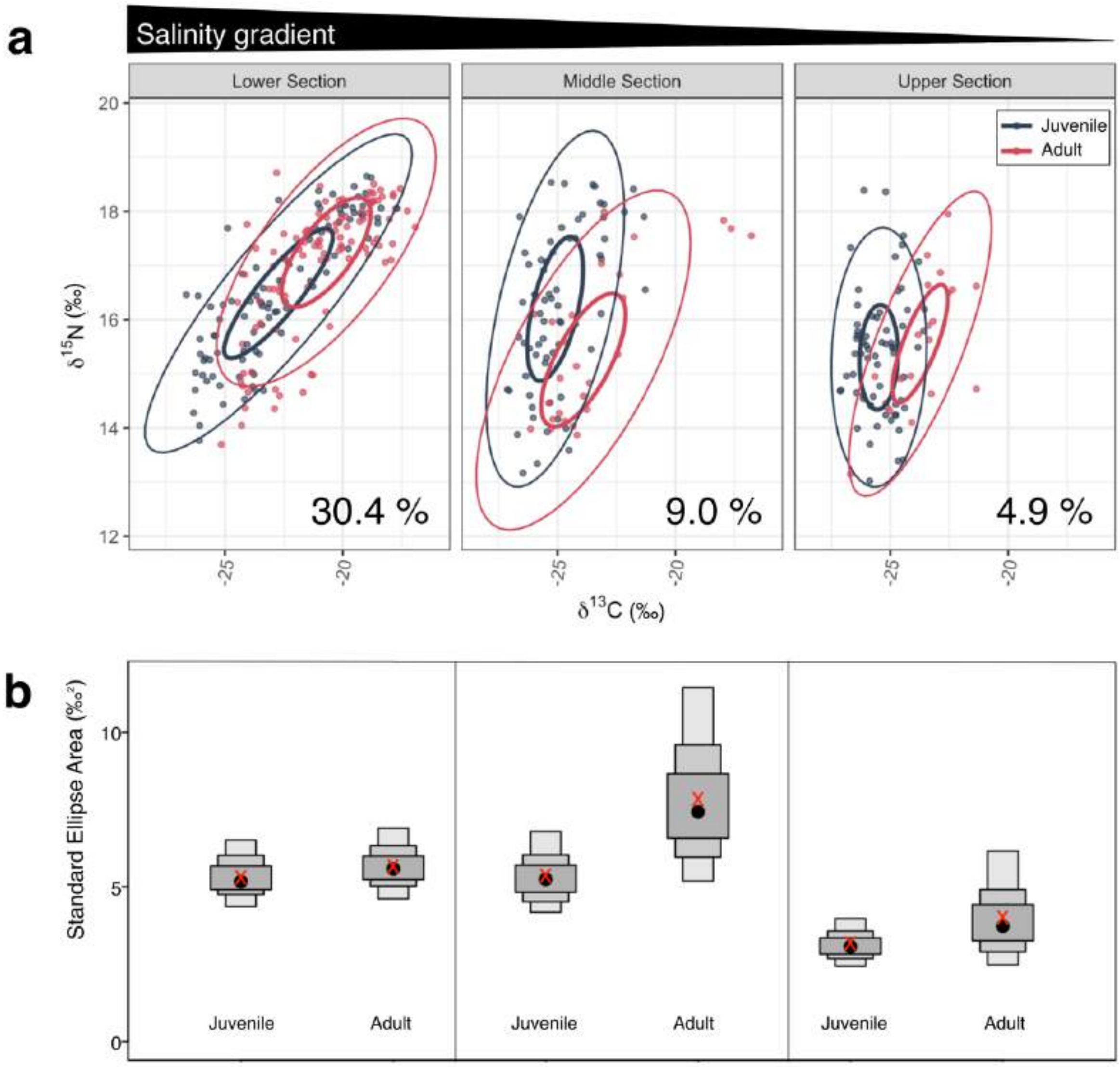

Fig. 2.4 Spatial variation in ontogenetic niches of juvenile and adult smelt along the salinity gradient. (a) Overlap of feeding niches across sections with standard ellipse area of 95% (outer ellipse) and 40% (inner ellipse) confidence intervals (CI). Overlaps were estimated using the 40% CI of both life stages. Percentages indicate overlap between isotopic niches (b) Density plots of SEA$_c$ (‰^{2}) per section based on stable isotope ratios of δ^{13}C and δ^{15}N. Grey shaded areas show 95%, 75% and 50% confidence intervals with black dots represent the mode values and red crosses the corrected value.

Spatial origin of food sources

We used Bayesian mixing models to explore the spatial origin of diet-derived carbon and nitrogen in juvenile and adult smelt. The main food items (identified through stomach content analysis and stable isotope analysis) in models for each section along the salinity gradient were used separately (see material and methods section). Isoplots showed a generally good alignment of $\delta^{13}C$ and $\delta^{15}N$ of smelt and its food sources (Fig. 2.5a). We furthermore observed higher $\delta^{13}C$ in smelt individuals (both juvenile and adult) from the lower sections, compared to the middle and upper sections, indicating local marine habitat use. The difference between the lower section and the mostly freshwater sections is also demonstrated by the proportions of the main food components found in smelt (Fig. 2.5b). Juvenile and adult smelt from the lower section have both elevated proportions of Clupeidae and the amphipod *C. volutator* in their isotopic signatures. Adult smelt from the middle and upper sections on the other hand displayed higher values in Gammaridae compared to those from the lower section (while no difference in juveniles was found). Eventually, the mixing model for the upper section demonstrates the importance of zooplanktonic food that they feed upon in the middle and upper sections.

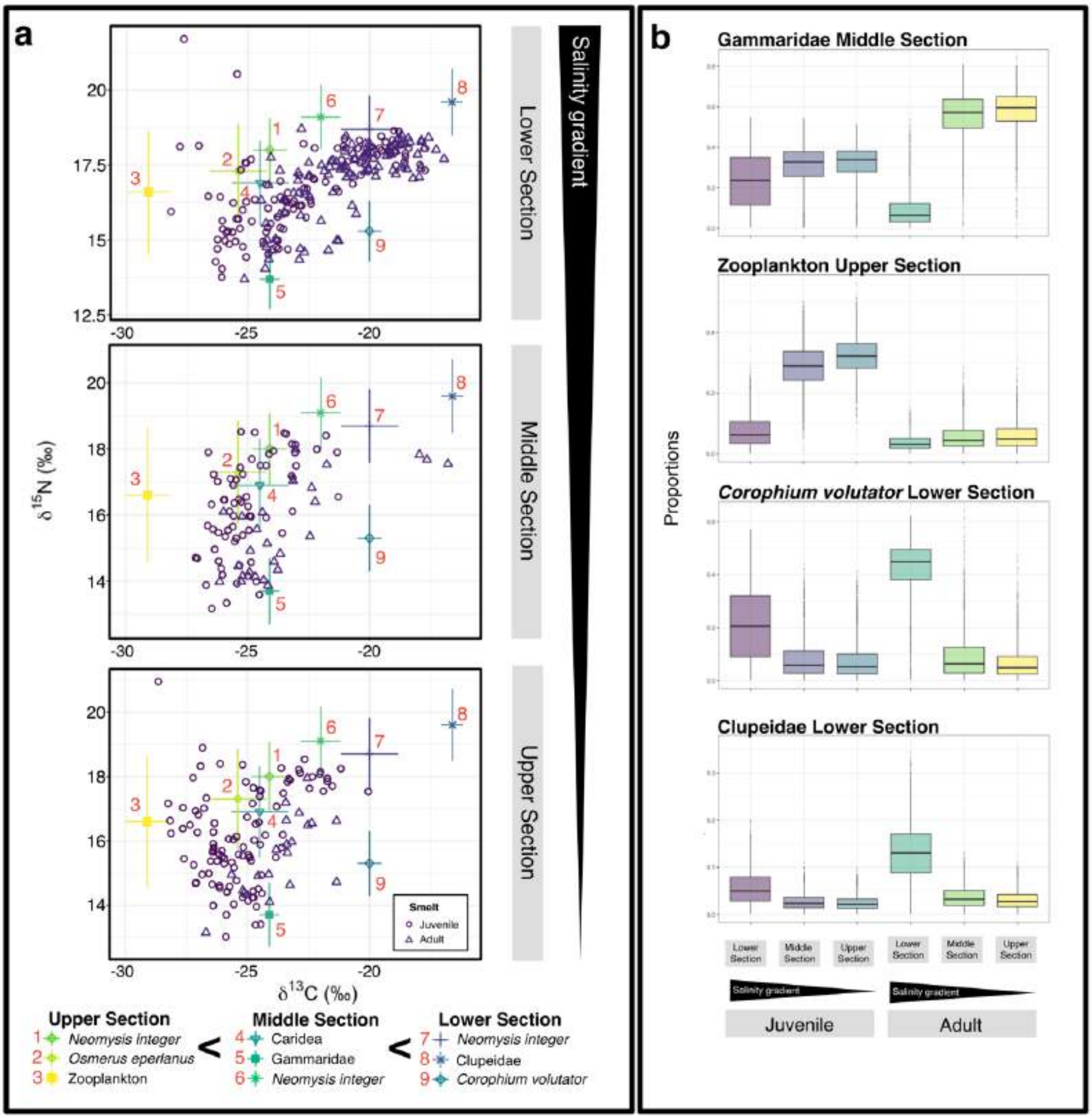

Fig. 2.5 Spatial feeding trends in smelt life stages using SIMMR output. (**a**) Isoplot of smelt (dots = juveniles, triangles = adults) from distinct sections with isotopic information of prey (containing TEF) from lower, middle and upper sections. Prey items (1-9) (red) differed in their isotopic values, ranging from low freshwater-derived sources in the upper section to high values derived from oligohaline/marine sources in the river mouth/lower section. (**b**) Proportions of most frequently assimilated prey type based on $\delta^{13}C$ and $\delta^{15}N$. Prey type of the respective section is shown above each panel.

We explored mixing models for the three spatial sections including both life stages (Fig. 2.6). Proportions of food items are summed according to the section where smelt were sampled. The result confirms the spatial feeding patterns that were observed. Adult smelt from the middle and upper sections mostly feed in the middle section (highest aggregated percentages) and mainly rely on Gammaridae. In contrast, adult smelt from the lower section mainly fed locally with the highest proportion of the amphipod *C. volutator*. A similar pattern appeared for juvenile smelt where individuals caught at the middle and upper sections mainly consumed local food, i.e. Gammaridae in the middle section and zooplankton in the upper section. Juveniles from the lower section derived their food mainly from the middle section, the lower section, relying on Gammaridae and *C. volutator*, respectively.

We estimated the highest proportions derived from local and upstream areas for juveniles, especially from the lower and middle sections (Fig. 2.6). We observed the highest variation in stable isotope estimated proportions in adult smelt caught at the lower section. In addition, adults further fed frequently on food derived in the middle but less on prey from the upper section.

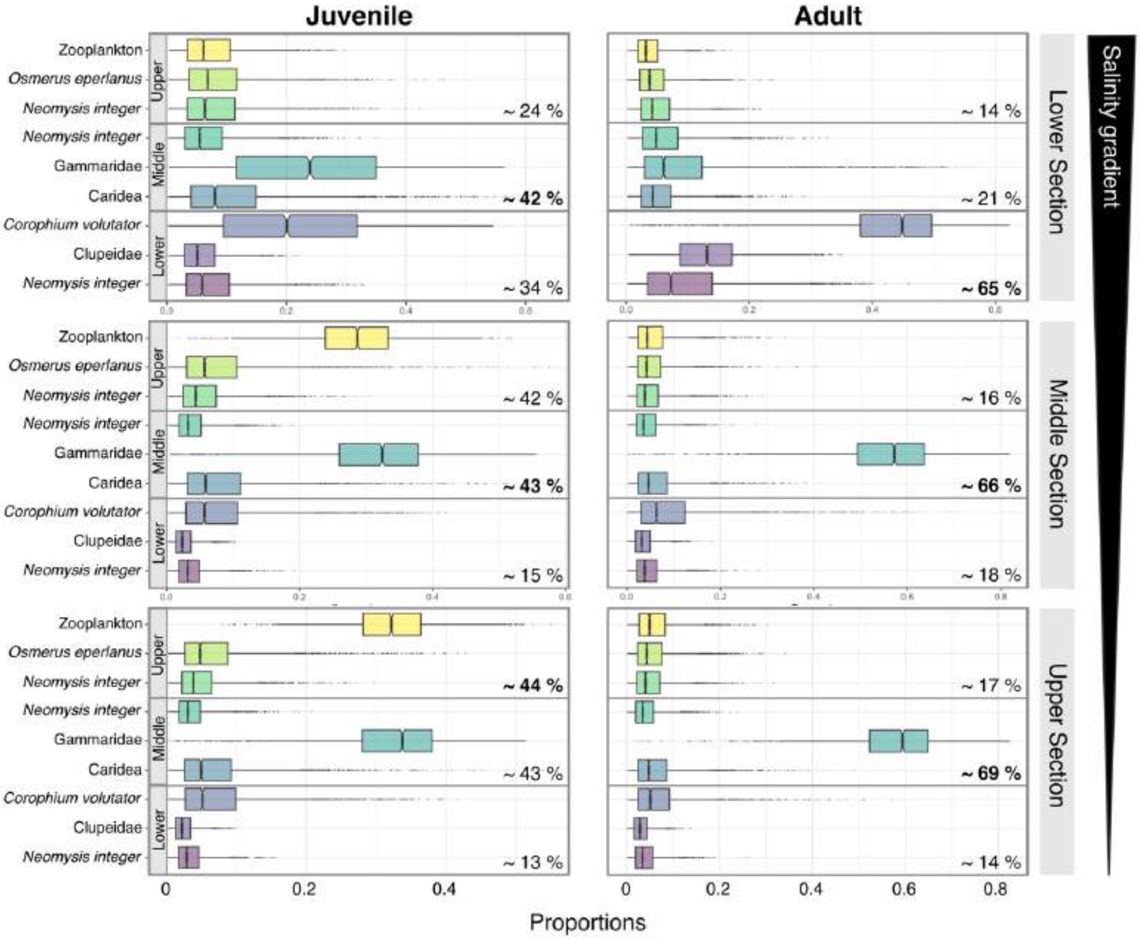

Fig. 2.6 Estimated proportions (%) revealed smelt movements in the Elbe estuary. Credibility interval plot of proportions of prey sources per section based on SIMMR for juvenile (left) and adult smelts (right) caught at lower, middle and upper sections along the salinity gradient. Proportions of prey groups were summed up per section to verify main feeding location of juvenile and adult smelt.

Discussion

Our study provides valuable insights into the feeding ecology and seasonal habitat exploitation of the European smelt (*Osmerus eperlanus*), a key species in the Elbe estuary. By utilizing stable isotope-based Bayesian mixing models of $\delta^{13}C$ and $\delta^{15}N$, along with stomach content analyses, we were able to assess both short-term and long-term dietary preferences, ontogenetic niche shifts and isotopic niche widths. This integrated approach allowed us to draw conclusions about spatial resource uses and migrations of smelt.

Diet composition reflect salinity regime

Stomach data provide a snapshot of a fish´s recent diet, whereas stable isotopes reflect the diet assimilated into the consumer´s muscle tissue over a longer time period (Harvey and Kitchell 2000), depending on individual growth and metabolic rates (Buchheister and Latour 2010). Integrating these methods reduces uncertainties (Nielsen et al. 2018) and enhances the interpretation of food web datasets (Layman et al. 2005, Davis et al. 2012). In our study, this approach enabled us for a more comprehensive characterization of smelt feeding ecology over a longer period of time.

Our analyses revealed that smelt from the river mouth exhibited a more diverse diet compared to those from the middle and upper sections of the Elbe estuary. Juvenile smelt in the lower section primarily consumed Mysidae species (*M. slabberi* and *N. integer*), whereas adults mainly fed on marine organisms such as clupeid larvae and the amphipod *C. volutator*. The calculated proportions of clupeids and *C. volutator* similarly showed increased assimilation of isotopes from these prey organisms in smelt tissue, especially in those of adult specimens. The isotope proportions of juveniles in the middle and upper estuary revealed that zooplankton from freshwater and gammarids from the middle of the estuary made up major components of the diet. In contrast, zooplankton appears to play a subordinate role as a food source for adults. Interestingly, zooplankton was nearly absent from the stomach contents of both life stages, despite being a significant component of the isotopic derived diet of juveniles. This discrepancy likely reflects an ontogenetic shift in diet, where juveniles switch from small prey organisms to larger prey with increasing growth (e.g., Franek 1988, Rochard and Elie 1994, Vinni et al. 2004, Taal et al. 2014). However, the absence of copepods in the stomachs of juvenile smelt could also be a consequence of fast digestion or of the high feeding pressure on copepods, making a switch to another prey source likely. Rapid depletion in copepod densities due to increased predation by fish has repeatedly been observed in the Elbe estuary (Thiel 2011).

Notably, at freshwater station Mühlenberger Loch (station 5), smelt larvae made up the largest proportion of the diet of juveniles and adults. Vinni et al. (2004) further demonstrated that smelt already show cannibalistic preferences at a total length of 7.8 cm, which promote higher growth rates in young fish.

To explore the ontogenetic shift from smaller to larger prey, we used standard ellipse areas (SEA_c) to analyze stable isotope data from juveniles and adults (see Jackson et al. 2011). We observed a significant shift in $\delta^{13}C$ and $\delta^{15}N$ values between the two life stages, though the isotopic niche shift was minor due to the high amount of shared prey organisms, such as mysids and amphipods. The analysis of isotopic overlaps on a spatial scale along the salinity gradient indicated that the overall pattern was mainly influenced by isotope data from the river mouth, with ellipses shifting and overlap reducing towards freshwater conditions. This suggests that juveniles and adults may be feeding on prey from different $\delta^{13}C$ pools in the more freshwater sections.

To account for potential outliers, we reanalyzed the isotopic niche overlap in the middle section, excluding adults with marine-derived $\delta^{13}C$ values ($n = 3$). The resulting overlap of 10.6% was similar to the previous calculation of 9.0%, though the isotopic niche width decreased significantly from 7.5‰^{2} to 3.8‰^{2} without outliers. This narrowing of the isotopic niche suggests that migratory smelt contribute to isotopic variability and, consequently, to isotopic niche width.

Elongated food chain in middle estuary

A unique feature of the middle section was the pronounced accumulation of $\delta^{15}N$ in juvenile smelt, which did not occur in other sections. Typically, larger prey organisms of higher trophic levels are found in adult´s stomachs, leading to higher $\delta^{15}N$ values (DeNiro and Epstein 1981, Fry 1988). However, the elevated $\delta^{15}N$ in juveniles here might be caused by anthropogenic influences such as wastewater (McClelland et al. 1997, Donázar-Aramendía et al. 2019), sewage treatment plants (Wayland and Hobson 2001, Cole et al. 2004, Morrissey et al. 2012) or agricultural runoff (Heaton 1986), all of which can increase nitrogen loads in the food web and further increasing $\delta^{15}N$ values (Cabana and Rasmussen 1996, McClelland et al. 1997, Cole et al. 2004). Sanders et al. (2018), however, identified nitrification hot spots in the Hamburg port area and the overall increased nitrate concentration that increased towards inland but did not measure high concentrations at the middle section of the Elbe estuary. Hence, the observed $\delta^{15}N$ enrichment may have other causes here.

The middle section, located within the maximum turbidity zone (MTZ) of the Elbe estuary, is characterized by high variation in salinities, turbid waters and nutrient fluxes from both upstream and downstream, driven by river runoff and the tidal inflow from the North Sea, respectively (Kamjunke et al. 2023). These conditions create a challenging environment that affects the entire aquatic food chain (Mosman et al. 2023). Limited food supply and mixotrophic feeding strategies in this area (Martens et al. 2024) likely contribute to the extended food chain, where mesozooplankton such as the copepod *Eurytemora affinis* switch to a more carnivorous diet (e.g., increased consumption of rotifers) under low food availability (Modéran et al. 2012). This extended food chain could lead to elevated $\delta^{15}N$ values in juvenile smelt (Layman et al. 2007, Biederbick et al. *unpubl.*, Chapter 4), indicating a reliance on local food supply and environmental conditions, while adults may avoid areas with low food availability or unfavorable environmental conditions (e.g., oxygen minimum zones) (Thiel et al. 1995).

Spatial habitat exploitation and movements

Our Bayesian models, applied separately to each fishing section for juveniles and adults, revealed major differences in dietary proportions between the river mouth and the more freshwater sections. Juveniles in the river mouth primarily assimilated prey from the middle and lower sections, while adults fed mainly within this region. In contrast, in the middle and upper sections, juveniles relied more on local and upstream food sources, while adults primarily consumed prey from the middle section. Due to the high proportions estimated from local and further upstream derived food sources in juvenile smelt, we conclude that their movements are potentially rather tidal depended. Our findings reveal distinct habitat exploitation patterns and migration behaviors in juvenile and adult smelt. However, due to the variability of food organisms in the respective sections, we cannot be completely sure that we have accounted for all important food sources. Still, our models offer robust insights into the spatial feeding dynamics of smelt across the estuary.

Juvenile and adult prey competition

In summary, our results enhance the understanding of the feeding ecology and seasonal habitat exploitation of smelt in the Elbe estuary. These insights could be applicable to trophic dynamics in other cool-temperate estuaries dominated by a key fish species.

During our sampling in May and June, both juveniles and adults were present throughout the study area, displaying distinct habitat exploitation strategies, as discussed above. Adults primarily fed in the river mouth and in the middle section of the Elbe estuary, while juveniles exploited local food sources. Nonetheless, we observed a large overlap of prey organisms, probably indicating intraspecific competition between the two life stages. As our findings illustrate the ability of adult smelts to leave areas with insufficient habitat conditions, the results promote a strategy of avoiding intraspecific competition when food supply is low. Additionally, estuaries are generally characterized by high biomass production and low species diversity (Platell et al. 2006), which consequently forces exploitation of the same prey species by consumers. We therefore assume that the influence of competition within the species is rather low. Previous studies showed that the opportunistic feeding of smelt generally correlates with the food availability of a few prey species that occur in high biomass rates (e.g., Popov 2006, Taal et al. 2014) which further fluctuate on a temporal and spatial scale (Pothoven et al. 2009, Taal et al. 2014).

In spring, high productivity rates (e.g., plankton blooms, high zooplankton densities) are particularly notable in the Mühlenberger Loch (station 5) and the adjacent Hahnöfer Nebenelbe, areas recognized as important nursery habitats for local fish species, especially smelt (Thiel et al. 1995, Thiel 2001). Thiel et al. (1995) observed here the highest smelt larvae densities especially in May. These areas are part of the Natura 2000 protected area network (Fricke et al. 2021) and are characterized by large biomasses of *Eurytemora affinis* in spring (Köpcke 2002). This copepod therefore serves as a key prey species for juvenile fish with increasing importance towards inland (Thiel 2001). The high predation pressure on mesozooplankton in these areas often leads to a top-down induced decline in copepod biomass during spring (Köpcke 2002, Thiel 2011), prompting a shift to cannibalistic feeding behavior in juvenile smelt. This dietary shift is supported by our analyses, which reveal a transition from zooplankton to cannibalism as a favored feeding strategy in juvenile smelt during this period. Our findings underscore the importance of the Mühlenberger Loch as both a nursery and feeding ground for various smelt life stages (Thiel et al. 1995, Thiel 2001), highlighting its crucial role in the local estuarine food web.

Conclusion

The findings of our study underline the importance of estuaries serving as essential nursery and feeding habitats for fish. Here we show that the feeding ecology of the key species smelt *Osmerus eperlanus* changes during its life cycle, as a result of changing diet during ontogeny and increasing movement ability in the adult stage. Juveniles showed a strong dependence on the local food supply, suggesting that they are more exposed to the prevailing environmental conditions than adults. Along the salinity gradient we observed increasing utilization of distinct resources in juveniles and adults highlighting increasing ecological differentiation between the life stages as salinity increases.

Due to its high biomass rates, smelt inhabits a key function in the Elbe estuary, serving as an important nutrient source for other fish, birds, and marine mammals (Taal et al. 2014, McCarthy et al. 2019). In northern Europe, anadromous smelt populations show an overall depletion (McCarthy et al. 2019), so does the Elbe smelt population in recent years (Illing et al. 2023, chapter 6). Smelt population decline can have multiple reasons, such as low food availability and impairment or even loss of suitable spawning and nursery habitats (Sendek and Bogdanov 2019). This study particularly emphasizes the relevance of intact nursery areas for the recruitment success of this important species. Based on our results, we conclude that particularly shallow areas are crucial for the development and growth of juvenile smelt and should be further considered as part of conservation strategies to protect the fish community in the Elbe estuary.

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Author contributions

EH: Writing – original draft, Writing – review and editing, Visualization, Methodology, Investigation, Formal Analysis, Conceptualization. JB: Writing – review and editing, SF: Writing – review and editing, Validation, Methodology, Conceptualization, RK: Writing – review and editing, , work (methodology), JT: Writing – review and editing, work (methodology), AF: funding acquisition, writing – review and editing, RT: funding acquisition, writing – review and editing, Kai Jensen: funding acquisition, writing – review and editing, PG: Writing – review and editing, Methodology, Formal Analysis, Conceptualization, Supervision, CM: Writing - review and editing, Conceptualization, Supervision, Funding acquisition.

Supplements

Tab. S2.1 Information on number of juvenile and adult smelt individuals caught at stations 1-5.

Section	Station number	Station	Juvenile (n)	Adult (n)
Lower	1	Medemgrund	60	49
	2	Brunsbüttel	56	50
Middle	3	Schwarztonnensand	68	25
Upper	4	Twielenfleth	43	4
	5	Mühlenberger Loch	36	13

Tab. S2.2 List of regressions to reconstruct prey biomasses based on measured fragments found in stomach contents of smelt (EXP= e^ function)

	Fragment	Regression TL (mm)	Regression weight (g)	Literatur
Pisces				
Osmerus eperlanus	Otolith diameter (OTO)	TL=-16.634+39.248*OTO		Debus and Winkler 1996
Osmerus eperlanus	Lower jaw (LJ)	TL=7.7098*LJ+4.7067		Debus and Winkler 1996
Pomatoschistus microps	TL		W = EXP(3.607 * ln(TL) + ln(0.0002))	Pihl and Rosenberg 1982
Caridea				
Crangon crangon	TL		W = EXP(2.84 * ln(TL) + ln(0.3603))	Pihl and Rosenberg 1982
Amphipoda				
Corophium volutator	Head diameter (H)		W=EXP((0.36+0.35*H)/10)	Mason 1986
Gammarus zaddachi	TL		W=0.0029×TL^ 2.88 *0.001	Wang and Zauke 2002
Mysidae				
Mesopodopsis slabberi	TL		W = 0.0000135*TL^2.744	Oesmann 1994
Neomysis integer	Eye	TL= -4.479+29.988*Eye	W=0.0000022715*TL3.46	Debus and Winkler 1996
Neomysis integer	Telson (TEL)	TL=1.6125+TEL*6.25		Debus and Winkler 1996
Neomysis integer	Standard		0.0035	Debus and Winkler 1996
Neomysis integer	TL		W=0.00000283*TL3.15	Debus and Winkler 1996
Neomysis integer	Standard	1.82 ± 0.19 cm	5.82±1.84 mg	Lindén et al. 2003
Copepoda				
Eurytemora affinis	TL		W=12.9*TL^2.92*0.000001	Christiansen 1988, Peitsch 1995
Others				
Annelida	Width, Length		W = -49.509 + 0.280 (Width)^2 + 9.205 (Length)^2	Marsh et al. 1989

Chapter 3

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

Fishing boat "Frida"
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Chapter 3 – Tracking fish life cycles

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

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Abstract

Estuaries are favored 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 $\delta^{13}C$ and $\delta^{15}N$. 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 cycles in estuaries. Our study further illustrates that migration patterns of fish using estuaries within their life 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

Chapter 3 – Tracking fish life cycles

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, a phenomenon, known as the *barrier effect* (Whitfield 2021). 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 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 shifts 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. 2024b, 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.

Material and methods

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).

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.

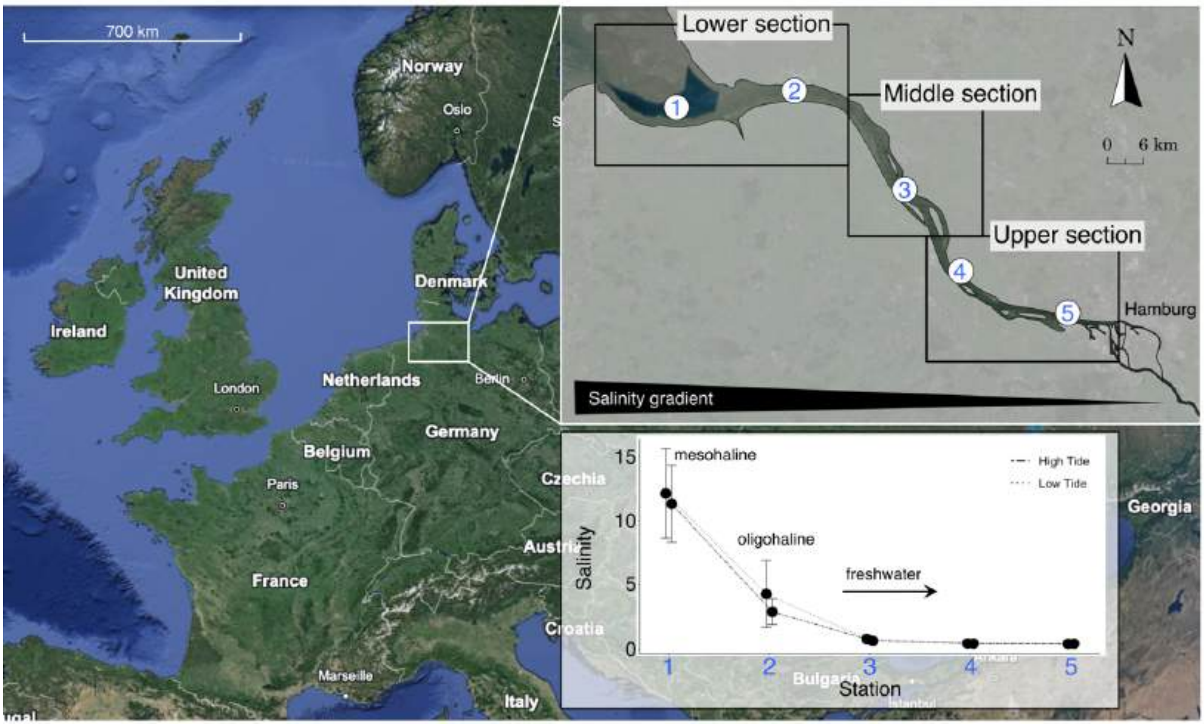

Fig. 3.1 The Elbe estuary in Northern Europe with sampling stations (1) Medemgrund, (2) Brunsbüttel, (3) Schwarztonnensand, (4) Twielenfleth and (5) Mühlenberger Loch and sections reflecting the salinity gradient of the main channel towards the city of Hamburg (map was created using Google Earth, 2024).

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:

$$\delta X = [\left(\frac{R_{Sample}}{R_{Standard}}\right) - 1] \qquad \text{Eq. 3.1}$$

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:

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

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 standard ellipses of $\delta^{13}C$ (40% CI) 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) and tidyverse (version 1.3.1).

Results

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 δ^{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 δ^{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 δ^{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) δ^{13}C signal.

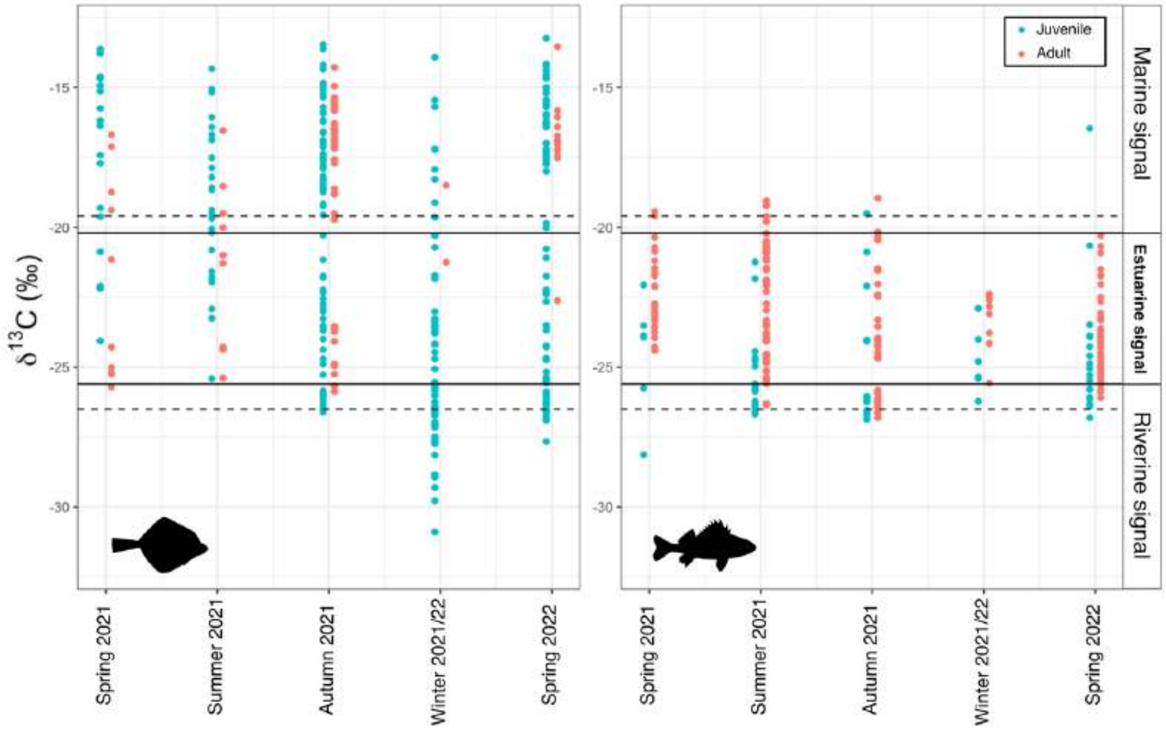

Fig. 3.2 δ^{13}C of European flounder and ruffe at different life stages (juvenile, adult) from spring 2021 to spring 2022. Black horizontal lines show predicted estuarine δ^{13}C signal in fish and SD (dashed lines) estimated from the δ^{13}C of mysids caught at station 1 (border to the sea) and station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

Ontogenetic niche shift

We did not find a clear relationship between fish length and isotope values in the flounders. For clarity, however, we have split the δ^{13}C data according to life stages. 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.

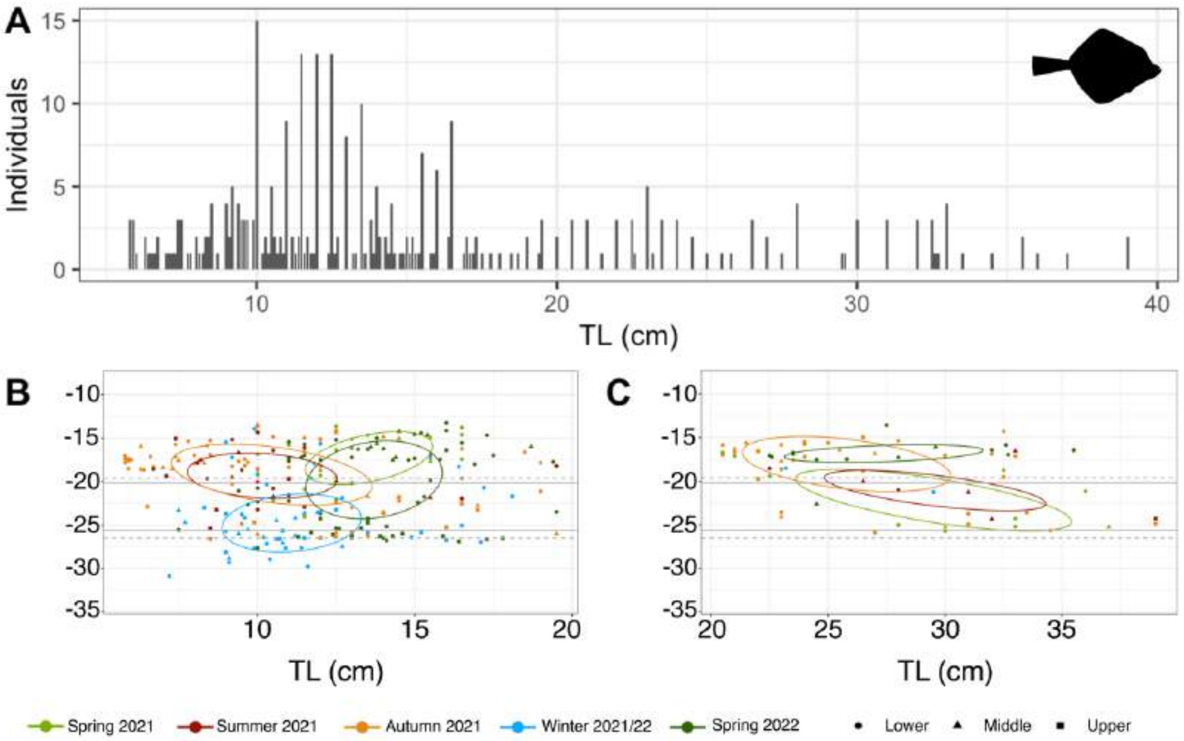

Fig. 3.3 (**A**) Visualization of measured total length (TL) in cm of flounder per number of caught individuals (see Tab. S3.1). (**B**) $\delta^{13}C$ ratios vs. TL of juveniles and (C) adults with standard ellipses (~40% CI) per season. Grey horizontal lines show predicted estuarine $\delta^{13}C$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

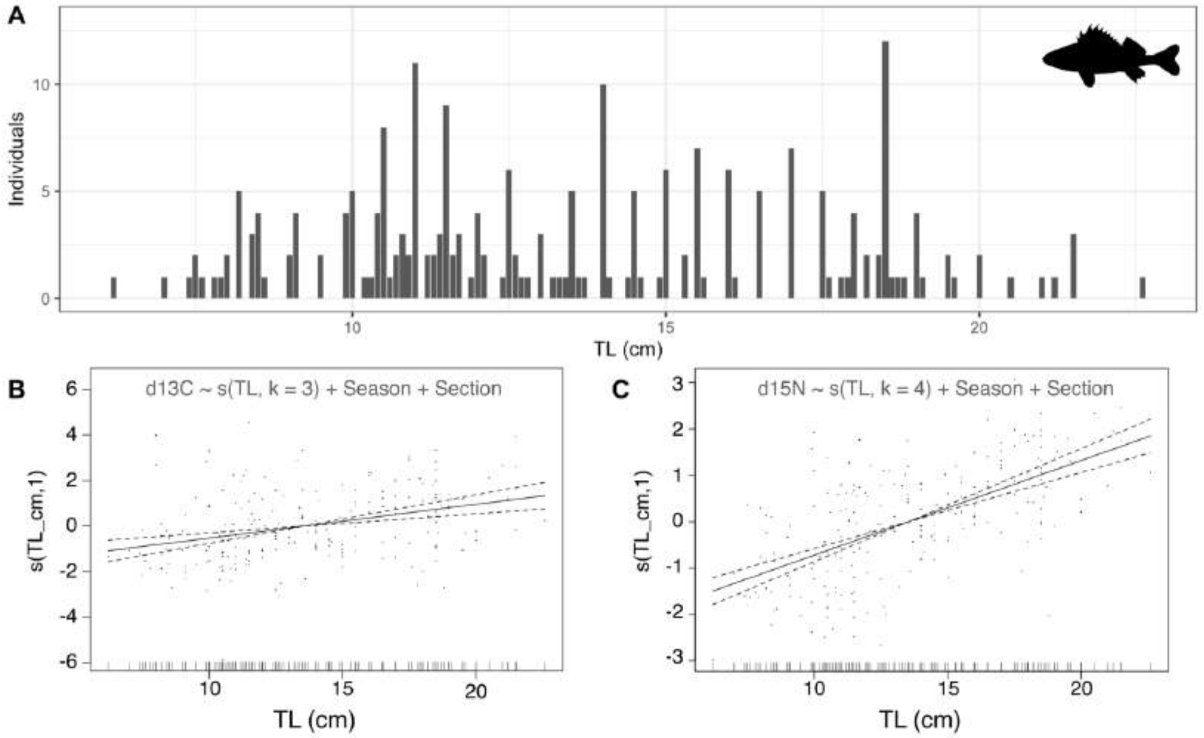

Fig. 3.4 Size-specific differences in stable isotope ratios in ruffe. (A) Total lengths (TL) in cm of caught ruffe during seasonal samplings. (B, C) Frequency distribution of TL and the relationships between TL and $\delta^{13}C$ as well as $\delta^{15}N$ based on GAM formula displayed above, respectively. 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.

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 δ^{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 ($p > 0.05$, $F = 0.64$, $n = 16$).

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 δ^{13}C values in the upper sections to higher δ^{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}.

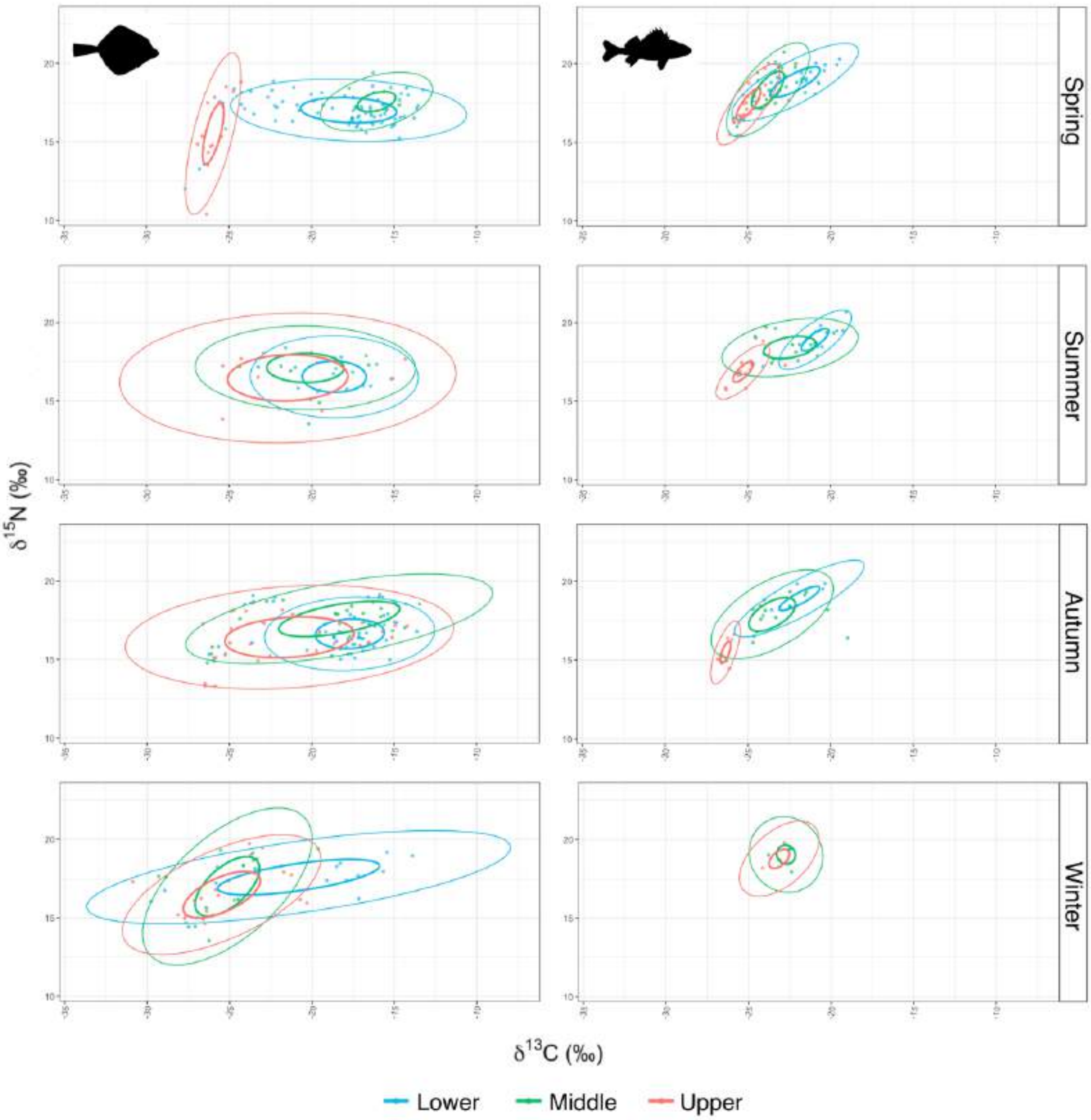

Fig. 3.5 Overlaps of isotopic ranges of $\delta^{13}C$ and $\delta^{15}N$ of flounder (left) and ruffe (right) caught at lower (blue), middle (green) and upper (red) sections during the different seasons. Outer and inner ellipses represent 95% and 40% confidence intervals, respectively.

Discussion

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) chose the term *facultative catadromous* to describe the life cycle of flounder based on the dependency of early life stages on estuarine habitats as nursery areas in the Seine, Gironde and Minho estuary. 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 occurring population(s) present. Therefore, we used the definition *facultative catadromy* according to the description of Daverat et al. (2012) in this study to describe flounder migration in the Elbe estuary.

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.

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 underlying this backward movement are still unknown but understanding them could be crucial to uncover the entire 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 this 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.

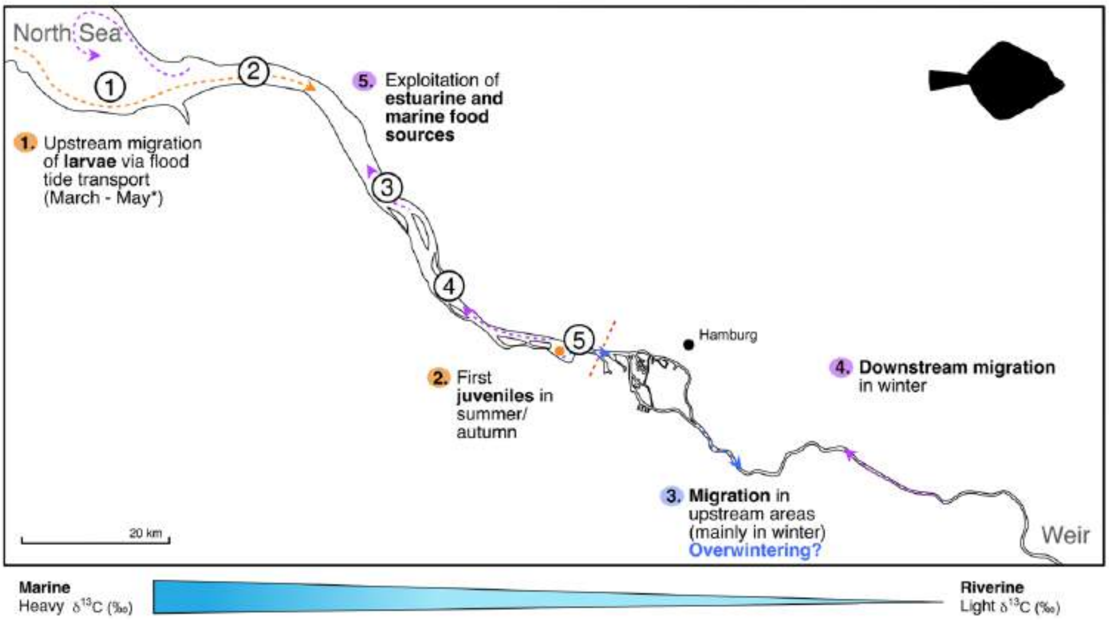

Fig. 3.6 Life cycle reconstruction of flounder in the Elbe estuary based on stable isotope data of seasonal $\delta^{13}C$ and literature (*Möller 1984, Bos et al. 1995). Arrows point towards migration direction of fish, starting during early life stages in late winter/spring from the river mouth to Mühlenberger Loch (orange), 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).

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 is not covered by our analysis. 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).

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).

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.

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.

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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Author contributions

EH: Writing – original draft, Writing – review and editing, Visualization, Methodology, Investigation, Formal Analysis, Conceptualization. JB: Writing – review and editing, SF: Writing – review and editing, Validation, Methodology, Conceptualization, RK: Writing – review and editing, , work (methodology), JT: Writing – review and editing, work (methodology), AF: funding acquisition, writing – review and editing, RT: funding acquisition, writing – review and editing, PG: Writing – review and editing, CM: Writing -review and editing, Conceptualization, Supervision, Funding acquisition.

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

	Ruffe						Flounder					
Season	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: $\delta^{13}C \sim s(TL\_cm, k = 3)$ + 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: $\delta^{15}N \sim s(TL\_cm, k = 6)$ + 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})	SEA_c (‰^{2})	Overlap (%) Lower	Overlap (%) Middle	Overlap (%) 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			/

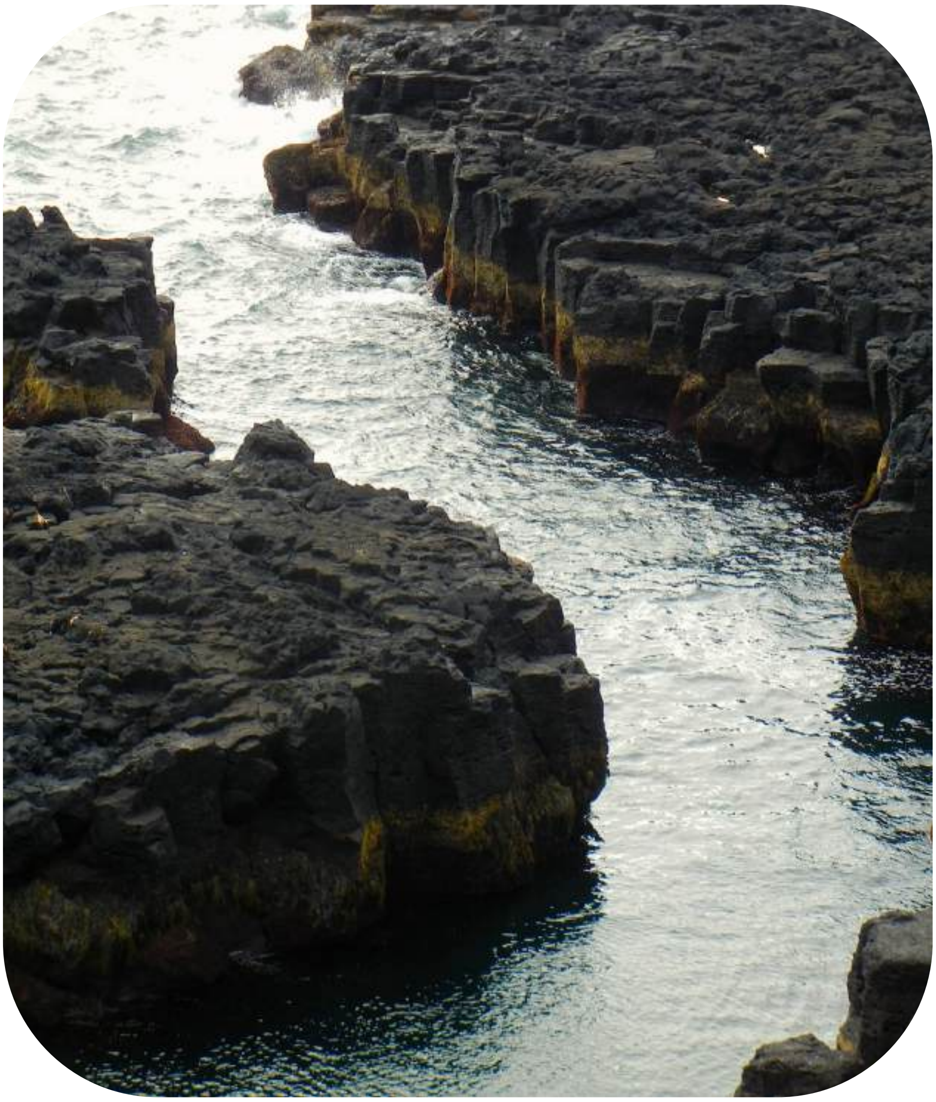
G. cernua	Spring								
		Lower	30	13.2	4.1	4.3	/	17.8	1.2
		Middle	26	10.1	3.3	3.4		/	23.9
		Upper	22	7.3	2.3	2.4			/
	Summer								
		Lower	14	4.5	1.7	1.9	/	21.3	0
		Middle	19	9.0	4.0	4.2		/	0
		Upper	14	5.1	1.8	1.9			/
	Autumn								
		Lower	6	2.4	1.9	2.4	/	12.4	0
		Middle	17	18.2	6.7	7.1		/	0
		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	Salinity ranges during sampling campaigns									
		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

Chapter 4

Spatial and temporal patterns of zooplankton trophic interactions and carbon sources in the eutrophic Elbe estuary (Germany)



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Chapter 4 – Zooplankton and carbon sources

Title: Spatial and temporal patterns of zooplankton trophic interactions and carbon sources in the eutrophic Elbe estuary (Germany)

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Abstract

Zooplankton in estuaries encounter complex physical and biogeochemical processes that affect the quantity, quality and origin of their food sources. The knowledge about how zooplankton deal with highly variable organic matter sources is sparse. Here, we investigated the spatial and temporal patterns of zooplankton trophic dynamics and carbon sources in the intensively dredged, eutrophic Elbe estuary. For this purpose, we applied elemental and stable isotope analysis (δ^{13}C and δ^{15}N) on particulate organic matter (POM) and dominant meso- and macrozooplankton species including ichthyoplankton from five stations along the entire salinity gradient of the estuary in 2022. The δ^{13}C values of POM (-29.2 to -23.0‰) indicated a conservative mixing of riverine and coastal carbon sources used by most taxa for their diet. *Eurytemora affinis* (-34.0 to -23.3‰) and *Mesopodopsis slabberi* (-22.2 to -20.0‰) exhibited a broader range in δ^{13}C than POM, suggesting selective feeding on single POM components depending on the season. In winter and autumn, under high suspended matter loads and limited availability of high-quality autochthonous phytoplankton, zooplankton showed increased tendency for carnivory (higher δ^{15}N values). Our study revealed a high trophic plasticity of estuarine Elbe zooplankton to buffer hydrological related alterations in their food source by dietary niche partitioning and a flexible switch in their feeding behavior.

Keywords: Elbe estuary, estuarine zooplankton, trophodynamics, stable isotopes, allochthonous and autochthonous carbon sources, selective feeding

Chapter 4 – Zooplankton and carbon sources

Introduction

Planktonic organisms play an important role in the production and transfer of organic matter and energy in aquatic ecosystems (Barnett et al. 2007, Koppelmann et al. 2009). Especially in estuaries, planktonic communities are affected by strong variability in environmental conditions, leading to complex food web structures. Estuaries are highly productive transition zones at the interface between freshwater and marine ecosystems and are characterized by physical (e.g., river discharge, tidal advection) and biogeochemical gradients (e.g. cycling of organic matter, nutrients and suspended solids) on various spatial and temporal scales (Hyndes et al. 2014, Geerts et al. 2017, Boynton et al. 2018, Kamjunke et al. 2023). These highly valuable habitats fulfil essential ecological functions such as transfer and sequestration of organic matter (Hyndes et al. 2014), recycling and filtering of nutrients (Boynton et al. 2018), refuge and nursery grounds for crustaceans and fish (Wilson 2002), and coastal protection (Koch et al. 2009). At the same time, estuaries are often severely impacted by anthropogenic stressors such as diking, dredging and eutrophication (Kerner 2007, Cloern et al. 2016).

Anthropogenic pressures and estuarine gradients impact the fate of organic matter transfer throughout different trophic levels, resulting in alterations of the food web structures and functions (Benfield 2012). Estuaries are defined by strong tidal mixing and high concentrations of nutrients and suspended particulate matter, which affect the production of autochthonous organic matter (Turner et al. 2022). Primary production is therefore often restricted to upstream freshwater areas, where light conditions are more favorable, and salinity stress and water turnover is reduced (Muylaert et al. 2005). Fresh phytoplankton biomass, the most nourishing food source for zooplankton (Müller-Solger et al. 2002), is therefore less accessible to primary consumers in turbid zones (Benfield 2012). In addition, excessive loads of organic matter in estuaries facilitate microbial colonization (Zimmermann-Timm et al. 1998), resulting in intense remineralization processes (Kamjunke et al. 2023), which increase microbial pathways in the pelagic food web (Stoecker and Capuzzo 1990, Lerner et al. 2022). This leads to strong fluctuations in the quality of food sources, which can shift from fresh phytoplankton to detrital, recalcitrant and less nutritious carbon sources (Gasparini et al. 1999, Müller-Solger et al. 2002) Despite this high importance, the role and dynamics of detrital sources and autochthonous organic matter in the trophodynamic of estuarine zooplankton are still not well understood. Fundamental knowledge of trophic interactions within estuarine planktonic communities is needed to understand the impact of increasing human pressures on estuarine food webs and to provide a tool for ecosystem-based management and conservation. Assessing the trophodynamics of estuarine zooplankton is difficult due to the potential diversity of different available organic sources of terrestrial, riverine and marine origin. Stable isotope analysis (SIA) is a powerful tool to disentangle the structure of planktonic food webs. Carbon isotope composition in consumers change little with the progression through a food web and thus reflect the time-integrated isotope composition of their diet (DeNiro and Epstein 1978). Moreover, carbon isotope composition in estuaries usually differs markedly between allochthonous inputs (e.g., terrestrial material), autochthonous riverine phytoplankton production and marine sources, allowing insights into organic matter sources at the base of the food web (Harmelin-Vivien et al. 2010). Trophic levels (TLs) for different organisms can be calculated based on their nitrogen isotope composition, which shifts in a predictable manner from one trophic level to the next (DeNiro and Epstein 1981, Post 2002). Moreover, stable isotopes also yield information about the width and overlap of the dietary niches of different organisms, and thus dietary niche differentiation (Newsome et al. 2007). SIA provides time-integrated dietary information over longer time periods, contrary to conventional methods, such as gut content analysis and feeding experiments, which offer short-term insights into dietary preferences (Dalerum and Angerbjörn 2005). The stable isotopic composition of organisms can therefore give a time- and space-integrated view of trophic interactions (Newsome et al. 2007) and is thus ideal for highly dynamic habitats such as estuaries. However, to date, few SIA studies of estuarine food webs have provided a spatially and seasonally resolved view across multiple taxa, as these studies often focus either on distinct areas (e.g., Taupp et al. 2017), seasons (e.g., David et al. 2016), or on a single taxon (e.g., Tackx et al. 2003).

The Elbe estuary, a highly turbid environment characterized by strong estuarine gradients and anthropogenic stressors, has so far received limited attention in determining planktonic food web structures. It is one of Europe's largest tidal estuaries located in north-west Germany, and serves multiple ecological functions, including refuge for many zooplankton and fish species (Bernát et al. 1994, Eick and Thiel 2014). It is also of significant socio-economic importance, particularly due to its connection to the seaport of the city of Hamburg. In recent decades, the water regime has experienced strong alterations, influenced by continuous channel deepening and dredging events, eutrophication, as well as climate change (Kerner 2007, Papenmeier et al. 2014). It is characterized by a semidiurnal, flood-dominated tidal wave that leads to steady resuspension of organic substances from benthic sources into the water column (Spieckermann et al. 2021). Most of the particulate organic matter in the Elbe estuary consists of allochthonous, decaying algae that originates from the shallow, non-dredged freshwater area upstream of the port (Geerts et al. 2017). Downstream of the port area, the organic matter source contains allochthonous material from adjacent coastal regions, primarily marine-like substances (Tobias-Hünefeldt et al. 2024), which are resuspended from deeper bottom water due to strong mixing forces (Spieckermann et al. 2021).

Although the Elbe estuary is well-studied in terms of organic and particle matter dynamics, the role and fate of autochthonous and allochthonous organic matter in its planktonic food web, along with the spatio-temporal dynamics of utilizing different organic matter sources by zooplankton, has never been fully investigated. So far, Kerner et al. (2004) have studied the carbon utilization of micro- and mesozooplankton species only in the freshwater area of the Elbe estuary using carbon isotopes. The authors found marked shifts in the use of the consumers' carbon sources on a seasonal scale. Here, we address this knowledge gap by investigating the trophodynamics of the planktonic food web along the entire salinity gradient of the Elbe estuary from seasonal samplings in 2022. We applied a carbon and nitrogen stable isotope analysis to dominant zooplankton taxa of different trophic positions and their main food sources from the Elbe estuary. This included the calanoid copepod *Eurytemora affinis* (Poppe, 1880) (Köpcke 2002), two sympatric mysids *Mesopodopsis slabberi* (Van Beneden, 1861) and *Neomysis integer* (Leach, 1814), the gammarid *Gammarus zaddachi* (Sexton, 1912) (Fiedler 1991), fish larvae of *Osmerus eperlanus* (Linnaeus, 1758) (Thiel 2001) as well as particulate organic matter as potential primary carbon source. The aim of this study was to determine (1) the isotopic signatures of the selected zooplankton species across spatio-temporal dimensions, as well as (2) the origin of primary carbon sources in their diet, and (3) to gain insights in the consumers' trophic positions and dietary niches.

Methods

Study area

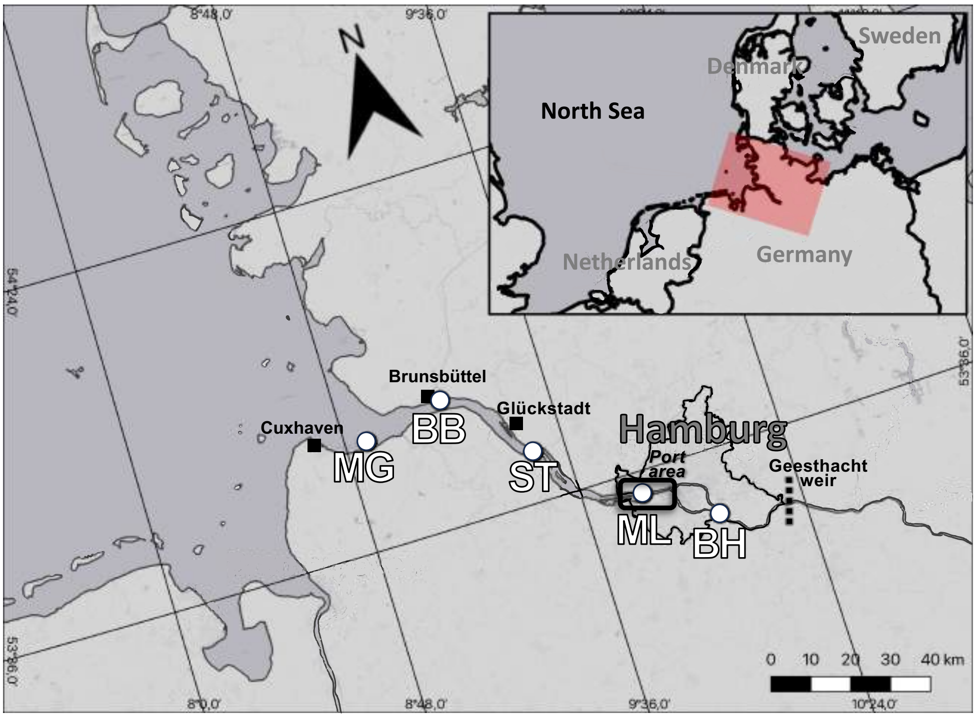


Fig. 4.1 Map of the Elbe estuary showing the five sampling locations. Station names abbreviated (see Table 4.1 for explanation). The weir at Geesthacht separates the estuary from the Elbe River. The background map has been provided by Esri, HERE, Garmin, ©OpenStreetMap contributors, and the GIS User.

The Elbe River is one of the major rivers in Europe, discharging through the Czech Republic and Germany into the German Bight in the North Sea. Its turbid estuary has a length of 142 km reaching from the weir at Geesthacht (Elbe-km 585, Fig. 4.1) to the river mouth at Cuxhaven (Elbe-km 727). The main channel is heavily dredged to enable access to the Port of Hamburg, the third largest port with overseas traffic in Europe, located 39 km downstream from the weir. The tidal range varies from 2 m at the weir to 3.5 m in the port area (HPA 2022). The water column is partially well-mixed (Pein et al. 2021) and is characterized by a long residence time of two to four weeks depending on river discharge (Amann et al. 2012). It includes a prominent maximum turbidity zone (MTZ) that extends 30 km and is located around Glückstadt (Papenmeier et al. 2014).

Tab. 4.1 Overview of the sampling sites and tidal phases during sampling.

Station (Abbreviation)	Coordinates		Elbe-km	Mean salinity ± SD	Tidal phase
	Latitude (°N)	Longitude (°E)			
Bunthäuser Spitze (BH)	53.45	10.07	609	0.4 ± 0.1	Low tide
Mühlenberger Loch (ML)	53.55	9.82	633	0.4 ± 0.1	High tide
Schwarztonnensand (ST)	53.71	9.47	665	0.7 ± 0.2	High tide
Brunsbüttel (BB)	53.89	9.19	692	6.1 ± 4.2	Low tide
Medemgrund (MG)	53.84	8.89	713	11.4 ± 7.1	Low tide

Elbe-km: Stream kilometer

Sample collection and processing

Sampling was performed during one-day cruises with the research vessel *Ludwig Prandtl* in the main channel at five stations along the entire salinity gradient in winter (February), spring (May), summer (June) and autumn (November) 2022 (Tab. 4.1). Sampling was scheduled to the same appointed time in the tidal cycle each time, ensuring consistent conditions between the campaigns. Stations were situated in the freshwater area upstream in the non-dredged channel (Bunthäuser Spitze (BH) at Elbe-km 609), within the port area (Mühlenberger Loch (ML) at Elbe-km 633), within the MTZ (Schwarztonnensand (ST) at Elbe-km 665), in the oligohaline zone (Brunsbüttel (BB) at Elbe-km 692) and mesohaline zone in the river mouth (Medemgrund (MG) at Elbe-km 727) (Fig. 4.1). At each station, net samples were taken by single horizontal tows at 1 m water depth using plankton nets of 100 µm (90 cm aperture, 3 m length) and 1000 µm (0.94 m aperture, 2.8 m net length) mesh size. Mesozooplankton and macrozooplankton was defined as organisms with a size between 100 – 1000 µm and 1 – 20 cm, respectively. Water samples were collected at 1 m water depth with multiple bucket hauls and filtered through pre-combusted and pre-weighted glass fiber filters (0.7 µm pore size, GF/F, Whatman, 450°C). The filtered water volume was adjusted at each station based on the concentration of suspended organic material to adequately coat the filters with biomass. At each station, three filters were taken to measure either chlorophyll *a* (Chl *a*) concentrations, suspended particulate matter (SPM) and the stable isotopic (SI) compositions of POM samples (one replicate each). For the POM filters, water samples were sieved through 100 µm mesh to remove large planktonic organisms. Filters and planktonic organisms from each haul were transferred to plastic trays and immediately stored at -80°C on board until further sorting and processing in the lab.

Temperature and salinity data were measured at each sampling site using an on-board in situ FerryBox system (see Petersen et al. 2011 for further details). River discharge data were obtained from the closest gauge station located upstream of the tidal limit in Neu Darchau (Elbe-km 536). Daily discharge rates for the corresponding sampling date were used which can be accessed through the Federal Waterways and Shipping Agency (WSV 2023).

In the lab, zooplankton samples were defrosted on ice, sorted for dominant taxa by hand using ultra-fine tweezers under a stereomicroscope and rinsed twice in ultrapure water for removal of adherent particles. Dominant mesozooplankton (*Eurytemora affinis*), macrozooplankton (*Mesopodopsis slabberi*, *Neomysis integer*, *Gammarus zaddachi*) and ichthyoplankton (*Osmerus eperlanus*) taxa were analyzed for stable isotopic composition. The individuals were sorted into separate plastic vials, freeze-dried at -80°C for 24h and grounded to fine powder before being transferred to tin capsules. Individuals of *E. affinis* were placed as whole animals directly into tin capsules after lyophilization. Triplicates of at least 100 individuals each of *E. affinis* and three for macrozooplankton and fish larvae were analyzed on elemental and stable isotopic composition, if sufficient specimens were collected. If biomass was insufficient, single samples were analyzed due to sample pooling instead.

Chl *a* was extracted by adding 10 ml of 90% acetone to the filters, which were then stored in darkness at 5°C for 24h before centrifugation (3000 rpm, 4°C, 15 min). The absorbance of the extracts was measured at a wavelength between 630 and 750 nm using a PerkinElmer photometer (LAMBDA XLS, Waltham, USA; model number: L7110189), following the method described by Jeffrey and Humphrey (1975). Filters that were not used for Chl *a* measurements were freeze-dried at -80°C for 24h. SPM content was determined by weighting the dry filters. For the measurement of elemental and stable isotope ratios of POM, aliquots were cut out of the filter. Parts of the subsamples were placed into tin capsules for analysis of δ^{15}N and C/N without prior treatment. The other aliquots were transferred to silver capsules and treated with HCl vapored for 2h under vacuum to remove carbonates for a separate C_{org} and δ^{13}C analysis, as carbonates potentially causing a bias in the organic carbon measurements (Jacob et al. 2005).

Analysis of elemental and stable isotope ratios

Data on elemental and stable isotopic composition of planktonic organisms were compiled from three elemental analyzer and isotope ratio mass spectrometer (IRMS) systems (see supplementary material (Tab. S4.1) for more details). SIA were performed using an elemental analyzer (Euro EA CHNSO, HEKAtech, Wegberg, Germany; Thermo/Carlo Erba NC 2500, Milan, Italy; PDZ Europa ANCA-GSL, Sercon Ltd., Cheshire, United Kingdom) interfaced to an IRMS system (IsoPrime 100, Elementar, Langenselbold, Germany; DeltaPlus Advantage, Thermo Fisher Scientific, Bremen, Germany; PDZ Europa 20-20, Sercon Ltd., Cheshire, United Kingdom). Isotope ratios of carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) were expressed as parts per thousands (‰) differences from a standard reference material:

$$\delta X = [(R_{Sample} / R_{standard}) - 1] \times 1000 \qquad \text{Eq. 4.1}$$

where **X** = ^{13}C or ^{15}N and the **R** the corresponding ratio $^{13}C/^{12}C$ and $^{15}N/^{14}N$. Vienna Pee Dee Belemnite (VPDB) for carbon and atmospheric N_2 for nitrogen were used as standard reference material. Helium was used as carrier gas. CO_2 and N_2 were used as working standards and were calibrated against international reference materials of the International Atomic Energy Agency (see supplementary material Tab. S4.1 for details).

Statistical analysis

Seasonal and spatial variability in $\delta^{13}C$, $\delta^{15}N$ and C:N of POM, meso- and macrozooplankton including ichthyoplankton were identified using a non-parametric test, Kruskal-Wallis test, since isotope ratios were not normally distributed. Post-hoc multiple pairwise comparisons (Dunn's test, Bonferroni corrected) were applied when differences were significant. C:N ratios were calculated based on their molar ratios. The ratio of chlorophyll *a* to SPM concentration (Chl *a* /SPM ratio) was used as an index for the phytoplankton availability that could potentially be consumed by the zooplankton (Irigoien and Castel 1995). Since it is difficult to separate phytoplankton from heterotrophic and detrital particulate matter including e.g., ciliates, flagellates, rotifers of similar size, POM was used as an indicator for a primary food source. $\delta^{15}N$ rations of the planktonic organisms were used to calculate trophic levels (**TL**s), assuming POM as an isotopic baseline TL =1 and a trophic enrichment factor (**TEF**) of 3.4‰. TLs for each station and season were calculated separately according to the equation of Post (2002):

$$TL = 1 + (\delta^{15}N_{\text{zooplankton}} - \delta^{15}N_{POM}) / TEF \qquad \text{Eq. 4.2}$$

The relationships between elemental and isotope ratios of the consumers and environmental variables were assessed by Pearson correlation analyses. The multivariate ellipse-based model SIBER (software version 2.1.9, Jackson et al. 2011) was applied to check for the species-specific isotopic niches. The trophic niches were calculated globally, as a minimum of at least five individual data points of $\delta^{13}C$ and $\delta^{15}N$ per taxon for each station and season are required to calculate either spatial or temporal niche space dynamics, which are not available in this study. Isotopic niche width (‰^{2}) was calculated by the standard ellipse function SEA (including 40% of the data) for each taxon, which was corrected for small sample size using a correction mode (SEA_c). In addition, the overlap of isotopic niches was calculated by applying a Bayesian estimate of standard ellipses. All statistical tests and visualizations were performed using R software, software version 4.3.2 (R Core Team 2024).

Results

Environmental conditions

Temperatures at the examined stations ranged between 5.1°C in winter and 21.4°C in summer 2022 (Tab. 4.2). Salinity exhibited a gradual increase downstream, with the weakest gradient observed in winter and the strongest in summer. At station ST, high SPM concentrations were measured, peaking in summer. Low SPM concentrations were generally observed at the freshwater station BH, with the lowest values occurring in autumn. In winter, SPM concentrations peaked at the river mouth (station MG), when river discharge was highest. Lowest river discharge rates occurred during summer. Chl *a* concentrations ranged from 3.9 µg l^{-1} at station BB in autumn to 152.7 µg l^{-1} at station BH in summer. In summer and spring, strong phytoplankton blooms occurred exclusively at the station BH, with a sharp decrease in Chl *a* concentrations downstream from the harbor area. Chl *a* concentrations were positively correlated with temperatures (Pearson, $n = 20$, $R = 0.45$, $p = 0.048$, see supplementary data Tab. S4.2) and remained low during autumn and winter (maximal up to 12.3 µg l^{-1}).

Tab. 4.2 Environmental conditions during the sampling campaigns in the Elbe estuary. Data were obtained along the entire salinity gradient (freshwater: BH, ML, ST; oligohaline: BB, mesohaline: MG) in each season in 2022.

Season	Station	Temperature (°C)	Salinity	SPM (mg l^{-1})	Discharge ($m^3 s^{-1}$)	Chl *a* (µg l^{-1})
Winter					1166	
	BH	5.1	0.3	23.4		7.6
	ML	5.5	0.3	80.1		6.2
	ST	5.8	0.4	70.8		10.7
	BB	5.6	0.8	124.3		10.3
	MG	5.6	2.7	164.3		12.0
Spring					345	
	BH	19.6	0.4	62.6		132.2
	ML	18.8	0.4	134.0		18.8
	ST	17.0	0.9	52.6		7.3
	BB	16.5	4.7	28.2		4.4
	MG	16.2	10.9	36.1		7.6
Summer					231	
	BH	21.4	0.5	37.5		152.7
	ML	20.5	0.5	57.9		22.6
	ST	19.3	0.7	253.6		17.8
	BB	18.6	9.8	40.3		7.0
	MG	18.0	20.0	30.6		10.9
Autumn					283	
	BH	10.9	0.5	7.6		6.4
	ML	12.8	0.5	40.1		6.0
	ST	12.7	0.8	130.7		12.3
	BB	12.5	9.2	33.8		3.9
	MG	12.4	12.0	48.2		5.0

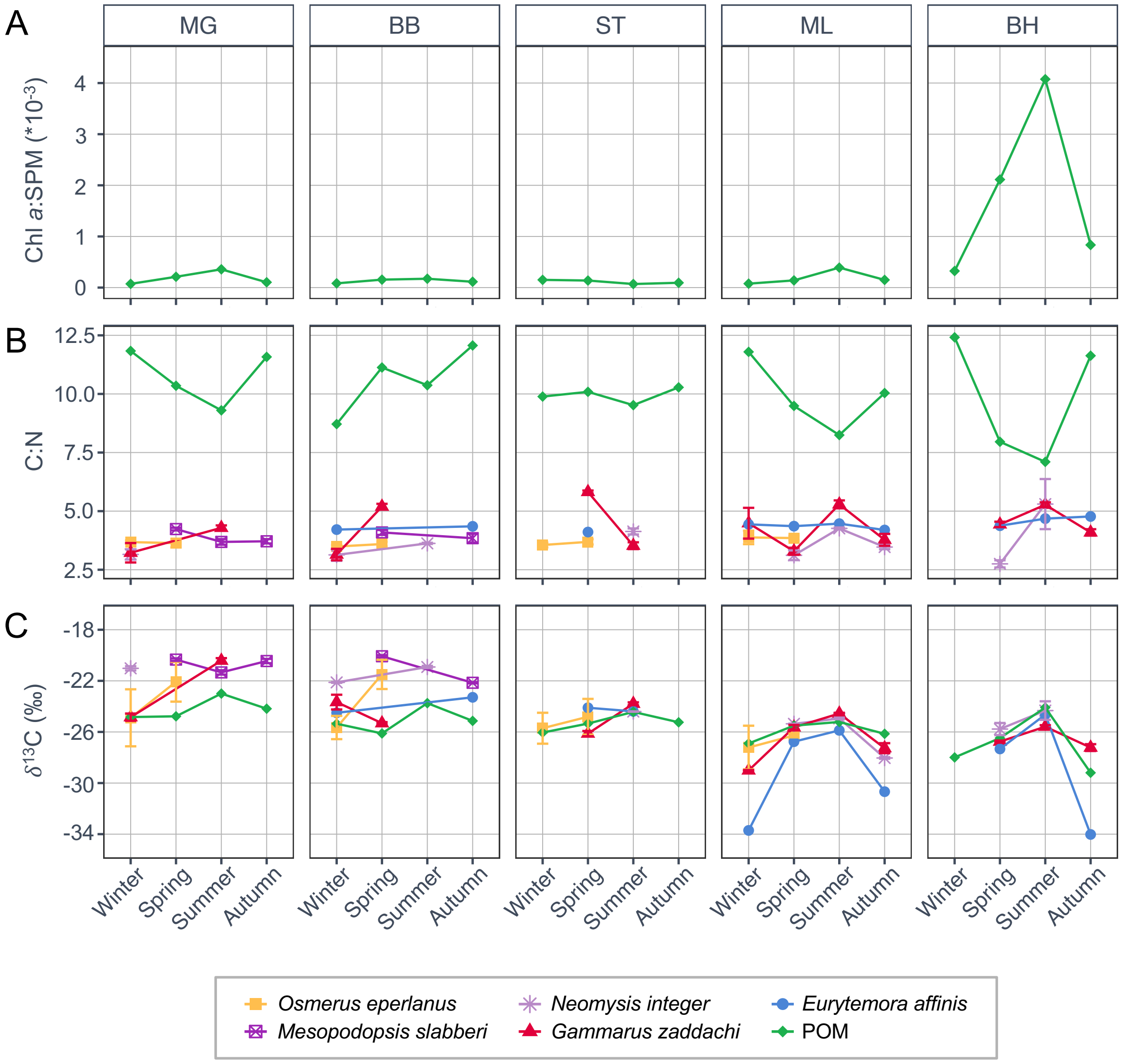
Abbreviations: SPM – suspended particulate matter; Discharge – river discharge; Chl *a* – Chlorophyll *a* concentration.

Spatial and temporal variation in quantity, quality and origin of particulate organic matter sources

The Chl *a*/SPM ratios were highest at station BH throughout the year with the largest ratio in summer (up to 4.1 $*10^{-3}$) (Fig. 4.2 A) caused by high Chl *a* concentrations (Pearson, $n = 20$, $R = 0.94$, $p < 0.001$, Tab. S4.2). Low Chl *a*/SPM ratios were consistently observed at station ST and BB during all seasons, where SPM levels were high. C:N ratios of POM decreased significantly with increasing Chl *a* concentrations (Pearson, $n = 20$, $R = -0.68$, $p < 0.001$). Generally, low C:N ratios of POM were measured in spring and summer, with a slight increasing trend seawards (e.g., from 7.1 at station BH to 10.4 at station BB in summer) (Fig. 4.2 B). At station ST, C:N ratios remained stable and high throughout the year. Similarly, $\delta^{13}C$ values of POM exhibited an increasing trend downstream (Fig. 2 C), with seasonal means ranging between -26.9 ± 2.2 ‰ at the uppermost freshwater station (station BH) and -24.2 ± 0.9 ‰ at the river mouth (station MG) (supplementary material, Tab. S4.3).

Salinity and temperature were both positively correlated with δ^{13}C values of POM (Pearson, n = 20; salinity: R = 0.59, $p < 0.01$; temperature: R = 0.46, $p < 0.05$). Moreover, in summer POM was significantly enriched in δ^{13}C compared to POM collected in winter (from -26.2 ± 1.3 to -24.1 ± 0.8 ‰; KW test, $p < 0.05$, Table 4.3). There was no significant difference in the δ^{15}N values of POM on the spatial and temporal scale (KW test, station: p = 0.9, season: p = 0.23, Tab. 4.3; Fig. 4.3 A).

Fig. 4.2 Seasonal and spatial changes in primary production processes and the origin of the carbon source at five stations along the entire salinity gradient (freshwater: BH, ML, ST; oligohaline: BB; mesohaline: MG) of the Elbe estuary in 2022. Mean values (± SD) are given when triplicate samples were measured. (**A**) Variations in the ratio of chlorophyll *a* to suspended particulate patter concentration. (**B**) C:N ratio and (**C**) δ^{13}C values of particulate organic matter (POM) and the zooplankton taxa.



Tab. 4.3 Results of the Kruskal-Wallis test (KW test) followed by a multiple comparison (post hoc, Dunn's test) comparing seasonal and spatial variations in $\delta^{13}C$ (‰), $\delta^{15}N$, C:N ratios and trophic levels (TL) of particulate organic matter (POM) and zooplankton taxa. Samples were collected at five stations along the entire salinity gradient of the Elbe estuary across all seasons in 2022.

Taxa			n	df	$\delta^{13}C$ (‰)	$\delta^{15}N$ (‰)	C:N	TL
POM	KW test	Season	20	3	H = 8.5 p-value: **<0.05***	H = 4.3 p-value: 0.2	H = 6.5 p-value: 0.08	
	Post hoc (p-value):	Season			**<0.05*** (Winter-Summer)			
	KW test	Station	20	4	H = 7.8 p-value: 0.1	H = 1.1 p-value: 0.9	H = 1.6 p-value: 0.8	
	Post hoc (p-value):	Station						
Eurytemora affinis	KW test	Season	11	3	H = 0.97 p-value: 0.8	H = 5.7 p-value: 0.1	H = 2.8 p-value: 0.4	H = 4.6 p-value: 0.2
	Post hoc (p-value):	Season						
	KW test	Station	11	3	H = 7.00 p-value: 0.07	H = 3.7 p-value: 0.3	H = 5.7 p-value: 0.1	H = 3.0 p-value: 0.4
	Post hoc (p-value):	Station						
	Post hoc (p-value):	Taxon			**<0.001***** (*M. slabberi*) **<0.05*** (*N. integer*)	<0.05* (*G. zaddachi*) **<0.001***** (POM)	**<0.001***** (*N. integer*) **<0.001***** (*O. eperlanus*)	**<0.001***** (POM)
Mesopodopsis slabberi	KW test	Season	15	2	H = 8.7 p-value: **<0.05***	H = 7.6 p-value: **<0.05***	H = 9.7 p-value: **<0.01****	H = 10.0 p-value: **<0.01****
	Post hoc (p-value):	Season			**<0.05*** (Spring-Autumn)	**<0.05*** (Spring-Summer)	**<0.05*** (Spring-Summer) **<0.05*** (Spring-Autumn)	**<0.01**** (Spring-Summer)
	KW test	Station	15	1	H = 0 p-value: 1	H = 8.7 p-value: **<0.01****	H = 0.5 p-value: 0.5	H = 4.0 p-value: **<0.05***
	Post hoc (p-value):	Station				**<0.01**** (MG-BB)		**<0.05*** (MG-BB)
	Post hoc (p-value):	Taxon			**<0.001***** (*O. eperlanus*) **<0.001***** (*N. integer*) **<0.001***** (*G. zaddachi*) **<0.001***** (*E. affinis*) **<0.001***** (POM)	**<0.001***** (*G. zaddachi*) **<0.001***** (POM)	**<0.01**** (POM)	**<0.01**** (*G. zaddachi*) **<0.001***** (POM)
Neomysis integer	KW test	Season	27	3	H = 19.9 p-value: **<0.001*****	H = 11.1 p-value: **<0.05***	H = 21.8 p-value: **<0.001*****	H = 9.3 p-value: **<0.05***
	Post hoc (p-value):	Season			**<0.01**** (Winter-Spring) **<0.01**** (Winter-Autumn) **<0.05*** (Summer-Autumn)	**<0.01**** (Winter-Spring) **<0.05*** (Spring-Summer)	**<0.01**** (Winter-Summer) **<0.001***** (Spring-Summer)	**<0.05*** (Winter-Spring)
	KW test	Station	27	4	H = 19.71 p-value: **<0.001*****	H = 13.6 p-value: **<0.01****	H = 3.9 p-value: 0.4	H = 18.1 p-value: **<0.01****
	Post hoc (p-value):	Station			**0.01*** (MG-ML) **<0.01**** (BB-ML)	**<0.01**** (BB-BH) **<0.05*** (ML-BH)		**<0.05*** (BB-BH) **<0.001***** (ST-BH)

	Post hoc (p-value):	Taxon			<0.001*** (_M. slabberi_)	<0.01** (_G. zaddachi_) <0.001*** (POM)	<0.05* (_G. zaddachi_) <0.001*** (_E. affinis_) <0.001*** (_N. integer_)	<0.05* (_G. zaddachi_) <0.001*** (POM)
Gammarus zaddachi	KW test	Season	39	3	H = 18.1 p-value: <0.001***	H = 11.9 p-value: <0.01**	H = 9.4 p-value: 0.05	H = 13.1 p-value: <0.01**
	Post hoc (p-value):	Season			<0.05* (Spring-Summer) <0.001*** (Summer-Autumn)	<0.01** (Winter-Spring)		<0.05* (Winter-Spring) <0.05* (Spring-Autumn)
	KW test	Station	39	4	H = 18.81 p-value: <0.001 ***	H = 15.7 p-value: <0.01**	H = 4.9 p-value: 0.3	H = 13.3 p-value: <0.01**
	Post hoc (p-value):	Station			<0.05* (MG-ML) <0.01** (MG-BH)	<0.01** (BB-BH) <0.05* (ML-BH)		<0.05* (BB-BH) <0.05* (ML-BH)
	Post hoc (p-value):	Taxon			<0.001*** (_M. slabberi_)	<0.001*** (_O. eperlanus_) <0.01** (_M. slabberi_) <0.01** (_N. integer_) <0.05* (_E. affinis_)	<0.05* (_O. eperlanus_) <0.05* (_N. integer_) <0.001*** (POM)	<0.001*** (_O. eperlanus_) <0.01** (_M. slabberi_) <0.05* (_N. integer_)
Osmerus eperlanus	KW test	Season	119	1	H = 15.9 p-value: <0.001***	H = 46.0 p-value: <0.001***	H = 14.1 p-value: <0.001***	H = 61.3 p-value: <0.001***
	Post hoc (p-value):	Season			<0.001*** (Winter-Spring)	<0.001*** (Winter-Spring)	<0.001*** (Winter-Spring)	<0.001*** (Winter-Spring)
	KW test	Station	119	3	H = 45.96 p-value: <0.001***	H = 3.3 p-value: 0.3	H = 44.7 p-value: <0.001***	H = 6.8 p-value: 0.08
	Post hoc (p-value):	Station			<0.001*** (MG-ML) <0.01** (BB-ST) <0.001*** (BB-ML) <0.001*** (ST-ML)		<0.05* (MG-ML) <0.001*** (BB-ML) <0.001*** (ST-ML)	
	Post hoc (p-value):	Taxon			<0.001*** (_M. slabberi_)	<0.001*** (_G. zaddachi_) <0.001*** (POM)	<0.05* (_G. zaddachi_) <0.001*** (_E. affinis_) <0.001*** (POM)	<0.001*** (_G. zaddachi_) <0.001*** (POM)

Abbreviations: df – degrees of freedom; n – number of samples; POM – particulate organic matter; TL – trophic level. Note: Significant differences are displayed in bold (p-value < 0.05: *, <0.01: **, <0.001: ***). Multiple comparisons (post hoc test) were performed with the pairwise non-parametric Dunn's test when significant differences were present.

Trophic transfer of carbon sources and stable isotopic composition of consumers

$\delta^{13}C$ values of the consumers ranged from -29.9 to -20.3 ‰ for *O. eperlanus*, -29.0 to -20.2 ‰ for *G. zaddachi*, -28.1 to -20.8 ‰ for *N. integer*, -22.2 to -20.0 ‰ for *M. slabberi* and from - 34.0 to -23.3 ‰ for *E. affinis* (Fig. 4.2 C). $\delta^{13}C$ values of the zooplankton displayed more variations than the POM. Similar to POM, most of the taxa collected upstream, had significantly more depleted $\delta^{13}C$ values compared to individuals from the river mouth (KW test, Tab. 4.3). Planktonic consumers were generally enriched in $\delta^{13}C$ in spring and summer compared to winter and autumn (KW test, Tab. 4.3). *M. slabberi* was most enriched and showed the least variation in $\delta^{13}C$, which was located only at station BB and MG. *Eurytemora affinis*, however, showed neither strong significant spatial nor seasonal variability in their $\delta^{13}C$ and $\delta^{15}N$ values but had the lowest $\delta^{13}C$ values (up to -34.0 ‰ at station BH), especially in winter and autumn (Fig. 4.2 C). Moreover, significant seasonal and spatial differences in the $\delta^{15}N$ values and trophic levels (TLs) of the planktonic consumers were present, except for *E. affinis* (KW test, Tab. 4.3). Overall, the $\delta^{15}N$ values and TLs of the zooplankton increased strongly downstream from station BH to BB (e.g., *G. zaddachi*, from 10.1 ± 1.5 to 12.9 ± 0.7 ‰ for $\delta^{15}N$, from 1.2 ± 0.3 to 2.2 ± 0.2 for TL, Tab. S4.3, Fig. 4.3). Generally, when examining the species-specific seasonal fluctuations in the TLs and $\delta^{15}N$ values, there was a significant increase for each taxon in winter and autumn compared to spring and summer (KW test, Tab. 4.3). In addition, *G. zaddachi* exhibited the most depleted and variable $\delta^{15}N$ values ranging between 8.1 to 17.1 ‰ (Fig. 4.3). Fish larvae of *O. eperlanus* exhibited the highest $\delta^{15}N$ values (up to 17.6 ± 0.6 ‰ at station ML) which decreased sharply in spring (up to 14.0 ± 0.5 ‰ at station ML), with TLs showing a similar trend. In addition, the consumers' TLs correlated globally negatively with Chl *a*/SPM ratio (Pearson, $n = 211$, $R = -0.49$, $p < 0.001$). The C:N ratio of the planktonic consumers showed rather seasonal than spatial trends (Fig. 4.2 B; KW test, Tab. 4.3), with a slight increase in ratios for most of the species in summer.

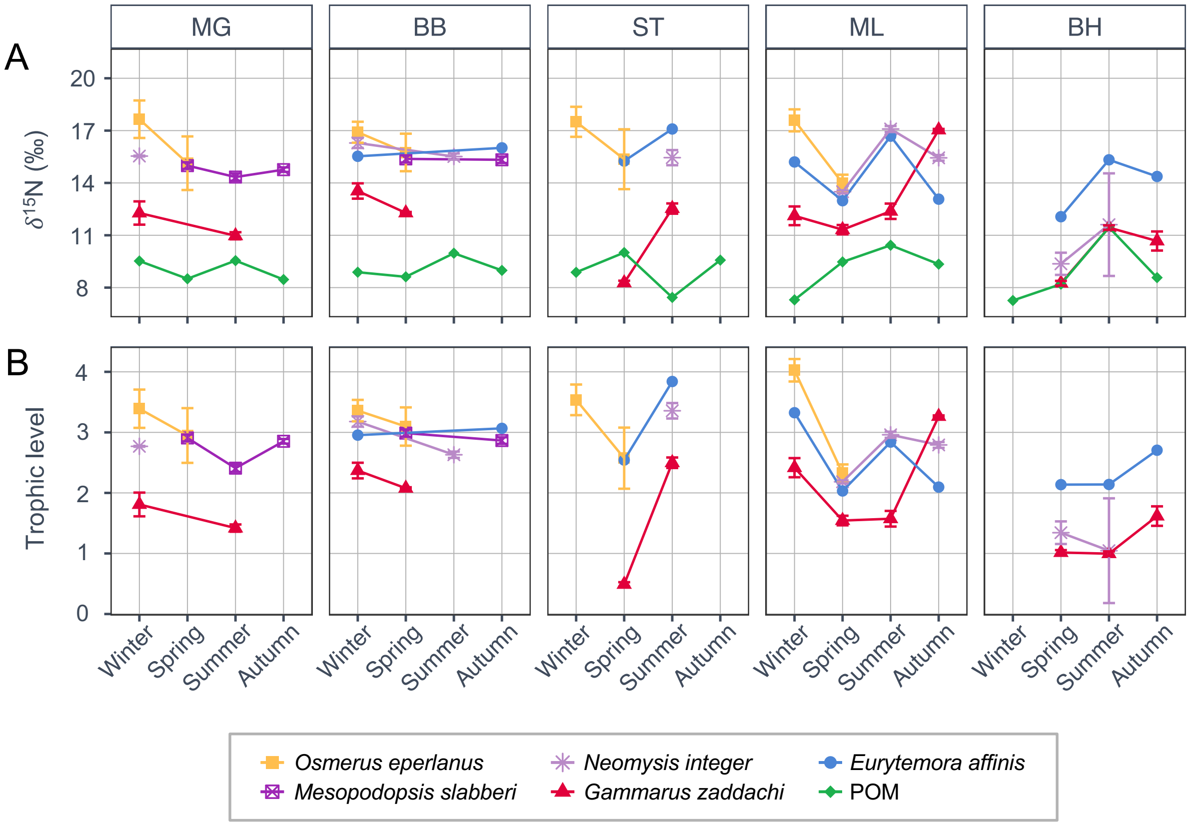


Fig. 4.3 Seasonal and spatial variability in (A) $\delta^{15}N$ values and (B) trophic levels (TL) of the zooplankton taxa and particulate organic matter (POM) collected at five stations along the entire salinity gradient (freshwater: BH, ML, ST; oligohaline: BB, mesohaline: MG) of the Elbe estuary in 2022. POM is not depicted in the lower plot (B) since it was set as baseline (TL = 1) for all seasons and stations. Mean values (± SD) are given when triplicate samples were measured.

Stable isotopic niches of the zooplankton

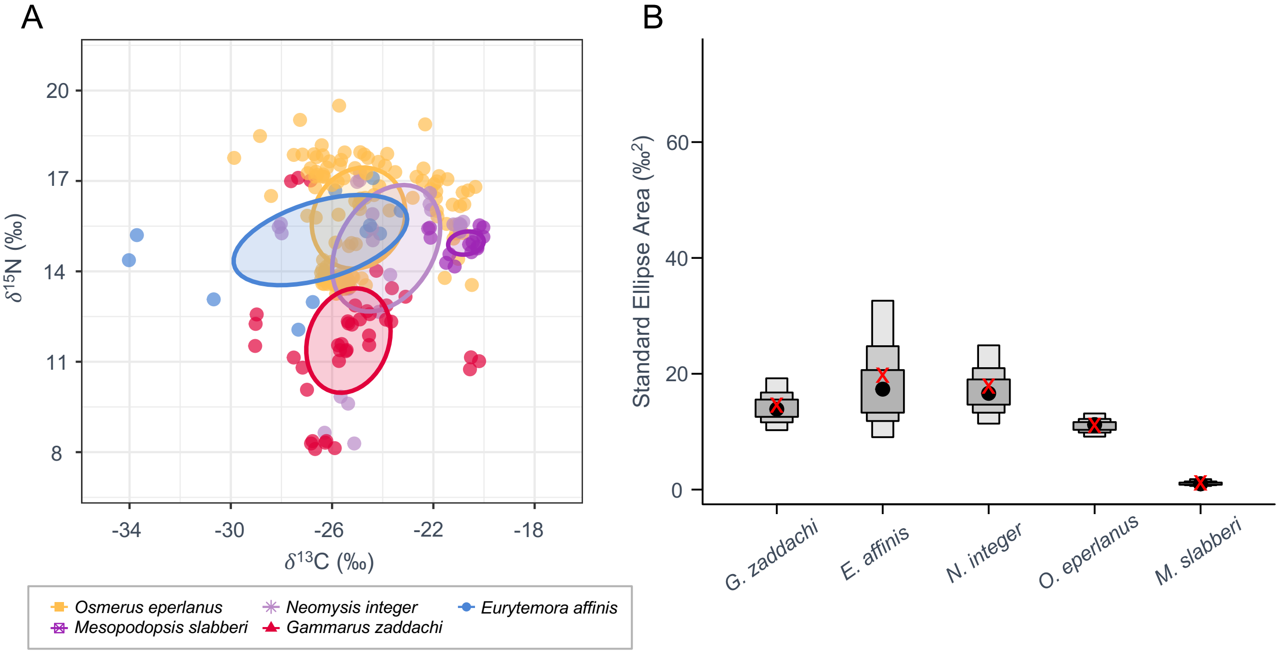


Fig. 4.4 Results of the overall niche space dynamics of the five dominant planktonic consumers sampled in the Elbe estuary in 2022. (A) Stable isotope biplot of δ^{13}C and δ^{15}N of the taxa including species-specific, small-size corrected standard ellipse areas (SEA$_c$) (including 40% of the data per species). (B) Density box plots of estimated Bayesian standard ellipse area (SEA$_b$) for the respective planktonic consumers, which indicate 50% (dark grey), 75% (grey) and 95% (light grey) credible intervals for mean estimations. Black dots depict the mean values of SEA$_b$, whereas the red cross represents the maximum likelihood estimate of SEA$_c$.

Stable isotopic niches calculated for each zooplanktonic group using the standard ellipse for small sample size (SEA$_c$) showed a high degree of overlap ranging between 17.7 and 31.7% between *E. affinis*, *N. integer* and *O. eperlanus* (Fig. 4.4 A, Tab. 4.4). *M. slabberi* exhibited a smaller and less variable isotopic niche, which did not overlap with those of the other taxa. *G. zaddachi* shared only one small overlap with the isotopic niche of *N. integer* (overlap 5.0‰^{2}, 17.7%, Tab. 4.4) and clustered at the bottom along the δ^{15}N axis. Species-specific estimated niche widths (SEA$_b$) ranged from the smallest niche of 1.2‰^{2} for *M. slabberi* to the widest niche of 19.8‰^{2} for *E. affinis* (Fig. 4 B). The width of SEA$_b$ of *G. zaddachi*, *E. affinis, N. integer* and *O. eperlanus* were similar in their mean values.

Tab. 4.4 Results of the SIBER analysis including δ^{13}C and δ^{15}N values of the planktonic consumers collected in the Elbe estuary in 2022. Sample size (n), total area (TA), standard ellipse area without (SEA) and with correction of small sample size (SEA$_c$) and the relative area of SEA$_c$ overlap (in ‰^{2} and %) between respective zooplankton species and the credible intervals of the estimated Bayesian standard ellipse area (SEA$_b$).

Species	n	TA (‰^{2})	SEA (‰^{2})	SEA$_c$ (‰^{2})	Credible intervals			SEA$_c$ overlap	
					50 %	95 %	99 %	(‰^{2})	(%)
Eurytemora affinis	11	28.6	17.8	19.8	14.0 – 21.4	8.5 – 32.5	6.0 – 40.7	*G. zaddachi:* 0 *N. integer:* 6.2 *O. eperlanus:* 7.4 *M. slabberi:* 0	*G. zaddachi:* 0 *N. integer:* 19.2 *O. eperlanus:* 30.8 *M. slabberi:* 0
Mesopodopsis slabberi	15	2.0	1.1	1.2	0.9 – 1.2	0.6 – 1.8	0.5 – 2.2	*G. zaddachi:* 0 *E. affinis:* 0 *N. integer:* 0 *O. eperlanus:* 0	*G. zaddachi:* 0 *E. affinis:* 0 *N. integer:* 0 *O. eperlanus:* 0
Neomysis integer	27	37.7	17.2	17.9	14.7 – 19.2	11.1 – 24.9	9.6 – 28.8	*G. zaddachi:* 5.0 *E. affinis:* 6.2 *O. eperlanus:* 7.1 *M. slabberi:* 0	*G. zaddachi:* 17.7 *E. affinis:* 19.2 *O. eperlanus:* 31.7 *M. slabberi:* 0
Gammarus zaddachi	39	43.4	14.2	14.6	12.4 - 15.4	10.2 - 19.2	9.0 – 21.3	*E. affinis:* 0 *N. integer:* 5.0 *O. eperlanus:* 0 *M. slabberi:* 0	*E. affinis:* 0 *N. integer:* 17.7 *O. eperlanus:* 0 *M. slabberi:* 0
Osmerus eperlanus	119	42.6	11.0	11.1	10.3 – 11.6	9.1 – 13.1	8.6 – 14.0	*G. zaddachi:* 0 *E. affinis:* 7.4 *N. integer:* 7.1 *M. slabberi:* 0	*G. zaddachi:* 0 *E. affinis:* 30.8 *N. integer:* 31.7 *M. slabberi:* 0

Discussion

Knowledge of zooplankton trophic interactions and carbon sources is incomplete for the intensively dredged, eutrophic Elbe estuary, which hampers understanding of food web structure and function. This study provides the first spatially and seasonally resolved view of the trophodynamics within the planktonic food web along the salinity gradient of the Elbe estuary based on carbon and nitrogen stable isotope analysis of dominant zooplankton taxa and of possible food sources. Our focus is particularly on the spatio-temporal patterns of POM as carbon sources for planktonic consumers in terms of food availability (quantity and quality) and origin, as well as trophic segregation and shifts in carbon source utilization among different planktonic consumers.

The origin and fate of the primary carbon source

Our results showed substantial seasonal and spatial variations in Chl *a* concentrations, Chl *a*/SPM ratios, C:N ratios and $\delta^{13}C$ of POM. We found an intense phytoplankton bloom with Chl *a* concentrations of up to 152.7 $\mu g\ l^{-1}$ exclusively at the uppermost freshwater station BH in spring and summer. When passing the Hamburg Harbor, a strong decline in phytoplankton biomass occurred with low Chl *a* concentrations at the downstream stations, which reflects results of earlier studies in the Elbe estuary (e.g., Wolfstein and Kies 1995, Pein et al. 2021, Kamjunke et al. 2023). This rapid drop in phytoplankton biomass downstream of the port area was caused by light limitation, resulting from a high load of suspended particulate matter that is accumulated and resuspended in the dredged section of the Elbe estuary due to the sudden change in the bathymetry and a respective decrease in the flow velocity (Kerner 2007, Geerts et al. 2017). This is in accordance with the SPM concentrations measured along the dredged area of the estuary from station ML to MG in our study, whereas highest SPM levels were found at the station ST in the maximum turbidity zone (Papenmeier et al. 2014). In February 2022, the MTZ shifted seawards as the SPM peak moved closer to the mouth of the estuary, likely due to the increase in river discharge rates in winter. The shallow tidal freshwater region upstream of the Hamburg Harbor is characterized by low turbidity and reduced water turnover throughout the annual cycle (Wolfstein and Kies 1995), which can favor intense phytoplankton blooms (Turner et al. 2022) and might explain the high Chl *a* concentrations observed exclusively at station BH during warm periods. The Chl *a*/SPM ratio, as a proxy for phytoplankton availability (Irigoien and Castel 1995), correlated positively with the Chl *a* concentration, and thus may account for the high ratios exclusively detected at station BH. Furthermore, the high load of SPM may not only have impacted the available light but also led to a loss of phytoplankton due to enhanced sedimentation of plankton aggregates as a result of their stickiness (Kiørboe and Hansen 1993). Phytoplankton produce sticky exudates during bloom conditions which increase their adhesion to other particles (Kiørboe and Hansen 1993). Steidle and Vennell (2024) suggested that the previously reported decline in Chl *a* concentration in the Hamburg Harbor may be attributed to phytoplankton adhesion to negatively buoyant suspended particles, which subsequently sink to light-limited water layers. Consequently, high loads of SPM in the Elbe estuary may result in distinct losses of primary producers as potential food source for planktonic consumers, particularly in the sections downstream from the port area, and can result in pronounced seasonal variations depending on river discharge rates.

C:N ratios give an indication of the quality of the organic matter sources, with values < 8 indicating fresh and high-quality POM and values > 8 representing detrital material, as algal detritus has increased C:N ratios due to diagenesis (Thornton and McManus 1994, Sterner and Elser 2003, Finlay and Kendall 2007). Indeed, the phytoplankton conditions in spring and summer coincided with a decline in C:N ratios < 8 for POM at station BH indicating a higher contribution of high-quality POM in the upstream section of the estuary during these seasons. The negative correlation between C:N ratio and Chl *a* concentration in the present enrichment downstream. The most seaward station (MG) was mesohaline and, thus, do not represent a stable isotopic signal of true marine material, which is generally difficult to obtain in estuaries (Middelburg and Herman 2007). In winter and autumn, POM was significantly study aligns with the seasonal patterns observed in other river systems (e.g., Rhone River, Harmelin-Vivien et al. 2010). When passing the port area, the C:N ratio of POM still showed a similar trend of decreasing values during spring and summer, but was distinctly enriched with values > 8, indicating a change in the organic matter composition to more detrital material and less fresh phytoplankton carbon sources. In the zone of maximum turbidity (station ST) we even observed poor POM quality year-round, as indicated by persistently high C:N ratios. These findings are in line with the spatio-temporal pattern in organic matter processing and degradation that has been reported for resuspended (Spieckermann et al. 2021) and particulate organic matter (Kamjunke et al. 2023) in the Elbe estuary. We hypothesize that the absence of high-quality food from the Hamburg Harbor seawards could be related to a shift from an autotrophic system at station BH towards a heterotrophic system along the dredged sections of the estuary. This shift is likely caused by light-limiting conditions and enhanced microbial processing, as associated with the high SPM load and reduced flow velocities (Kerner 2007, Geerts et al. 2017).

Particulate organic matter in the Elbe estuary was likely composed of several sources with a predominance of detrital terrestrial organic matter, as indicated by intermediate $\delta^{13}C$ values ranging from -26.9 ± 2.2 ‰ (at station BH) to -24.2 ± 0.9 ‰ (at station MG), which is consistent with $\delta^{13}C$ values reported for other European estuaries (Middelburg and Herman 2007). Marine organic sources often have $\delta^{13}C$ values between -18 and -22 ‰, whereas riverine material, derived from riverine algae and terrigenous material, typically exhibit values between -32 and -20 ‰ (Thornton and McManus 1994, Finlay and Kendall 2007). Although the organic matter was probably diluted due to strong tidal mixing processes, we observed a trend of ^{13}C-depleted in ^{13}C, especially in the upper part of the estuary. High river discharge rates during winter were likely responsible for the increase in ^{13}C-depleted POM, which might have carried an enhanced amount of terrestrial material into the center of the river. δ^{13}C values of POM are substantially influenced by species composition (Cloern et al. 2002, Finlay and Kendall 2007) and seasonal changes in the phytoplankton community structure are well known for the Elbe estuary (Wolfstein 1996). In particular, for the year 2022, Martens et al. (2024) observed a transition from the predominance of diatoms in summer to mixotrophic flagellates in winter in the Elbe estuary as a result of unfavorable light conditions. Since flagellates are more ^{13}C - depleted than diatoms (Gearing et al., 1984), a shift in the phytoplankton community towards a higher contribution of flagellates in the POM could have led to depleted δ^{13}C values observed during winter and autumn. In contrast, POM showed neither spatial nor temporal patterns in the δ^{15}N, which is in line with findings from Middelburg and Herman (2007) across multiple European estuaries. As in our study, the authors explained the consistent δ^{15}N values by a rapid nitrogen turnover driven by intense microbial activity paired with continuous lateral inputs from adjacent marshlands. Nevertheless, other components like respiration, photosynthetic rates as well as the dissolved inorganic carbon and nitrogen pool also influence the isotopic composition of aquatic primary producers (Finlay and Kendall 2007), which were not assessed in this study.

Zooplankton diet and trophic segregation

Most of the examined zooplankton taxa showed similar δ^{15}N values, indicating that they shared a diet of the same trophic origin. Only the gammarid *G. zaddachi* exhibited clearly lower δ^{15}N values, probably caused by lower nitrogen enrichment factors than herbivores or carnivores due to ammonia excretion (Mancinelli 2012). On the other hand, fish larvae of *Osmerus eperlanus* showed significantly higher δ^{15}N values in winter, approximately one trophic level higher than in other seasons and compared to other the taxa. During this time, the fish larvae were only a few weeks old and likely fed from the yolk sac, indicating the isotopic signature of the adults, which the fish can retain for several weeks due to long tissue turnover rates (Vander Zanden et al. 2015). Similar to δ^{13}C studies from other European estuaries (Van Den Meersche et al. 2009, Modéran et al. 2012, David et al. 2016), the values of the examined consumers in our study ranged between -34 and -20‰. Overall, the zooplankton species followed the general isotopic pattern of POM, which showed an enrichment of δ^{13}C during seasons of high primary production and downstream towards the river mouth, indicating that the zooplanktonic generally relied on the local POM as their primary carbon source. Most of the taxa showed an opportunistic feeding strategy consuming similar food sources, as reflected by their broad isotopic niche width and the large overlap between niches. Processes such as strong tidal mixing, microbial diagenesis of organic matter or species-specific isotopic fractionation hamper the precise identification of the primary carbon sources for the consumers. Compound-specific stable isotope analysis or a combined biomarker approach using fatty acids would help in determining the origin of carbon sources in the consumers' diet when multiple sources are available and their isotopic signals might be blurred (Cloern et al. 2002, Finlay and Kendall 2007). *Mesopodopsis slabberi* and *Eurytemora affinis* differed markedly in the range of $\delta^{13}C$ compared to POM indicating that these species did not directly consume POM in total but were likely feeding on selective components of the POM that exhibit more seasonal and spatial differences in isotopic fractionation compared to bulk POM (Bouillon et al. 2000). This feeding pattern might be also reflected in their position and compactness of their isotopic niche. Specifically, *M. slabberi* displayed $\delta^{13}C$ values ranging from -22.2 to -20.0‰, generally up to 4‰ more ^{13}C-enriched than those of the POM, exhibiting a narrow and unique dietary niche which do not overlap with other taxa. Food-niche partitioning probably allows *M. slabberi* to reduce food competition and to co-exist with the sympatric mysid *Neomysis integer* (Winkler et al. 2007). Similar to Modéran et al. (2012), our results show that *M. slabberi*, only detected in the river mouth, fed on marine phytoplankton as a main carbon source. This species was absent in winter because it only migrates into the inner part of estuaries for reproduction and growth in spring (Hamerlynck and Mees 1991). Nevertheless, we cannot definitively exclude microphytobenthos as potential food source, as it can constitute a considerable proportion of the total pelagic POM through resuspension from tidal flats (De Jonge and Van Beusekom 1992).

Eurytemora affinis followed the seasonal trend of POM with markedly lower $\delta^{13}C$ values in winter and autumn. During periods of limited primary production or low food quality, *E. affinis* might have presumably consumed ^{13}C-depleted constituents of the POM, probably derived from detrital source causing a broad isotopic niche width for *E. affinis*. The ability of *E. affinis* to select its prey among suspended inorganic particles (Gasparini and Castelt 1997, Tackx et al. 2003) and to feed on detrital organic sources to cover its nutritional requirements are in line with the results of Kerner (2004) for the Elbe estuary and has also been reported for other European estuaries (David et al. 2006, 2016, Modéran et al. 2012). We provide evidence, that selective feeding can be an important strategy for the zooplankton in the Elbe estuary to optimize the use of available carbon sources to avoid competition and to survive stressful periods (i.e. winter and autumn) when food quality and availability (i.e. at the MTZ) is low. However, when SPM levels increase and phytoplankton can no longer be selectively preyed, a switch to alternative food sources, like protozoans, will be performed (Gasparini and Castelt 1997). Such shifts from herbivorous to omnivorous feeding are widespread in turbid estuaries (e.g., David et al. 2006, Van Den Meersche et al. 2009, Modéran et al. 2012). Suspended organic particles, in particular detrital matter, are often populated by bacteria, protozoans and small metazoans which can be passively ingested in estuarine food webs (Stoecker and Capuzzo 1990). These changes are accompanied by a rise in the consumers' $\delta^{15}N$ values, and thus lead to an increase in food chain lengths (Lerner et al. 2022). In fact, we observed a significant rise in the consumers' $\delta^{15}N$ values and TLs seawards peaking at the MTZ, as well as during winter and autumn, when the carbon dynamics in the Elbe estuary shifted to a predominantly heterotrophic system due to limiting primary production. Feeding on heterotrophic sources results in lower C:N ratios for the consumers (Elser et al. 2000) as presented by the seasonal trends in the C:N ratios of consumers in this study. Mixotrophic pathways have also been reported for phytoplankton taxa in the Elbe estuary, especially for taxa in the MTZ or during winter (Martens et al. 2024). The ability to use alternative sources of material is a crucial aspect of the trophic plasticity of planktonic consumers, helping to stabilize and maintain food web structures (e.g., David et al. 2006, Modéran et al. 2012, Lerner et al. 2022). This adaptability likely plays a central role for zooplankton in the Elbe estuary to cope with the environmental forces and to avoid competition.

Conclusion

Our results highlighted that temporal and spatial variations in the quality and quantity of food for zooplankton in the Elbe estuary are influenced by primary production processes and the amount of suspended particulate matter in the water column resuspended by strong tidal mixing processes. Autochthonous algal material was mainly produced in the un-dredged freshwater area of the Elbe estuary upstream of Hamburg Harbor, which probably was subject to intense heterotrophic decomposition downstream of the port area. The stable isotopic signatures of POM indicate a mixture of coastal and riverine derived organic matter in the carbon source, which was predominated by the input of terrigenous matter from adjacent marshlands, especially during periods of high river discharge. The microbial trophic pathways in the planktonic food web impacted the trophodynamics both temporally and spatially, reflected by a considerable increase in δ^{15}N and, consequently, also an increase in trophic levels of the consumers. The investigated planktonic organisms were generally able to cope with strong variations in food quality and quantity due to opportunistic feeding behavior. Selective feeding, portioning of dietary niches and switching from herbivorous to omnivorous nutrition allow species to co-exist and to optimize the use of allochthonous and autochthonous organic material. This trophic plasticity of the zooplankton may thus be an essential feature to withstand alterations in the hydrology of the Elbe estuary related to human disturbances (i.e. variations in turbidity and flow velocity) and natural estuarine gradients. We provided new insights into the utilization of alternative trophic pathways of the Elbe estuary and redefined the trophodynamic of the planktonic food web. This study helps to understand the impact of increasing human pressures on estuaries by providing a powerful tool for ecosystem-based management and conservation.

Data availability

The data underlying this article are available in the research data repository of the Universität Hamburg, at https://doi.org/10.25592/uhhfdm.14727.

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CRediT authorship contribution statement

Declaration of interest

The authors have no conflicts of interest to declare.

JB: Writing – original draft, Writing – review and editing, Visualization, Methodology, Investigation, Formal Analysis, Conceptualization. CM: Writing -review and editing, Conceptualization, Supervision, Funding acquisition. EH: Writing – review and editing, Investigation, Resources, Validation. VR: Writing – review and editing, Investigation, Resources, Validation. NL: Writing – review and editing, Investigation, Resources. TH: Writing – review and editing, Investigation, Resources. JD: Writing – review and editing, Methodology, Formal Analysis, Conceptualization, Supervision. RK: Writing – review and editing, Methodology, Formal Analysis, Conceptualization, Supervision.

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Supplements

The following supplementary material is available at ICESJMS online.

Tab. S4.1 Overview of the different Isotope Ratio Mass Spectrometry systems, including the elemental analyser and the reference material used to analyse the elemental and stable isotopic composition of the zooplankton species in the Elbe estuary.

Species	Elemental analyser	Isotopic mass spectrometer	Reference material for CO_2 and N_2	Reference material for isotope ratios	Measurement accuracy	Laboratory
Particulate organic matter, *Eurytemora affinis*	Euro EA CHNSO, HEKAtech, Wegberg, Germany	IsoPrime 100, Elementar, Langenselbold, Germany	IAEA CH6, CH7, N1, N2; IVA soil, sucrose	Vienna PeeDee Belemnite (VPDB), atmospheric nitrogen (N_2)	EA: 0.05% C, 0.005% N IRMS: <0.2‰ for $\delta^{15}N$	Institute for Geology, Universität Hamburg, Germany
Mesopodopsis slabberi, Neomysis integer, Gammarus zaddachi	NA 1110, Thermo/Carlo Erba, Milan, Italy	DeltaPlus Advantage, Thermo Fisher Scientific, Bremen, Germany	IAEA N1, N2, NO3; NBS-600, NBS 22; Acetanilide, Caffein	VPDB, N_2, Vienna Canyon Diablo Troilite (VCDT)	IRMS: ± 0.25‰ for $\delta^{15}N$ and ± 0.2‰ for $\delta^{13}C$	GEOMAR, Central lab for chemical Analysis (ZLCA), Kiel, Germany
Osmerus eperlanus	PDZ Europa ANCA-GSL, Sercon Ltd., Cheshire, United Kingdom	PDZ Europa 20-20, Sercon Ltd., Cheshire, United Kingdom	IAEA 600; USGS 40, 41, 42, 43, 61, 64, 65	VPDB, N_2	IRMS: ± 0.17‰ for $\delta^{13}C$, ± 0.08‰ for $\delta^{15}N$	UC Davis Stable Isotope Facility, University of California, United States

Tab. S4.2 Results of the Pearson correlation test between environmental parameters, including the elemental and stable isotopic composition of particulate organic matter, measured along the five stations in the Elbe estuary in 2022. Correlation coefficients are presented above the diagonal, while the p-values are below the diagonal.

	$\delta^{13}C$	$\delta^{15}N$	C:N	Q	Temp	Salinity	Chl a	SPM	Chl a/SPM
$\delta^{13}C$		0.49*	-0.38	-0.34	0.46*	0.60**	0.10	0.24	0.04
$\delta^{15}N$	0.03		-0.53	-0.39	0.47*	0.07	0.33	-0.18	0.41
C:N	0.09	0.16		0.32	-0.61**	0.16	-0.68**	-0.14	-0.60**
Q	0.14	0.09	0.17		-0.86***	-0.33	-0.20	0.18	-0.23
Temp	0.03	0.04	0.004	<0.001		0.21	0.45*	-0.05	0.41
Salinity	0.006	0.78	0.5	0.16	0.38		-0.24	-0.32	-0.19
Chl a	0.69	0.16	<0.001	0.39	0.048	0.31		-0.06	0.94***
SPM	0.31	0.46	0.56	0.45	0.85	0.17	0.79		-0.23
Chl a/SPM	0.86	0.07	0.006	0.33	0.07	0.43	<0.001	0.33	

Abbreviations: Q – river discharge rate; Temp – temperature; Chl a – chlorophyll a; SPM – suspended particulate matter.
Note: Significant differences are displayed in bold (p-value < 0.05: *, <0.01: **, <0.001: ***).

Tab. S4.3 Seasonal and spatial mean values of stable isotope data for POM and the zooplanktonic organisms collected at the five stations in the Elbe estuary during all seasons in 2022. Trophic levels were calculated based on δ^{15}N values of POM, which were used as trophic baseline (Trophic level (TL) = 1). Means are given with standard deviations for n ≥ 3, while for n = 2 the range of values is shown.

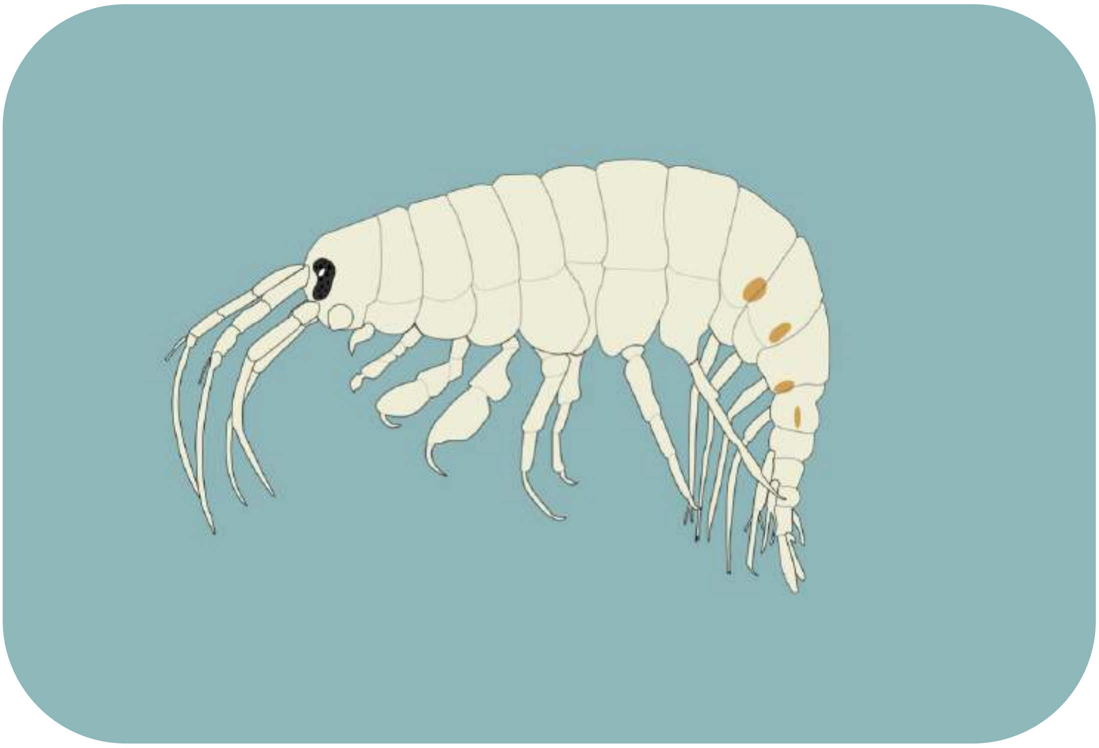
Species	n			δ^{13}C (‰)	δ^{15}N (‰)	C:N ratio	TL
POM	20			-25.5 ± 1.4	9.0 ± 1.1		1
	5	Season	Winter	-26.2 ± 1.3	8.4 ± 1.0	10.9 ± 1.6	1
	5		Spring	-25.6 ± 0.7	9.0 ± 0.8	9.8 ± 1.2	1
	5		Summer	-24.1 ± 0.8	9.8 ± 1.5	8.9 ± 1.3	1
	5		Autumn	-26.0 ± 1.9	9.0 ± 0.5	11.1 ± 0.9	1
	4	Station	BH	-26.9 ± 2.2	8.9 ± 1.8	9.8 ± 2.6	1
	4		ML	-25.9 ± 0.7	9.1 ± 1.3	9.9 ± 1.5	1
	4		ST	-25.3 ± 0.7	9.0 ± 1.1	9.9 ± 0.3	1
	4		BB	-25.1 ± 1.0	9.1 ± 0.6	10.6 ± 1.4	1
	4		MG	-24.2 ± 0.9	9.0 ± 0.6	10.8 ± 1.2	1
Eurytemora affinis	11			-27.2 ± 3.9	14.9 ± 1.6	4.4 ± 0.2	2.7 ± 0.6
	2	Season	Winter	-24.5 – -33.7	15.2 – 15.5	4.2 – 4.4	3.0 – 3.3
	3		Spring	-26.1 ± 1.7	13.5 ± 1.7	4.3 ± 0.2	2.2 ± 0.3
	3		Summer	-25.0 ± 0.8	16.4 ± 0.9	4.6 ± 0.2	2.9 ± 0.9
	3		Autumn	-29.3 ± 5.5	14.5 ± 1.5	4.4 ± 0.3	2.6 ± 0.5
	3	Station	BH	-28.7 ± 4.8	13.9 ± 1.7	4.6 ± 0.2	2.3 ± 0.3
	4		ML	-29.3 ± 3.6	14.5 ± 1.8	4.4 ± 0.1	2.6 ± 0.6
	2		ST	-24.1 – -24.4	15.3 – 17.1	4.1 – 4.2	2.5 – 3.8
	2		BB	-23.3 – -24.5	15.5 – 16.0	4.2 – 4.4	3.0 – 3.1
	0		MG	NA	NA	NA	NA
Mesopodopsis slabberi	15			-20.9 ± 0.8	15.0 ± 0.4	3.9 ± 0.2	2.8 ± 0.2
	0	Season	Winter	NA	NA	NA	NA
	6		Spring	-20.2 ± 0.2	15.2 ± 0.3	4.2 ± 0.1	2.9 ± 0.1
	3		Summer	-21.3 ± 0.2	14.4 ± 0.2	3.7 ± 0.1	2.4 ± 0.1
	6		Autumn	-21.3 ± 0.9	15.0 ± 0.3	3.8 ± 0.2	2.9 ± 0.1
	0	Station	BH	NA	NA	NA	NA
	0		ML	NA	NA	NA	NA
	0		ST	NA	NA	NA	NA
	6		BB	-21.1 ± 1.1	15.3 ± 0.2	4.0 ± 0.2	2.9 ± 0.1
	9		MG	-20.7 ± 0.5	14.7 ± 0.3	3.9 ± 0.3	2.7 ± 0.2
Neomysis integer	27			-24.1 ± 2.3	14.4 ± 2.5	3.7 ± 0.8	2.5 ± 0.8
	6	Season	Winter	-21.6 ± 0.6	15.9 ± 0.4	3.1 ± 0.2	3.0 ± 0.2
	6		Spring	-25.6 ± 0.4	11.4 ± 2.3	2.9 ± 0.3	1.8 ± 0.5
	12		Summer	-23.6 ± 1.7	14.9 ± 2.5	4.3 ± 0.8	2.5 ± 0.1
	3		Autumn	-28.0 ± 0.1	15.5 ± 0.2	3.5 ± 0.1	2.8 ± 0.1
	6	Station	BH	-25.1 ± 1.0	10.5 ± 2.3	4.0 ± 1.6	1.2 ± 0.6
	9		ML	-26.1 ± 1.5	15.4 ± 1.6	3.6 ± 0.5	2.6 ± 0.4
	3		ST	-24.4 ± 0.1	15.4 ± 0.5	4.1 ± 0.1	3.4 ± 0.1
	6		BB	-21.5 ± 0.7	15.9 ± 0.5	3.4 ± 0.3	2.9 ± 0.3
	3		MG	-21.0 ± 0.2	15.5 ± 0.1	3.2 ± 0.2	2.8 ± 0.1
Gammarus zaddachi	39			-25.4 ± 2.1	11.8 ± 2.2	4.3 ± 0.9	1.8 ± 0.7

	9	Season	Winter	-25.8 ± 2.5	12.7 ± 0.8	3.6 ± 0.8	2.2 ± 0.3
	12		Spring	-26.0 ± 0.6	10.0 ± 1.9	4.7 ± 1.0	1.3 ± 0.6
	12		Summer	-23.6 ± 2.0	11.8 ± 0.7	4.6 ± 0.8	1.6 ± 0.6
	6		Autumn	-27.2 ± 0.3	13.8 ± 3.5	3.9 ± 0.3	2.4 ± 0.9
	9	Station	BH	-26.5 ± 0.7	10.1 ± 1.5	4.6 ± 0.5	1.2 ± 0.3
	12		ML	-26.6 ± 1.8	13.2 ± 2.4	4.2 ± 0.8	2.2 ± 0.7
	6		ST	-25.0 ± 1.3	10.4 ± 2.3	4.7 ± 1.3	1.5 ± 1.1
	6		BB	-24.5 ± 1.0	12.9 ± 0.7	4.2 ± 1.1	2.2 ± 0.2
	6		MG	-22.6 ± 2.4	11.6 ± 0.9	3.8 ± 0.6	1.6 ± 0.2
Osmerus eperlanus	119			-24.8 ± 2.1	15.8 ± 1.7	3.7 ± 0.2	2.9 ± 0.6
	37	Season	Winter	-26.0 ± 1.5	17.4 ± 0.8	3.6 ± 0.2	3.6 ± 0.3
	82		Spring	-24.3 ± 2.1	15.1 ± 1.5	3.7 ± 0.1	2.6 ± 0.5
	0		Summer	NA	NA	NA	NA
	0		Autumn	NA	NA	NA	NA
	0	Station	BH	NA	NA	NA	NA
	27		ML	-26.6 ± 1.0	15.1 ± 1.8	3.9 ± 0.2	2.8 ± 0.8
	59		ST	-25.1 ± 1.5	16.0 ± 1.8	3.6 ± 0.1	2.9 ± 0.6
	25		BB	-22.8 ± 2.2	16.1 ± 1.1	3.6 ± 0.1	3.2 ± 0.3
	8		MG	-23.1 ± 2.2	16.1 ± 1.9	3.7 ± 0.1	3.1 ± 0.4

Chapter 5

Is acidification of common estuarine macroinvertebrates in stable isotope approaches necessary to analyze aquatic food webs?

> “Before all masters, necessity is the one most listened to, and who teaches the best.”
> Jules Verne



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Chapter 5 – Acidification in stable isotope analysis

Title: Is acidification of common estuarine macroinvertebrates in stable isotope approaches necessary to analyze aquatic food webs?

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Abstract

We evaluated the effect of in situ acidification of common estuarine macroinvertebrates from the Elbe estuary on stable isotope ratios as the non-removal of inorganic carbon can significantly influence aquatic food web analyses. 10% HCl solution was used to remove inorganic carbon from crustaceans that potentially biases the true ratio of assimilated dietary carbon. Acidified samples were dried and filled into silver and tin capsules. A control group without treatment was used to ensure proper comparison of no acid and acid washed samples. We detected significant differences in the $\delta^{13}C$ values of all investigated crustaceans except for the mysid shrimp *Mesopodopsis slabberi*. On the contrary, acidification impacts on $\delta^{15}N$ were only observed in *Gammarus* spec. samples. A carbonate proxy was additionally computed to evaluate the necessity of acidification because high values indicate high inorganic carbon in the tissue that may alter true $\delta^{13}C$ values. Our results indicate that the necessity of acid treatment of common estuarine macroinvertebrates before stable isotope analysis depends on the species-specific carbonate content. Acid treatment is therefore not required for all species when analyzing aquatic food webs. Based on our and results from other studies, we created a basic decision tree to guide future research in assessing the need of acidification and to provide further options to remove the effects of inorganic carbon from stable isotope analysis.

Keywords: Stable isotope analysis, carbonate, sample acidification, aquatic food web analysis, macroinvertebrates, estuary

Chapter 5 – Acidification in stable isotope analysis

Introduction

Stable isotope analysis of $\delta^{13}C$ and $\delta^{15}N$ is a widely used tool in ecology that enables researchers to achieve fundamental insights into food web functioning and animal behavior (Layman et al. 2012). Topics to be addressed by stable isotope analysis include, among others, the examination of trophic levels (Fry 2002), food chain lengths (Layman et al. 2012) and migration routes (Hobson and Wassenaar 2008). Nitrogen stable isotopes are commonly used in food web studies to evaluate trophic positions of organisms as $\delta^{15}N$ increases stepwise in the food chain, a phenomenon known as the *trophic level effect* (Fuller et al. 2012). $\delta^{13}C$ on the other hand acts as a potential dietary tracer that reflects either a resource pool or the isotopic signature of food assimilated in the consumer's tissue (Mc Cutchan et al. 2003, Schlacher and Connolly 2014).

The results of stable isotope analysis of animals that bind inorganic carbon such as chitin ($C_8H_{13}O_5N$) or calcium carbonate ($CaCO_3$) must be treated with caution, because biogenic carbon can influence $\delta^{13}C$ values as it potentially reflects the isotope signal of inorganic carbon of the surrounding environment (Yokoyama et al. 2005, De Lecea et al. 2011). Ensuring the measurement of pure organic carbon requires the pre-analysis removal of calcified structures such as bones, shells, teeth, or other skeletal structures in animals containing inorganic carbon (Schlacher and Connolly 2014).

Before conducting a food web analysis, the effect of inorganic carbon on the isotopic values should be proofed. If the amount of inorganic carbon in the tissues is low, it can be assumed that it can be neglected after an assessment based on previous studies to determine whether the analysis is significantly affected or not (Jacob et al. 2005, Jaschinsky et al. 2008, De Lecea et al. 2011, Connolly and Schlacher 2014).

If there is verified impact, there are two ways to remove inorganic carbon. First, calcified structures may be dissected mechanically before the drying process. This approach is especially appropriate for larger organisms, such as mussels, caridean crustaceans or fish larvae where calcified compartments like shells, exoskeletons or bones can be easily removed (Schlacher and Connolly 2014). However, the effectiveness of mechanical dissection of inorganic carbon depends on the animal´s size and its morphology which may prevent the removal of hard structures (Pires-Teixeira et al. 2020). Second, acidification techniques can be used to chemically remove inorganic carbon from samples, whereby different acids can have inconsistent effects on the $\delta^{13}C$ and $\delta^{15}N$ values. H_2SO_3 and H_3PO_4 were observed to cause heavier $\delta^{13}C$ values and affect $\delta^{15}N$ values (Kennedy et al. 2005, Brodie et al. 2011, Kazanidis et al. 2019). Acidification using HCl is generally considered to produce coherent and reliable results compared to other treatments (Brodie et al. 2011), however HCl may still have a significant effect on $\delta^{15}N$ (Kennedy et al. 2005, Jaschinsky et al. 2008). A solution to mitigate this bias is to prepare separate samples of carbon and nitrogen isotopic values for further analysis, however making this method both time consuming and expensive (Serrano et al. 2008). Additionally, the low weight of small macroinvertebrates and the loss of biomass during the drying process also limits the sample size, which makes a separate treatment for stable isotope analysis less practical. Before conducting a food web analysis using stable isotopes, the necessity of acid treatment of the samples should therefore be carefully verified.

Estuaries represent a challenging habitat for their biota due to often strongly variable environmental conditions characterized by permanent salinity gradients, diel tidal cycles, periodically occurring oxygen minimum zones or high turbidity levels (Elliot et al. 2007, Colombano et al. 2021, Mosman et al. 2023). As a result, estuarine systems are often characterized by low species richness, making individual species more important in terms of occupying trophic niches and their role in food web functioning (Whitfield 1999, Whitfield and Harrison 2020). At the same time, estuaries are among the most productive ecosystems due to their elevated nutrient loading rates that can support immense biomasses of fish and crustaceans (Constanza et al. 1993, Elliot and Hemingway 2002, Stompe et al. 2023).

Macroinvertebrates are less diverse in estuaries but occur in high densities where they serve as important prey for many consumers such as fish (Platell et al. 2006, Tweedley et al. 2012), through which their carbon isotope ratio can be measured in the assimilated $\delta^{13}C$ values of predators (Mc Cutchan et al. 2003, Michener and Lajtha 2007). Acidification treatment of these prey species may be a suitable solution, however its necessity for reliable stable isotope analysis is still controversially discussed (e.g., Jaschinsky et al. 2008, Serrano et al. 2008, Pires-Teixeira et al. 2020). We here studied the effect of acidification on the results of stable isotope analysis of estuarine macroinvertebrate species from a temperate estuary, that has to our knowledge not been performed before. We anticipate that food web analyses in estuaries using stable isotope analyses will become increasingly relevant in the future, so our results provide an important contribution.

We investigated the effect of acidification versus no acid treatment on $\delta^{13}C$ and $\delta^{15}N$ in four common macroinvertebrates from the Elbe estuary, i.e. *Gammarus* spec., *Mesopodopsis slabberi*, *Neomysis integer* and *Palaemon longirostris*. Our results indicate that acid treatment of common estuarine macroinvertebrates before stable isotope analysis depends on the species-specific carbonate content and is not required for all species when analyzing aquatic food webs. Based on our and results from other studies, we created a decision tree to guide future research in assessing the need of acidification and to provide further options to remove the effects of inorganic carbon from stable isotope analysis.

Materials and Procedures

We derived samples of common estuarine macroinvertebrates during a research cruise conducted by a commercial stow-net vessel in the Elbe estuary in spring 2022. Samples were collected with a ring net that has an opening of 0.9 m and a mesh size of 1000 µm to collect common prey species for fish, including *Palaemon longirostris*, *Gammarus* spec. as well as mysid species *Mesopodopsis slabberi* and *Neomysis integer*. After collection individuals were immediately rinsed with milli-Q and stored in tubes at -80°C. For further analysis, samples were dried for 24 hours using a freeze dryer and afterwards homogenized using a cell lyser. Samples of each species were separated into `acid washed´ and `no acid´ groups with 10 samples for each treatment and species. Acid washed samples were placed in ceramic dishes using a spatula and carefully sprinkled with 0.2 µL of 10% HCl solution following the procedure of the `Champagne test´ (Jaschinsky et al. 2008). Samples were subsequently dried for one hour at 60°C in the oven and the acid treatment was repeated afterwards. Acid washed samples were dried again for 12 hours at the same temperature to assure that inorganic carbon has been completely removed. Afterwards, samples were first filled into silver capsules (0.8 – 1.2 mg) and then folded into tin capsules. The untreated dried samples were weighed directly into tin capsules at the same quantity immediately after homogenization.

Acid washed and untreated samples were analyzed by the UC Davis Stable Isotope Facility in California using a continuous flow isotope ratio mass spectrometer IRMS (Europa Hydra 20/20; Europa Scientific, Cambridge, UK). The standard δ notation of ^{13}C and ^{15}N is used to report the isotopic ratios of both elements relative to the international standards VPDB (Vienna Pee Dee Belemnite) for carbon and atmospheric air for nitrogen using the formula:

$$\delta X = [\left(\frac{R_{Sample}}{R_{Standard}}\right) - 1] \qquad \text{Eq. 5.1}$$

where X is the stable isotope ratio per mille (‰) and R the mass ratio of the standard or the sample ($^{13}C/^{12}C$ and $^{15}N/^{14}N$).

Differences between acid washed and no acid samples in terms of stable isotopes were assessed with paired t-tests per species after we had verified the normality of the data.

We furthermore computed a carbonate proxy calculated using C:N ratios of no acid ($C{:}N_{no\ acid}$) and acid-washed samples ($C{:}N_{acid\ washed}$) to determine the effect of inorganic carbon on the difference between acidified and untreated samples regarding δ^{13}C and δ^{15}N (Jacob et al. 2005, Ng et al. 2007):

$$\text{Carbonate proxy} = \left(\frac{C{:}N_{no\ acid}}{C{:}N_{acid\ washed}}\right) - 1 \qquad \text{Eq. 5.2}$$

The $CaCO_3$ content within the sample is assumed to be low when the proxy is zero and high with increasing values (Ng et al. 2007). The carbonate proxy was plotted against the difference between untreated and acid washed samples of each stable isotope.

Assessment and Discussion

Our study demonstrates that the necessity of acidification of common estuarine macroinvertebrates for stable isotope analysis is species dependent. $\delta^{13}C$ ratios were significantly lower after acidification in macroinvertebrate species ranging from 0.2‰ in *Neomysis integer*, 0.5‰ in *Palaemon longirostris* and 1.7‰ in *Gammarus* spec. (Fig. 5.1, Tab. 5.1). Acid treatment resulted in lower $\delta^{15}N$ ratios in *N. integer* and *P. longirostris* but was only significant in *Gammarus* spec. samples, where treatment resulted in 0.9‰ higher $\delta^{15}N$ values (Tab. 5.1). In contrast, we observed no significant effect of acidification on the mysid shrimp *Mesopodopsis slabberi*, where treated and untreated samples in mean stable isotope ratios varied only marginally. Depleted isotope values in our analysis can be likely explained by the chemical removal of ^{13}C- and ^{15}N-rich organic compounds, such as amino acids and proteins, during the acidification (Ostle et al. 1999, Ng et al. 2007).

We generally observed overall higher data dispersion in isotope values of acid washed samples compared to untreated samples. This higher variation in the data may be a direct effect of the sample treatment and has been shown for ground shrimp samples, especially in $\delta^{15}N$ values (Bunn et al. 1995). However, other authors suggested that the variation in $\delta^{15}N$ values is a result of compound-specific leaching rates of organic N during acid treatment (Goering et al. 1990).

Our analysis shows further a decrease in $\delta^{13}C$ values for most species after acidification. Macroinvertebrates that form exoskeletons accumulate $CaCO_3$ by extracting heavy HCO_3^- from the surrounding seawater, resulting in higher $\delta^{13}C$ levels (Yokoyama et al. 2005). The removal of inorganic carbon using acid washing therefore often leads to depleted $\delta^{13}C$ levels after treatment (Søreide et al. 2006).

The additional work steps in the laboratory due to the acid washing and subsequent drying also increase the sources of error when processing the samples, which can potentially be reflected in the variability of individual samples. Increasing data variation can lead to a loss of statistical reliability and thus to misinterpretation of the results (Bunn et al. 1995).

Tab. 5.1 Results of the statistical analysis of differences between stable isotope ratios of $\delta^{13}C$ and $\delta^{15}N$ (in ‰ ± SD) in acid-washed and no acid treated samples of common estuarine invertebrates using paired t-tests (df = 10 for all comparisons).

Species	Acid washed $\delta^{13}C$ (‰)	No acid $\delta^{13}C$ (‰)	Test output	Acid washed $\delta^{15}N$ (‰)	No acid $\delta^{15}N$ (‰)	Test output
Gammarus spec.	-25.9 ± 0.2	-24.2 ± 0.1	t = -24.359**	12.1 ± 0.2	11.2 ± 0.05	t = 11.711**
Mesopodopsis slabberi	-20.2 ± 0.3	-20.2 ± 0.02	ns	15.0 ± 0.3	15.1 ± 0.03	ns
Neomysis integer	-25.9 ± 0.2	-25.7 ± 0.02	t = -2.6312*	14.8 ± 0.2	14.8 ± 0.03	ns
Palaemon longirostris	-26.4 ± 0.7	-25.9 ± 0.7	t = -5.1012**	13.3 ± 1.0	13.4 ± 0.8	ns

df = degrees of freedom; *p < 0.05; **p < 0.001; ns = non-significant

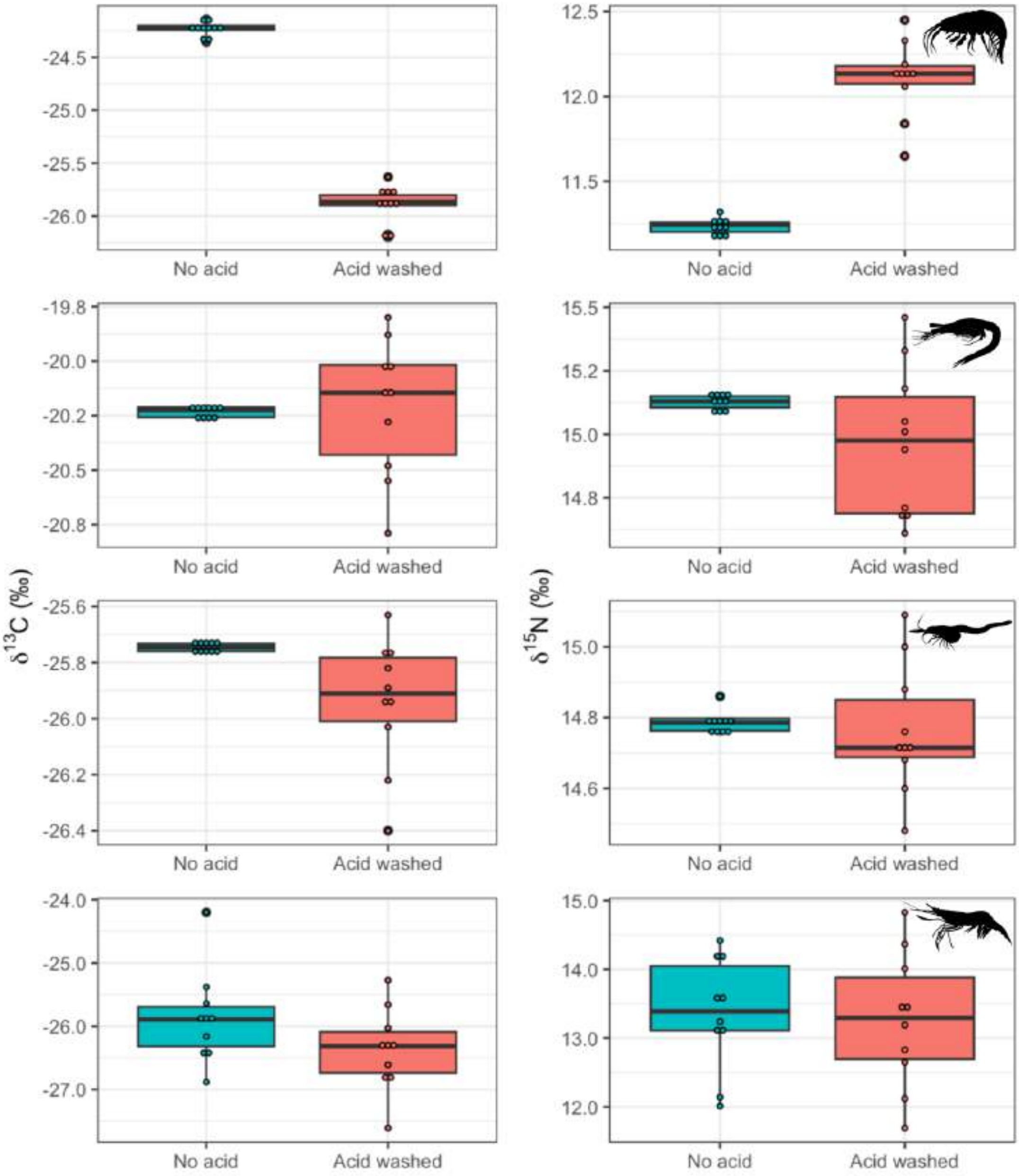

Fig. 5.1 Differences between $\delta^{13}C$ (left) and $\delta^{15}N$ (right) values between acid washed (red) and no acid (blue) washed samples of common estuarine macroinvertebrates (from top to the bottom): *Gammarus* spec., *Mesopodopsis slabberi*, *Neomysis integer,* and *Palaemon longirostris*. Boxplots show mean values (solid horizontal bars) and standard deviation (SD) (upper and lower horizontal bars) with points representing individual measurements.

The biplot visualization of $\delta^{13}C$ and $\delta^{15}N$ displays the differences between acid washed and untreated samples and the related data variability (Fig. 5.2). Clearly, an analysis without acidification resulted in inflated values of both isotopes in *Gammarus* spec., while no strong effect is observed for *M. slabberi* and *N. integer*. The values of *P. longirostris*, however, show a high variability in both untreated and acid washed samples, suggesting a high natural variation but no strong effect of acidification.

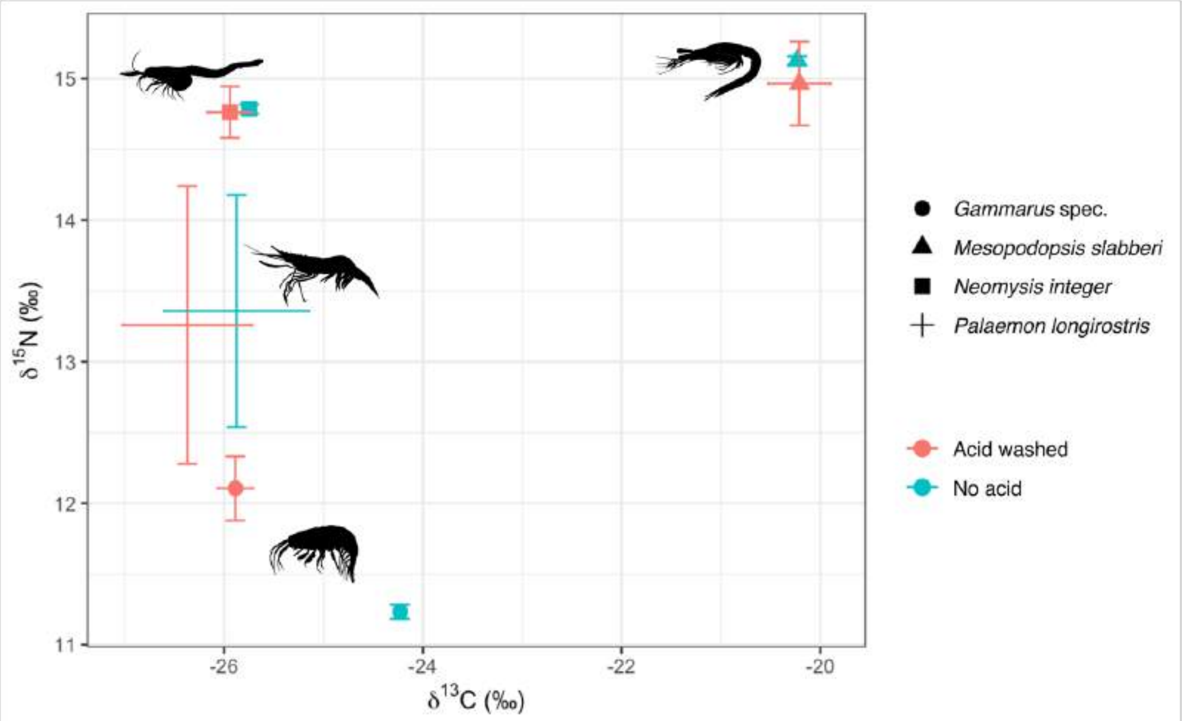

Fig. 5.2 Stable isotope biplot of δ^{13}C and δ^{15}N (± SD) values with acid washed and no acid treatment of common estuarine macroinvertebrates *Gammarus* spec., *Mesopodopsis slabberi*, *Neomysis integer* and *Palaemon longirostris*.

Although the effect of acidification on stable isotope values has been studied over the last 40 years there is still uncertainty about its importance since the results deviate between studies. Investigations on plankton showed no effects of acid treatment in some studies (Chanton and Lewis 1999) but did in others (Pires-Teixeira et al. 2020). Carabel et al. (2006) observed a size-dependent effect in which only small (< 300 µm) and large (> 750 µm) plankton were affected by acid treatment. Studies on macroinvertebrate species indicated a species-specific effect of acidification. For instance, the results of Yokoyama et al. (2005) showed low δ^{13}C and δ^{15}N values and a significant effect of acid washing on δ^{13}C in ghost shrimp (*Nihonotrypaea* spec). Studies on mud shrimp (*Crangon septemspinosa*) (Bosley and Wainright 1999) and the related brown shrimp (*Crangon crangon*) (Jaschinsky et al. 2008) revealed no effect of acidification, while Bunn et al. (1995) observed an effect on δ^{15}N values in speckled shrimp *Metapenaeus* spec. In situ acidification of the mysid shrimp *Praunus flexuosus* by Jaschinsky et al. (2008) indicated no significant difference in the isotopic values but a decrease of both δ^{13}C and δ^{15}N. Acid treatment of amphipods such as gammarids consistently showed significant effects on δ^{13}C, but no differences in δ^{15}N with depleted values in both isotopes (Jaschinsky et al. 2008, Kolasinski et al. 2008).

Further the carbonate proxies of the macroinvertebrates were calculated as the effect on δ^{13}C becomes higher with increasing carbonate in the tissue (Serrano et al. 2008). Carbonate proxies showed no negative mean values in our study on common estuarine macroinvertebrates of the Elbe estuary (Fig. 5.3). Single negative carbonate proxy values revealed that the biogenic N content decreases more than the C content due to acid

washing, which leads to higher C/N ratios in decalcified samples (Ng et al. 2007). As this negative effect is assumed to be independent of the $CaCO_3$ content of the sample, the values must not be corrected (Jacob et al. 2005).
We calculated the highest carbonate proxy value for *Gammarus* spec. (0.119 ± 0.03‰) and lowest values for mysid shrimps *Neomysis integer* (0.002 ± 0.03‰) and *Mesopodopsis slabberi* (0.007 ± 0.02‰) (Fig. 5.3). Mean $CaCO_3$ proxy for decapod *Palaemon longirostris* was 0.02 ± 0.04‰. The estimation of the carbonate proxy is an important tool to evaluate the necessity of acidification in food web analysis using stable isotopes. Serrano et al. (2008) recommend acidification of only carbonate-rich organisms prior to $\delta^{13}C$ analyses. Biotic hard structures with a carbonate proxy > 0.05 should be acid washed, whereas the efficiency of acid treatment should be proofed per taxa when the estimated proxy lies between 0.03 and 0.05 (Kolasinski et al. 2008).

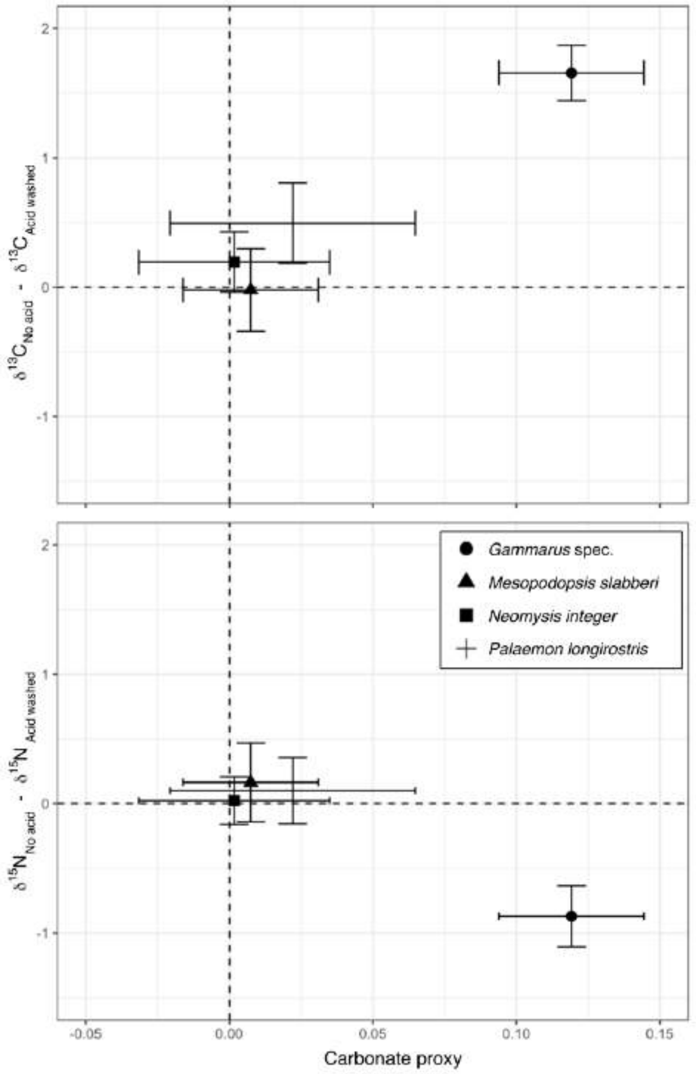

Fig. 5.3 Carbonate proxy (mean ± SD) showing the effects of sample $CaCO_3$ content on $\delta^{13}C$ and $\delta^{15}N$ of no acid and acid washed samples of common estuarine macroinvertebrates *Gammarus* spec., *Mesopodopsis slabberi*, *Neomysis integer* and *Palaemon longirostris*.

Several authors emphasized the importance of a standardized procedure for handling inorganic carbon in food web studies using stable isotopes and recommended acid treatment of samples only when "absolutely necessary". (e.g., Jaschinsky et al. 2008, Serrano et al. 2008, Pires-Teixeira et al. 2020). As mentioned in the introduction, there are also other procedures to remove inorganic carbon. But the decision on what procedure to use or not is sometimes challenging as the realization of a study often depends on various practical issues (e.g., material availability, conditions in the field) as well as structural limitations (e.g., time management, staff capacity). Therefore, we here provide a basic decision tree (Fig. 5.4) based on previous studies (e.g., Jacob et al. 2005, Jaschinsky et al. 2008, Brodie et al. 2011, De Lecea et al. 2011, Schlacher and Connolly 2014, Pires-Teixeira et al. 2020) that 1) clarifies whether acidification to remove inorganic carbon is necessary for further analysis, and 2) provides alternative options.

Following our guideline, we recommend to first decide on the research question, especially if a study targets upper trophic levels only or additionally lower trophic levels of the food web. Other studies with comparable analyses, research questions or similar target species should be considered. For instance, in ecological studies of higher trophic levels such as fish or other consumers, lower trophic level organisms are often analyzed without any treatment (e.g., Cunjak et al. 2005, Ravinet et al. 2013) presumably because it is assumed that a consumer assimilates both organic and inorganic $\delta^{13}C$ from its prey while ingesting the whole individual that is later reflect in its own $\delta^{13}C$ values. Whereas in studies dealing with gammarids and lower trophic levels, the removal of inorganic carbon already appears to be a standard procedure (e.g., Søreide et al. 2006, Rothhaupt et al. 2014, Remy et al. 2017). After assessing the necessity to remove inorganic carbon based on previous studies, either further treatment options are evaluated, or the stable isotope analysis is performed without intermediate steps. Next, the carbonate proxy of the investigated species is determined, either through an own experimental setup, already existing data, or the outcomes of comparable publications. For organisms for which no data are available morphological and physiological features (e.g., shell-forming mollusks or chitinous exoskeletons of crustaceans) can be used to verify the carbonate content (Schlacher and Connolly 2014, Pires-Teixeira et al. 2020). If the carbonate proxy is low (< 0.05), the effect of inorganic carbon on the $\delta^{13}C$ values can be neglected and the analysis can be performed without further treatment of the samples (Jacob et al. 2005, Serrano et al. 2008). Instead, if the proportion of inorganic carbon is presumably high (carbonate proxy > 0.05) (Serrano et al. 2008), mechanical removal of the hard structures is considered the next option (Mateo et al. 2008). Especially in large invertebrates it is recommended to mechanically remove compartments such as exoskeletons or bones before the analysis to avoid acid treatment and proceed directly to stable isotope analysis (Mateo et al. 2008). When this mechanical procedure is not an option, chemical treatment of the specimens via acidification is attempted. We strongly agree with other authors that a standardized protocol for acidification should be used to ensure comparability of data (e.g., Jaschinksy et al. 2008, Serrano et al. 2008).

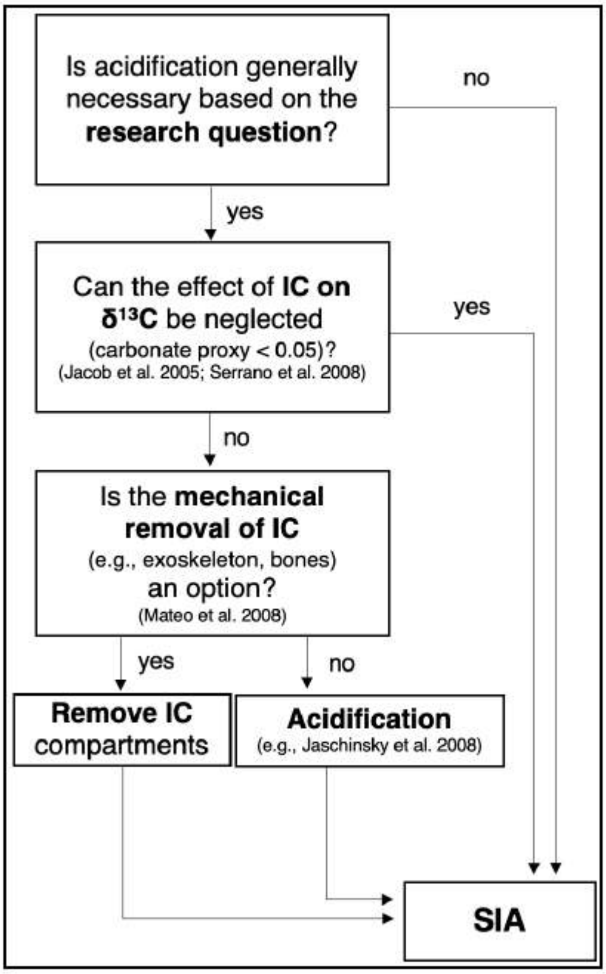

Fig. 5.4 Decision tree to evaluate the most appropriate method to handle potential effects of inorganic carbon (IC) in food web analysis using stable isotope analysis (SIA) based on previous studies.

Based on our analysis of estuarine macroinvertebrates, which includes empirical statistics of δ^{13}C and δ^{15}N of acid-treated and untreated samples, calculation of the carbonate proxy, and following our decision tree-based guidelines, we conclude that acidification of Gammarus spec. is necessary, while the removal of inorganic carbon from mysids (*M. slabberi* and *N. integer*) can be neglected due to the low carbonate proxies. For *P. longirostris*, we recommend removing the exoskeleton mechanically prior to analysis to avoid acid treatment.

Comments

The findings of our study revealed that acidification can serve as a reliable tool for the removal of inorganic carbon, but also has the disadvantage that acid treatment generally leads to a higher variability of the data. Overall, a standardized approach to inorganic carbon removal is needed to ensure comparability of data from different ecological studies. Our work provides guidance on whether and how biogenic carbon can be removed from samples and contributes to the standardization of methods. We agree with the opinions of other colleagues from previous studies that acidification should only be performed when other procedures are inappropriate.

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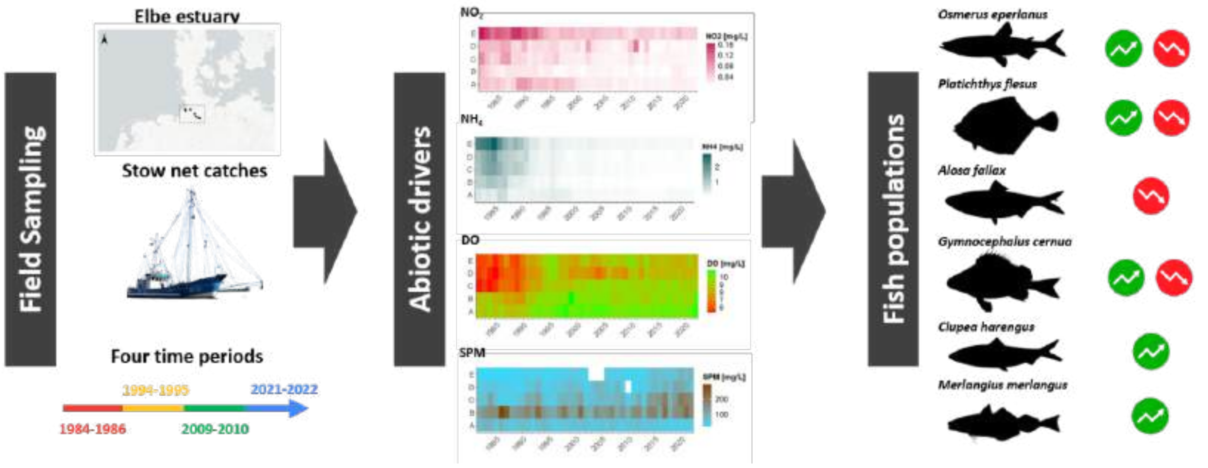
Declaration of interest

The authors have no conflicts of interest to declare.

EH: Study design, visual conceptualization, data acquisition, statistical analysis, editing and writing, AP: laboratory work and statistical analysis, CM: Writing, review and editing, supervision.

Chapter 6

Environmental factors affecting the fish fauna structure in a temperate mesotidal estuary: a 40-year long-term study in the Elbe estuary, southern North Sea



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Title: Environmental factors affecting the fish fauna structure in a temperate mesotidal estuary: a 40-year long-term study in the Elbe estuary

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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 such a habitat, we created a long-term time series over the last four decades (1984–2022) combining fish species compositions and abundances with environmental conditions along the course of the temperate mesotidal Elbe estuary. We detected major changes in the species richness and 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 abundances, specially *Osmerus eperlanus*, until 2010. Anthropogenic hydromorphological interventions, however, 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 abundances dropped by over 91% compared to 2010 to an all-time low in the data. This reduction was primarily a result of a decline in juveniles of the key species smelt (*Osmerus eperlanus*) in the system along with declines of *Alosa fallax, Platichthys flesus, Gymnocephalus cernua, Abramis brama* and other species. On the contrary, marine species (*Clupea harengus* and *Merlangius merlangus*) abundances increased. Overall, the time-series provides insight into the strong impact of human intervention surpassing yet climatic impacts in the system, that are however expected to lead to further stressors.

Keywords: Estuary, fish assemblage, guild composition, fish stocks, smelt

Chapter 6 – Long-term study on Elbe fishes

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 abundance
- 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 particulate matter
TOC	total organic carbon
wTemp	water temperature

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 (e.g., salinity, temperature, oxygen concentration, turbidity, river runoff), 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. 2017). 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 and 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 and Gaumert 1982, Thiel 2011, Eick and Thiel 2014, Scholle and Schuchard 2020). However, the Elbe estuary still harbours internationally important ecological populations and habitats (Thiel and Thiel 2015), like a number of other temperate estuaries worldwide (Ducrotoy et al. 2019). For instance, in the freshwater and oligohaline regions of the Elbe estuary important nursery areas of different fish species were found (Thiel 2001). The Elbe estuary provides also essential feeding and spawning grounds as well as migration routes for a number of fish species (Nellen and Thiel 1994, Thiel et al. 1996, Thiel 2003) constituted with around 80 fish species the most species-rich estuary in Europe (Thiel 2011).

Generally, 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). In previous studies fish species in the Elbe estuary were grouped into life cycle guilds (e.g., Thiel 2011) according to their biogeographic affinity (e.g., Thiel et al. 2003, von Beusekom et al. 2017) for two or three selected time periods, but an overview of the entire period from the 1980s to the present day is still lacking. This in part due to fact that monitoring data of fish composition and abundances available from state funded monitoring used varying fishing methods rendering catch data from before 2000 and recent data incomparable. 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.

To fill the knowledge gaps shown above, the aim of our study was to analyze 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 and Potter 2001, Thiel et al. 2003, Thiel 2011, Eick and Thiel 2014). The aims of the study include (1) the assessment of changes in the life cycle categories, (2) the quantification of changes in the abundances of the fish species and (3) the identification of long-term shifts of environmental factors and their influence on the fish species abundances. 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.

Material and methods

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 and Potter 2001, Eick and Thiel 2014, Pein et al. 2023). In past studies, the Elbe estuary was categorized as a well-mixed estuary (Muylaert and Sabbe 1996, Amann et al. 2012, Eick and 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 and 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.

We selected five stations along the salinity gradient at regularly spaced distances within the Elbe estuary (Fig. 6.1): 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.

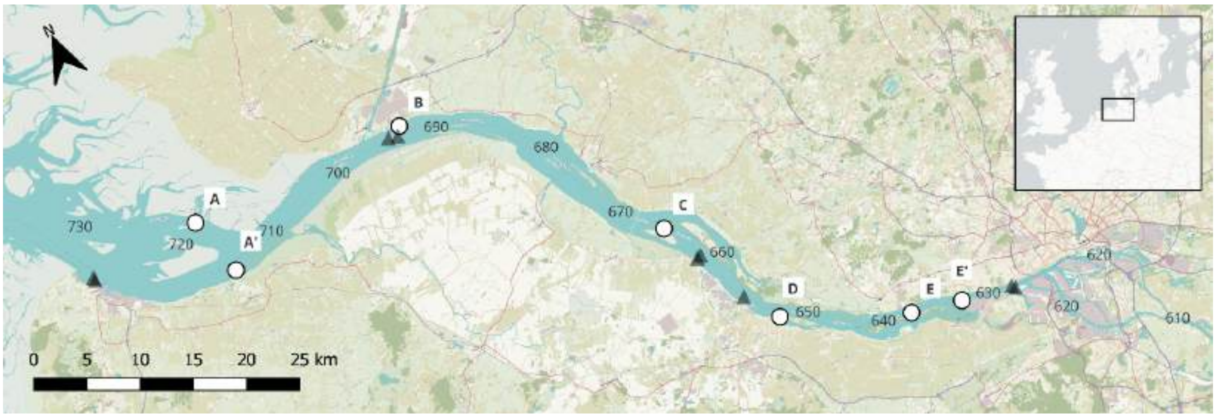

Fig. 6.1 Elbe estuary with the sampled stow net stations (white circles): Medem Reede/Medemgrund at Ekm 713/716 (A'/A), Brunsbüttel at Ekm 692 (B), Schwarztonnensand at Ekm 665 (C), Twielenfleth at Ekm 651 (D) and Neßsand/Mühlenberger Loch at Ekm 633/639 (E'/E). Stations from which environmental data were obtained from the FGG Elbe data portal were indicated with triangles (Map sources: NextGIS, CartoDB).

Fish sampling and calculation of abundances

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 using commercial stow net vessels having mesh sizes between 5 and 10 mm at the cod end. 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 and Potter 2001, Thiel et al. 2003, Eick and 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 and Nielsen (1999), Kottelat and Freyhof (2007), Knebelsberger and Thiel (2014) and Froese and Pauly (2022).

To calculate fish abundances (individuals per 1 million m^3), we used net opening dimensions and the water volume per haul. Water flow through the stow net was measured using mechanical flow meters (Hydro-Bios® and/or General Oceanics®) during periods II, III and IV. 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 of the Elbe estuary occurred, approximation of the water volume based on these two periods was regarded as sufficiently accurate.

Harmonization of fish abundance 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 and one tide was sampled per station (Möller 1988). 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 each tidal phase (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 and 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 4h for period I (Möller 1988), 4h during period II (Thiel et al. 2003), 3h (combined) during period III (Eick and Thiel 2014) and 3h during period IV. 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 and Thiel 2014) a higher spatial and temporal resolution of the sampling data were available. In order to ensure the best possible comparability of the abundance 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.

Life cycle categories and geographical distribution categories

Fish species in the Elbe estuary were grouped into life cycle guilds (LCG) following Thiel and Potter (2001), Thiel et al. (2003), Elliott et al. (2007), Franco et al. 2008, Thiel et al. (2011) and Eick and Thiel (2014): S: marine stragglers (e.g., *Ammodytes tobianus*), O: marine estuarine-opportunists (e.g., *Clupea harengus*), E: solely estuarine (e.g., *Pomatoschistus microps*), C: catadromous (e.g., *Anguilla anguilla*), A: anadromous (e.g., *Osmerus eperlanus*), F: freshwater (e.g., *Sander lucioperca*). These definitions were based on the most recent work by Potter et al. (2013), who classified life cycle categories of estuarine fish species worldwide. To ensure exact comparison with previous data (Thiel et al. 1995, Eick and 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 abundances of marine species and an increase of abundances of freshwater species (Thiel 2011, Eick and Thiel 2014). In the past century, the anadromous smelt *Osmerus eperlanus* has been dominant fish species in the Elbe estuary since it occurs in the entire estuary with high abundances and biomasses (Ehrenbaum 1894, Lillelund 1961, Möller 1988, Thiel et al. 1995, Eick and Thiel 2014). The invasive *Neogobius melanostomus* was assigned to the freshwater LCG.

Collection of environmental data

Environmental data originated from long-term routine monitoring programs by the local authorities and were downloaded from the 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 supplements Tab. S6.1).

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 (interval measurements) with usually one to four measurements per month were available. Measurements from the longitudinal profile and interval measurements were combined in order to calculate monthly and annual means for each environmental variable at the designated station.

Statistical analyses

Non-metric multidimensional scaling (NMDS) was applied to fourth root transformed abundance data on a Bray-Curtis distance matrix using 'metaMDS' function in the R-package 'vegan' (Oksanen et al. 2022). 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. Permutational multivariate analysis of variance (PERMANOVA) was used to compare dispersion of the group variable 'period' using pairwise adonis function in 'vegan'. In a two-dimensional NMDS ordination stress values below 0.20 are regarded as acceptable (Quinn and Keougth 2002). Over the entire time series, fish abundance 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 NMDS computation (period I n = 79, period II n = 63, period III n = 126, period IV n = 42). Influences of environmental factors on the fish species composition were analysed by using post-hoc vector fitting function 'envfit' to the NMDS-ordination following a workflow repeatedly used in estuarine fish species compositions (Troast et al. 2022, Lundstrom et al. 2022, Harrison-Day 2024).

Results

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: *Osmerus eperlanus, Clupea harengus, Alosa fallax, Gasterosteus aculeatus, Gymnocephalus cernua, Platichthys flesus, Sprattus, sprattus, Blicca bjoerkna, Abramis brama, Syngnathus rostellatus, Merlangius merlangus, Sander lucioperca, Pomatoschistus spp., Liparis liparis* and *Anguilla anguilla* (Fig. 2, Tab. A.2). While smelt (*O. eperlanus*) 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 (*A. fallax*) was the second most abundant species during the 1980s, while in the 1990s *A. fallax* 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 (*C. harengus*) 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 (*G. cernua*), which was the second most abundant species during 2009-2010 (1980s: 15th, 1990s: 5th, 2020s: 7th) and three-spined stickleback (*G. aculeatus*) which was the second most abundant species during the 1990s (1980s: 4th, 2009-2010: 11th, 2020s: 9th). While whiting (*M. merlangus*) was the third most abundant species in the 2020s, the ranks of M. merlangus were lower in the 1980s (42nd), 1990s (10th), 2009-2010 (26th).

The mean abundance per haul of several species exhibited varying patterns over time (Fig. 6.2, Tab S6.2). Specifically, there was an increase in abundance 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 abundance of fish species such as *O. eperlanus, S. lucioperca, G. cernua, L. fluviatilis, A. brama, B. bjoerkna* and *S. rostellatus* 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 abundance per haul of *O. eperlanus* 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. *A. anguilla* expressed a gradual decrease over time, displaying the highest abundance during the 1980s and the lowest during 2020s. The abundance of *A. fallax* decreased from the 1980s from 1,700 to 52 in the 1990s. With the period 2009-2010 abundance of *A. fallax* increased to 1,000 and decreased with the latest period (2021-2022) to 12. Mean abundance of *P. flesus* increased in the 1990s up to 600, but exceeding to the time periods 2009-2010 (240) and 2021-2022 (33) the abundance was reduced. *Pomatoschistus* spp. had the highest abundance during the 1980s (165), which decreased to 15 in the 1990s and 0.7 during 2009-2010, while the abundance of *Pomatoschistus* spp. increased in the time period 2021-2022 (26). Additionally, during 2021-2022 herring (1,500) and whiting (314) displayed their highest mean abundances in the analyzed time frame.

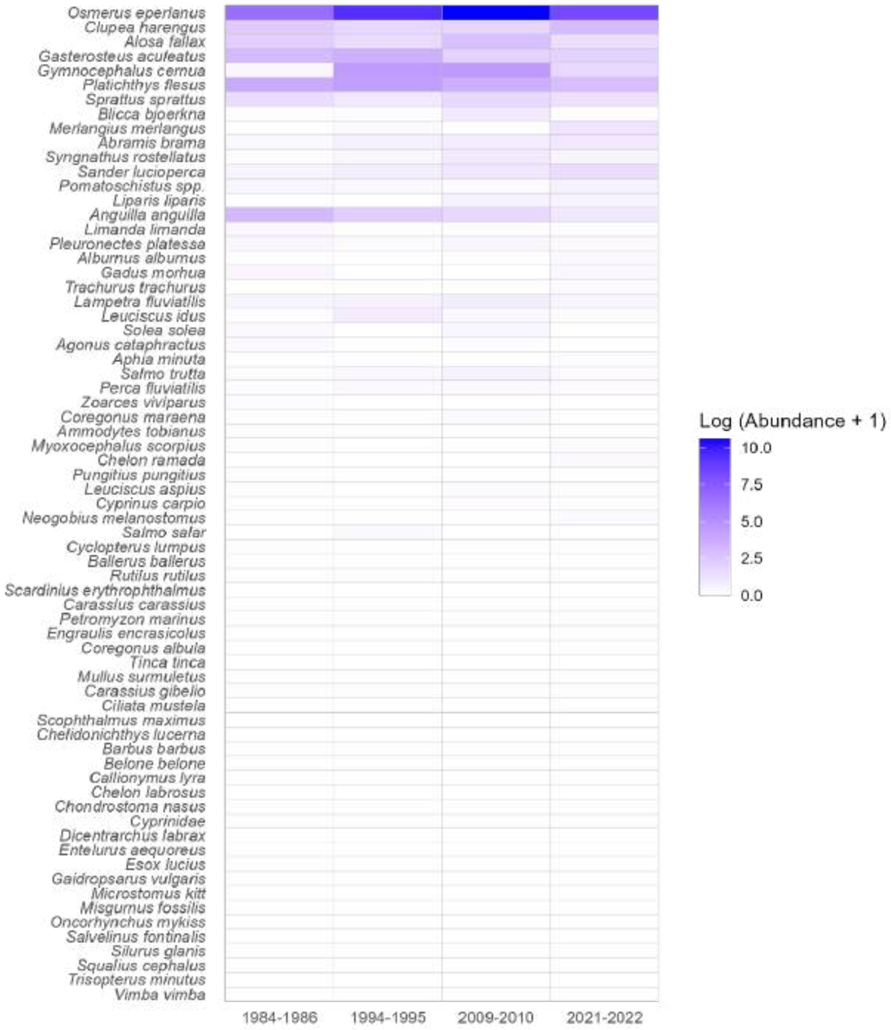

Fig. 6.2 Fish taxa found in in the Elbe estuary at the distinctive time periods 1984-1986, 1994-1995, 2009-2010 and 2021-2022. Changes of mean abundances per haul (Log transformation) indicated by heatmap. Log-transformation of abundance data for enhanced visualization (large ranges of abundances between species), untransformed data see Tab. S6.2.

Spatiotemporal abundance variance of dominant fish species

Differences in the distribution of species abundance 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 (*O. eperlanus*, *C. harengus*, *A. fallax*, *G. aculeatus*, *G. cernua*, *P. flesus)* as well as important predatory fish species (*S. lucioperca*, *M. merlangus*), *L. fluviatilis* as endangered anadromous species and *A. anguilla* as endangered catadromous species across various stations in the Elbe estuary were chosen to indicate their variance across spatiotemporal scales (Fig. 6.3). Abundances of *O. eperlanus*, changed most substantially in the upstream regions being completely absent on 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 abundances were observed the Hamburg region. At E/E' with a median abundance of 25,000 during period IV, the smelt population was reduced by nearly 85 %, compared to period III with a median abundance of 162,000. In the same time period, the median abundance at D was reduced from 76,000 to 5,000 while at C the abundance was reduced from 33,000 to 2,200.

Generally, *C. harengus* exhibited its highest abundances in the downstream areas, specifically at stations A/A' and B near Cuxhaven and Brunsbüttel. Throughout all four time periods, *C. harengus* was found up to the mid estuarine section (C). Abundance of *C. harengus* was notably lower during the 1980s compared to surveys conducted in the 2020s, where the abundance reached its peak in that region. Only during the periods of 2009-2010 and 2021-2022 the presence of *C. harengus* extended till the upper estuary (D), with higher abundance observed in the 2020s. *A. fallax* exhibited highest abundances, particularly during the 1980s at D and C. Anyway, the highest median abundance (27) of *A. fallax* 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 abundances of *A. fallax* were observed at E/E' (spawners) and B (juveniles).

G. aculeatus abundance was highest during the 1990s period, especially at C, where the abundance of *G. aculeatus* was highest. During the most recent period (2021-2022) abundances of *G. aculeatus* were considerably lower at all five stations compared to period III (2009-2010). During the 1980s, *G. cernua* 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 abundances. However, during the 1990s and the 2009-2010 period, the abundance of *G. cernua* was highest in the freshwater region, particularly at D and E/E'.

P. flesus was found at its highest abundance during the 1990s with A/A' (median abundance: 3,000). Lower median abundances of *P. flesus* 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 abundances of *S. lucioperca* 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 abundance of zander increased from 7.5 to 17 at E/E'. At the more downstream areas zander abundances were lower during all four time periods. *M. merlangus* was found exceptionally in the downstream regions in the river mouth at A/A' and B, where the higher abundances were found at the outermost station A/A', especially during the 2021-2022 survey. During period IV at A/A' the median abundance of *M. merlangus* reached 263, while the median abundance at the other periods was 0, since *M. merlangus* occurred only sporadically during those time periods.

Generally, the abundance of river lamprey decreased with our recent sampling in the 2020s. The highest median abundances of river lamprey (*L. fluviatilis*) were found in the river mouth are, especially during the 2009-2010 period at A/A' (2.1). At B the median abundances were lower: 0.7 (1980s) and 0.8 (1990s). During period III at D the maximum abundance of *L. fluviatilis* recorded was 177, followed by 89 at C and 82 at D. The maximum abundance of *L. fluviatilis* in the 2021-2022 period was found at B with 11, which had also decreased. Abundances of *A. anguilla* decreased consistently at all five stations across the Elbe estuary over the four observed time periods.

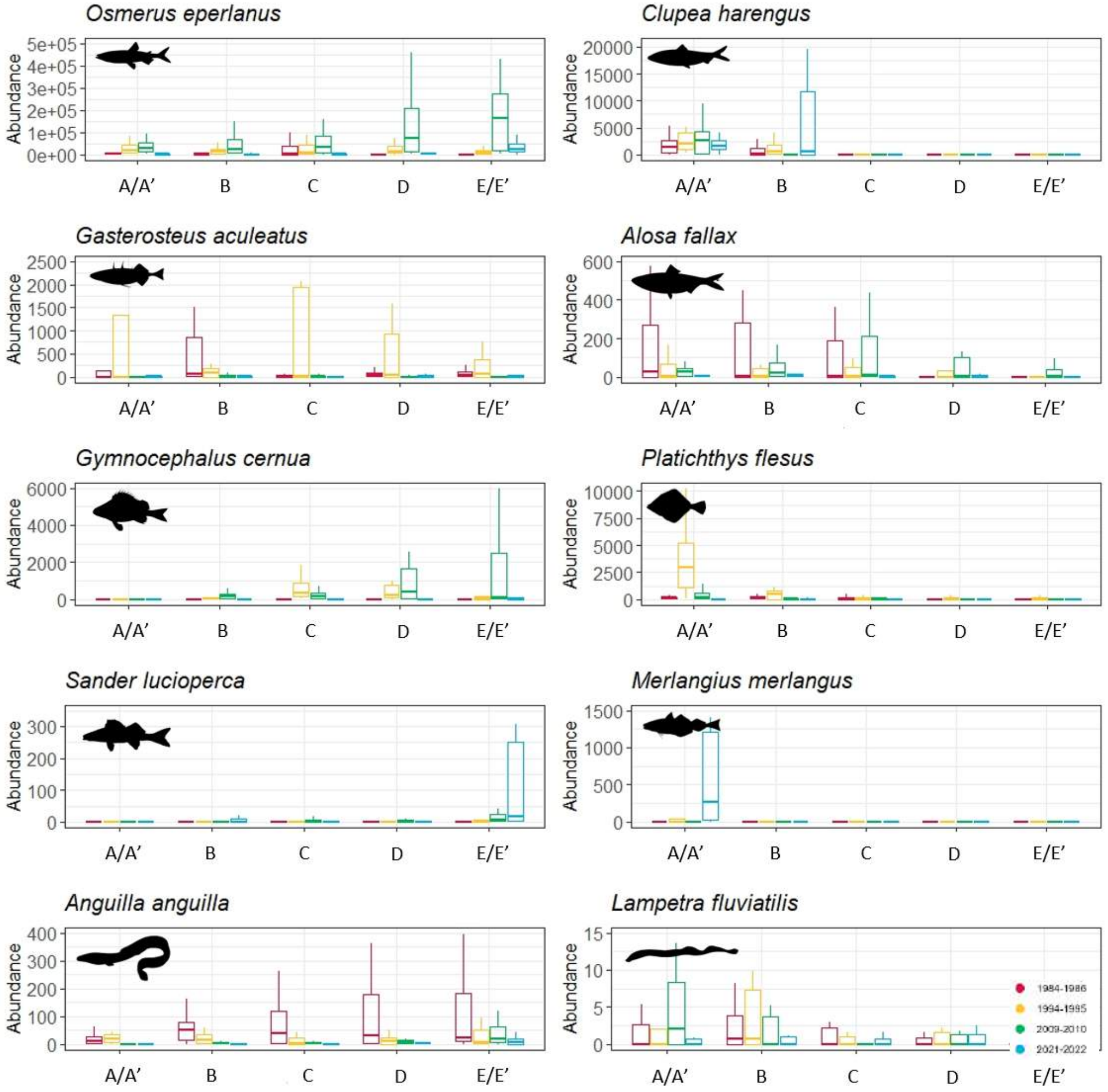

Fig. 6.3 Abundances of ten of the key fish species along the longitudinal course of the Elbe estuary. A line at zero indicating complete absence of the species at the specific station during the referring time period. Medem Reede/Medemgrund at Ekm 713/716 (A/A'), Brunsbüttel at Ekm 692 (B), Schwarztonnensand 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.

Spatiotemporal variations of life cycle categories

Marine stragglers were found only in the downstream areas (A/A' and B), with relatively low and consistent abundances, with less than 0.1% (Fig. 6.4, Tab. S6.3). The highest abundance 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 abundances 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 abundance 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 abundances of the estuarine-opportunists LCG were lower.

In the river mouth section (A/A') the abundance 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, abundances 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 abundances around 0.5 (< 0.1%). The number of catadromous species consistently decreased throughout the entire estuary from the 1980s to the 2020s. The mean abundance 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 abundances were less pronounced compared to the upstream areas. From 2009-2010 period to 2021-2022 period the abundance of freshwater species declined notable in the freshwater regions of E/E' and D.

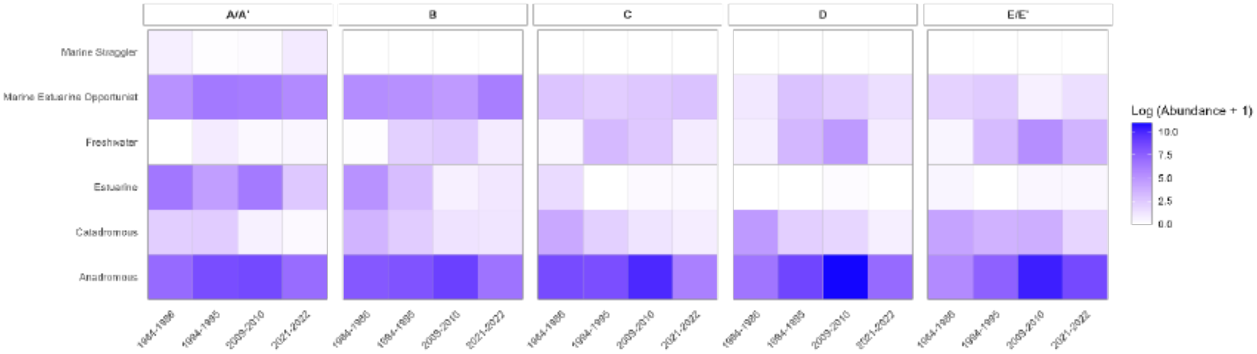

Fig. 6.4 Spatiotemporal variation of mean abundances of LCG at four distinct time periods along the salinity gradient of the Elbe estuary: Medem Reede/Medemgrund at Ekm 713/716 (A/A'), Brunsbüttel at Ekm 692 (B), Schwarztonnensand at Ekm 665 (C), Twielenfleth at Ekm 651 (D) and Neßsand/Mühlenberger Loch at Ekm 633/639 (E/E'). Log-transformation of abundance data for enhanced visualization (large ranges of abundances between guilds), untransformed data see Tab S6.3.

Long term shifts of environmental factors

Over the course of the past 40 years, the Elbe estuary underwent changes in environmental conditions (Fig. 6.5). 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 and 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.

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. 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.

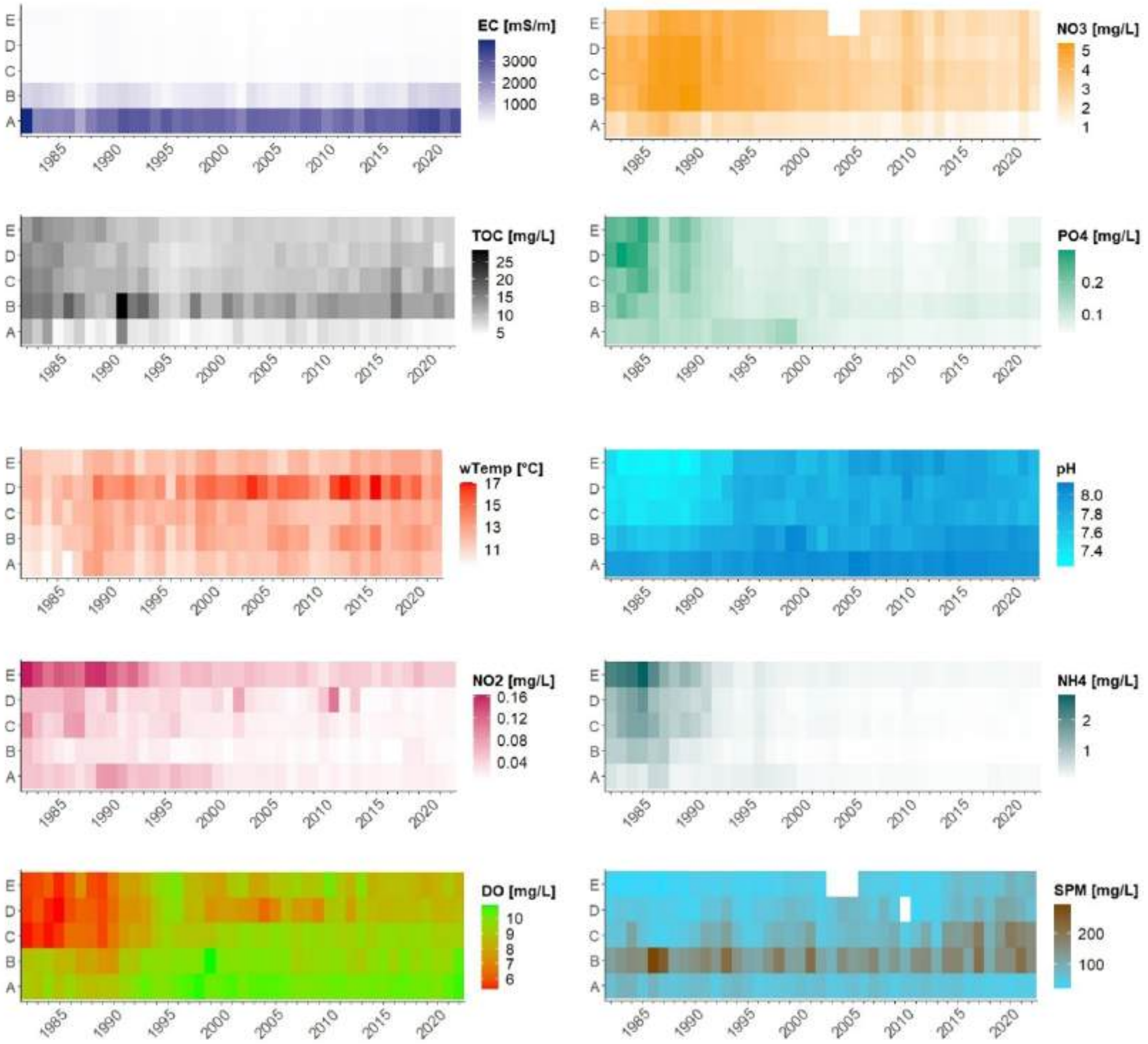

Fig. 6.5 Environmental parameter electric conductivity (EC), nitrate (NO_3), nitrite (NO_2), ammonium (NH_4), phosphate (PO_4), total organic carbon (TOC), suspended particular matter (SPM), water temperature (wTemp), dissolved oxygen (DO) and pH as heatmap (annual mean) from 1982 to 2022. A = Medem Reede/Medemgrund (Ekm 713), B = Brunsbüttel (Ekm 692), C = Schwarztonnensand (Ekm 665), D = Twielenfleth (Ekm 651), E = Neßsand/Mühlenberger Loch (Ekm 633).

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.

Influence of environmental factors on the fish fauna

The NMDS ordination plots represent the fish species, the composition at each site at the respective period and environmental gradients. Marine stragglers (*A. cataphractus, C. lumpus* or *C. lucerna)* and marine-estuarine opportunists (*M. merlangus, G. morhua* or *C. harengus*) were mainly associated with samples from the river mouth (A/A'). Diadromous fish (mainly *O. eperlanus, A. fallax* and *G. aculeatus*) were ordinated more centralized while freshwater species (e.g., *P. fluviatilis, A. brama, L. idus*) were mainly focused on the upper sections D and E/E' (Fig. 6.6). SIMPER analysis (Tab. 6.1) revealed that the largest influence on the ordination is caused by variation of abundance from *O. eperlanus* (21.5%). The species that had the second most influence on the dissimilarity matrix was *C. harengus* (8.9%) followed by *G. aculeatus* (7.9%), *G. cernua* (7.4%), *A. fallax* (7.2%), *S. sprattus* (5.7%), *P. flesus* (5.0%), *A. anguilla* (4.4%), *S. lucioperca* (3.5%) and *A. brama* (3.0%).

Tab. 6.1 Cumulative percentages of the ten fish species responsible for the most variation (75%) in the Elbe estuary (SIMPER-Analysis).

Species	Cumulative contribution [%]
Osmerus eperlanus	21.5
Clupea harengus	30.4
Gasterosteus aculeatus	38.3
Gymnocephalus cernua	45.7
Alosa fallax	52.9
Sprattus sprattus	58.6
Platichthys flesus	63.6
Anguilla anguilla	68.0
Sander lucioperca	71.5
Abramis brama	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 PERMANOVA showed that the largest dissimilarity was between the periods 1984-1986 and 2009-2010 (R^2 = 0.146, $p < 0.001$) and smallest dissimilarity was between the periods 1994-1995 and 2009-2010 (R^2 = 0.052, $p < 0.001$). Dissimilarities were identified between 2021-2022 and 1984-1986 (R^2 = 0.77, $p < 0.001$) as well as 1994-1995 (R^2 = 0.076, $p < 0.001$) and 2009-2010 (R^2 = 0.074, $p < 0.001$) were smaller according to post-hoc PERMANOVA. Strongest influence on the species composition of the fish fauna in the Elbe estuary was attributed by the factor electric conductivity (EC) (r^2 = 0.571, $p < 0.001$). EC acts as a proxy for the salinity, and the salinity gradient is ordinated along the stations with the highest salinity (with the corresponding fish species) at the estuary mouth (A/A') and freshwater sites near Hamburg (E/E'). Water temperature had the second strongest influence ($r^2 = 0.421$, $p < 0.001$) on the composition of fish species. Nitrogen compounds NH_4 ($r^2 = 0.274$), NO_3 ($r^2 = 0.316$) and NO_2 ($r^2 = 0.232$) were correlated significantly ($p < 0.001$) with the calculated Bray-Curtis distance. Also, TOC had a significant influence ($r^2 = 0.166$, $p < 0.001$). Dissolved oxygen ($r^2 = 0.237$) showed significant influence ($p < 0.001$) on the fish composition in the samples. SPM ($r^2 = 0.097$, $p < 0.001$) and pH ($r^2 = 0.084$, $p < 0.001$) were observed with significant influence on the fish species composition.

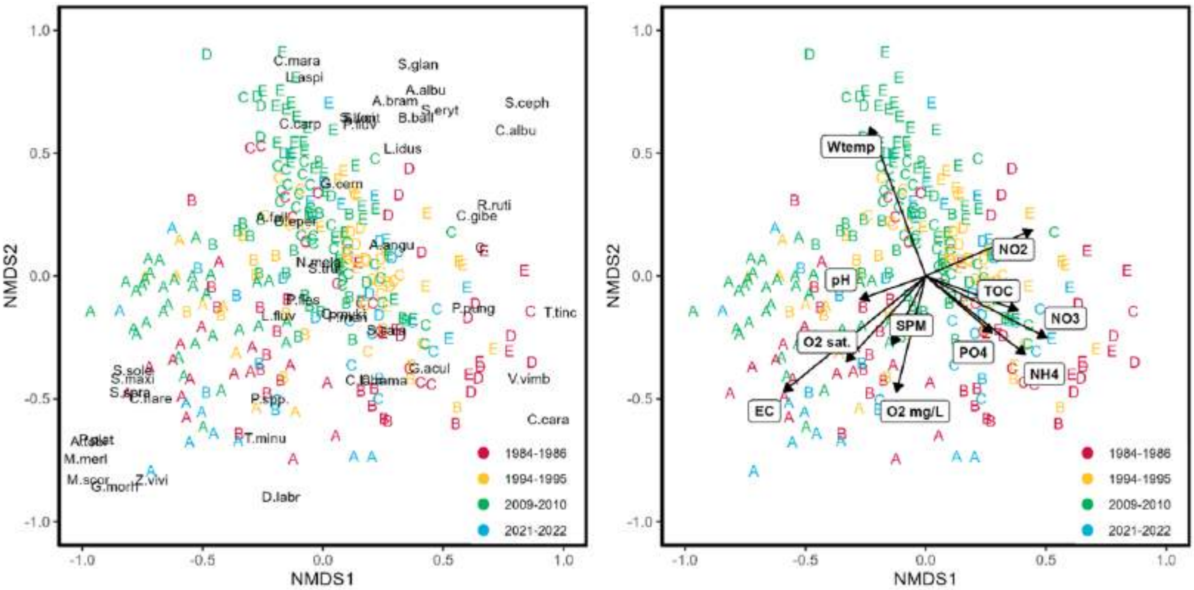

Fig. 6.6 NMDS ordination (stress = 0.165), separated for visualization by left: species as abbreviations; and right: environmental variables as vectors: EC (electric conductivity), SPM (suspended particular matter), NO_2 (nitrite), NO_3 (nitrate), NH_4 (ammonium), PO_4 (phosphate), TOC (total organic 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).

Discussion

Changes of life cycle categories

In this study we aimed to describe changes in the abundance and the species composition in a temperate mesotidal estuary over a long-term time-series and determine the driving factors for fish occurrences.

As being among the most abundant species, *O. eperlanus* and *A. fallax* 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. The recent degradation of general habitat quality including deterioration of nursery habitats (oxygen deficiencies and increased turbidity), together with a reduction in shallow water areas in the freshwater regions (Scholle and Schuchard 2020, Illing et al. 2024), has led to a decline in the abundance of anadromous (juvenile) fish in the Elbe estuary. From period III to period IV the mean abundance 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 catadromous LCG was mainly represented by *A. anguilla*, 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 and not specific to factors found exclusively in the Elbe (Behrmann-Godel and Eckmann 2003, Briand et al. 2007, Decker 2016, Kullmann et al 2017).

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. Due to enlarged channel diameter, larger quantities of marine waters can enter the Elbe estuary during high tides (Zhu et al. 2014, Ralston and 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 and Dewailly 1995). These alterations suggest an ongoing transition of the fish guild composition towards those found in a macrotidal estuary.

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 E and C, which acts as a proxy for salinity. Stations with higher salinities at the river mouth and lower sections (A and B, see Fig. 6.1) had higher abundances of marine stragglers (*Z. viviparus, C. lyra, A. cataphractus*) and marine-estuarine opportunists (*C. harengus, G. morhua, M. merlangus*), which confirms the findings of the spatiotemporal structure found in previous studies (e.g., Thiel and Potter 2001, Eick and 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 and Potter (2001). With diadromous species such as *O. eperlanus, A. fallax, G. aculeatus* in the fish community, species composition changes during migration periods and juvenile nursery time. Smelt abundances peaked between early June and late August, when the population consists predominantly of juvenile young-of-the-year specimens. Abundances of three spined stickleback (*G. aculeatus*) tended to be highest in colder temperatures during winter and early spring samples. In the freshwater sections bream (*A. brama*) abundances tended to be higher at warmer temperatures during summer months. Upstream migration of anadromous fish can be induced by factors such as water temperature (Aprahamian et al. 2003) and river runoff (Lillelund 1961). As fish spawning and nursing (high abundances of juveniles) underlies seasonal patterns significant influence of the water temperature as a proxy for seasonal fish species composition seems reasonable.

In the 1980s water quality in the Elbe estuary was poor due to wastewater sewage (ARGE Elbe 1984, Möller and Scholz 1991), which was expressed by high concentrations of nitrogen compounds (NH_4, NO_2, NO_3), TOC and phosphates. The concentrations of those compounds were highest in the freshwater and upper oligohaline areas of the estuary (Fig. 6.2). NO_2 is particularly toxic to fish by reducing oxygen uptake ultimately causing death (Russo and Thurston 1977) by suffocation which is even more unfavorable 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, NH_4 values that, depending on temperature and pH becomes fish toxic NH_3, 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 and 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 and Scholz 1991, Thiel et al. 1995). During 1985 highest catches of juvenile smelt were in areas with more than 5 mg/L DO (Möller and 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.

Dredging impacts on nursery habitats

While moderate suspension loads are a natural feature of many inland water sheds (Bruton 1985), elevated SPM concentrations can lead to a variety of problems. If suspended solids in the water column exceed a species-specific threshold, sublethal physiological and behavioral stress responses can be triggered (Bruton 1985) but lethal consequences have been described by other authors (e.g., Newcombe and Jensen 1996, Wilber and Clarke 2001). Findings from Thiel and Pezenburg (2001) showed that elevated SPM concentration in the Mühlenberger Loch region can induce alterations in the gill epithelial tissue (increase of mucocytes) of juveniles from *O. eperlanus* and therefore could cause raised mortality rates and stunted growth. Chiasson (1993) observed increased activity after exposition of 10 - 40 mg/L SPM of related smelt (*O. mordax*) under rearing conditions. In the Mühlenberger Loch region (E/E') the SPM concentration doubled in the period 2021-2022 compared to 2009-2010 and 1994-1995 and even tripled compared to 1984-1986. The highest annual mean SPM ever recorded was found during the year 2020 (92.01 mg/L), which primarily were caused by channel alterations (tidal pumping) and maintenance dredging paired with reduced river runoff (Kerner 2007). This potentially affected the reproduction success and recruitment of specimens from the year-1 age group caught during the 2021 sampling campaigns. Thiel and Pezenburg (2001) argued that SPM containing more mineral compounds tend to cause stronger irritations in the gill tissue as SPM containing more organic materials. Hecht and van der Lingen (1992) found that turbidity affects feeding behavior 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. Recent observations from liver transcriptomes of juvenile zander in the system indicated signs of starvation in the maximum turbidity zone which was supported by lower body conditions in that region (Koll et al. 2024a).

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 and Vennell 2024) leading to lower abundances of zooplankton and benthic invertebrates (Shoup and 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 abundances in recent years. Generally, copepod densities are highest in the Mühlenberger Loch area around E/E' (Fiedler 1991, Kafemann et al. 1996, Thiel 2001), making this area the favored nursery habitat for fish larvae, as prey organisms of the optimal size are available here. However, Bilkovic (2009) 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. 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 favor 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. However, the amount of shallow water areas in the Elbe estuary has dramatically been reduced without appropriate compensation, either through construction (Mühlenberger Loch), or siltation. Not only the harbor region faces sedimentation problems. Especially those stagnant areas with slow flow velocities are subject to increased siltation, which is intensified due to increasing suspended sediments in the water column, leading to a decrease of shallow waters. These deficiencies are even enhanced by the increase of suspended solids in the water column from channel deepening and maintenance dredging, which was designated as possible influence on smelt in the Elbe estuary by Scholle and Schuchard (2020). The SPM concentration especially in the freshwater and oligohaline areas of the Elbe estuary has increased, which also altered the fish species composition in our most recent fish sampling period (2021-2022). Kerner (2007) argued that the sediment transport of the Elbe estuary changed significantly after the annual amount of dredged material increased from 4.3 Mio m^3 to over 8 Mio m^3 after 2004. Increasing SPM concentration at C (Schwarztonnensand) showed that the reach of the maximum turbidity zone (MTZ) 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). Model calculations showed that channel deepening appeared to be the main enhancing factor for an upstream transport of sediments (van Maren et al. 2014). 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.

Due to climate change and displacement of precipitation, drought situation had intensified over the last decade in Germany (Petrovic et al. 2022) and river discharges into the Elbe estuary have reduced in recent years, except for one major flooding event in June 2013 (Pein et al. 2023). From 2014 to 2022 there have been no records of annual high runoff events with an effluent of more than 1500 m^3/S (FGG Elbe 2024). River runoff drives the downstream transport of organic and inorganic loads towards the ocean (Pein et al. 2021), and a reduced freshwater flow results in an increased tidal influence. The reduced river runoff leads to a longer retention time of the particles and thus causes an accumulation of suspended particles in the water column. The increased turbidity could have affected the fishes in the Elbe estuary on various levels: firstly, the suspended solids could have caused irritation in the gill tissue, secondly, foraging behavior could have been impaired and finally, overall food availability could have been reduced.

Recent effects of environmental factors on fish species abundances

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 abundance. The mean abundance per haul of *O. eperlanus* reduced by 92.4%, other species such as *A. fallax* were reduced by 98.7%, *G. cernua* by 95.3%, *P. flesus* by 86%, *G. aculeatus* by 78%, *A. brama* by 86.9%, *L. liparis* 96.6%, *S. rostellatus* by 97.8%. Overall smelt abundance decreased in the entire estuary, but the most notable absence was observed in the mid and upper sections (C and D). While oxygen concentration fell below 4 mg/L only on few occasions in those regions, the turbidity at C (Grauerort Ekm 660) increased significantly. Specifically, after the year 2014 high turbidity values (above 1000 FNU) were measured on reoccurring instances. Before 2014 highest turbidity measured in that region was approx. 500 FNU (FGG Elbe 2024). Illing et al. (2024) found that smelt larvae from the Elbe estuary have higher prey intake in lower turbidity, where optimum feeding was found between 100-200 NTU and survival rate of smelt larvae decreased at above 300 NTU. This indicates coherencies between increased upstream turbidity and a decrease of juvenile smelt abundancies.

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. Compared to the previous time periods, the mean abundance per haul of herring was highest during the 2021-2022 survey. The herring abundance (represented by juveniles) increased especially in the more upstream areas C and D. During period I and period II no herring occurred in the more upstream regions D and E, while during period III and IV distribution of herring continued until station D. The more upstream occurrence of marine-estuarine opportunists leads to the conclusion that the marine influence had increased over the time in the upstream areas of the Elbe estuary. Another marine estuarine-opportunist species that benefited in the most recent period was whiting (*M. merlangus*). During spring and summer 2021, highest abundances of juvenile whiting were found at B and A/A'. Whiting is a lusitanian fish species and according to Thiel et al. (2024) abundances of whiting have recently increased in the German North Sea areas. Although high abundances 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.

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 abundances 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 abundances 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.

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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Author contributions

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

Supplements

Tab. S6.1 Stations from where environmental data were extracted from the FGG-Elbe (2024) database with the designated fishing stations.

Fishing station	FGG-Elbe Station	Ekm
A/A′	Cuxhaven Kugelbake	727
	Cuxhaven	725.5
B	Brunsbüttelkoog	694
	Brunsbüttel Elbehafen	693
C	Grauerort	660.6
	Grauerort Anleger	660.5
D	Schwingemündung Stadersand	655
E/E′	Seemannshöft	628.9

Tab. S6.2 Fish taxa found in in the Elbe estuary at the distinctive time periods 1984-1986, 1994-1995, 2009-2010 and 2021-2022 with their life cycle guilds (LCG), biogeographic affinities (Biog.), absolute mean abundances per haul in individuals per 10^{-6} m^{-3} (n) and relative abundances (%).

Fish Taxa	LCG	Biog.	1984-1986		1994-1995		2009-2010		2021-2022		Combined	
			n	%	n	%	n	%	n	%	n	%
Osmerus eperlanus	A	boreal	12144.3	76.4	29378.1	89.7	167938.0	97.0	12836.1	84.4	55574.1	93.70
Clupea harengus	O	boreal	841.2	5.3	651.3	2.0	914.6	0.5	1523.6	10.0	982.7	1.66
Alosa fallax	A	lusitanian	1691.4	10.6	52.1	0.2	953.1	0.6	12.7	<0.1	677.3	1.14
Gasterosteus aculeatus	A	boreal	404.3	2.5	1235.4	3.8	130.5	<0.1	28.8	0.2	449.7	0.76
Gymnocephalus cernua	F	palearctic	1.8	<0.1	533.6	1.6	971.8	0.6	45.5	0.3	388.2	0.65
Platichthys flesus	O	lusitanian	151.2	1.0	636.0	1.9	236.5	0.1	33.0	0.2	264.2	0.45
Sprattus sprattus	O	lusitanian	142.9	0.9	107.4	0.3	513.0	0.3	168.1	1.1	232.9	0.39
Blicca bjoerkna	F	palearctic	0.3	<0.1	0.4	<0.1	484.6	0.3	<0.1	<0.1	161.8	0.27
Merlangius merlangus	O	lusitanian	<0.1	<0.1	15.8	<0.1	0.2	<0.1	314.2	2.1	110.1	0.19
Abramis brama	F	palearctic	1.5	<0.1	5.7	<0.1	370.5	0.2	48.5	0.3	106.5	0.18
Syngnathus rostellatus	E	lusitanian	6.0	<0.1	34.1	0.1	342.8	0.2	7.5	<0.1	97.6	0.16
Sander lucioperca	F	palearctic	7.6	<0.1	10.9	<0.1	107.6	<0.1	103.7	0.7	57.4	0.10
Pomatoschistus spp.	O	lusitanian	165.9	1.0	15.6	<0.1	0.7	<0.1	26.1	0.2	52.1	<0.1
Liparis liparis	O	boreal	0.2	<0.1	0.5	<0.1	173.2	<0.1	5.9	<0.1	44.9	<0.1
Anguilla anguilla	C	atlantic	119.1	0.7	30.3	<0.1	22.3	<0.1	4.1	<0.1	43.9	<0.1
Limanda limanda	O	boreal	27.4	0.2			<0.1	<0.1	<0.1	<0.1	27.4	<0.1
Pleuronectes platessa	O	lusitanian	7.2	<0.1	14.7	<0.1	7.0	<0.1	1.4	<0.1	7.6	<0.1
Alburnus alburnus	F	palearctic			<0.1	<0.1			5.1	<0.1	5.1	<0.1
Gadus morhua	O	boreal	12.8	<0.1	0.2	<0.1	0.1	<0.1	2.4	<0.1	3.9	<0.1
Trachurus trachurus	S	lusitanian	<0.1	<0.1			<0.1	<0.1	3.0	<0.1	3.0	<0.1
Lampetra fluviatilis	A	boreal	1.4	<0.1	2.2	<0.1	6.4	<0.1	1.2	<0.1	2.8	<0.1
Leuciscus idus	F	palearctic	0.3	<0.1	7.3	<0.1	1.6	<0.1	0.4	<0.1	2.4	<0.1
Solea solea	O	lusitanian	1.3	<0.1	0.3	<0.1	3.0	<0.1	<0.1	<0.1	1.5	<0.1
Agonus cataphractus	S	boreal	2.4	<0.1			0.3	<0.1	<0.1	<0.1	1.4	<0.1
Aphia minuta	M	lusitanian							1.1	<0.1	1.1	<0.1
Salmo trutta	A	boreal	0.4	<0.1	0.6	<0.1	1.9	<0.1	0.3	<0.1	0.8	<0.1
Perca fluviatilis	F	palearctic	0.2	<0.1	1.5	<0.1	0.6	<0.1	0.5	<0.1	0.7	<0.1
Zoarces viviparus	S	boreal	0.5	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	0.5	<0.1
Coregonus maraena	E	boreal			<0.1	<0.1	0.5	<0.1			0.5	<0.1
Ammodytes tobianus	S	boreal	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	0.5	<0.1	0.5	<0.1
Myoxocephalus scorpius	S	boreal	0.4	<0.1	<0.1	<0.1	0.1	<0.1	0.7	<0.1	0.4	<0.1
Chelon ramada	C	lusitanian							0.4	<0.1	0.4	<0.1
Pungitius pungitius	F	palearctic	0.4	<0.1	<0.1	<0.1	<0.1	<0.1			0.4	<0.1
Leuciscus aspius	F	palearctic	<0.1	<0.1	<0.1	<0.1	0.5	<0.1	0.3	<0.1	0.4	<0.1
Cyprinus carpio	F	palearctic	<0.1	<0.1			0.3	<0.1			0.3	<0.1
Neogobius melanostomus	F	palearctic							0.3	<0.1	0.3	<0.1
Salmo salar	A	atlantic	<0.1	<0.1	0.4	<0.1	0.1	<0.1	<0.1	<0.1	0.3	<0.1
Cyclopterus lumpus	M	boreal	0.2	<0.1							0.2	<0.1
Ballerus ballerus	F	palearctic	<0.1	<0.1	<0.1	<0.1	0.2	<0.1			0.2	<0.1
Rutilus rutilus	F	palearctic	0.3	<0.1	0.2	<0.1	0.1	<0.1	0.1	<0.1	0.2	<0.1

Scardinius erythrophthalmus	F	palearctic	0.2	<0.1							0.2	<0.1
Carassius carassius	F	palearctic	0.1	<0.1			<0.1	<0.1			<0.1	<0.1
Petromyzon marinus	A	boreal	<0.1	<0.1			0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Engraulis encrasicolus	S	lusitanian					<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Coregonus albula	F	palearctic	<0.1	<0.1					<0.1	<0.1	<0.1	<0.1
Tinca tinca	F	palearctic			<0.1	<0.1			<0.1	<0.1	<0.1	<0.1
Mullus surmuletus	M	lusitanian							<0.1	<0.1	<0.1	<0.1
Carassius gibelio	F	palearctic							<0.1	<0.1	<0.1	<0.1
Ciliata mustela	O	boreal			<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Scophthalmus maximus	S	lusitanian	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Chelidonichthys lucerna	O	lusitanian					<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Barbus barbus	F	palearctic					<0.1	<0.1			<0.1	<0.1
Belone belone	M	lusitanian	<0.1	<0.1							<0.1	<0.1
Callionymus lyra	S	lusitanian					<0.1	<0.1			<0.1	<0.1
Chelon labrosus	O	lusitanian	<0.1	<0.1	<0.1	<0.1					<0.1	<0.1
Chondrostoma nasus	F	palearctic					<0.1	<0.1			<0.1	<0.1
Cyprinidae	F	palearctic					<0.1	<0.1			<0.1	<0.1
Dicentrarchus labrax	M	lusitanian			<0.1	<0.1					<0.1	<0.1
Entelurus aequoreus	S	lusitanian	<0.1	<0.1			<0.1	<0.1			<0.1	<0.1
Esox lucius	F	palearctic	<0.1	<0.1							<0.1	<0.1
Gaidropsarus vulgaris	S	lusitanian					<0.1	<0.1			<0.1	<0.1
Microstomus kitt	S	boreal					<0.1	<0.1			<0.1	<0.1
Misgurnus fossilis	F	palearctic	<0.1	<0.1							<0.1	<0.1
Oncorhynchus mykiss	F	nearctic			<0.1	<0.1	<0.1	<0.1			<0.1	<0.1
Salvelinus fontinalis	F	nearctic					<0.1	<0.1			<0.1	<0.1
Silurus glanis	F	palearctic					<0.1	<0.1			<0.1	<0.1
Squalius cephalus	F	palearctic	<0.1	<0.1			<0.1	<0.1			<0.1	<0.1
Trisopterus minutus	M	lusitanian	<0.1	<0.1							<0.1	<0.1
Vimba vimba	F	palearctic					<0.1	<0.1			<0.1	<0.1

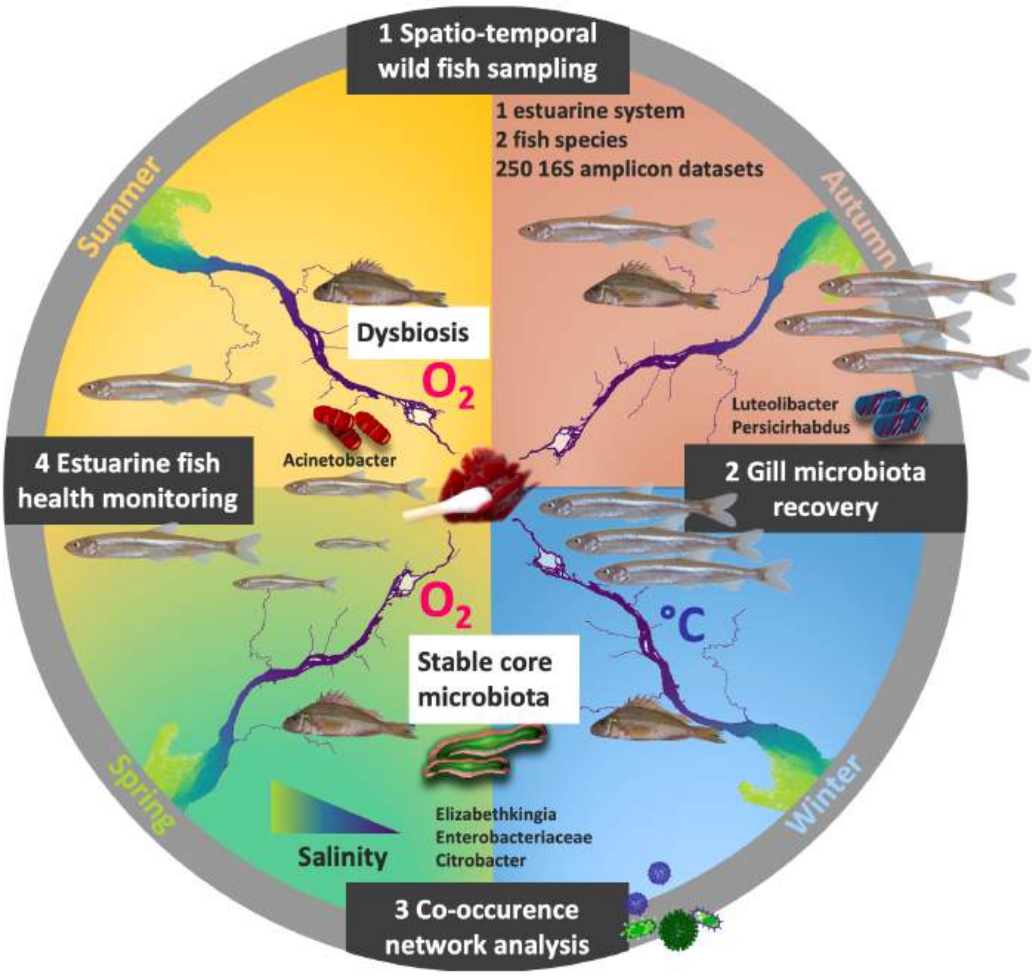
Tab. S6.3 Mean abundances (above) and relative abundances (below) of fish LCGs in the Elbe estuary comparing the four time periods at five sampling regions along the salinity gradient.

	A/A'				B				C				D				E/E'				Combined			
Mean abundances		Ekm 713/716				Ekm 692				Ekm 665				Ekm 651				Ekm 633/639						
	1984-1986	1994-1995	2009-2010	2021-2022	1984-1986	1994-1995	2009-2010	2021-2022	1984-1986	1994-1995	2009-2010	2021-2022	1984-1986	1994-1995	2009-2010	2021-2022	1984-1986	1994-1995	2009-2010	2021-2022	1984-1986	1994-1995	2009-2010	2021-2022
Marine Straggler	1.1	0.1	0.2	1.6	< 0.1	< 0.1	< 0.1	< 0.1													0.4	< 0.1	0.1	0.5
Marine Estuarine Opportunist	178.3	597.5	517.6	255.5	220.8	191.6	116.1	464.8	14.8	9.3	12.9	17.0	1.9	16.7	9.1	3.5	7.2	10.4	1.0	3.5	70.5	137.6	109.4	124.0
Estuarine	660.8	99.2	567.0	12.2	173.5	21.4	1.0	2.0	4.5		0.3	0.4	< 0.1	< 0.1	0.2	< 0.1	0.6	< 0.1	0.5	0.5	139.9	24.1	94.8	2.5
Anadromous	1233.9	4763.9	5756.7	1109.8	2774.3	3508.7	8324.0	787.4	5182.8	4475.1	22003.4	419.5	727.3	6874.5	54446.3	1217.3	254.4	1873.4	31152.4	5603.2	1695.5	3582.6	20280.4	1522.9
Catadromous	8.8	10.2	0.9	0.3	33.2	10.0	2.7	2.4	59.6	8.2	2.6	1.3	118.0	8.6	5.7	1.1	78.0	36.8	43.7	6.0	49.6	12.3	9.3	1.9
Freshwater		1.5	0.4	0.5	0.1	8.0	11.1	1.5	0.5	25.4	12.0	1.5	1.2	29.3	121.4	1.5	0.6	23.9	215.5	33.7	0.5	14.7	60.1	6.2

	A/A'				B				C				D				E/E'				Combined			
Relative abundances		Ekm 713/716				Ekm 692				Ekm 665				Ekm 651				Ekm 633/639						
	1984-1986	1994-1995	2009-2010	2021-2022	1984-1986	1994-1995	2009-2010	2021-2022	1984-1986	1994-1995	2009-2010	2021-2022	1984-1986	1994-1995	2009-2010	2021-2022	1984-1986	1994-1995	2009-2010	2021-2022	1984-1986	1994-1995	2009-2010	2021-2022
Marine Straggler	0.1	< 0.1	< 0.1	0.1	< 0.1	< 0.1	< 0.1	< 0.1													< 0.1	< 0.1	< 0.1	0.1
Marine Estuarine Opportunist	8.6	10.9	7.6	18.5	6.9	5.1	1.4	36.9	0.3	0.2	0.1	3.9	0.2	0.2	< 0.1	0.3	2.1	0.5	< 0.1	0.1	3.6	3.4	1.8	11.9
Estuarine	31.7	1.8	8.3	0.9	5.4	0.6	< 0.1	0.2	0.1		< 0.1	0.1	< 0.1	< 0.1	< 0.1	< 0.1	0.2	< 0.1	< 0.1	< 0.1	7.5	0.6	1.7	0.2
Anadromous	59.2	87.1	84.1	80.4	86.6	93.8	98.5	62.6	98.5	99.1	99.9	95.4	85.7	99.2	99.8	99.5	74.6	96.3	99.2	99.2	80.9	95.1	96.3	87.4
Catadromous	0.4	0.2	< 0.1	< 0.1	1.0	0.3	< 0.1	0.2	1.1	0.2	< 0.1	0.3	13.9	0.1	< 0.1	0.1	22.9	1.9	0.1	0.1	7.9	0.5	< 0.1	0.1
Freshwater		< 0.1	< 0.1	< 0.1	< 0.1	0.2	0.1	0.1	< 0.1	0.6	0.1	0.3	0.1	0.4	0.2	0.1	0.2	1.2	0.7	0.6	0.1	0.5	0.2	0.2

Chapter 7

Spatio-temporal fish gill microbiota analysis as indicators in estuarine fish health monitoring



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Chapter 7 – Fish gill microbiota

Title: Spatio-temporal fish gill microbiota analysis as indicators in estuarine fish health monitoring

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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 the abundance and virulence of pathogenic bacteria are rapidly increasing. The microbial composition of the gill mucus reflects environmental conditions and represents a major 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 the takeover of opportunistic pathogens (*Acinetobacter, Shewanella, Aeromonas*). By correlating bacterial abundances with environmental and physiological parameters in a co-occurrence network approach, we describe plasticity in microbiota composition, identify biomarkers of fish health and reconstruct movement patterns of the fish. Our results will help to shape future non-invasive and cost-effective monitoring programs, and identify factors that might be controlled in the estuary to promote fish and stock health.

Key words: 16S, metabarcoding, network analysis, hypoxia, holobiont

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 cross talk between host and microbiome, might create opportunists or pathogens from otherwise commensal species (Kelly and Salinas 2017). Changes in salinity (François-Étienne et al. 2023), temperature (Matanza and Osorio 2018, Ghosh et al. 2022, Yang et al. 2022, Morshed and Lee 2023) and dissolved oxygen (DO) (Fan et al. 2020, Shi et al. 2021, Wang et al. 2021, Song et al. 2023) have been shown to impact, or otherwise be modulated by, both the host physiology and the nature of the microbial biom, increasing the likelihood of pathogenic species and reducing fish health and survivability. Of particular concern are recent reports of hypoxia-induced immune suppression, making 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 have already intensified pathogen loads and disease outbreaks in aquaculture (Vezzulli et al. 2013, Harrison et al. 2022, Samsing and Barnes 2024) and estuaries (Siboni et al. 2023).

Estuarine ecosystems constitute important nursery areas and population sources for fish (Seitz et al. 2014, Pasquaud et al. 2015, Tournois et al. 2017), and are characterized by intense fluctuations in these physicochemical conditions (Elliot and 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 (Diaz and Rosenberg 2008, Little et al. 2017, Breitburg et al. 2018, Cottingham et al. 2018, Lauchlan and Nagelkerken 2020, 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, van Maren et al. 2015, Reese et al. 2019), expansion of 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. 2024a, Scholle and 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 (Wei et al. 2018, Suzzi et al. 2022, Suzzi et al. 2023a, Suzzi et al. 2023b, de Macedo et al. 2024, Koll et al. 2024a). 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 (Pratte et al. 2018, Sylvain et al. 2020, Minich et al. 2020, 2022, Itay et al. 2022, Amill et al. 2024, Varela et al. 2024). The fish gill, with critical roles in respiration, osmoregulation and waste exchange, forms not only a unique habitat for the microbes but further constitutes a major route for pathogen invasion (Salinas 2015, Pratte et al. 2018). A gill associated lymphoid tissue (GIALT) (Salinas 2015) composed of adaptive and innate immune cell populations enables cross-talk between microbes and host (Gomez et al. 2013, Kaetzel 2014) and discrimination between beneficial and pathogenic bacteria keeping the microbiome in homeostasis (Nakanishi et al. 2015, Yu et al. 2021).

In this study, we aimed now to determine spatio-temporal variation in composition in a seasonal time-series of estuarine fish gill microbiota. Here we focus on two fish species with highly different lifestyles and habitat usage to generate a larger picture on 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 and 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 and Thiel 2015), gathering in autumn at the mouth of the estuary and migrating upstream as temperatures drop to 3 - 6° C (Freyhof and Kottelat 2007). Within a month they reach spawning grounds upstream Hamburg and spawn until March (Borchardt 1998, Eick 2015, Thiel and Thiel 2015). Hatched larvae drift to shallow habitats downstream of the port, where oxygen deficiency can increase mortality (Diercking and Wehrmann 1991, Thiel et al. 1995, Thiel and Thiel 2015). Adults avoid low-oxygen areas and accumulate where oxygen exceeds 5 mg/l (Möller and Scholz 1991). In spring, all life stages occupy the estuary before the majority of adults migrate to the ocean (Thiel and Thiel 2015). The ruffe (*Gymnocephalus cernua*, L.) on the other hand, is a rather undemanding bentho-pelagic freshwater species common in oligo- and mesohaline (0 – 12 psu) and eutrophic estuaries (Gutsch and Hoffman 2016). It is the third common fish in the estuary (Eick and Thiel 2014) and important benthic predator of invertebrates (Hölker and Thiel 1998). Ruffe migrate to shallow waters in summer and deeper areas in winter (Gutsch and Hoffman 2016). They mature at 1 - 3 years and spawn in early and possibly again migrate to shallow waters in summer and deeper areas in winter (Gutsch and 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 (Ogle 1998, Gutsch and Hoffman 2016).

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 health situation of fish stocks in this understudied estuarine habitat.

Materials and methods

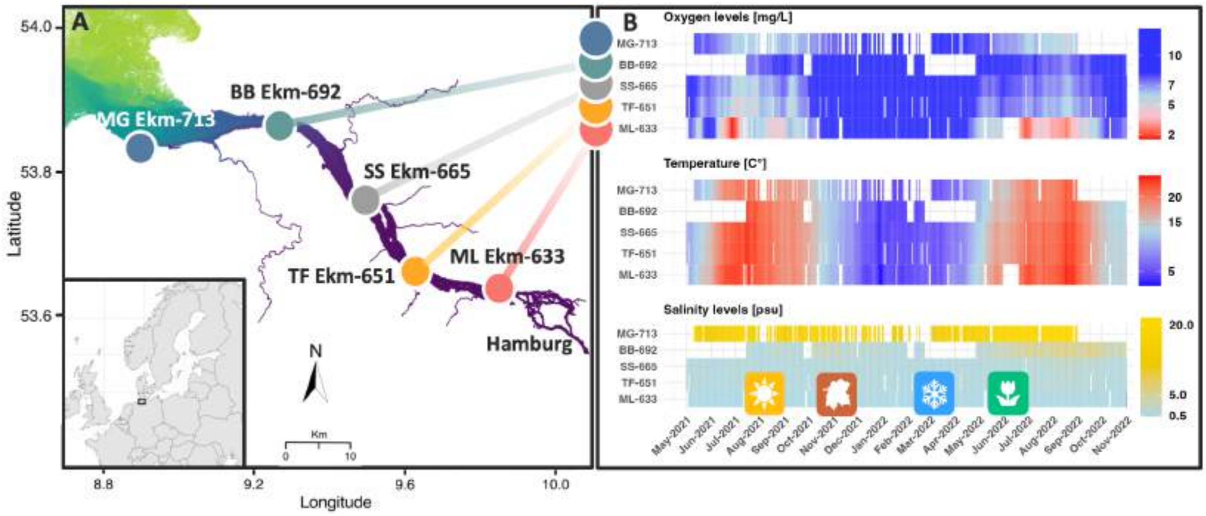


Fig. 7.1 Overview map of sampling locations along the course of the estuary (**A**) and abiotic conditions (oxygen levels, temperature and salinity) in the water column in a seasonal and spatial view (**B**). Sampling stations are marked by colored dots while seasonal sampling periods are indicated by symbols.

Sample collection

Fish were caught with a stow-net fishing vessel (opening area of 135 m^2, mesh size of 10 mm at the cod end) at five stations along the Elbe estuary (Fig. 7.1) in summer (25.-29.08.21), autumn (17.-21.11.21), winter (08.-12.03.22) and spring (31.05-04.06.22). Sampling stations were chosen within different estuarine sections classified by dominating abiotic drivers (Amann et al. 2012): Station ML-Elbe kilometer (Ekm) 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) 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), hepatosomatic (HSI), splenosomatic (SSI) and gonadosomatic 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.

DNA extraction and sequencing

DNA extraction, sequencing and bioinformatics were performed as explained elsewhere (Koll et al. 2024a). 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.

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. S7.7). ASV table and sample data were parsed to phyloseq (v.1.45.0) (McMurdie and 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. S7.1) 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 and 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.

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) (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.

Statistical analyses

First, we visualized the gill bacterial community according to the fish species, sampling season and location using stacked barplots (Fig. 7.1). 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 (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 and 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. S7.4, relative abundance of overall ASV counts were visualized as heatmap in Fig. S7.2B.

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 and Anderson 1999) via vegan *capscale* function following the workflow described in 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. S7.3).

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. 7.4E, Fig. 7.4B). We than applied a weighted gene co-expression network analysis (WGCNA) (v.1.77- 1) (Langfelder and Horvath 2008) to infer networks of co-abundant bacterial taxa and relate them to physiological and environmental data in an integrated heatmap analysis approach (Strand et al. 2021, Koll et al. 2024a) (Fig. 7.5, Fig S7.6, S7.7). 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.

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 bacterioplankton (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. S7.1).

Bacterial gill communities deviate from bacterioplankton

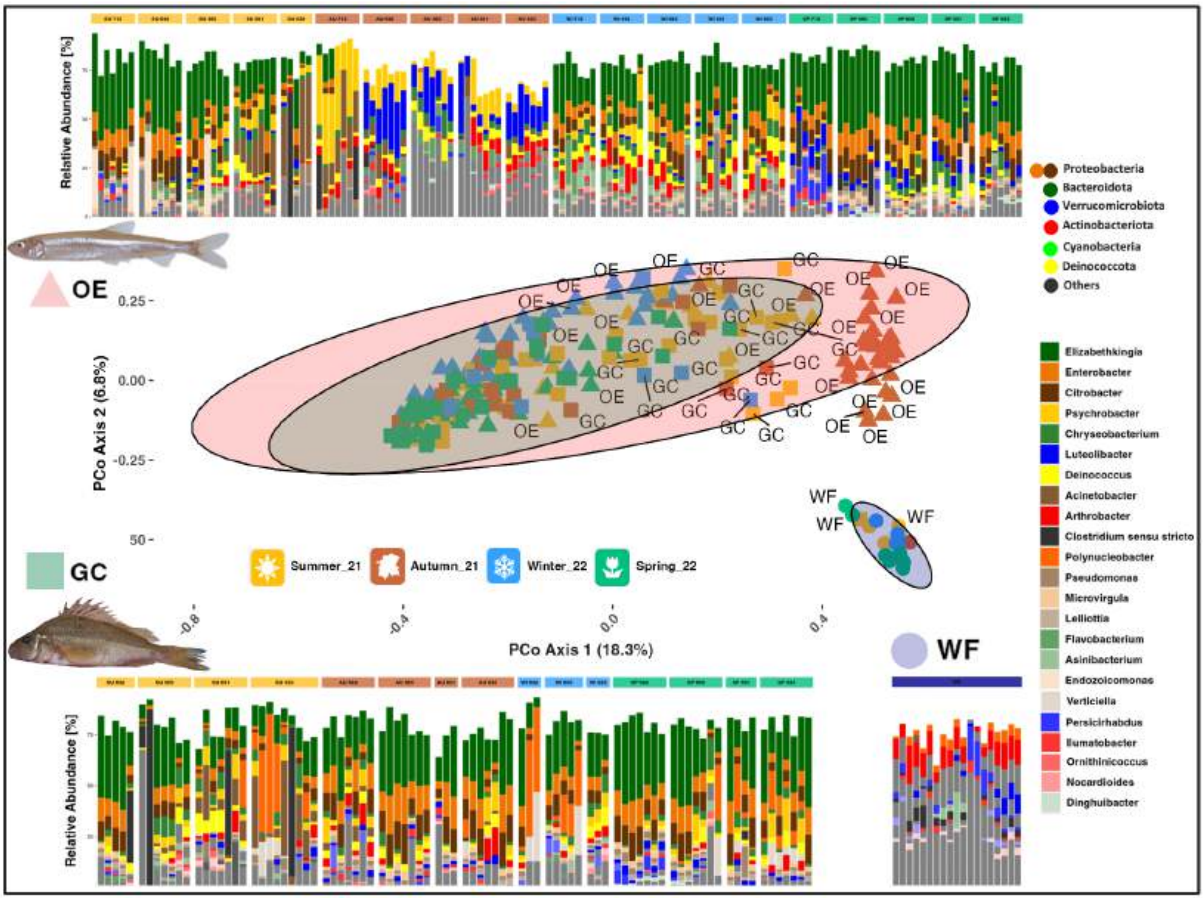


Fig. 7.2 Overview of spatio-temporal patterns in bacterioplankton and bacterial gill mucus communities. Principal coordinates analyses (PCoA) of gill bacterial community based on averaged subsampled Bray-Curtis dissimilarity (middle panel). Ellipses display the sample kinds (OE, GC, WF), coloration by season of sampling. Stacked barplots (top and bottom panel) depict relative abundance of the most abundant genera within the three biomes (OE upper panel, GC and 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.

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.

S.7.1). 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%).

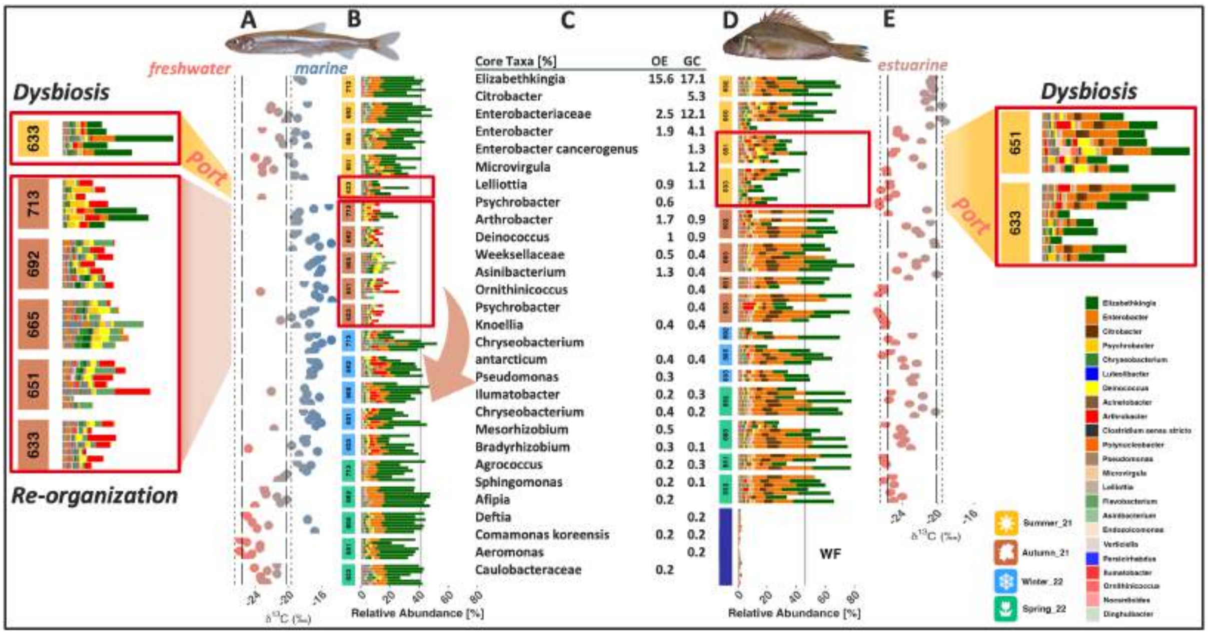
Analyses of alpha diversity showed significantly higher richness and Shannon indices in free-living bacteria samples compared to the fish mucus communities, but no overall significant difference between the two fish species (Fig. S7.3A and B)

The principal coordinates analysis in Fig. 7.2 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 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 (Fig. S7.2A). 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. S7.2). 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 section).

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. S7.4).

Stable estuarine core gill microbiota



Core Taxa [%]	OE	GC
Elizabethkingia	15.6	17.1
Citrobacter		5.3
Enterobacteriaceae	2.5	12.1
Enterobacter	1.9	4.1
Enterobacter cancerogenus		1.3
Microvirgula		1.2
Lelliottia	0.9	1.1
Psychrobacter	0.6	
Arthrobacter	1.7	0.9
Deinococcus	1	0.9
Weeksellaceae	0.5	0.4
Asinibacterium	1.3	0.4
Ornithinicoccus		0.4
Psychrobacter		0.4
Knoellia	0.4	0.4
Chryseobacterium antarcticum	0.4	0.4
Pseudomonas	0.3	
Ilumatobacter	0.2	0.3
Chryseobacterium	0.4	0.2
Mesorhizobium	0.5	
Bradyrhizobium	0.3	0.1
Agrococcus	0.2	0.3
Sphingomonas	0.2	0.1
Afipia	0.2	
Deftia		0.2
Comamonas koreensis	0.2	0.2
Aeromonas		0.2
Caulobacteraceae	0.2	

Fig. 7.3 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. 7.3). Only a fraction of these taxa was present in the bacterioplankton (33% OE, 20% GC) accounting for < 1% of the bacterioplankton relative abundance (Fig. 7.3D). *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. 7.3B, D, highlighted).

Drivers in gill mucus bacterial composition

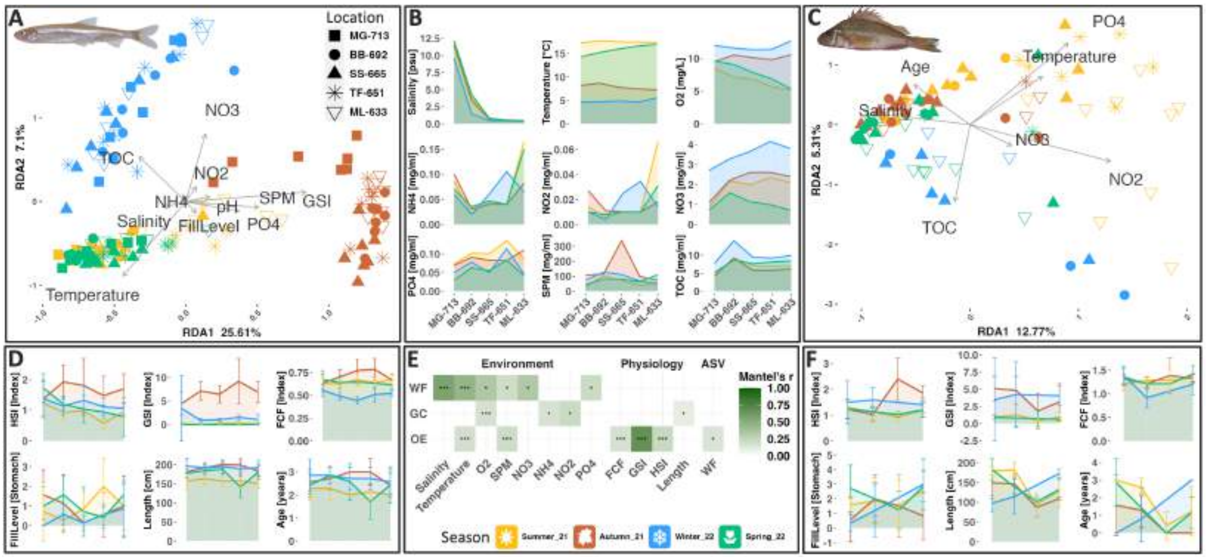


Fig. 7.4 Environmental and physiological drivers in gill mucus bacterial composition. Average Bray-Curtis distance-based redundancy analyses (dbRDA) on gill mucus bacterial community in two host species *O. eperlanus* OE (**A**) and *G. cernua* GC (**C**). Datapoints indicate 16S rRNA gill mucus microbiota 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. 7.4 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. S7.2) 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. 7.4 E, all results Tab. S7.3). The results showed associations ($r = 0.20 – 0.55$) between bacterioplankton and environmental parameters (salinity, temperature, O_2, SPM, NO_3, PO_4), however these were weaker for both fish species. OE was most strongly correlated with GSI ($r = 0.67$). There was overall no significant association between the bacterioplankton community and GC, and a slight overall trend between OE and free-living bacteria. A spatio-temporally resolved analysis for this species showed that only the microbiota of the autumn smelt were significantly associated with bacterioplankton (Fig. S7.4B).

Movement patterns and 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 and 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. As described above, the bacterial core taxa in this group were rare (Fig. 7.4B), while mantel tests indicated a significant association to the bacterioplankton communities (Fig. S7.4B). Analyzing the bacterial taxa shared between 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. S7.4A). 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. 7.4 A, B). In contrast, GC samples range all year within the signal boundaries of the estuary and show a strong spatial pattern for sampling station along the course of the estuary (Fig. 7.4 D, E).

Bacterial network response

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. 7.5C, Fig S7.6). Both fish mucus assemblage networks resulted in highly overlapping sub-networks correlated to the dominant environmental drivers.

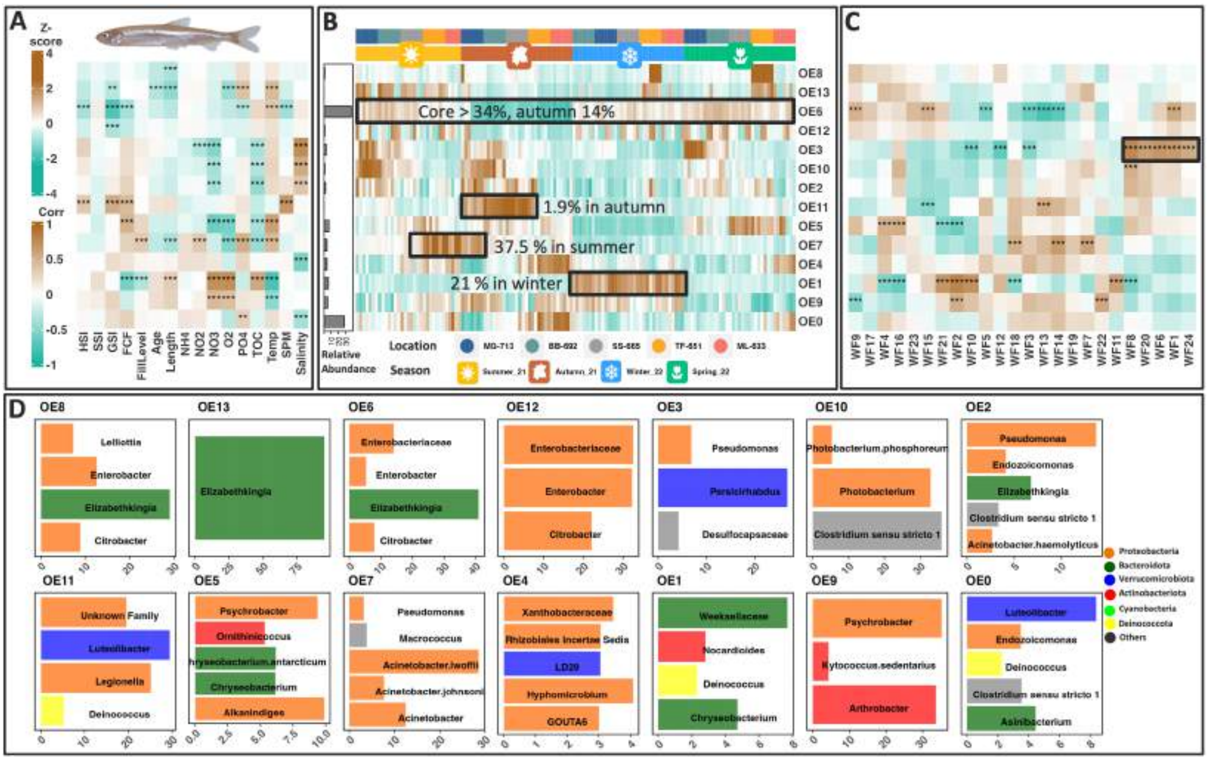


Fig. 7.5 Relationship between bacterial co-occurrence networks, biometric measurements and abiotic conditions for Elbe estuarine smelt OE. Integrated heatmap of gill bacterial mucus network analysis showing correlation between ASV module eigengenes (**B**: OE-1-13), physiological traits (**A**: HSI, 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. S7.5.

The prevalence determined core-taxa were also reflected within networks of both species in the dominant modules OE6 and GC8 overlapping in 70.5% and 90.1% in abundance with prevalence determined taxa (Fig. 7.5 and Fig. S7.5) and by 80% and 96% with each other. 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 and 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 number of uncorrelated taxa raised to 49%.

Salinity

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

The mesohaline conditions on the contrary are correlated with *Persicirhabdus* (33.9%), *Ilumatobacter* (10.5%) and *Halioglobus* (10.4%) in OE3 (r = 0.72, P ***, 17 – 0 % in upstream direction), Less abundant, 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%).

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 %).

Desoxygenation

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***, NO_2 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.

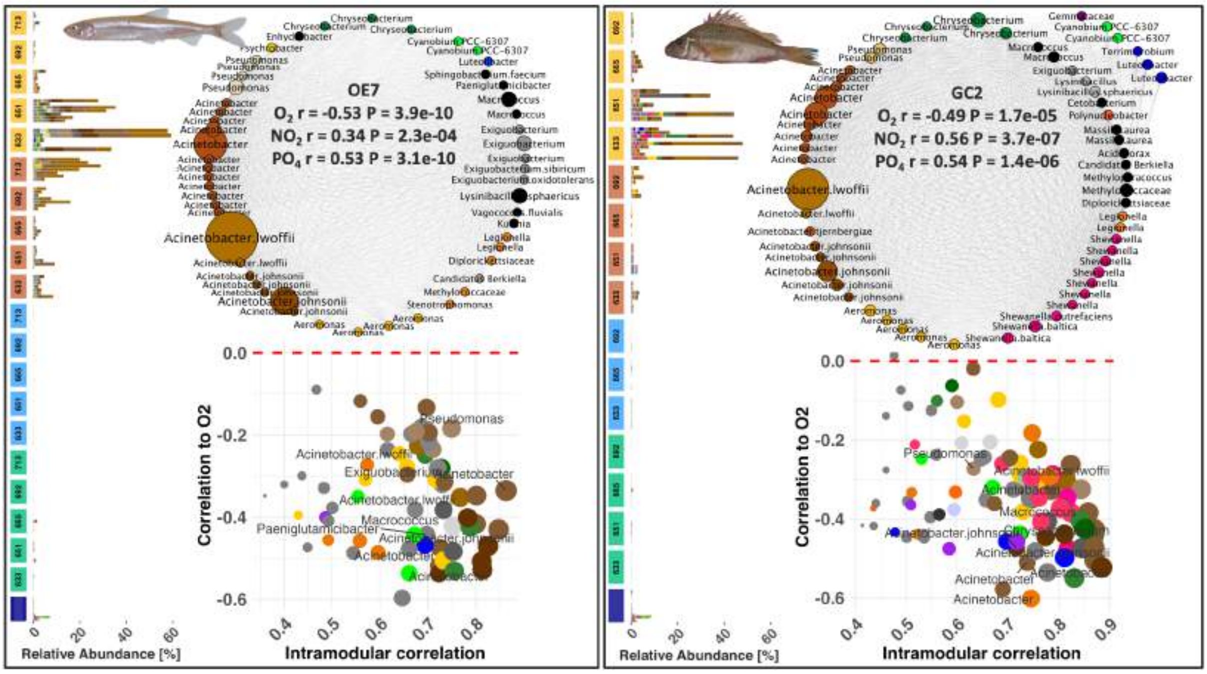


Fig. 7.6 Opportunistic pathogens thrive under prolonged hypoxia in the Hamburg Port area (Ekm 651 – 633). Dominant bacterial species in smelt OE and ruffe GC network modules with highest negative correlation to oxygen values are shown as network modules OE7 and GC2. Stacked barplots depict relative abundance of these taxa in spatio-temporal course. Dotplots indicate individual correlation of ASVs to DO levels and intra-module correlation.

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.

Life history strategies and microbiota plasticity

Albeit surrounded by water, a clear distinction existed between bacterioplankton and gill mucus microbiota communities suggesting selective colonization (Legrand et al. 2018, Pratte et al. 2018, Minich et al. 2020, Rosado et al. 2021, Koll et al. 2024a). 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 here, *Luteolibacter*, a marine and freshwater taxon (Ji et al. 2021) associated with bacterial dysbiosis in fish (Sun et al. 2021, Mondal et al. 2022, Kakakhel et al. 2023) became dominant. The taxon was shown to antagonize pathogen growth contributing to microflora resilience by niche colonization and might further be involved in epithelial tissue repair (Nakatani and Hori 2021) which could serve beneficial roles during deep-reorganization in migrating smelt. In the second, slower step, the estuarine core taxa 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 and 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, Yang et al. 2023), potentially preventing toxic ammonia buildup at the gills (van Kessel et al. 2016, Legrand et al. 2018) 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 (Han et al. 2021, Amin et al. 2022, Scheifler et al. 2023).

Estuarine gill core microbiota

Core taxa are assumed to serve beneficial roles in the host (Sehnal et al. 2021) and the dominant taxa here, *Elizabethkingia* (Jacob and Chenia 2011), *Enterobacteriaceae, Lelliottia* (Salgueiro et al. 2020), *Arthrobacter* (Tsoukalas et al. 2023), *Deinococcus* (Amill et al. 2024), *Asinibacterium, Knoellia* (Zou et al. 2023) are all well described from external fish microbiota. *Asinibacterium* and *Enterobacter* taxa are even considered probiotic, inhibiting pathogen growth (Halet et al. 2007, Schubiger et al. 2015). The genus *Citrobacter* contains pathogens of marine and freshwater fish (Sato et al. 1982, Liu et al. 2024), but was found here as a constant compartment of the gill microbiota in both fish species.

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. 2024a). 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 (Roeselers et al. 2011, Sharpton et al. 2021, Luan et al. 2023). The size of the core microbiota with ~30 genera appeared to be low in estuarine fish compared to hundreds in other studies (Sharpton et al. 2021, Lorgen-Ritchie et al. 2022), but at 30 – 50% high in terms of abundance similar to gut microbiota studies (Wilkes Walburn et al. 2019). Determining the average core abundance proportions in this larger dataset enabled the identification of animals with strong deviation. In both species, we found groups of animals with core abundance <20%, which could indicate profound reorganization or dysbiosis and possible disease states (Sehnal et al. 2021). In this context, analyzing the prevalence of potentially pathogenic taxa in overlap with signs of dysbiosis of the overall microbiota composition is probably more informative, as potential pathogens may also be part of the core community of apparently healthy individuals (Itay et al. 2022, Yajima et al. 2023).

Deoxygenation and dysbiosis in estuarine fish

Dysbiosis, the disturbance of microbiome homeostasis (abnormal taxonomic structure and metagenomic function) by uprise of opportunistic taxa (Egan and 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 and Joyce 2023). Understanding these processes and identifying biomarkers for dysbiotic processes before physical signs of infection are important not only in fish farming (Mougin and 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, Vasemägi et al. 2017, Zhang et al. 2018, Mougin and Joyce 2023, Liu et al. 2024, Xavier et al. 2024). 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 and in agreement with recent reviews, did not consider them suitable for detecting perturbations in microbial homeostasis (Xavier et al. 2024).

We observed a strong increase in opportunistic *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 (Visca et al. 2011, Kozińska et al. 2014, Malick et al. 2020, 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 (Cao et al. 2018, Bi et al. 2023). 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 opportunistic *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. No physiological measurements (i.e. lesions) of gills or other tissues were performed in this study 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 we identified strong correlations of the immune response and cellular stress response in the gill and liver of another estuarine species in response to an increase in *Shewanella* and *Acinetobacter* (Koll et al. 2024a).

Conclusion

Our data showed the potential of non-invasive gill microbiomes for long-term monitoring the health of estuarine fish in the context of changes occurring in estuaries 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, that an easily identifiable core microbiome associated with healthy fish. Further, for each species, we show that changes in these core microbiota, either as a consequence of migration, seasonality or stress can increase the likelihood of opportunistic and pathogenic species which may impact fish survivability. Whilst, additional targeted studies, would be necessary to clarify the pathogenic nature of these strains, and the capacity of the gill microbiome to recover after alleviation of stress inducing conditions, 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 estuarine fish and the water column, might provide a solution by identifying areas of reduced impact that can be restored or conserved, ensuring that estuaries continue to provide this much needed ecosystem function.

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

Declaration of interest

The authors have no conflicts of interest to declare.

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

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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.

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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.

Supplements

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

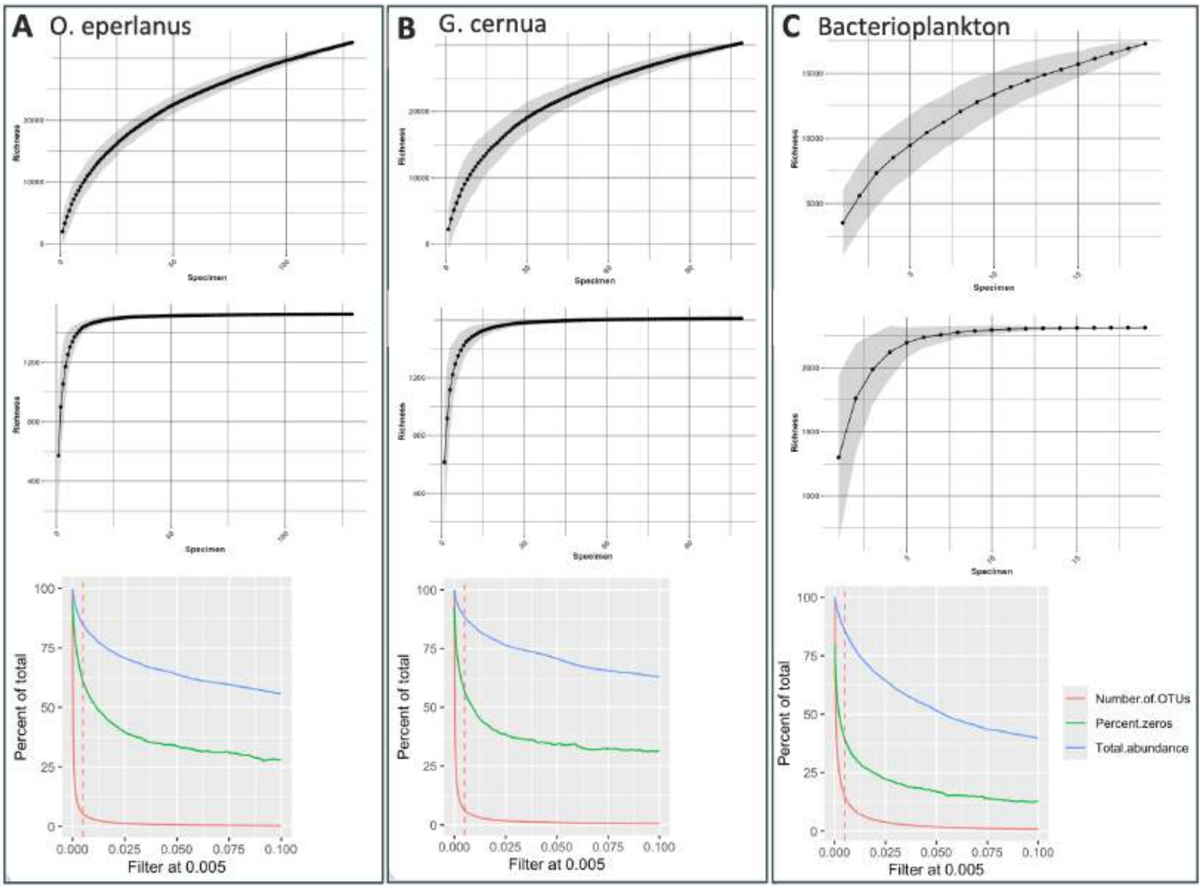

Fig. S7.1 Bacterial ASV accumulation over number of sampled specimens for OE (**A**), GC (**B**) and bacterioplankton (**C**) for unfiltered data (upper row) and 0.005% overall abundance filtered data (middle row). Bottom row depicts the number of ASVs, percent zeros and total abundance of counts in the individual datasets along different abundance filter levels.

Tab. S7.1 Relative abundance (%) of sequences belonging to phyla in gill microbiome and bacterioplankton (higher 1% per sample group).

Phylum	Bacterioplankton	OE	GC
Proteobacteria	41 ± 5	51 ± 10	56 ± 13
Bacteroidota	19 ± 7	28 ± 12	25 ± 9
Actinobacteriota	19 ± 9	7 ± 5	6 ± 6
Verrucomicrobiota	8 ± 3	5 ± 8	3 ± 2
Firmicutes		4 ± 7	5 ± 13
Deinococcota		2 ± 2	2 ± 2
Desulfobacterota	1.5 ± 1.1		
Acidobacteriota	2.3 ± 1.8		
Cyanobacteria	1.4 ± 1.9		
Nitrospirota	1.9 ± 1.4		
Gemmatimonadota	1.4 ± 0.6		
Chloroflexi	1.2 ± 0.9		

Tab. S7.2 Envfit results for ordistep and VIF selected reduced dbRDA models per dataset

Sample Kind	Parameter	R^2	Sig. P	
OE gill microbiome	Temperature	0.682	0.001	***
	Salinity	0.022	0.236	
	FillLevel	0.018	0.257	
	GSI	0.534	0.001	***
	NH4	0.006	0.7	
	NO2	0.026	0.174	
	NO3	0.476	0.001	***
	PO4	0.12	0.001	***
	pH	0.023	0.219	
	TOC	0.309	0.001	***
	SPM	0.203	0.001	***
GC gill microbiome	Temperature	0.179	0.002	**
	Salinity	0.11	0.012	**
	Age	0.098	0.012	**
	NO2	0.292	0.001	***
	NO3	0.039	0.206	
	PO4	0.438	0.001	***
	TOC	0.258	0.001	***
Bacterioplankton	Temperature	0,962	0,001	***
	Salinity	0,669	0,002	**
	PO4	0,107	0,377	
	pH	0,874	0,001	***

Tab. S7.3 Significant association between average Bray-Curtis distance matrices for gill mucus and bacterioplankton communities and Euclidean distances matrices for environmental and physiological measurements estimated via Mantel test.

Sample Kind	Parameter	Mantel	Sig.
OE gill microbiome	Temperature	0.18	0.0001
	SPM	0.20	0.0001
	FCF	0.17	0.0004
	GSI	0.67	0.0001
	HSI	0.23	0.0001
	Bacterioplankton	0.14	0.0018
GC gill microbiome	O2	0.17	0.0001
	NH4	0.17	0.0055
	NO2	0.22	0.0016
	Length	0.10	0.0062
Bacterioplankton	Temperature	0.45	0.0003
	Salinity	0.55	0.0002
	O2	0.33	0.0015
	SPM	0.19	0.0327
	PO4	0.21	0.0219
	NO3	0.33	0.0022

Tab. S7.4 Indicator species via multipatt with indicator value >0.7 and P-value <0.05 after 999 permutations. Shown are taxa with >1% relative abundance (>0.2% for spatial comparisons).

Biomes/Season/Location	Phylum	Order	Lowest Taxonomic level	Rel. Abundance [%]
Bacterioplankton	Actinobacteriota	Frankiales	*Sporichthyaceae*	3.4
			hgcI clade	1.1
		Microtrichales	CL500-29 marine group	2.3
	Bacteroidota	Sphingobacteriales	NS11-12 marine group	1.3
		Flavobacteriales	*Cryomorphaceae*	1.1
Fish gill mucus	Bacteroidota	Flavobacteriales	*Elizabethkingia*	16
	Proteobacteria	Enterobacterales	*Enterobacteriaceae* (4 ASVs)	9.4
			Enterobacter	2.2
			E. cancerogenus	1.1
			Citrobacter	3.4
			Lelliottia	1
		Burkholderiales	*Microvirgula*	1.1
	Verrucomicrobiota	Verrucomicrobiales	*Luteolibacter*	1.6
	Actinobacteriota	Micrococcales	*Arthrobacter*	1.4
GC	Proteobacteria	Burkholderiales	*Verticiella*	1.9
			Polynucleobacter	1.3
		Rickettsiales	*Candidatus Megaira*	0.6
GC Summer	Firmicutes	Clostridiales	*Clostridium* sensu stricto 1	4.3
	Proteobacteria	Pseudomonadales	*A. lwoffii*	2.4
			Acinetobacter	1.2
			A. johnsonii	1
GC Winter	Proteobacteria	Burkholderiales	*Polynucleobacter*	3.2
GC Spring	Bacteroidota	Flavobacteriales	*Chryseobacterium*	1
GC BB	Verrucomicrobiota	Verrucomicrobiales	*Persicirhabdus*	0.2
	Proteobacteria	Pseudomonadales	*Halioglobus*	0.2
GC TF	Firmicutes	Staphylococcales	*Macrococcus*	1.5
GC ML	Bacteroidota	Cytophagales	*Hymenobacter*	0.2
	Proteobacteria	Pseudomonadales	*Psychrobacter*	0.2
OE Summer	Proteobacteria	Pseudomonadales	*Acinetobacter johnsonii*	1.2
OE Winter	Bacteroidota	Flavobacteriales	*Chryseobacterium*	1.2
OE Spring	Verrucomicrobiota	Verrucomicrobiales	*Luteolibacter*	1.3
			Persicirhabdus	1
	Bacteroidota	Flavobacteriales	*Chryseobacterium*	1
OE TF	Bacteroidota	Flavobacteriales	*Elizabethkingia*	1.4
	Proteobacteria	Enterobacterales	*Enterobacter*	0.6
			Citrobacter	0.4
			Lelliottia	0.4
			Enterobacteriaceae	0.2
OE ML	Verrucomicrobiota	Verrucomicrobiales	*Luteolibacter*	0.2

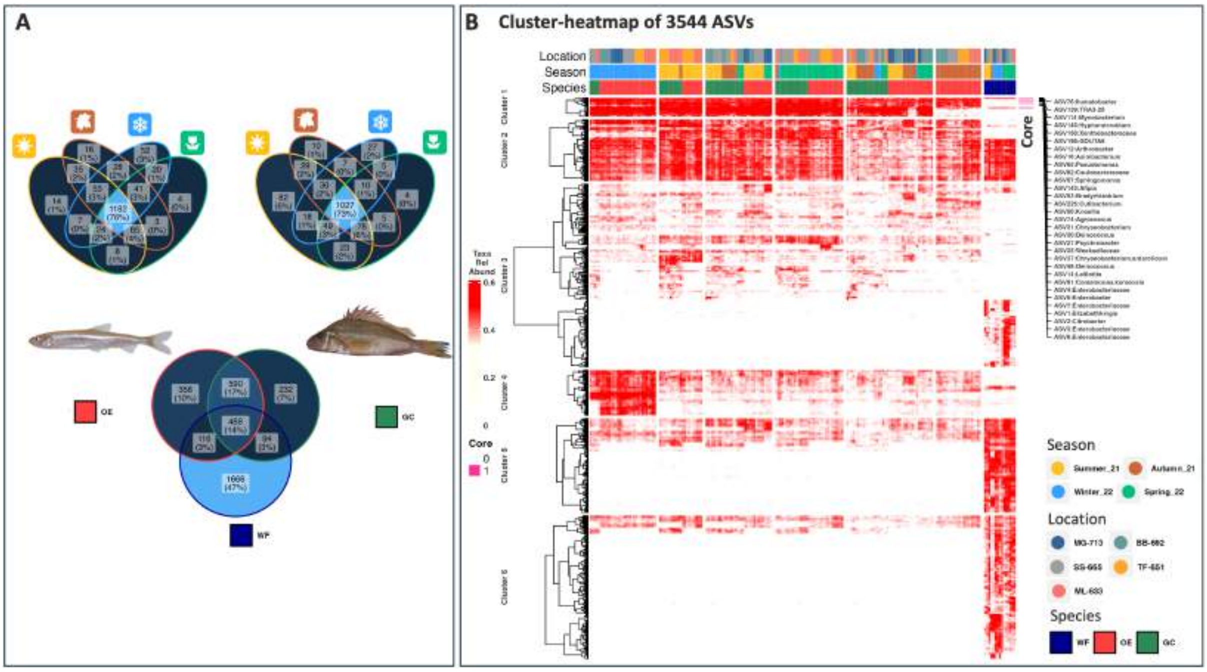

Fig. S7.2 Presence-absence analysis and abundance visualization of bacterial taxa within the biomes. Venn Diagrams depict presence/absence of bacterial ASVs found in bacterioplankton (WF), smelt (OE) and ruffe (GC) gills and in fish swabs at seasonal samplings (A). Cluster-heatmap of the relative abundance of the bacterial taxa within all datasets with core taxa in fish gill mucus (B). While color depicts low abundance, red high abundance. The maximum value of the color code is set at 0.6% to enable meaningful visualization.

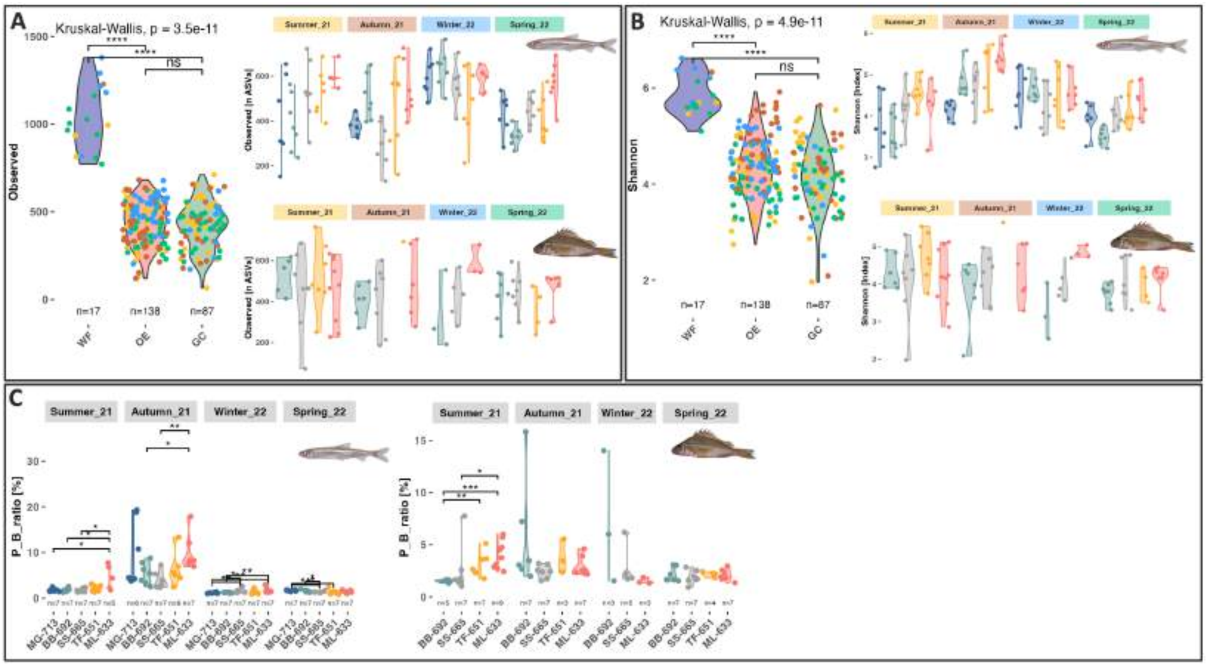

Fig. S7.3 Alpha diversity metrices in bacterioplankton and fill gill mucus. Overall observed richness (A) and Shannon indices (B) are shown for the three biomes (WF = bacterioplankton, OE = smelt, GC = ruffe) and with spatio-temporal resolution for the fish associated bacteria communities. The ratios of Proteobacteria and Bacteroidetes in spatio-temporal resolution are shown for both fish species (C).

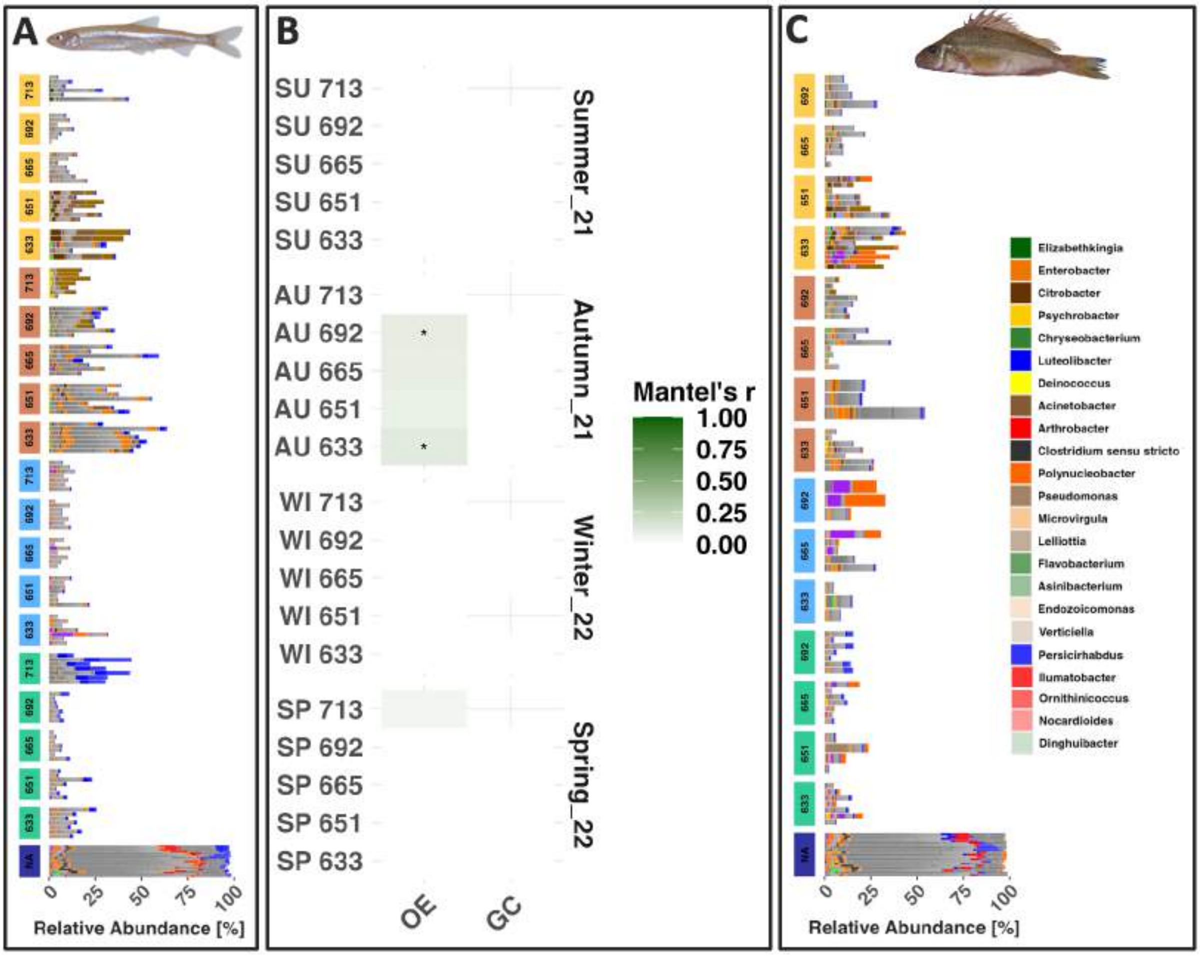

Fig. S7.4 Similarity between bacterioplankton and fish gill communities. Compositional barplot (A, C) show relative abundance of bacterial taxa shared between the bacterioplankton and the fish communities excluding the fish core microbiome. Mantel tests (B) show matrix correlation between overall bacterioplankton communities and spatio-temporal fish gill communities.

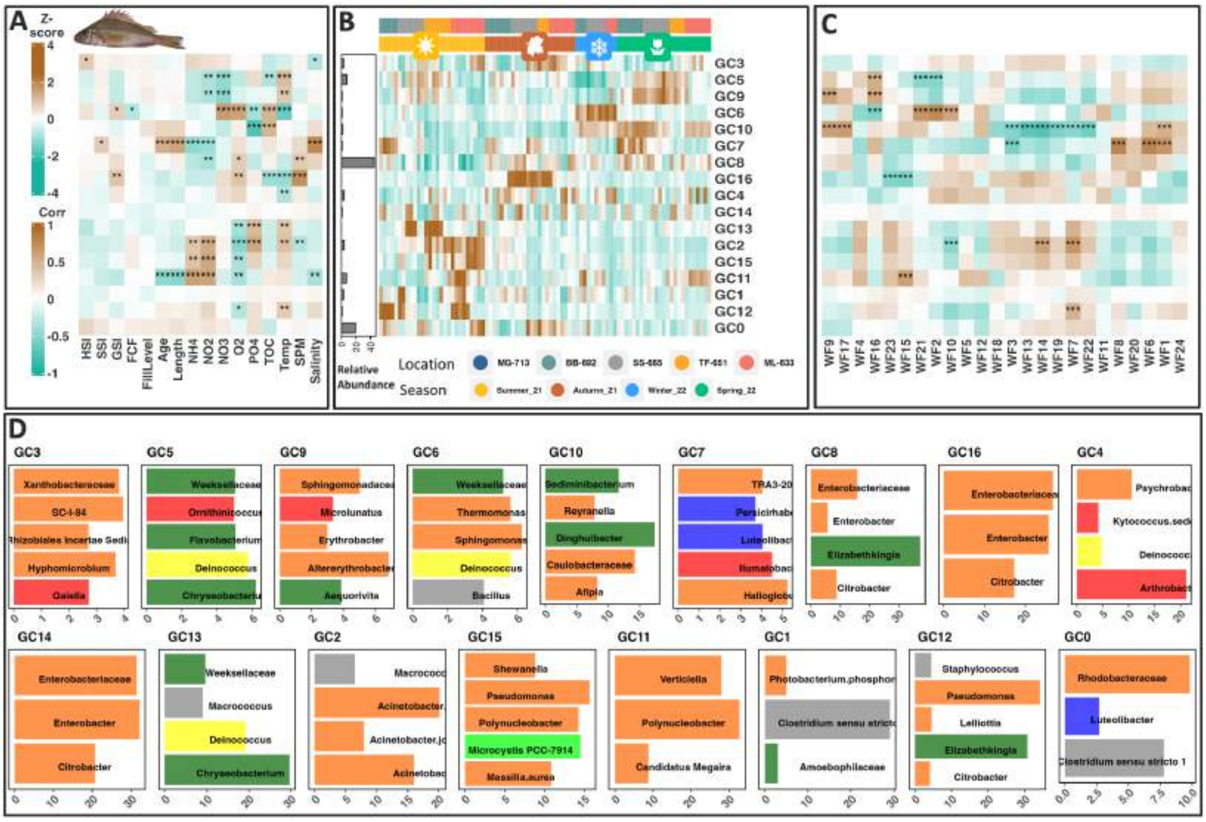

Fig. S7.5 Relationship between bacterial co-occurrence networks, biometric measurements and abiotic conditions for Elbe estuarine ruffe GC. Integrated heatmap of gill bacterial mucus network analysis showing correlation between ASV module eigengenes (**B**: GC-0-16), physiological traits (**A**: HSI, SSI, GSI, FCF, FillLevel, 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 = 89) ordered by season and facets within season by sampling location in upstream direction as columns. Overall relative abundance per module is shown 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.

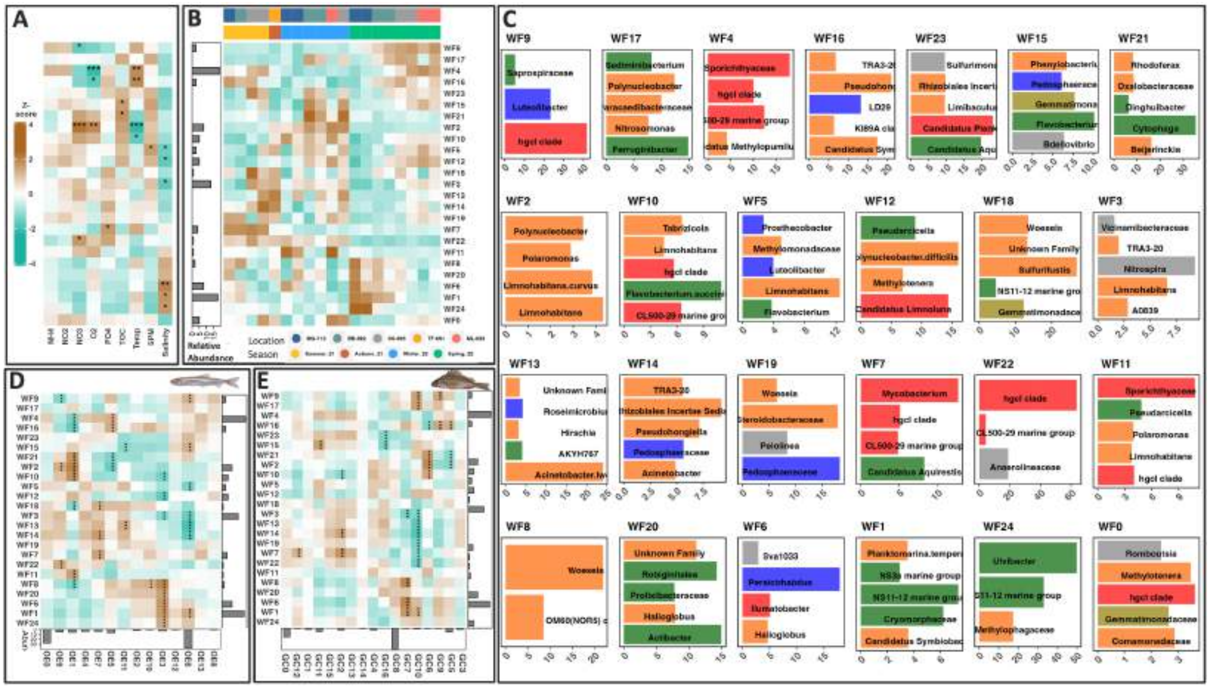

Fig. S7.6 Relationship between bacterial co-occurrence networks and abiotic conditions for free-living bacteria in the Elbe estuary. Integrated heatmap of the bacterioplankton network analysis showing correlation between ASV module eigengenes (B: WF-0-24) and relevant abiotic factors (A: NH_4, NO_2, NO_3, O_2, PO_4, TOC, temperature, SPM, salinity). **B** shows Z-score of eigengene values per module as rows for individual water samples (n = 19) ordered by season and facets within season by sampling location in upstream direction as columns. Overall relative abundance per module is shown the barplot on the left side. **A** shows FDR corrected Pearson correlation strength between eigenASVs and external traits (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 (**C**) colored by phylum identity. Module-correlation between biomes and individual relative abundance of module taxa are shown in **D** and **E**.

Measures to counter contamination in the workflow:

Strains of Acinetobacter were described as potential contaminants of microbiome studies repeatedly (François-Étienne et al. 2023, Salter et al. 2014, Weyrich et al. 2019). To ensure contamination-free data sets in this wild-sampling study, we added mock community samples randomly at every extraction and sequencing batch indicating no contamination throughout the workflow (Fig. S7.7). Furthermore, we suspect a low risk of contamination between fish samples in abundant bacteria, as the gill is protected by the opercular, and we see progressing trends in bacterial composition at stations or sampling events where only one species was caught.

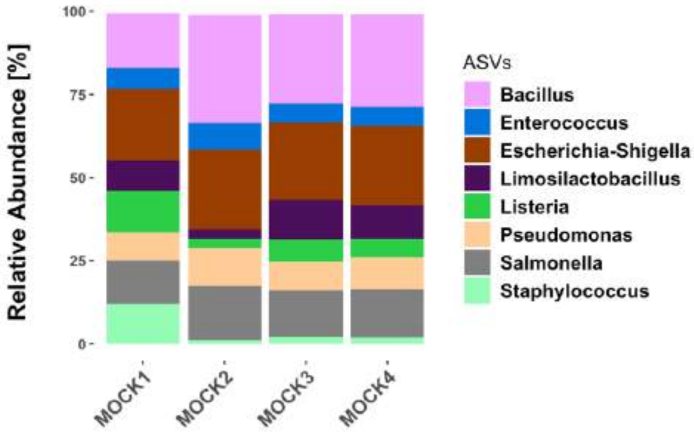

Fig. S7.7 Mock communities. Sterile swabs used for gill mucus recovery were immersed in the MOCK community mix (Zymo) and randomly included during every extraction batch and send for sequencing treated as a normal sample. According to the manufacturer's instructions, the composition should be: *Listeria monocytogenes* - 12%, *Pseudomonas aeruginosa* - 12%, *Bacillus subtilis* - 12%, *Escherichia coli* - 12%, *Salmonella enterica* - 12%, *Lactobacillus fermentum* - 12%, *Enterococcus faecalis* - 12%, *Staphylococcus aureus* - 12%, *Saccharomyces cerevisiae* - 2% and *Cryptococcus neoformans* - 2%.

Chapter 8

General discussion

"There is something almost magical to the notion that our bodies, minds and ideas have roots in the crust of Earth, water of the oceans, and atoms in celestial bodies. The stars in the sky and the fossils in the ground are enduring beacons that signal, though the pace of human change is ever accelerating, we are but a recent link in a network of connections as old as the heavens."

Neil Shubin – The Universe Within

8. General discussion

The aim of this thesis was to improve our understanding of the ecological relationships and spatio-temporal interactions of key fish species and lower trophic levels in the food web of the Elbe estuary using stable isotope analysis of $\delta^{13}C$ and $\delta^{15}N$.
Previous studies dealing with the Elbe estuary, in which stable isotope approaches were applied, mainly focused on questions regarding nitrification processes and the N-cycle using $\delta^{15}N$ and $\delta^{18}O$ isotopes (e.g., Dähnke et al, 2008, Deutsch et al. 2009, Dähnke et al. 2010, Sanders et al. 2018) or the hydrological origin of the Elbe water using $\delta^{2}H$ and $\delta^{18}O$ (e.g., Barth et al. 2006, Koeniger et al. 2022). Some studies dealt with suspended matter (Middelburg and Herman 2007) and organic matter compartments of the sediment, especially of the maximum turbidity zone (MTZ) (e.g., Schoer 1990, Schwedhelm et al. 1988). Rare comprehensive studies on trophic diversity in the Elbe estuary in the planktonic food web using $\delta^{13}C$ were carried out by Kerner et al. (2004). Taupp et al. (2016) recorded the isotopic niches of common benthic invertebrates in the MTZ of the Elbe estuary using $\delta^{13}C$ and $\delta^{15}N$. This doctoral thesis is therefore the first study to investigate this estuarine food web with a special focus on estuarine fishes at this scale using stable isotope analysis.

8.1 Key findings

Intraspecific habitat exploitation of key species smelt - In this chapter we found that foraging of dominating smelt *Osmerus eperlanus* strongly depends on prey availability in the Elbe estuary. We identified a high overlap of used prey in both juvenile and adult smelt with *Neomysis integer* and *Gammarus* spec. serving as key prey items. However, our data indicated a switch in feeding preferences during ontogeny in both stomach content and stable isotope data with a shifting trend towards piscivorous feeding with increasing fish length. We further observed changes in isotopic niche overlaps and isotopic niche widths along the course of the river. Intensive cannibalistic foraging was observed in the freshwater area of the Mühlenberger Loch by both life stages. However, stable isotope approaches of juvenile tissue samples from this area showed high proportions of assimilated isotopes derived from zooplanktonic origins, thus further underlines an ontogenetic shift. It became apparent that juveniles are exposed to the respective habitat with the given conditions and food supply, while adults are able to avoid areas with unfavorable settings. Our results highlight the importance of estuaries serving as nursery and feeding areas for different life stages of migrating fish.

Tracking life history of estuarine fishes - Here, we tracked annual migration and habitat exploitation of flounder *Platichthys flesus* and ruffe *Gymnocephalus cernua* using stable isotopes. Facultative catadromous flounder reached the estuary as young of the years (YOY) between summer and autumn and exploit the upper freshwater area of the Elbe estuary in winter, revealing a wide movement radius. Potamodromous ruffe was rather site-specific and hardly migrated between the local sections, which was also reflected in a significantly smaller isotopic niche compared to flounder. Overall, two distinct isotope patterns and a temporal delay in the arrival of juvenile flounder were observed, indicating that two populations use the estuary as a nursery area that is further promoted by literature. Our data on ruffe, though, showed that these fish do not leave our study area during the course of the year, which may indicate an adapted brackish water population of this species. Nevertheless, further genetic studies are necessary to confirm our assumption.

Spatial and temporal patterns of zooplankton - In this study we found that the habitats upstream and downstream of the city of Hamburg differed significantly in terms of carbon quality and thus primary productivity. Therefore, the upstream area at Bunthaus was characterized by its high-quality riverine organic matter (OM) and high phytoplankton blooms in spring and summer, while the heavy anthropogenically influenced area downstream was significantly less productive. Especially in winter and autumn, zooplankton showed increased carnivory feeding that is further reflected in higher $\delta^{15}N$ values, due to limited high-quality autochthonous phytoplankton availability and high suspended matter loads. Overall, our study showed that the zooplankton community of the Elbe estuary is capable of responding seasonally to hydrologically induced changes through dietary shifts and niche partitioning.

Acidification of macroinvertebrates in stable isotope approaches - The impact of inorganic carbon signatures of calcified structures forming prey on stable isotope analysis is a common but often overseen problem in aquatic food web studies. However, calcified structures in organisms may rather reflect inorganic carbon signatures from surrounding water than $\delta^{13}C$ derived from diet potentially leading to misinterpretation of stable isotope data. Acidification is a known but intricate method to remove calcified structures prior stable isotope analysis. Here, we analyzed acid treated and untreated samples of four macroinvertebrate species based on their $\delta^{13}C$ and $\delta^{15}N$ values. We found that acidification is particularly effective for organisms with high carbonate content, such as gammarids, while the influence of inorganic carbon signals on mysid shrimp (*Neomysis integer, Mesopodopsis slabberi*) which contain low carbonate proxies can be neglected in food web studies. For large-sized macroinvertebrates, such as tested *Palaemon longirostris,* we recommend the mechanical removal of the exoskeleton prior to the analysis so an acidification can be avoided (compare with Mateo et al. 2008). Based on other ecological studies, we furthermore created a decision tree to evaluate the most appropriate method to minimize the impact of inorganic carbon signatures in isotope-based food web studies.

Long term study of environmental factors affecting the fish fauna structure - We analyzed the effects of environmental factors on the fish fauna over the last 40 years using abundance data and abiotic measurements from four sampling periods in the Elbe estuary. Changes in environmental factors led to alterations in fish species compositions and life cycle guilds of mainly anadromous, marine-estuarine opportunistic and catadromous fish species. Major changes in environmental conditions and species composition were observed in freshwater and oligohaline areas, where important nurseries for estuarine fishes are located. We found that fish abundance, particularly of smelt *Osmerus eperlanus*, followed an increasing trend from the 1980s to 2010 as water quality improved. However, hydromorphological changes recently led to higher suspended particulate matter (SPM) concentrations, reduced river runoff and low oxygen levels, which again led to a drastic decline in fish abundances, especially in smelt, flounder, twaite shad and ruffe. Marine species such as herring and whiting, though, increased in abundance due to the amplified inflow of saline water from the North Sea as a result of channel modifications in the estuary.

Fish gill microbiota as indicators for fish health - The interactions between host and its microbiome are important for fish health. Investigating the microbiome of the gill mucus therefore provides information about the state of health, as the gills are in direct exchange with the environment. Overall, the core microbiota of the estuarine fish studied, did not differ significantly from each other and comprised only about 30 genera, suggesting that only a small number of generalist species can cope with estuarine conditions. While the microbiome of smelt gradually adapted to the estuary during the migration from the sea in autumn, the microbiota composition of the stationary ruffe was primarily characterized by spatial drivers. Hypoxia events and high nutrient loads led to dysbiosis states, which are largely characterized by the takeover of opportunistic pathogens (*Acinetobacter*, *Shewanella*, *Aeromonas*).

8.2 Estuaries as habitats for fishes

Estuaries are often associated in public discourse with their ability to absorb and store large amounts of carbon (e.g., Abrantes et al. 2015, Krauss et al. 2018, Bulmer et al. 2020) or with their economic importance as waterways for large commercial vessels (e.g., Hafen Hamburg Marketing e.V. 2014). These ecosystem services primarily address human-related global issues, but estuaries are also essential habitats as part of life cycle strategies in fishes (Whitfield et al. 2022). The connectivity of different habitat types (e.g., tidal wetlands, seagrass beds, salt marshes) with adjacent ecosystems such as the sea or river systems also makes these systems appealing to numerous fish species (Whitfield et al. 2022). As a result, estuaries serve as nursery, feeding and spawning habitats as well as refuges and migration routes (Haedrich 1983, Elliot and Hemingway 2002).

The exploitation of these ecosystem services by fish is determined by their ability to cope with fluctuating environmental conditions, particularly temperature and salinity (Able 2005). Accordingly, the species composition of estuarine fish communities is characterized by their tolerance to these environmental fluctuations (Whitfield et al. 2022), which in turn determines local guild compositions (Whitfield et al. 2022). However, habitat use by fish could become more difficult in the upcoming years, as it is assumed that increasing anthropogenic influences and climate change (Cloern et al. 2016) will amplify the pressure on estuarine communities in the future (Robins et al. 2016, Colombano et al. 2021).

Stable isotope analyses can contribute to a better understanding of population dynamics (e.g., Semmens et al. 2009, Guelinckx et al. 2008), migratory behavior (e.g., Hobson 1999, Hobson and Wassenaar 2008, Trueman et al. 2012) and trophic interactions (e.g., Hayden et al. 2016, de Carvalho et al. 2017, Poiesz et al. 2021). Enhanced knowledge can therefore help to develop necessary protection concepts and limit future pressures from anthropogenic sources.

8.3 Fish migration patterns and stock development

Smelt *Osmerus eperlanus* - Anadromous fish are the most successful species in the Elbe estuary and make up over 98% of the total fish population, with the smelt *Osmerus eperlanus* alone accounting for 96% (Eick and Thiel 2014). On a globally scale more than 20% of the worldwide fish species follow an anadromous migration pattern, which mainly occurs in the northern hemisphere (Morais and Daverat 2016). Anadromous fishes live in marine waters during their adult phase and migrate into freshwater areas to spawn (Myers 1949, Morais and Daverat 2016) (Fig. 8.1A). In the Elbe estuary the spawning migration of smelt takes place between February and March (Borchardt 1988), whereby pre-spawning adults starting to aggregate in the river mouth between November and February (Kottelat and Freyhof 2007, Thiel and Thiel 2015). The migration mechanism is thereby triggered by temperature (Kottelat and Freyhof 2007) leading to an optimal synchronization of offspring development and local food production (Sepúlveda et al. 1993). During spring, different age classes are sharing the same habitats and estuarine resources (Borchardt 1988). Juvenile and adult smelt feed extensively on gammarids and mysids (chapter 2). This is further reflected by an overall isotopic niche overlap of 16.4% and an isotopic overlap of 52.4% while including 95% CI. However, we suggest that this overlap is not equivalent to intraspecific competition, as the effect is diminished by the dietary shift during ontogeny and the high productivity of just a few species such as *Neomysis integer* (Mees et al. 1993, 1995, Fockedey and Mees 1999) or *Gammarus* spec. (Riedel-Lorjé and Gaumert 1982) in the estuary (Elliott and McLusky 2002). The short-term and long-term information from stomach and stable isotope analysis further indicate that feeding preferences generally rely on food availability rather than on the selectivity of the consumer (chapter 2) that was also found in smelt from the Baltic Sea (e.g., Popov 2006, Taal et al. 2014). Predation on offspring occurs in both juveniles and adults especially in the freshwater area of the Mühlenberger Loch in the Elbe estuary. Cannibalistic feeding is a known characteristic in smelt´s diet (e.g., Henderson and Nepszy 1989, Northcote and Hammar 2006), promoting higher growth rates especially in juveniles (Vinni et al. 2004). In Finland lakes, young smelt started to feed on offspring with a total length of approximately 8 cm (Vinni et al. 2004).

Studies on related rainbow smelt (*Osmerus mordax*) in Lake Champlain, US and Canada, reveal a mortality between 38-93% on age group 0 due to cannibalism with highest pressures in June (Parker Stetter et al. 2007). Smelt abundances from the Elbe estuary also peaked between June and late August with juvenile young-of the-year (YOY) specimens dominating the contribution of this species. However, based on the high abundances and dominance of smelt (chapter 6), we assume that cannibalism plays a minor role regarding recruitment success in the Elbe estuary.

Thiel and Potter (2001) described three main reasons that explain the success of smelt in the Elbe estuary. Firstly, the spawning sites are located upstream in a region that is not affected by the deepening of the main channel (Sepúlveda et al. 1993). Secondly, the so-called *umbrella* structure of the eggshell allows temporary attachment to the substrate, whereupon, thirdly, the hatched larvae drift downstream with the tides to the nursery areas (Ehrenbaum 1894, Lillelund 1961, Thiel and Potter 2001). However, young smelt primarily depend on the local food supply and remain in one place for an extended period of time, which increases their vulnerability with regard to environmental conditions (chapter 2). Our study further indicates that adult smelt, in contrast, can leave an area due to increased mobility if the environmental conditions are unfavorable.

Flounder *Platichthys flesus* - Flounder is the only European flatfish that swims into estuaries and can live under freshwater conditions for an extended amount of time (Summers 1979, Hemmer-Hanson et al. 2007). Complementary to smelt, it follows a facultative catadromous migration pattern with spawning taking place in marine or brackish waters (Daverat et al. 2012, Morais and Daverat 2016). Primarily, the estuary serves as a nursery area for larvae and juveniles (Henderson and Holmes 1991, Kerstan 1991, Mendes et al. 2020). The period of spawning in the adjacent North Sea lies between winter and early spring (Summer 1979, Muus and Nielsen 1999), so that the first larvae are transported via tidal flood transport into the Elbe estuary in winter (Bos et al. 1995) (Fig. 8.1B). The first juveniles disperse in the estuary in summer and autumn. In addition to strongly varying total lengths, which split into two groups, we observed two isotope clusters that are independent of life stage, indicating two distinct populations (chapter 3). Bos et al. (1995) already reported the arrival of two AG0 groups in the Elbe estuary: one in mid-March and a second AG 0 group in mid-April. The authors referred to studies by Campos et al. (1994), who described two North Sea populations with different hatching areas west of the island of Texel, Netherlands, and northwest of the island of Helgoland, Germany. Simulations of distributions of early life stages of flounder by Barbut et al. (2020) are consistent with these assumptions. However, underlying data from the years 1997-2006 were used here, which provide only limited information about the current spawning areas. Our assumptions can therefore be partially explained by previous literature, but recent studies are needed to finally confirm our results.

Overwintering behavior of young flounder at the upstream freshwater sections was detected by our study for the first time as δ^{13}C below our investigated isotopic ranges of the sampling sites reveal riverine food sources that were assimilated in the tissue (chapter 3). As the tidal influenced part of the Elbe River is limited upstream by the weir of Geesthacht (Kerner 2004), the upstream movement of fish is potentially also limited here. Bos and Thiel (2006) observed that early life stages of flounder prefer low salinities, which could further act as a trigger for upstream movements in the estuary. However, information on overwintering strategies in juvenile flounder is scarce. Bell (2009) analyzed overwintering behavior of related winter flounder (*Pseudopleuronectes americanus*) in three North American estuaries and detected overall little starvation during overwintering. As the mortality of juvenile fish is generally highest during wintertime of their first year of life (Bell 2009), fish often invest their energy in growth in summer and switch to energy storage in late autumn and early winter (Heintz and Vollenweider 2006, Slater et al. 2007). Therefore, the ability to ingest prey during winter, when food availability is usually low (Thiel 2001), maintains energy storage and decreases the mortality risk, especially the risk of size-specific mortality (Pratt and Fox 2002, Eckmann 2004, Bell 2009). In the Elbe estuary, studies have shown that the common daily ratios >20% of body weight of juvenile flounders can be reduced to <5% by food reduction (Thiel 2001, 2011). Contrary to our results, Bos (1999) found that the first juvenile flounder arrive in the upper freshwater areas of the estuary in June. We therefore assume that the juveniles reach this upper part earlier but occur in highest densities in winter. The term *overwintering* can therefore be discussed here, and further studies are required to finally determine the movements of juvenile flounder. Based on our isotope data, we conclude that mainly juvenile and minor adult flounders exploit these freshwater river sources (chapter 3).

Ruffe *Gymnocephalus cernua* - Ruffe as a potamodromous species fulfills its entire life cycle in freshwater and undertakes migrations to feeding, overwintering and spawning habitats in order to feed, refuge or reproduce here (Morais and Daverat 2016). Ruffe from the Elbe estuary showed high site fidelities and resident behavior throughout the year (chapter 3) with highest abundances at the freshwater regions between Twielenfleth and Mühlenberger Loch (chapter 6). However, ruffe is known to be a robust, adaptative species, that can tolerate a wide range of environmental and ecological factors (Svirgsden et al. 2015). Therefore, this species occurs in various habitats (e.g., lakes, rivers, tidal estuaries and non-tidal estuaries) (Hölker and Thiel 1998) and can quickly establish in non-native regions, such as North American lakes where invasive ruffe was introduced by accident (Gutsch and Hoffman 2016). In general growth performance of ruffe is better in estuaries rather than in freshwater systems (Hölker and Thiel 1998).

Since ruffe of the Elbe estuary have hardly reached or exceeded the isotopic limits of our sampling sites, there is a possibility that a brackish water population exists in this area that is isolated from the pure freshwater ruffe occurring further upstream (chapter 3, Fig. 8.1).

Previous studies demonstrated that different populations of ruffe developed distinct modifications depending on their habitat requirements (e.g., Vetemaa and Saat 1996, Svirgsden et al. 2015). These include, for example, larger eggs that contain more energy (Albert et al. 2006, Svirgsden et al. 2015) and higher salinity tolerances of embryos from brackish populations compared to freshwater populations (Vetemaa and Saat 1996, Albert et al. 2006). Investigations of Vetemaa and Saat (1996) on brackish water populations from the Pärnu Bay, Baltic, Sea, Estonia, showed that ruffe still reproduces at salinities ranging between 6 and 8 ppt at bays with freshwater inflow. The mortality during gastrulation of freshwater population from the Pärnu Lake, Estonia, on the other side, increased already at 6 ppt (Vetemaa and Saat 1996). Since the ruffe in the Elbe estuary is dependent on these described adaptations under different osmotic pressures to maximize the survival probability of the offspring (e.g., buoyancy of the eggs, production of larger larvae) (Svirgsden et al. 2015), a distinct brackish water population is likely. Furthermore, the high site fidelity, a small movement radius compared to the studied flounder (chapter 3), and the occurrence of ruffe in surrounding freshwater areas (Thiel and Thiel 2015) support our hypothesis. Nevertheless, further investigations, such as genetic analyses, are necessary to confirm this assumption.

Contrary to diadromous species (see sections above), ruffe do not migrate long distances but perform local spawning migrations to shallow waters between March and May and overwinter in deep waters (Gutsch and Hoffman 2016). In the Elbe estuary ruffe occurs both in the side and main channels (Thiel et al. 1995), probably migrating to adjacent habitats at higher depths in winter. Stable isotope analysis of ruffe from Allouez Bay, USA, found depleted $\delta^{13}C$ values that probably indicate foraging migration to another habitat outside the studied wetland area (Sierszen et al. 1996). Our study could not verify these migrations (see section 8.3), however, stable isotope data of $\delta^{13}C$ and $\delta^{15}N$ per total length reveal an ontogenetic shift in ruffe (chapter 3), indicating a switch from small to larger prey with increasing body size (Hammerschlag-Peyer et al. 2011). Although ruffe is generally known as a benthic feeder (Ogle et al. 1995, Sierszen et al. 1996), planktivorous prey plays a particularly important role in the diet of juveniles in estuaries (Hölker and Thiel 1998). As a generalist feeder ruffe can feed on a variety of prey sources and can switch habitat use and prey consumption after being introduced to non-native ecosystems, such as reported in Mildevatn Lake, Norway (Kalas 1995). In the Elbe estuary juveniles switch from copepods (Ladiges 1935) to mysid shrimps and amphipods with a body length >7 cm (Hölker and Thiel 1998). Adult ruffe increasingly feeds on mud shrimp *Crangon crangon* (Hölker and Temming 1996) and fish (mainly smelt and gobies) (Hölker and Hammer 1994).

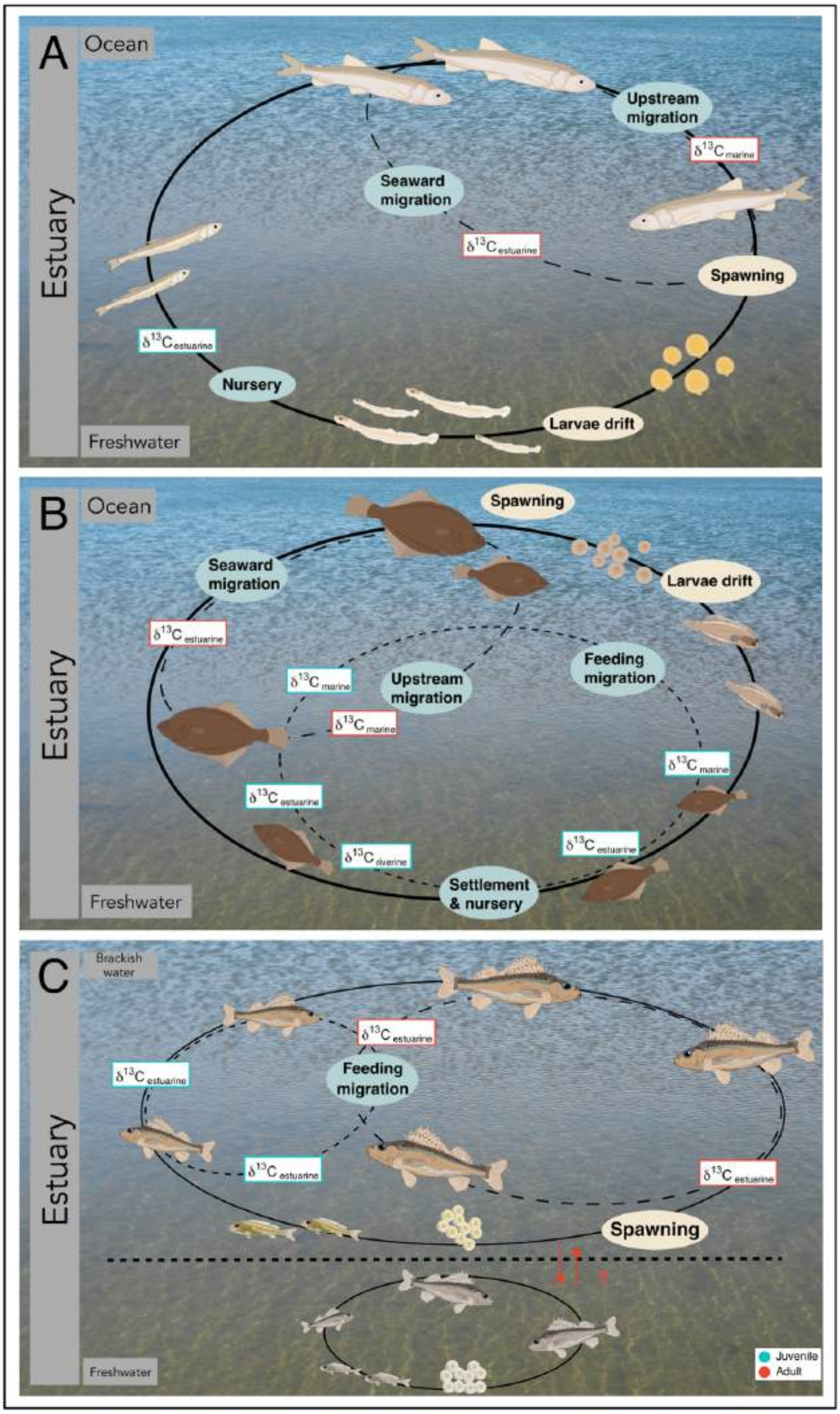

Fig. 8.1 Life history strategies of estuarine fishes in the Elbe estuary using $\delta^{13}C$ ratios of juvenile and adult life stages (solid lines). $\delta^{13}C$ indicate marine or estuarine origins, further enables to track upstream or seaward migrations. Variabilities within estuarine ranges further reveal inner estuarine feeding migrations (dashed lines): (**A**) Anadromous smelt (*Osmerus eperlanus*), (**B**) Facultative catadromous flounder (*Platichthys flesus*) and (**C**) Potamodromous ruffe (*Gymnocephalus cernua*).

Overall, we conclude that anadromous fish species are the most successful in the Elbe estuary, which can be confirmed by previous studies (Thiel and Potter 2001, Eick and Thiel 2014). But this is not the case for other temperate estuaries in Europe. In several European estuaries, gobies dominate in abundance, e.g. in the Canche estuary, France (Selleslagh and Amara 2008), in the upper Scheldt estuary, Belgium (Maes et al. 2005) and in the Mondego estuary, Portugal (Martinho et al., 2007a, b, Dolbeth et al. 2008). Breine et al. (2011) documented the European flounder (*Platichthys flesus*) as the dominant species in the Zeeschelde estuary, Belgium.

Although diadromous smelt and flounder and potamodromous ruffe as shown in our studies are known to be well adapted to exploit the habitat during their life cycle, they have faced dramatical threats in recent years. Estimations on the population development during the last 40 years showed an overall decline in their fish biomass. Especially fishes that use the Elbe estuary as a nursery area declined dramatically (chapter 6). Mean abundance of anadromous species, of mainly of smelt (*Osmerus eperlanus*), twaite shad (*Alosa fallax*), three-spined stickleback (*Gasterosteus aculeatus*) for instance declined from 20,000 ind./Mio. m^3 in 2009/10 to 1,500 ind./Mio. m^3, thus representing a decline of >92% in their abundance. Smelt abundances peaked between June and late August when juvenile YOY specimens are dominating the contribution of this species. Even the abundance of flounders and ruffe decreased recently (chapter 6). The underlying reasons for these changes include both anthropogenic and natural stressors, which have intensified in recent years (see section 8.5).

8.4 Strengths and limitations of stable isotope analysis in this study

The Elbe estuary is one of the largest estuaries in Europe and forms the link between the Elbe River and the North Sea (Schwentner et al. 2021), therefore it connects different food webs with distinct carbon (C) sources. Since biogeochemical processes and nutrient inputs influence isotope ratios, these different C sources can be detected by local $\delta^{13}C$ signatures (Peterson and Fry 1987, Morais and Daverat 2016). Riverine C is thus usually significantly lower in its $\delta^{13}C$ ratios than marine C, which reflects heavier isotope ratios (Peterson and Fry 1987).

Mobile consumers, such as fishes, ingest food in one habitat and assimilate the respective carbon signature via diet in their tissues, which they in turn carry along to other habitats (Hobson 1999, Durbec et al. 2010). These distinct riverine $\delta^{13}C$ signals on the one side and mesohaline/marine $\delta^{13}C$ ratios on the other make estuaries particularly suitable for reconstructing fish migrations using stable isotopes (see chapter 3).

In the Elbe estuary, the particulate organic matter (POM) values of $\delta^{13}C$ decrease from -24.2 ± 0.9‰ to -26.9 ± 2.2‰ from the river mouth to the freshwater area (Bunthaus) above the city of Hamburg (chapter 4), whereby the intermediate isotopic compositions due to the predominance of detrital terrestrial OM is comparable to other European estuaries (Middleburg and Herman 2007). To classify the isotopic ranges of the study area to track fish migrations, POM baselines were not usable as the samples were taken during one-day trips and do not necessarily represent the whole season. Furthermore, these surveys did not necessarily coincide with our fishing trips, which impaired comparability. However, mysid shrimps, mainly *Neomysis integer*, serves as a suitable indicator for local $\delta^{13}C$ as the species occurs along the course of estuary and is a major prey for local fish (Sepúlveda et al. 1993). As described by Guelinckx et al. (2006), we tracked migrations of resident ruffe *Gymnocephalus cernua* and facultative catadromous flounder *Platichthys flesus* using $\delta^{13}C$ values of the preferred prey, represented by *N. integer*. Other studies performing stable isotope approaches have also proven that macroinvertebrates such as Hydropsychidae (e.g., Durbec et al. 2010) are particularly effective for identifying migrations in fish species (e.g., Cunjak et al. 2005). According to the respective research question, filter feeders such as mussels and snails can further be used as baselines to trace pelagic and benthic feeding in fish (e.g., Vander Zanden et al. 1997, Post 2002, Ankjærø et al. 2012, Rakauskas et al. 2013).

Using mysid shrimps to isotopically characterize the study area of the Elbe estuary is appropriate for tracking large annual migrations on the longitudinal scale of the river by flounder but is less effective for small-scale migrations that are frequently performed by ruffe (chapter 3). To track these small-scale movements of ruffe we suggest that a higher local resolution of baselines might improve the interpretation of stable isotope data here. Harrod et al. (2005), for instance, detected local migrations of European eel *Anguilla anguilla* along the salinity gradient of a tidal Atlantic lake using $\delta^{13}C$, $\delta^{15}N$ and C:N of small-scale baselines of an invasive gastropod. Therefore, it can be concluded that dense local baselines from lower trophic levels are beneficial to improve the resolution of isotopic data to further detect movements of resident fishes.

Mysid shrimps, as well as other macroinvertebrates, form carapace structures, that may reflect the isotopic composition of the surrounding water rather than dietary derived carbon (Yokoyama et al. 2005, De Lecea et al. 2011). Subsequent stable isotope analysis can be severely affected unless this inorganic carbon (IC) is removed, e.g., via acidification (chapter 5). However, based on our findings we concluded that mysid shrimps are not strongly affected by inorganic carbon signals, making an effect neglectable. As the necessity of acid treatment correlates with species-specific carbonate content, gammarids, containing a high amount of carbonate (> 0.05) (Serrano et al. 2008), should be prepared prior to the actual analysis. Though, the effect that non-treated macroinvertebrates have on food web analyses often depends on the research question. In studies where IC was not removed (e.g., Cunjak et al. 2005, Ravinet et al. 2013), the focus was mostly on a large-scale classification of upper trophic levels, such as fish. Presumably, it is hypothesized that the consumer, while ingesting the whole animal, stores both inorganic and organic C in its tissues, which is further reflected in its $\delta^{13}C$ ratios. For small-scale movements as described above, a potential impact on the analysis should be considered. Acidification is recommended in studies dealing with lower trophic levels and has long been a standard procedure here (e.g., Søreide et al. 2006, Rothhaupt et al. 2014, Remy et al. 2017).

8.5 Spatial challenges for estuarine fish

Based on our investigations on carbon sources and its quality (chapter 4), trophic interactions (chapter 2 and 4), fish migrations (chapter 3), and abiotic parameters (chapter 6), we identified four areas in the Elbe estuary which are of crucial relevance in understanding the estuarine food web: 1) the mouth of the river, 2) the maximum turbidity zone (MTZ), 3) the Mühlenberger Loch and 4) the freshwater area upstream of the city of Hamburg (Fig. 8.2).

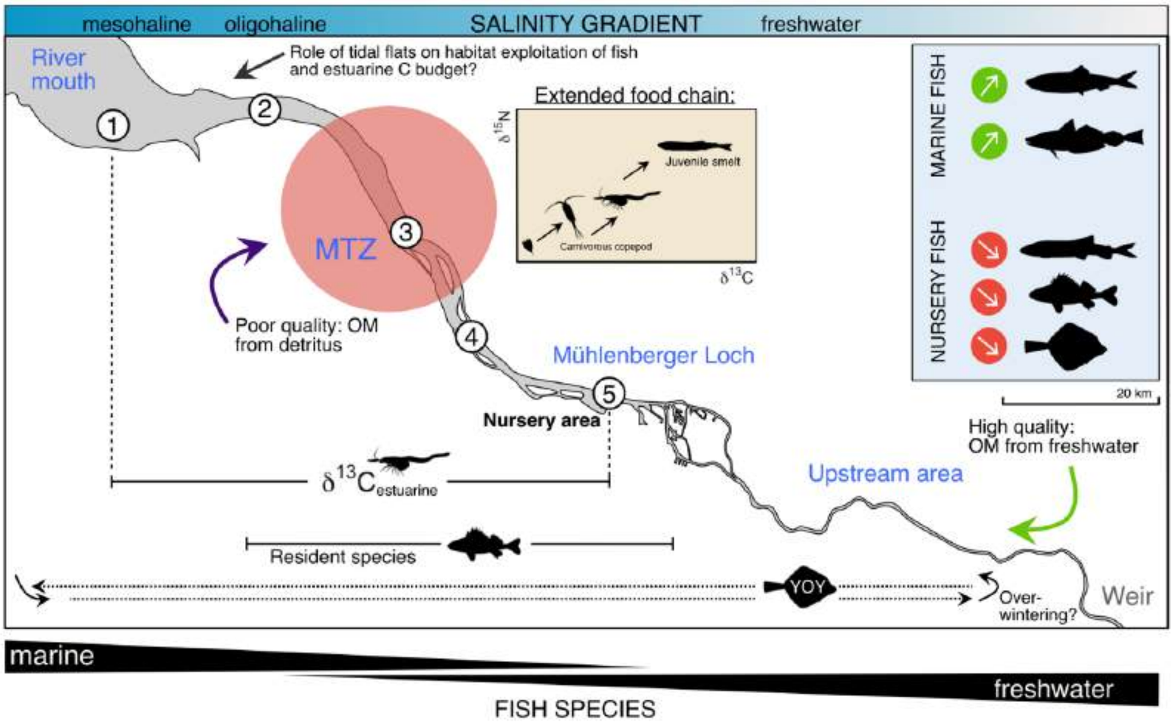

Fig. 8.2 Summary of spatial characteristics of the Elbe estuary with four important areas: river mouth, maximum turbidity zone (MTZ), Mühlenberger Loch and upstream area (blue) with station numbers 1-5. Stable isotope analysis at the MTZ promoted elongation of the local food chain (chapter 2). Estuarine $\delta^{13}C$ values of mysid shrimps (mainly *Neomysis integer*) indicate homing behavior in ruffe and migration of juvenile (here: YOY = young-of-the-year) flounder up to freshwater regions upstream in winter (chapter 3). Organic matter (OM) composition reveals poor quality POM downstream and high-quality POM upstream of the port of Hamburg (chapter 4). Blue box shows recent population trends of marine species and migrating species that exploit the estuary as a nursery area (chapter 6).

River mouth – The river mouth area serves as an entrance for migrating fish species to exploit ecosystem services (e.g., feeding or spawning) (Elliot and Hemingway 2002). As marine organic sources at the bottom of the trophic food chain often have high $\delta^{13}C$ values between -18 and -22‰ (Thornton and McManus 1994, Finlay and Kendall 2007), marine fishes generally contain higher marine-derived $\delta^{13}C$ values than resident estuarine and freshwater species (Cunjak et al. 2005).

The local fish community is mainly shaped by physicochemical factors and biological interactions (Neto et al. 2023), whereby temperature and salinity act as main drivers in these areas (e.g., Breine et al. 2011, Thiel and Potter 2001). In European estuaries, marine fishes usually dominate in species number and abundance (Maes et al. 1998, Potter et al. 2001, Thiel 2011, Eick 2015), unlike in the Elbe estuary, which is dominated by anadromous fish species (see section 8.3). Marine species are most abundant in the river mouth and decrease with decreasing salinity upstream, while freshwater species show a reverse trend (Thiel and Potter 2001). The success of settlement of certain fish species in estuaries is related to their respective ability to osmoregulate (Thiel and Potter 2001). Studies on gene transcription and biochemical data on delta smelt *Hypomesus transpacificus*, for example, suggest that salinity is the major factor affecting generalized stress response in juveniles (Hasenbein et al. 2013).

Marine fish species account for the greatest biodiversity in the Elbe estuary (e.g., Potter and Thiel 2001, Eick and Thiel 2014). In the river mouth, mainly marine stragglers (e.g., *Agonus cataphractus, Chelidonichthys lucerna*) and marine-estuarine opportunists (e.g., *Clupea harengus, Merlangius merlangus*) were found (chapter 6), containing isotopically higher $\delta^{13}C$ values (e.g., *Clupea harengus*: -19.6 ± 1.8‰, *Sprattus sprattus*: -20.4 ± 0.7‰, *Gadus morhua*: -17.6 ± 0.7‰) than resident fishes (Hauten et al. *in prep.*). Especially marine-estuarine opportunists can occur in higher densities, mainly during their juvenile life stage by using the habitat for nursery and as a refuge area (Thiel 2011). Our long-term study indicated a positive trend on the occurrence of these marine fishes (mainly *C. harengus* and *M. merlangus*) (chapter 6), caused partially by the increased inflow of saline water due to the channel widening and deepening of the tidal Elbe in recent years (Zhu et al. 2014, Ralston and Geyer 2019).

Obviously, the occurrence and abundance of these species fluctuate temporally and spatially. In spring and summer 2021, for instance, most juvenile whiting was caught in the mesohaline and oligohaline area up to Brunsbüttel (chapter 6). Juvenile herring also showed a larger distribution area and occurred as far as Schwarztonnensand and Twielenfleth, which was not the case in previous sampling periods. Rising sea levels and reduced river discharge as a result of climate change are expected to lead to a further increase in these saline species in the future (Pein et al. 2023).

Although diadromous populations are currently declining in the study area (chapter 6), these species in particular can move to other catchment areas temporarily (McDowall 1995) but would potentially be able to successfully recolonize their habitat if water quality improved (Eick and Thiel 2014). This is also reflected by the recovery of smelt *O. eperlanus* in the 1990s with improving water quality after its rapid decline in the 1980s due to high pollution loads in the Elbe estuary (Potter and Thiel 2001) (chapter 6).

Our investigations on the dietary preferences of anadromous smelt revealed that the greatest diversity of prey species is found in the mouth of the river, indicating a richer food supply than in areas further upstream (chapter 2). However, we also observed highest isotopic niche widths and overlaps in this area both decreasing towards inland. These outcomes indicated that the prevailing salinity conditions shape the local feeding preferences of smelt in the estuary (chapter 2). We assume that smelt might partially migrate laterally to adjacent temporary tidal areas of salt marshes to exploit resources here. Previous studies have shown that native fish species enter these areas during their life cycle to take advantage of the food supply and habitat (Prahalad et al. 2019). Especially the adjacent salt marshes of the Wadden Sea area serve as important nursery areas for fishes, including smelt, where biota benefits from lower predation and competition pressure compared to pelagic habitats (Friese 2020). Salt marshes could therefore play a key role here and could have a significant influence on local fish production (Jones et al. 2021). However, migratory behavior of estuarine fishes between salt marshes and the Elbe estuary have been scarcely investigated and should be included in future studies.

Maximum turbidity zone (MTZ) - The MTZ defines the oligohaline freshwater-seawater interface in an estuary where sediments and organic matter (OM) accumulate, and flocculation of suspended matter leads to high turbidity rates (Fockedey and Mees 1999). At the same time, it is the area with the highest salinity variability (Taupp and Wetzel 2014) which amplifies major osmoregulatory stress in aquatic consumers (Dahl 1956, Taupp et al. 2017). The area is further characterized by the input of both marine and riverine carbon and nutrient sources, whereby the habitat is usually less productive as the high turbidity rates promote light limitations that further hamper photosynthetic processes (Hoffman et al. 2008, Kundu et al. 2021). In the Elbe estuary primary production is low in the MTZ with year-round poor POM quality (chapter 4).

In the local food chain, these circumstances promote an increase of mixotrophic phytoplankton species on a low trophic level especially during winter (Martens et al. 2024). On the zooplankton level, calanoid copepods *Eurytemora affinis*, switch from detrital feeding to a more carnivorous diet (chapter 4) with rotifers as favored diet, that was further reported in the Charente estuary, France (Modéran et al. 2012). These additional steps in the trophic structure imply the elongation of the food chain that leads to an enrichment of δ^{15}N values in consumers (Layman et al. 2007). As *Neomysis integer* feeds partly on *E. affinis*, (Fockedey and Mees 1999) an enrichment of δ^{15}N in the food chain of the MTZ would be promoted (Fockedey and Mees 1999). However, findings from Fockedey and Mees (1999) revealed that mysids in this region consume a large proportion of Rotifera as well, similar to *E. affinis*. This finding is consistent with similar δ^{15}N values of *N. integer* and *E.affinis* that could be observed in our study (chapter 4), indicating a similar food source use of both species. We therefore conclude that the impact of *N. integer* on the isotopic enrichment in the food chain is potentially low, although the fact that both samplings (Fockedey and Mees

1999, chapter 4) represent one sampling day and do not cover all variabilities in this dynamic system. However, we measured enriched $\delta^{15}N$ values in juvenile smelt caught in the MTZ compared to the other areas of the estuary (chapter 2). $\delta^{15}N$ values of adult smelt, in contrast, were not enriched, indicating the ability of large fish to avoid areas with inappropriate environmental conditions and low food supply. The MTZ can be therefore termed as one of the most challenging habitats for the estuarine community, further affecting the food web structure and leading to behavioral adaptations of the consumers.

Besides these trophic alterations, fishes face also direct threats (Hasenbein et al. 2013), such as reduced oxygen update due to alterations in gill epithelial tissue, as observed in juvenile smelt (Thiel and Pezenburg 2001), affecting the survival of fish eggs and larvae (Yang et al. 2019) and the feeding success in visual predators (Hecht and van der Lingen 1992). The latter is confirmed by a recent study by Koll et al. (2024a) using transcriptomic analyses, according to which zander (*Sander lucioperca*) as a visual predator suffers from starvation in the MTZ. Ruffe on the other hand can locate prey even under dim light conditions due to its well-developed lateral system (Disler and Smirnov 1977), a particularly extensive canal system in the head region (Janssen 1997, Hölker and Thiel 1998) and modified eyes with *tapetum lucidum* (Ahlbert 1969). Previous studies on North American delta smelt *Hypomesus transpacificus* revealed that the larvae do not forage in pristine water as smelt needs the visual contrast to detect its prey (Baskerville-Bridges et al. 2004). Further, other studies on wild delta smelt showed a positive relationship between its occurrence and turbidity (Moyle et al. 1992, Feyrer et al. 2007, Tigan et al. 2020). Illing et al. (2024) on the other hand found that the food intake of European smelt larvae decreases with increased turbidity. Therefore, it can be assumed, that foraging success of smelt larvae relies on certain turbid conditions that might be another reason of smelt being so successful in the Elbe estuary.

As other environmental factors, turbidity varies on a spatial and temporal scale, thus depending on e.g., weather conditions, tidal fluxes, water flow, algal growth, light and water depth (Hasenbein et al. 2013). Even though increased turbidity levels are highest in the MTZ, other regions of the Elbe estuary are also affected. Particularly the region downstream of the Port of Hamburg, that is further demonstrated by increased suspended organic matter (SPM) values in recent years (FGG 2024). Therefore, annual SPM concentrations tripled from 1984-86 and doubled from 2009/10 to 2021/22 (chapter 6), which further hamper primary production (e.g., Hoffman et al. 2008). Human induced alterations in rivers, such as waterway maintenance dredging and shipping traffic are considered to be the causes of increased turbidity in anthropogenically modified estuaries (Wilber and Clarke 2001, Smith and Friedrich 2011, Illing et al. 2024). Kerner (2007) examined the topographical causes by channel deepening after 1999. The deepening of the main channel therefore resulted in higher sedimentation depositions between 0.7 and 1.5 m in shallow water areas from 1999 to 2004. Covering sediment further leads to less settlement of biota in these dredged regions (Kerner 2007). The extraction of fine-grained sediment by maintenance dredging in the Elbe estuary has increased significantly in recent decades from 4.5 million m^3/year (2008 -2013) to 8.5 million m^3/year (2014-2019) (Weilbeer et al. 2021, Illing et al. 2024). This may explain our findings from comparative surveys in the Elbe estuary from 1984-2022 that show an increasing trend in SPM values ranging between 18.6 and 30.7 mg/L in the 1980s to today's values between 71.1 and 92.1 mg/L (chapter 6).

Mühlenberger Loch - The Mühlenberger Loch and adjacent Hahnöfer Nebenelbe are located at Ekm 632-635 in the freshwater section of the Elbe estuary and known as the main nursery area for fishes (Kafemann 1992, Thiel 2011). Compared to other shallow areas, the Mühlenberger Loch is considered the most species-rich with a high proportion of AG0 juveniles in the region (Dierking 1992, Kafemann 1992) and highest annual fish production in the entire estuary with > 200 kg/ha (Thiel 2001). It further serves as a bird sanctuary which is protected as part of the Natura 2000 network (Fricke et al. 2021). Although the area is of high ecological importance as it preserves essential ecosystem functions for the estuarine community, its topography has been altered by human interventions in the past. This includes the dike construction works in the 1960s that led to a closure of the connection to the southern river Süderelbe section (Dörjes and Reineck 1981). In 2000, the Mühlenberger Loch was partially sealed during the construction of the Finkenwerder Airbus facility (GOEP 2022, Hütter 2023).

Due to low velocities and temporarily flooded tidal flats, particles as well as planktonic organisms remain in this bay significantly longer compared to the main channel, where tidal forces occur more intensively (Steidle and Vennell 2024). In particular, the mudflats in the south-eastern part of the Mühlenberger Loch are significantly more productive than the fine and coarse silt areas in the adjacent waterway (Kafemann 1992), leading to highest densities of key zooplankton species *Eurytemora affinis* here (Fiedler 1991, Kafemann et al. 1996, Thiel 2001). During springtime *E. affinis* populations collapse due to massive predation by higher trophic levels (top down), especially juvenile fishes (Köpcke 2002, Thiel 1990, Thiel 2011). Stable isotope analysis of juvenile smelt showed high proportions derived from mesozooplankton in this area and a switch to larger prey afterwards (chapter 2). In other estuarine shallow zones from the UK, especially mysid shrimp *N. integer* exerts the high predation pressure on *E. affinis*, while predation by fishes plays only a minor role (Irvine et al. 1995).

The comparative abundance data of fish over the last 40 years showed that especially fishes that use the Elbe estuary as a nursery area have strongly decreased in abundance overall (chapter 6, section 8.3). Although smelt were still the most abundant species caught in the Mühlenberger Loch during our 2021/22 sampling, their abundance decreased by ~85%

compared to the 2009/10. Mature twaite shad, which spawns in the adjacent Neßsand area (Magath 2013), was also caught most frequently here but declined overall in its abundance (chapter 6).

Hypoxia events (<2 mg O_2/L) are often considered a severe environmental threat that correlates with eutrophication (Carpenter et al. 1998, Geerts et al. 2017). The occurrence of hypoxia in oceans, coasts and estuaries has been found to double every ten years since 1960 (Diaz and Rosenberg 2008). As smelt is known to be sensitive to pollution and hypoxia it serves as an indicator species in terms of water quality (Thomas 1998, McCarthy et al. 2019). According to our findings, juvenile smelt are much more sensitive to changes in the environment than adults (chapter 2). Earlier studies have shown that growth of juvenile smelt is reduced at oxygen concentrations <4.5 mg/L (Sepúlveda 1994). Persistent critical oxygen levels <3 mg/L can even lead to the destruction of an entire year class that may affect the whole population in the estuary (Köhler 1981, Möller and Schulz 1991, Thiel et al. 1995). As hypoxic events have been detected in this area, particularly during the summer months, we assume that low oxygen concentration is a major threat especially for juvenile fishes (chapter 6). Furthermore, the impacts of climate change could further amplify anoxic conditions, as recent studies suggest (e.g., Colombano et al. 2021). According to these, more frequent and more extreme OMZs are to be expected in shallow coastal waters such as estuaries. Therefore, especially young fishes can suffer from adverse conditions that leads to higher mortality rates in the harbor region leading to less recruitment of local fish populations.

Freshwater area upstream of the city of Hamburg – The freshwater area upstream of the city of Hamburg was not examined by fishing activities during this study. However, in chapter 4 we investigated carbon origins and trophic interactions in zooplankton including the upstream station Bunthaus. We showed that the particulate organic matter (POM) quality along the course of the river differs significantly, which further impacted higher trophic levels (chapter 4). POM from the Elbe estuary that derived from fluvial origins are of higher quality than POM derived from marine sources (chapter 4) supporting high phytoplankton and zooplankton productivity (Malone et al. 1988, Barros et al. 2022). Besides this, the freshwater area upstream of the city of Hamburg is unaffected by frequent dredging activities that are mainly threatening the downstream area. Certain fish species use this area for spawning (e.g., Sepúlveda et al. 1993, Lòzan and Kausch 1996, Thiel and Potter 2001, Thiel and Thiel 2015) or feeding (Bos 1999).

The spawning grounds of the smelt are located upstream near Köhlbrandt at the Süderelbe and between Bunthaus and Fliegenberg (Lillelund 1961, Köhler 1981, Möller and Diekwisch 1991, Sepúlveda et al. 1993). Previous studies have described the upstream area as an important nursery area for flounders and other fish species, especially in the summer months (Thiel and Bos 1998, Bos 1999). Our investigations showed particularly high chlorophyll values here, especially in spring and summer, with peak values of up to 153 μg/L (chapter 4). Due to high quality C and high primary productivity as well as good habitat conditions, such as shallow water regions, low turbidity and year-round reduced water turnover, we assume that this habitat is still exploited by fishes. However, recent studies have focused primarily on areas located downstream of the Hamburg port, but more recent studies are essential to assess the current importance of these habitats for fishes.

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E. Haufen

Hamburg, den 10. Oktober 2024

Contribution of authors

Chapter 2: 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 Koll, Jesse Theilen, Andrej Fabrizius, Ralf Thiel, Kai Jensen, Peter Grønkjær and Christian Möllmann

This chapter is a draft currently being prepared for publication in a scientific journal. The contribution of the authors is as follows: **Elena Hauten (EH)**, Peter Grønkjær (PG) and Christian Möllmann (CM) developed the objective of this study, whereby PG and CM supervised the work. **EH** conducted the field work (methodology) together with Jesse Theilen (JT) und Rapahel Koll (RK). Laboratory work was done by **EH**. Data analyses was developed by **EH**, PG and Steffen Funk (SF) with the advice of CM, and conducted by **EH**. Data visualization was created by **EH**. Ralf Thiel (RT), Kai Jensen (KJ), Andrej Fabrizius (AF) and CM provided the funding acquisition of the study including methodology and the publishing process. All co-authors reviewed and edited the original draft written by **EH**.

Confirmation of correctness

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Place, date

Christian Möllmann

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

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Christian Möllmann

Chapter 4: Spatial and temporal patterns of zooplankton trophic interactions and carbon sources in the eutrophic Elbe estuary (Germany)

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Christian Möllmann

Chapter 5: Is acidification of common estuarine macroinvertebrates in stable isotope approaches necessary to analyze aquatic food webs?

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This chapter is a draft submitted to *Limnology and Oceanography: Methods* (02/2024) and currently under submission. The contribution of the authors is as follows: **Elena Hauten (EH)** designed the experimental design for this study and collected macroinvertebrate individuals in the field. Amami Perera (AP) prepared the samples in the laboratory and conducted statistical analysis under advice of **EH**. AP gave comments on the first draft written by **EH**. **EH** analyzed and visualized the data for the publishing process. Christian Möllmann (CM) coordinated the funding acquisition and contributed to the writing process by reviewing and editing the original draft written by **EH**. This study was supervised by CM.

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Christian Möllmann

Chapter 6: Environmental factors affecting the fish fauna structure in a temperate mesotidal estuary: a 40-year long-term study in the Elbe estuary

Authors: Jesse Theilen, Victoria Sarrazin, Elena Hauten, Raphael Koll, Christian Möllmann, Andrej Fabrizius and Ralf Thiel

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Chapter 7: Spatio-temporal fish gill microbiota analysis as indicators in estuarine fish health monitoring

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