KLAIPĖDA UNIVERSITY

Tobia POLITI

# BIOGEOCHEMICAL INTERACTIONS AMONG BENTHIC MACROFAUNA, MICROBIAL COMMUNITIES AND MACROPHYTES IN EUTROPHIC COASTAL LAGOONS

DOCTORAL DISSERTATION

Natural sciences,
ecology and environmental sciences (N 012)

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# SĄVEIKA TARP DUGNO BESTUBURIŲ, MAKROFITŲ IR MIKROORGANIZMŲ IR JOS VAIDMUO EUTROFINĖSE LAGŪNOSE

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XXXIV CICLO
IN CO-TUTELA CON KLAIPEDA UNIVERSITY

# Biogeochemical interactions among benthic macrofauna, microbial communities and macrophytes in eutrophic coastal lagoons

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# Abstract

Surface sediments are interesting spots to analyze the paradigm of biodiversity and ecosystem functioning due to the multiple physic and chemical gradients that shape the interactions among microbial communities, macrofauna and primary producers. Sediments receive large inputs of organic matter and are sites of intense biogeochemical processes, mediated by microbial communities and facilitated by macrofauna, ultimately resulting in nutrients uptake by benthic primary producers or their recycling to the water column. The relationship between diversity and ecosystem functioning was analyzed at different spatial scales in the benthic compartment of two shallow eutrophic lagoons, the Curonian Lagoon (Lithuania) and the Sacca di Goro (Italy). Special attention was given to the benthic nitrogen (N) cycle, due to the critical role of this element in aquatic ecosystem functioning and to the complex regulation of its various oxic and anoxic reactions, carried out by diverse microbes and strongly influenced by macrofauna and primary producers. Investigations were carried out at different spatial scales included whole lagoon (macro-scale) as well as single macrofauna individuals and holobionts' microbiomes (micro-scale). At the two lagoons the benthic functioning was evaluated by quantifying rates of whole system respiration and production via gas exchange, nutrient cycling and exchange at the sediment-water interface. Multivariate statistical analyses were used to reveal the interactions between the dominant macrofauna species and net solute fluxes and speculate about underlying processes. Such approach allowed to reconstruct how different macrofauna functional groups shape benthic N cycling in different macro areas of the Sacca di Goro, and determine net loss, net recycling or different level of coupling between processes (e.g., ammonification and nitrification, or nitrification and denitrification). In the Curonian Lagoon the whole scale approach was used to verify whether macrofauna act as a natural buffer against redox-dependent phosphorus recycling during short-term events of oxygen shortage. Manipulative experimental approaches addressed specific processes at the microscale, in sediments colonized by different macrofauna functional groups, and along gradients of density. Such approaches included intact or reconstructed sediment incubation, metabolic measurements of single macrofauna individuals, and the use of $^{15}$N-labeled inorganic N forms to measure specific microbial transformations (denitrification, anammox, nitrate ammonification, N-fixation) in sediments or in macrofauna microbiota. Moreover, molecular tools were used to analyze microbial diversity (16S rRNA metabarcoding) and activity (marker genes and transcripts) in holobionts. Three organisms that are abundant in the Curonian lagoon were considered: the burrowing larvae of *Chironomus plumosus,* the filter feeder bivalve *Dreissena polymorpha* and the phytophagous gammarid *Pontogammarus robustoides*. Results suggest

that in the Sacca di Goro lagoon macrofauna play an important role, in regulating N transformations. However, its importance also depends on the prevailing environmental factors (i.e., salinity, hydrodynamics and background nutrient concentrations). Whereas in the Curonian Lagoon bioturbation did not significantly affect the nutrient metabolism and the stability of reductive-oxidative reactions during anoxia events. Molecular studies revealed that Chironomid larvae burrows are hot-spots of microbial communities involved in N cycling and that these organisms, via bioirrigation, significantly enhance both the recycling of ammonium and N removal via denitrification. Mussels primarily enhance the recycling of N to the water column, both via direct excretion and by stimulating dissimilatory nitrate reduction to ammonium. The latter is likely an effect of mussel's biodeposits. For these two organisms the quantification of functional genes showed a significantly higher potential for microbial denitrification, nitrate ammonification and $N_2$-fixation in macrofauna as compared to the surrounding environment. As chironomid and dreissenid densities in eutrophic lagoons are large, animals-associated microbes may account for a substantial (and so far, overlooked) N import and recycling. *P. robustoides* was finally demonstrated to have an important role in the survival of *Chara contraria* in the eutrophic Curonian Lagoon. The gammarid facilitates *C. contraria* via active grazing on the macroalgae-associated epiphytes combined with ammonium excretion, thus supporting the growth of the characeans.

## Key words

Biogeochemical cycles, Biodiversity, Estuarine systems, Ecosystem functioning, Eutrophication.

# Reziumė

Paviršinės dugno nuosėdos yra idealus pavyzdys, analizuojant biologinės įvairovės ir ekosistemų funkcionavimą dėl čia esančių daugybės fizinių ir cheminių gradientų, kurie lemia mikroorganizmų bendrijų, makrofaunos ir pirminių gamintojų sąveiką. Įprastai dugno nuosėdų paviršiuje kaupiasi organinė medžiaga, vyksta intensyvūs mikroorganizmų vykdomi biogeocheminiai procesai, kuriuos reguliuoja makrofaunos veikla. Dėl jos keičiasi maistmedžiagių ir kitų elementų apykaita tarp dugno nuosėdų ir priedugnio vandens bei jų prieinamumas pirminiams gamintojams. Doktorantūros darbe sąveika tarp įvairovės ir ekosistemos funkcionavimo buvo analizuojama skirtingose dimensijose – nuo mikro- iki makroskalės, dviejose sekliose eutrofininėse lagūnose – Kuršių mariose (Lietuva) ir Sacca di Goro (Italija). Išskirtinis dėmesys buvo skirtas azoto (N) ciklui dugno nuosėdose dėl šio elemento svarbos ekosistemos funkcionavimui ir jo mikrobiologinių virsmų kompleksiškumo, kurį lemia makrofauna ir pirminiai gamintojai. Azoto ciklo virsmų tyrimai apėmė ekosistemos lygmenį (makro), individo bei holobionto (mikro). Visos dugno bendrijos funkcionavimas kiekybiškai įvertintas išmatuojant kvėpavimo, dujų ir maistmedžiagių asimiliacijos bei produkcijos greičius. Tuo tarpu daugiamatė statistinė analizė buvo taikoma identifikuojant sąveiką tarp vyraujančių makrofaunos grupių ir skirtingų junginių apykaitos greičių. Pastarasis metodas suteikė galimybę aprašyti pagrindinius mechanizmus kaip makrofauna gali reguliuoti N biogeocheminius procesus (amonifikaciją, nitrifikaciją ir denitrifikaciją) Sacca di Goro lagūnos paviršinėse dugno nuosėdose. Kuršių mariose daugiamatė statistinė analizė padėjo įvertinti ar makrofaunos vaidmuo svarbus reguliuojant redukcijos-oksidacijos reakcijų stabilumą ir fosforo judrumą formuojantis trumpalaikėms deguonies trūkumo sąlygoms. Eksperimentiniai metodai buvo taikomi nustatyti specifiniams procesams mikroskalėje, kurie vyksta nuosėdose, paveiktose skirtingų makrofaunos funkcinių grupių. Darbe naudoti eksperimentiniai metodai apėmė nesuardytos struktūros ar rekonstruotų dugno nuosėdų mikrokosmų inkubaciją, makrofaunos individų metabolizmo matavimus ir žymėtų azoto ($^{15}N$) izotopų taikymą, identifikuojant specifinius mikrobiologinius virsmus (denitrifikaciją, anamoks procesą, nitratų disimiliacinę redukciją iki amonio (DNRA), $N_2$ fiksaciją) paviršinėse nuosėdose ir makrofaunos individuose. Taip pat molekuliniai metodai buvo pritaikyti identifikuojant mikrobų įvairovę (16S rRNR metabarkodavimas) ir kiekybiškai vertinant genų, koduojančių skirtingus azoto mikrobiologinius virsmus, gausumą ir jų aktyvumą paviršinėse dugno nuosėdos bei holobiontuose. Makrofaunos vaidmuo holobionte buvo analizuojamas tik Kuršių marių dominuojančiose funkcinėse grupėse – uodo trūklio lervose (*Chironomus plumosus*) ir dvigeldžiuose moliuskuose (*Dreissena polymorpha*). Gauti rezultatai parodė, kad makrofaunos bendrijos, aptinkamos Sacca

di Goro lagūnoje, atlieka svarbų vaidmenį reguliuojant N virsmus skirtingose sedimentacinėse aplinkose. Visgi šių bendrijų svarba taip pat priklauso ir nuo vyraujančių aplinkos veiksnių (druskingumo, hidrodinamikos ir maistmedžiagių koncentracijos). Tuo tarpu Kuršių mariose aptinkama makrofaunos bendrija pasižymi mažu aktyvumu rausiant dugno nuosėdas. Tai sąlygoja jos mažą poveikį maistmedžiagių apykaitai bei redukcinių-oksidacinių reakcijų stabilumui deguonies trūkumo sąlygomis. Molekuliniai tyrimai atskleidė, kad *C. plumosus* sukonstruoti ir ventiliuojami urveliai pasižymi itin didele mikroorganizmų bendrijos įvairove, kuri vykdo N virsmus. Galima teigti, kad urvelio ventiliacija, kurios metu su priedugnio vandeniu patenka deguonis ir nitratai, svarbūs elementai nitrifikacijos ir denitrikacijos procesui, o pasišalina kenksmingi metabolizmo produktai, teigiamai veikia mikroorganizmų įvairovę. Dėl šios priežasties lervų gausiai išraustos dumblo nuosėdos funkcionavo kaip akumuliacinė zona nitratams iš vandens storymės. Tuo tarpu *D. polymorpha* kolonijos, įprastai aptinkamos smėlio nuosėdų paviršiuje, intensyviai transformavo azoto junginius ir išskyrė juos į aplinką. Svarbiausi N kaupimosi priedugnio vandenyje mechanizmai – moliusko ekskrecija ir mikroorganizmų vykdoma DNRA. Apibendrinant kiekybinę genų gausumo analizę *C. plumosus* eksperimentuose, matyti, kad tirtų N virsmų genetinis potencialas yra didesnis lervos holobionte nei supančioje aplinkoje. Atsižvelgiant į didelį lervų ir moliuskų gausumą Kuršių mariose, holobionto vaidmuo galėtų būti svarbus reguliuojant N dinamiką šioje eutrofinėje ekosistemoje. Taip pat buvo nustatyta, kad kietašarvės šoniplaukos (*Pontogammarus robustoides*) atlieka svarbų vaidmenį mažojo maurabragio (*Chara contraria*) išgyvenamumui Kuršių marių litoralėje. Šios šoniplaukos, palengvina *C. contraria* augimą, nuėsdamos epifitinius, siūlinius dumblius nuo augalo šakelių, antra – ekskrecijos metu išskirdamos amonį, kuris yra svarbi maistmedžiagė pačiam maurabragiui.

## Reikšmingi žodžiai

Biogeocheminiai ciklai, bioįvairovė, estuarinės sistemos, eutrofikacija, ekosistemų funkcionavimas

# Sintesi

Gli strati superficiali del sedimento sono caratterizzati da gradienti fisici e chimici che regolano le interazioni tra comunità microbiche, macrofauna e produttori primari. Rappresentano quindi siti ideali per lo studio del paradigma ecologico "biodiversità e funzionamento dell'ecosistema". Il sedimento riceve costantemente carichi di materia organica che stimola i processi biogeochimici . Questi processi sono mediati dalle comunità microbiche e facilitati dall'attività della macrofauna bentonica, e possono determinare una rigenerazione di nutrienti verso la colonna d'acqua e/o assorbimento da parte dei produttori primari bentonici. Le relazioni tra biodiversità e funzionamento dell›ecosistema sono state analizzate a diverse scale spaziali nel comparto bentonico di due lagune eutrofiche poco profonde, la Laguna di Curi (Lituania) e la Sacca di Goro (Italia). Particolare attenzione è stata data al ciclo dell›azoto (N), per il suo ruolo critico nel funzionamento dell›ecosistema acquatico e per la complessità generata dalle varie reazioni ossiche e anossiche svolte da diversi batteri e fortemente influenzate dalla macrofauna e dai produttori primari. Questo studio è stato condotto a diverse scale spaziali e ha incluso studi su ampia scala a livello dell'intera laguna, così come attività sperimentali su singoli individui appartenenti alla macrofauna e microbiomi costituiti da olobionti (microscala). Nelle due lagune il funzionamento del sistema bentonico è stato studiato quantificando i tassi di respirazione e di produzione attraverso misure di scambio di gas e nutrienti disciolti all›interfaccia sedimento-colonna d'acqua. È stato quindi applicato un approccio di tipo statistico per identificare le possibili interazioni tra le specie dominanti (macrofauna) e i flussi netti dei soluti. Questo approccio ha permesso di capire come diversi gruppi funzionali della macrofauna influenzano il ciclo bentonico dell›azoto in diverse macro aree della Sacca di Goro e di identificare i fattori che determinano la rimozione e la rigenerazione dell'azoto e i processi biogeochimici accoppiati (es. ammonificazione e nitrificazione, o nitrificazione e denitrificazione) . Nella Laguna dei Curi è stato utilizzato un simile approccio per verificare il ruolo della macrofauna come tampone naturale contro la rigenerazione e/o il rilascio, redox-dipendente, del fosforo durante eventi di anossia. Altri approcci sperimentali di tipo manipolativo hanno investigato alcuni processi specifici sulla microscala, in sedimenti colonizzati da diversi gruppi funzionali di macrofauna e lungo un gradiente di densità crescente. Tali approcci includono l›incubazione di sedimenti intatti o ricostruiti, misure di metabolismo di singoli individui di macrofauna e l›uso di forme di azoto inorganiche marcate ($^{15}N$) per misurare i processi batterici (denitrificazione, anammox, ammonificazione di nitrato, fissazione di N) nei sedimenti o nel microbioma della macrofauna. Inoltre, sono stati utilizzati metodi di tipo molecore per analizzare la diversità (16S rRNA metabarcoding) e l'attività microbica

(geni marcatori e trascrizioni) negli olobionti. Sono stati presi in considerazione tre organismi abbondanti nella Laguna dei Curi: larve scavatrici di *Chironomus plumosus*, il bivalve filtratore *Dreissena polymorpha* e il gammaride fitofago *Pontogammarus robustoides*. I risultati suggeriscono che nella Sacca di Goro la macrofauna svolge un ruolo importante nella regolazione delle trasformazioni dell'azoto. Tuttavia, la sua importanza dipende anche dai fattori ambientali prevalenti (cioè salinità, idrodinamismo e concentrazioni di nutrienti). Nella Laguna dei Curi la bioturbazione non ha influenzato significativamente le dinamiche dei nutrienti e la stabilità delle reazioni di ossido-riduzione durante gli eventi di anossia. Gli studi molecolari hanno rivelato che le gallerie scavate dai chironomidi sono hot-spot per le attività di comunità microbiche coinvolte nel ciclo dell'azoto e che questi organismi, tramite la bioirrigazione, aumentano significativamente sia la rigenerazione dell'ammonio che la rimozione dell'azoto tramite la denitrificazione. I bivalvi aumentano principalmente la rigenerazione di N nella colonna d'acqua, sia attraverso l'escrezione diretta, sia stimolando la riduzione dissimilatoria di nitrato ad ammonio. Quest'ultimo è probabilmente un effetto dei biodepositi e delle escrezioni solide. Per questi due organismi la quantificazione dei geni funzionali ha mostrato un potenziale significativamente maggiore di denitrificazione microbica, ammonificazione del nitrato e fissazione di $N_2$ nella macrofauna rispetto all'ambiente circostante. Se si tiene in considerazione l'alta densità di chironomidi e bivalvi nelle lagune eutrofiche, i batteri associati a questi organismi possono determinare un sostanziale (e finora trascurato) incremento nei processi di fissazione e rigenerazione di N. Con questo studio è stato anche dimostrato che *P. robustoides* ha un ruolo importante nella sopravvivenza della macroalga *Chara contraria* nella Laguna dei Curi. I gammaridi facilitano *C. contraria* attraverso il pascolo attivo degli epifiti che coprono le macroalghe e attraverso l'escrezione di ammonio, supportando così la crescita delle characee in ambienti eutrofici.


## Parole chiave

Cicli biogeochimici, Biodiversità, Estuari, Funzionamento degli ecosistemi, Eutrofizzazione.

# Contents

## List of original publications

The material of this study was presented in 5 original publications, published in peer-reviewed scientific journals, and 1 manuscript, referred in the text by their Roman numbers:

I. **Politi, T.,** Zilius, M., Castaldelli, G., Bartoli, M., & Daunys, D. (2019). Estuarine macrofauna affects benthic biogeochemistry in a hypertrophic lagoon. Water, 11(6), 1186. doi.org/10.3390/w11061186.

II. Samuiloviene A., Bartoli M., Bonaglia, S., Cardini U., Vybernaite-Lubiene I., Marzocchi U., Petkuviene J., **Politi T**., Zaiko A., & Zilius M. (2019). The effect of chironomid larvae on nitrogen cycling and microbial communities in soft sediments. Water, 11(9), 1931. doi.org/10.3390/w11091931.

III. Marzocchi, U., Bonaglia S., Zaiko A., Quero G. M., Vybernaite-Lubiene I., **Politi T**., Samuiloviene A., Zilius M., Bartoli M., & Cardini U. (2020). Zebra mussel holobionts fix and recycle nitrogen in lagoon sediments. Frontiers in Microbiology, 11. doi.org/10.3389/fmicb.2020.610269

IV. **Politi T.,** Barisevičiūte R., Bartoli M., Bonaglia S., Cardini U., Castaldelli G., Kančauskaitė A., Marzocchi U., Petkuviene J., Samuiloviene A., Vybernaite-Lubiene I., Aiko A., & Zilius M. (2021). A bioturbator, a holobiont, and a vector: The multifaceted role of *Chironomus plumosus* in shaping N-cycling. Freshwater Biology. doi.org/10.1111/fwb.13696.

V. **Politi, T.,** Zilius, M., Bartoli, M., & Bučas, M. (2021). Amphipods' grazing and excretion loop facilitates Chara contraria persistence in a eutrophic lagoon. Aquatic Botany, 171, 103378. doi.org/10.1016/j.aquabot.2021.103378

VI. **Politi, T.,** Zilius, M., Daunys D., Forni, P., Bartoli, M., Biogeochemical buffers in eutrophic coastal lagoon along an oxic-anoxic transition. (Submitted to Estuarine, Coastal and Shelf Science, April 13, 2022).

## Author's contribution

I. Politi Tobia contributed to the study design, pre-processing, post-processing, data analysis, and wrote the manuscript draft.

II. Politi Tobia performed the sampling. Contributed to experimental work in laboratory, to the writing of the original draft preparation, and to review and editing the final manuscript.

III. Politi Tobia took part in the sampling, contributed to experiments and chemical analyses. He also contributed to the writing of the original draft preparation, and to review and editing the final manuscript.

IV. Politi Tobia performed the sampling and carried out the incubation experiments; contributed to the study design, to the first draft of the manuscript to the discussion and interpretation of data, and revised and approved the manuscript for submission.

V. Politi Tobia took part in conceiving the ideas and designed methodology. Performed sampling, carried out the incubation experiments, led the data analysis. Contributed to the discussion and interpretation of data, writing and editing of the manuscript for submission.

VI. Politi Tobia contributed to the study design, performed the sampling, data collection, pre-processing, post-processing, and data analysis, and wrote the manuscript draft.

## Additional publications by the author of this thesis

I. Zilius, M., Vybernaite-Lubiene, I., Vaiciute, D., Overlingė, D., Grinienė, E., Zaiko, A., Bonaglia S., Liskow I., Voss M., Andersson A., Brugel S., **Politi, T.**, & Bukaveckas, P.A. (2021). Spatiotemporal patterns of $N_2$ fixation in coastal waters derived from rate measurements and remote sensing. Biogeosciences, 18(5), 1857-1871. https://doi.org/10.5194/bg-18-1857-2021

II. Broman, E., Zilius, M., Samuiloviene, A., Vybernaite-Lubiene, I., **Politi, T.**, Klawonn, I., Voss M., Nascimento F.J.A. & Bonaglia, S. (2021). Active DNRA and denitrification in oxic hypereutrophic waters. Water Research, 116954. https://doi.org/10.1016/j.watres.2021.116954

III. Morkūnė R., Bučas M., Kataržytė M., **Politi T.,** Vaičiūtė D., Vizzini S., Martin G., 2022 “Littoral trophic networks in macrophyte habitats in a transitional Baltic ecosystem: stable isotope ratios reveal food sources for benthic grazers”. Water. (submitted).

# Abbreviations

| Abbreviation | Explanation |
|---|---|
| **Anammox** | Anaerobic ammonium oxidation |
| **B & EF** | Benthic and Ecosystem Functioning |
| **DFe** | Dissolved Iron |
| **DIN** | Dissolved inorganic Nitrogen |
| **DIP** | Dissolved inorganic Phosphorus |
| **DMn** | Dissolved Manganese |
| **DNRA** | Dissimilative Nitrate Reduction to Ammonia |
| **DSi** | Dissolved Silica |
| **$Fe^{3+}$** | Iron oxyhydroxides |
| **$H_2S$** | Sulfide |
| **N** | Nitrogen |
| **$N_2$** | Di-Nitrogen (Gas) |
| **$NH_4^+$** | Ammonium |
| **$NO_3^-$** | Nitrates |
| **$O_2$** | Oxygen |
| **OM** | Organic matter |

# 1

# Introduction

## 1.1 “Old” and “new” paradigms of ecosystem functioning

The relationships among organisms composing a community in an ecosystem and the functioning of that ecosystem becomes more central in recent ecological studies. This subject is not novel as it was tackled years ago in terrestrial ecosystems with the main aim to understand what regulates primary production (Balvanera et al., 2006; and references therein). The concern was to forecast the consequences of simplified biodiverse agricultural ecosystems on primary production, and specifically to ensure high production in low diverse cultivated plant communities (Tilman., 1999; Balvanera et al., 2006). In the past the paradigm of functioning of marine ecosystems was a very applied field of research: again, the main focus was to keep the industry of fisheries active and productive. In both aquatic and terrestrial ecosystems, scientists were therefore trying to understand how to keep the provision of ecosystem services as food production elevated.

In recent time, this paradigm has become more complex for different reasons. Scientific advancements have demonstrated a diversified range of ecosystem services provided by aquatic ecosystems besides food provision as the regulation of the chemical composition of the atmosphere, the filtration, sequestration, storage and accumulation of nutrients and pollutants by micro-organisms, algae, plants, and animals or the maintenance of habitats diversity (Barbier et al., 2011). However, the provision

of such ecosystem services is threatened by an increasing rate of species loss due to multiple and environmentally unsustainable human pressures. Moreover, ecosystem functioning is a trait of ecosystems that is not clearly and unequivocally defined as it is measured by different authors in different ways (e.g., Loreau et al., 2001; Biles et al., 2002; Jax, 2005; Godbold et al., 2011; Kristensen et al., 2014; Norkko et al., 2015). With ecosystem functioning it is possible to refer to ecosystem processes, to biogeochemical cycling and to production processes as well, functioning is the more generally used term that incorporates the importance of abiotic properties (e.g., sedimentary pools of organic matter) as well as the goods and services of the ecosystem (Hooper, 2005; Beaumont et al., 2007). Thus, it cannot be reduced to the standing stock of primary producers or to photosynthetic rates as the majority of ecosystems on earth are in the dark (e.g., the dark ocean water column and sediments, covering more than 60% of the planet's surface). New approaches to measure functioning are therefore necessary and can be different for different ecosystems. According to recent definitions, ecosystem functioning includes a set of bio- geo- and chemical processes and transformations occurring within an ecosystem whereas carrying capacities, resistance or resilience are ecosystem attributes.

This thesis work focuses on a benthic ecosystem, which are the surface sediment of estuaries. Surface sediments of shallow areas with variable salinity are interesting spots to analyze the interactions among microbial communities, meio- and macrofauna and primary producers. Here, large inputs of organic matter (OM) fuel a heterotrophic activity whose efficiency depends on the coupling of processes and results in nutrient regeneration to the water column (Canuel & Hardison, 2016). Shallow estuarine sediments are highly heterogenic largely regulated by hydrodynamic factors, offering the possibility to analyze benthic functioning along environmental gradients (Hooper et al., 2005). The functioning of benthic ecosystems was evaluated with a biogeochemical approach, quantifying rates of whole system metabolism via gas exchange [oxygen ($O_2$), carbon dioxide, molecular dinitrogen ($N_2$)] and nutrient cycling [inorganic N, phosphorus (P) and silica (Si)]. A special attention was given to the benthic N cycle as it involves multiple redox reactions, carried out by diverse microbes and strongly influenced by the activity of macrofauna and primary producers. Understanding how N cycles in different benthic ecosystems is an alternative but appropriate approach to analyze the relationship between biodiversity and functioning.

## 1.2 Benthic N cycling, a model cycle linking microbes, macrofauna and primary producers

Most nutrient cycles involve direct or indirect interactions among different organisms. N cycle is a "model" cycle that allows to explore how micro- and macroorgan-

isms interact along changing abiotic conditions. The biogeochemical cycles of the element, in fact, is made of different redox reactions, including strictly aerobic and strictly anaerobic processes, that in sediment can be separated by micrometers and coupled to each other (Canfield & Thamdrup, 2009). N cycle, which is mostly microbial, is largely influenced by macrofauna and primary producers (Welsh, 2003; Ieno et al., 2006; Karlson, Bonsdorff & Rosenberg, 2007). Such influence can be due to excretion, uptake, $O_2$ release or non-local transport, but also to intimate associations among microbial communities, macrofauna and plants.

The processes involved in N cycling are reported in Fig. 1 (modified from Canfield, Kristensen & Thamdrup, 2005). Molecular N ($N_2$) can be fixed by diazotrophic microorganisms and transformed to bioavailable N, and hence counteract N loss by denitrification. Organic N forms can be mineralized to inorganic N in the water column and in the sediment. Both organic and inorganic N forms can diffuse from the water column to the sediment and vice versa. Nitrogen can be buried in the deep sediment as organic N and as adsorbed and mineral-bound ammonium ($NH_4^+$) in sediments. Then, organic N in the surface sediment and water column can be transformed into $NH_4^+$ through ammonification or N mineralization. Later, $NH_4^+$ may be oxidized by nitrifying microorganisms under oxic conditions, in the water column or in the oxic sediment. $NH_4^+$ and nitrite ($NO_2^-$) oxidations form $NO_3^-$ via nitrification; in the sediment $NO_3^-$ could be transported downward by diffusion to the anoxic sediment. Here, $NO_3^-$ could be reduced to $N_2$ by denitrification or to $NH_4^+$ by dissimilatory nitrate reduction to ammonium (DNRA). Denitrification, together with the reduction to $N_2$ coupled to the oxidation of $NH_4^+$ with $NO_2^-$ (anammox), contribute to the loss of N from the system to the atmosphere.

The focus on N cycling and macrofauna seems particularly promising with respect to the evaluation of complexity-functioning hypotheses for a few main reasons. First, N cycling is based on a large number of different reactions and processes, mediated by specialized heterotrophs as well as by autotrophs. Then, macrofauna, which includes a diverse group of invertebrates, interact with the different components of aquatic ecosystem, including water, sediments, phytoplankton, microphytobenthos, macrophytes and microbial communities. On the basis of feeding guilds, macrofauna can be divided into five categories: suspension feeders, surface and sub-surface deposit feeders, herbivores and carnivores (Rosenberg, 2001; Welsh, 2003; Mermillod-Blondin & Lemoine, 2010). Among them, burrowing invertebrates constitute the major bioturbators of the sediment. Bioturbation have implications on habitat formation or alteration, on other species or on microbial communities that drive biogeochemical N transformations. Furthermore, macrofauna can change sediment distribution and particle erosion through burrowing, feeding and movement, and thus it may enhance both the direct regeneration of N to the water column and, indirectly, the surface area available for microbial activity (Aller, 1988). Finally, the availability of methods to

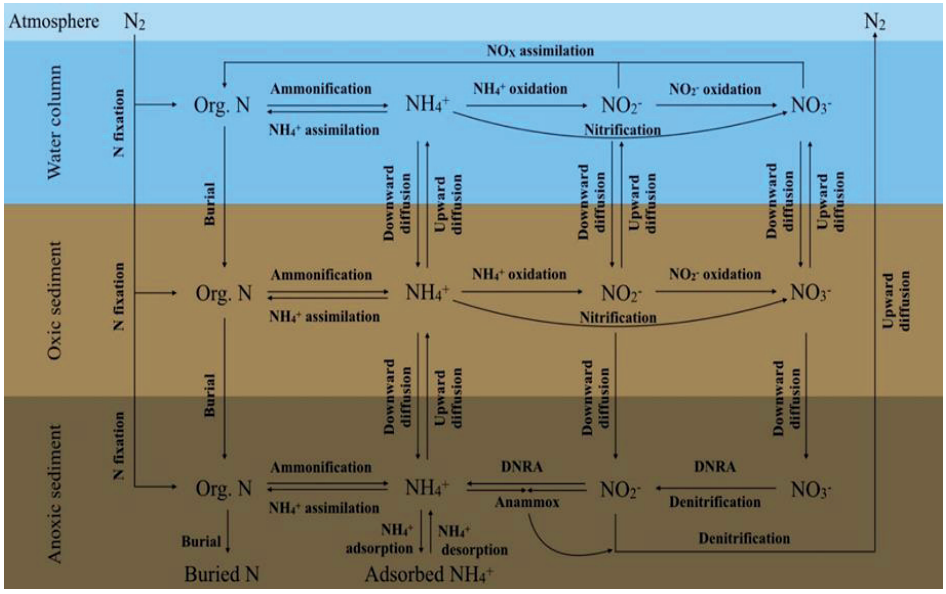



*Figure 1:* The complexity of microbial nitrogen cycling in aquatic environments, showing the major transformations within and between anoxic sediment, oxic sediment, the water column, and the atmosphere. (Modified from Canfield, Kristensen & Thamdrup, 2005)

analyze in detail N processes in laboratory experiments offer a unique possibility to test biogeochemical feedbacks and interactions. Similarly, macrofauna affect other biogeochemical cycles as that of phosphorus (P), which mobility is strongly dependent upon the sediment redox. n some estuaries, phosphate ($PO_4^{3-}$) can also be limiting or co-limiting primary producers during winter months or when N concentrations are elevated (Pinckney et al., 2001; Conley et al., 2009). Under oxic conditions P is readily immobilized by adsorption to solid iron oxyhydroxides ($Fe^{3+}$) in the sediment, but when ($Fe^{3+}$) is reduced under anoxic conditions, P is released back into solution becoming available to primary producers (Sundby et al., 1992; Gunnars & Blomqvist, 1997). Pelagic primary production is therefore strongly regulated by $O_2$ oscillations in the bottom water (Conley et al., 2009). The overall dynamics of P cycle are directly and indirectly influenced by the activity of macrofauna (Welsh, 2003). Bioturbation and burrows bioirrigation in particular may thus facilitate the immobilization of P in oxidized, $Fe^{3+}$-rich burrow walls, and simultaneously macrofauna may promote P recycling via excretion and advective transport from anoxic pore water.

## 1.3 Approaches to measure benthic biodiversity and biogeochemical functioning

In the last decades, an increasing number of ecological studies have addressed the effects of biodiversity loss on ecosystem functioning (Schulze & Mooney, 1993; Loreau et al., 2002; Thébault & Loreau, 2003). However, most studies analyzed only a few aspects of biodiversity and ecosystem functions, investigating how diversity within communities may affect the processes that play important roles in ecosystems, as for example primary production, OM decomposition or nutrient cycling (Gamfeldt & Hillebrand, 2008). For what concerns benthic environments, and in particular shallow water systems, a wide range of studies have been conducted on microbial ecology, population dynamics of primary producers, meio and macrofauna ecology and biogeochemical cycles (Gray & Elliott, 2009). The first attempts to understand the direct effects of macrofauna on benthic biogeochemical processes involved laboratory studies that allowed to control and to change a wide range of experimental conditions (Banta et al., 1999; Ferguson & Eyre, 2013). Typically, these investigations were carried out removing part of the environmental variability by sediment sieving and with the construction of micro- and mesocosms containing an increasing number of organisms of a single trophic level. These studies considered only the heterotrophic component of the benthic system (Pelegrí & Blackburn, 1995; Nizzoli, et al. 2006; Bonaglia et al., 2013; Zilius et al., 2022). Results from these works were extremely predictive in defining specie-specific regulatory mechanisms, as the measured biogeochemical processes were directly or indirectly related to the biomass of the targeted species (i.e. directly via excretion) and to the biomass-dependent bioturbation (i.e. indirectly via the increase of sediment-water interfaces in burrows). They also helped in understanding features about physiology, *in situ* density effects, respiration, excretion, etc, and their relationships with the physical environment. Although studies on a single trophic level represented the first attempts to join community and ecosystem (material and energy flow) ecology, they were insufficient to understand the role of the species in the ecosystem functioning in natural environments, since the latter are more complex and comprise multiple trophic levels (Hooper et al., 2005; Duffy, 2009).

Recently, the research in this field has shifted from the traditional experimental approaches towards more complex designs, merging traditional biogeochemical approaches with stable isotopes and molecular tools that help understanding how species interact among multiple trophic levels and how this can affect the ecosystem functioning. For instance, experiments performed using larger spatial and temporal scales) taking in consideration different trophic levels may lead to a more comprehensive picture of the direct and indirect effects of species diversity on ecosystem processes. In multi-trophic systems, these effects are difficult to predict, since the targeted process rates could increase, decrease or stay the same (Thébault & Loreau, 2003; Vaughn,

2010). A few articles analyzed the multiple interactions between organisms and the abiotic components in freshwater and brackish environments (Welsh, 2003; Chase & Knight, 2006; Mermillod-Blondin & Lemoine, 2010; Naldi et al., 2020). These studies described the activity of different functional groups of macrofauna related to primary producers' growth. They demonstrated that macrofauna enhanced the growth of macrophytes, either by removing epiphytes from macrophyte leaves or mobilizing dissolved nutrients that were readily taken up by macrophytes. These examples show the importance of using holistic and inclusive approaches to have deeper knowledge about a particular ecosystem and its function in a particular period of time. Taking into account species belonging to different trophic levels seems to be the key factor for this approach (Ieno et al., 2006).

Investigations targeting multiple organisms and multiple trophic levels require large experimental and analytical efforts and can take advantage of qualitative and quantitative modeling. In fact, combining experimental with modeling approaches, the indirect and hidden interactions among the species, not measurable with experiments alone, could be better understood and more realistic scenarios could be provided (Gamfeldt & Hillebrand, 2008). Whole system scales of analysis can be derived from upscaling processes measured at the microscale or via different techniques including ecological modeling or multivariate statistical analysis. Data on biodiversity can be matched with properties of ecosystems (solutes or energy fluxes, concentrations, standing stocks) in order to infer causal relationships within a certain probability. Such inferential approach does not open the black box of ecosystem functioning but allows for large-scale analysis.

On the contrary, accurate process rate measurements, in manipulated communities, allow experimental determination of biogeochemical pathways and their regulation. Even more, the inclusion of molecular tools in such experimental research allow us to analyze ecological relationships among micro and macro-organisms (from intimate or random associations, including symbioses). The application of models and the use of statistical tools would be useful to describe at a larger scale the ecosystem functioning and to predict further changes (Loreau et al., 2001; Thébault & Loreau, 2003; Cardinale et al., 2012). At the opposite, fine, isotope-based approaches coupled with molecular investigations allow in-depth analysis of ecological interactions at the small scale, for example at the scale of single and isolated macrofauna organisms. This is another new and promising frontier in ecological studies as it may reveal novel and understudied ecological interactions among microbes and macrofauna. In this thesis the major efforts were addressed to across-scales studies, moving from the macroscale (system lagoon studies) to the microscale (Holobionts, the assemblage of the host and its associated microbial community; Dittami et al., 2021). Benthic compartment was studied using different approaches, alone or in a synergistic combination. An approach was inferential and adopted to analyze macrofauna diversity–benthic processes rela-

tionships at the macro scale (e.g., whole estuarine scale). Another approach addressed mechanisms and was based on reconstructed sediments added with macrofauna and on the accurate analysis of different microbial processes along a macrofauna density gradient. Another approach combined the fine biogeochemical measurements with the genetic analysis of microbial diversity and of target functional genes. A last approach focused on the understudied ecological interaction *facilitation*, and targeted nutrient mass budgets in illuminated sediments with macrofauna and primary producers.

## 1.4 Estuarine sediments as natural laboratories

Estuaries and coastal lagoons are biogeochemical reactors, actively transforming dissolved and particulate matter along the terrestrial-ocean continuum. They are generally shallow ecosystems, where sediments with their diversified communities play a major metabolic role. Microbes in sediments act upon the pools of settled OM and oxidize them, regenerating mineral compounds, through an array of aerobic and anaerobic processes that are vertically stratified within an often hostile matrix (Canuel & Hardison, 2016). Macrofauna contribute both to the pelagic-benthic coupling and to the benthic-pelagic coupling and facilitate the activity of microbes and of photosynthetic organisms (Mermillod-Blondin & Lemoine, 2010; Ferguson & Eyre, 2013). Pelagic-benthic coupling occurs when part of the phytoplanktonic primary production is displaced to the sediment surface as biodeposits by filter feeders. The ecological fate of biodiepostits includes burial and incorporation in subsurface sediments, ingestion by surface or deposit feeding macrofauna, mineralization via combined action of macroinvertebrates, meiofauna and microbes and recycling to the pelagic compartment. Some of the phytoplankton associated to biodeposits remain alive and in illuminated sediments becomes an active layer of microphytobenthos, acting as a trap of nutrients diffusing from the water column and subsurface sediments (Newell et al., 2002; Newell, 2004). Macrofauna, through its various functional groups, links the pelagic and benthic compartments and ensures circularity of nutrient cycles (Kristensen et al., 2012). Moreover, macrofauna play an important role in sediment chemistry. To contrast pore water toxicity [e.g., high Ammonia ($NH_3$) and Sulfide ($H_2S$) level] and low $O_2$ levels, macrofauna rework and bioirrigate sediments. Reworking results in mixing old, refractory organic particles with recent, labile ones, and promotes more effective mineralization activity via the so-called priming effect. Bioirrigation is the active pumping of water containing oxidized molecules or ions as $O_2$ or $NO_3^-$ within burrows. Bioirrigation creates oxidized niches within anoxic and chemically reduced sediments and increases the sediment volume where more efficient aerobic microbial degradation processes can occur. Macrofauna therefore increase the complexity of the benthic system, including the sediment landscape (e.g., they increase the microhabi-

tats and the small-scale heterogeneity via burrows, deposits, and their own body) and the zonation and coupling of redox processes (e.g., not simply vertical as in microbes alone-dominated systems). The presence of macrofauna in the peculiar compartment represented by sediments, and even more in fine, impermeable sediments where diffusion regulates the exchange of solutes, cannot simply be considered as an addition of a group of organisms. Macrofauna increase the biocomplexity of the system, stimulating the interplay of behavioral, biological, chemical, and physical interactions that affect, sustain, or modify the biotic and abiotic compartment (Michener et al., 2001, Mermillod-Blondin & Lemoine, 2010; Kristensen et al., 2012). In shallow water ecosystems, the functioning of benthic compartment depends on the interactions between the physico-chemical environment and the community of micro and macroorganisms. The sedimentary environment displays steep redox gradients and multiple interfaces, where fully oxic layers may be adjacent to sulfidic sediments. Biological communities have evolved adaptations, creating a network of coupled processes and multiple feedbacks. The sedimentary environment is complex and sometimes difficult to colonize but it is simultaneously full of opportunities for organisms, as it may be more stable than the water column, and it may receive a large flux of energy. The study of benthic ecosystem functioning is also challenging for scientists, as sediment can be manipulated in the laboratory in order to validate key ecological questions generally addressed in terrestrial environments, among which those related to diversity and functioning. Relatively heterogeneous and biologically diverse subsystems within the estuaries are affected by the interplay of freshwater and saline water. Thus, estuarine sediments offer unique opportunities for these kinds of studies for multiple reasons. They represent natural laboratories where the effects of the loss of species or of invasions on the physical, chemical and biological level can be tackled. They allow the study of natural gradients, such as salinity, sediment granulometry, OM or nutrient contents. Such gradients need to be carefully addressed as they affect processes and the interactions among macrofauna and biogeochemical cycles. Estuaries and lagoons have spots where contrasting conditions can be simultaneously tested and where it is possible to evaluate how the abiotic settings shape benthic communities and how different benthic communities shape the same biogeochemical process. Estuarine sediments, regardless of the area from where they are studied, display steep vertical chemical gradients, which increase complexity and which amplify the biogeochemical role of different macrofauna functional groups. This allows to test understudied ecological interactions (i.e., facilitation), which are likely widespread and important at the ecosystem level.

## 1.5 Macrofauna: fantastic ecosystem engineers and ecological facilitators

Macrofauna produce deep impacts on benthic functioning, including direct and indirect effects. Macroinvertebrates are grouped depending on their functional differences (e.g., surface or deep burrowers) or on their feeding modes (e.g., filter feeders or deposit feeders) (Welsh, 2003; Kristensen et al., 2012). Each group, depending on functional or feeding mode, produces a variety of effects on the benthic ecosystems (Welsh, 2003; Ieno et al., 2006; Karlson, Bonsdorff & Rosenberg, 2007). All burrowers rework sediments, displace and redistribute particles along the vertical sediment horizons and mix recent and old OM with a stimulatory effect on microbial decomposition. Burrowing macrofauna include aerobic organisms that need to ventilate their burrows and bio-irrigate with $O_2$-rich bottom water in the sediment. Bioirrigation maintains the sediments oxidized and within sedimentary aerobic microbial communities otherwise limited by $O_2$ shortage. Macrofauna provide different biogeochemical services to sediments including the regeneration of buffer capacity to contrast negative effects of organic matter or anaerobic end products accumulation as $H_2S$. Besides indirect stimulation of different microbial processes, macrofauna excrete nutrients that are released and recycled and so available to microbes and primary producers. Macrofauna are in constant contact with sediments and with the microbial communities inhabiting sediments (Kristensen, 2000; Bertics & Ziebis, 2009; Stief, 2013). The mosaic of oxic and anoxic niches created by the network of burrow walls expands the sediment–water interface, where important biogeochemical processes, namely aerobic mineralization, nitrification and denitrification and iron reoxidation can occur (Pelegri & Blackburn, 1995; Mayer et al., 1995; Bartoli et al., 2001; Welsh, 2003). Simultaneously, burrow ventilation by macrofauna can increase the supply of fresh organic material, redistribute electron acceptors in the deeper sediment and subsequently change the balance between aerobic and anaerobic respiration (Banta et al., 1999; Welsh, 2003). Exchange of solutes between the pore water and the overlying water column may be increased by several times (Mermillod-Blondin et al., 2004; Kristensen, Delefosse & Quintana, 2014). Experiments showed that intense bioturbation can change the balance between aerobic and anaerobic respiration, with the dominance of the former pathway. Therefore, bioturbation stimulates nitrification and sediment $O_2$ uptake to an equivalent extent, while coupled nitrification-denitrification could be stimulated twice as much as $O_2$ consumption (Stief & De Beer, 2006). Macrofauna activity influences N cycling in burrow microenvironments and N flux across sediment-water interface due to excretion of labile N-compounds (e.g., urea) (Pelegrí & Blackburn, 1995; Mayer et al., 1995). Other examples are the mussels and clams, sedentary filter-feeders that may occur at dense, spatially heterogeneous and multi-species aggregations that form biogeochemical hotspots with strong effects at

the sediment surface. Via filter-feeding, sorted, ingested particulate resources may be excreted as dissolved OM that is more bioavailable owing to fragmentation and chemical degradation during digestion (Hopper et al., 2021) Additionally via respiration and deposition of feces and pseudofeces (Middelburg & Levin, 2009) They become critical to ecosystem productivity and biogeochemical cycling (Allen et al., 2012; Vaughn & Hoellein, 2018; Lopez et al., 2020).

In dark, not illuminated sediments, macrofauna contribute with microbial communities to the regeneration of nutrients associated with settling OM via the processes described above. In illuminated sediments macrofauna often interact with primary producers. Different studies demonstrated positive interactions among macrofauna and macrophytes as the latter provide habitat and refuge to macrofauna. On the other hand, macrofauna can feed on epiphytic organisms, maintaining cleaned fronds and allowing plant survival also under eutrophic conditions. Macrofauna can also enhance the chemical quality of sediments (with positive feedbacks to roots of aquatic plants) and the regeneration of nutrients (supporting microbial organic matter oxidation or via direct excretion) favoring plant uptake. Ecological studies traditionally focused on negative ecological interactions as predation and competition whereas recent studies highlight the occurrence of facilitation in communities. It is interesting and challenging to reveal how macrofauna can facilitate primary producers.

# 2

# Aims and working hypotheses

The general aim of this thesis was *to explore the relationship between benthic diversity and ecosystem functioning in estuarine sediments*. Specific objectives were developed in order to highlight the interaction among microbes, benthic macrofauna and primary producers:

1. To assess whether macrofauna communities provide measurable biogeochemical buffer capacity to sediments of a eutrophic boreal lagoon that specifically delays reactive P release.
2. To analyze how different macrofaunal communities, including cultivated clams, affect benthic respiration and nutrient regeneration in contrasting macroareas of a Mediterranean eutrophic lagoon.
3. To identify and quantify the facilitation of nectobentosmacrofauna (amphipods) on charophyte meadows via *grazing–excretion loop*, promoting the survival of the charophyte in eutrophic ecosystems.
4. To reveal the functional role of chironomid larvae in organic sediments and their contribution to N cycling.
5. To combine molecular and biogeochemical tools to analyze the microbiome diversity of single chironomid larvae and zebra mussel individuals and its contribution to N transformations.

With respect to the specific objectives, it was hypothesized that burrowing macrofauna may counteract short-term, negative effects of anoxia by increasing the sink role of sediment for reactive P in hypereutrophic systems (objective 1), the interplay between different dominating macrofauna functional groups and benthic features shape differentially benthic N cycling (objective 2), that grazers support the survival and growth of slow growing characeans in eutrophic settings (objective 3), that macrofauna facilitates the activity of microbial communities living in sediments or associated to their body, and that such facilitation results in quantitatively important biogeochemical transformations (objective 4 and 5).

In this thesis benthic biodiversity includes the diversity of microbial communities in sediments, measured via molecular tools or indirectly, via the quantification of specific microbial processes in particular those related to N transformations. It includes also a few macrofauna organisms and macroalgae. Ecosystem functioning is evaluated via a biogeochemical approach, including measurements of primary production, whole system, microbial and macrofauna respiration or specific microbial processes, analyzed via stable isotopes ($^{15}N$). Biodiversity and ecosystem functioning were analyzed at different scales, including the whole estuarine system, the specific areas colonized by specific macrofauna and in laboratory reconstructed microcosms or in single macrofauna holobionts with their associated microbiome. For each scale, different approaches were used, from inferential statistics to biogeochemical or molecular measurements.

## 2.1 Novelty

There are a few elements of novelty in this thesis that are shortly summarized here. The first element is the multiple scale approach. Ecologists in the field of B & EF advocate the need of large-scale studies that are necessary to reveal whether the biodiversity issue (or better the species loss issue) affects large ecosystem functioning. Such aim goes far beyond a 4-year PhD thesis; however, experimental results strongly stress the importance of macrofauna diversity in estuarine sediments functioning. Multiple evidences (e.g., higher and faster nutrient recycling, much larger N removal via denitrification or P retention in the presence of surface and deep burrowers) stress the key role macrofauna performs ensuring biogeochemical functioning and promoting ecosystem resilience and stability (e.g., counteract short-term, negative effects of anoxia).

Simultaneously, and this is another element of novelty, experiments tackled the small scale of analysis, measuring microbial functional diversity, functional genes, and associated processes at the level of single macrofauna individuals. Such approach has implied the interaction with- and the support of colleagues from Klaipeda University as well as from other institutes and disciplines (microbial ecology, molecular

biology). The molecular approach revealed the incredible diversity of microbes growing on exterior and intestine of macrofauna, actively mediating element cycling and likely taking advantage of the much larger mobility of macrofauna across sediments and water column, which means access to much larger amounts of solutes and substrates. This encouraged to reconstruct microbial N cycling pathways at the holobiont scale (i.e., quantifying processes at the level of the microbes living in association with single-macrofauna individuals), calculate the contribution of macrofauna metabolic activity and upscale measured processes on the basis of macrofauna densities. For the organisms considered in this study (e.g., chironomid larvae and the zebra mussel) results are quantitatively important due to their high densities and widespread distribution in the lagoon.

A last element is methodological as, thanks to the INBALANCE project where I was involved, a sequential measurements approach was developed. It included a combination of incubations, starting from intact and unmanipulated cores with macrofauna, where I first measured net exchange of solutes, then specific N cycling pathways traced with labeled N. In parallel to those measurements, I have implemented single individuals' incubations to disentangle the contribution of macrofauna from that of the whole benthic community, including sediments, microbes, and meiofauna. This allowed partitioning macrofauna metabolism (respiration and excretion) and specific N processes associated with holobionts. All these activities were accompanied by identification of microbial communities and quantification of their genetic potential via molecular tools.

## 2.2 Scientific and applied significance of the results

Traditionally the role of macrofauna on the biogeochemical functioning of benthic ecosystems has been analyzed with two distinct approaches: 1) the use of various statistical techniques to interpret solute flux data with macrofauna diversity and 2) manipulative experiments on reconstructed or intact sediments targeting individual species or simplified communities. Results from this thesis add new perspectives and try to merge classical and new methods to overcome their limitations. In particular, the first approach misses a detailed understanding of the mechanisms underlying the stimulatory effects of macrofauna on various microbial processes. In fact, most studies are based on very simple biogeochemical measurements (e.g., net fluxes of $O_2$ or nutrients budgets) and their conclusions are inferential but do not open the black-box of the benthic system. On the other hand, manipulative experiments tackle mechanisms and single processes that represent oversimplified natural ecosystems and are seldom upscaled to check whether their results are valid and fit whole system analyzes. In this thesis, the two approaches were simultaneously applied to explore the

functioning of the benthic compartment. The statistical inferences between macrofauna and nutrients dynamics were carried out at the macro scale, results were validated bydownscaling and testing the processes via manipulative experiments using single macrofauna organisms, and by detailed analysis of microbes-macrofauna interactions in holobionts. Moreover, biogeochemical approaches were based on combinations of different techniques involving $^{15}N$ and targeting specific microbial processes, so they went into the details of the N cycle and not only on net exchanges. Last, but not least, biogeochemical techniques were flanked by *state of the art* molecular approaches targeting microbial diversity and functional genes expression. This complex methodological approach has an important scientific and applied significance as it goes in the direction of complex and integrated experimental and statistical approaches as new frontiers in the field of B & EF. The topic itself merges traditionally distinct lines of research, on population/community ecology and on biogeochemistry, that followed for decades parallel paths. Such a new paradigm requires a new approach integrating different disciplines.

## 2.3 Scientific approval

Results of this study were presented in 5 international and 1 regional conferences:

1. 11th National Conference on Marine Science and Technology “Marine and Coastal research” (“Jūros ir krantų tyrimai”), Klaipėda, Lithuania, May 2018. (Poster presentation)
2. ASLO 2018; Association for Science of Limnology and Oceanography; Aquatic Sciences Meeting, Victoria, British Columbia, Canada, September 2018. (Poster presentation)
3. ASLO 2019; Association for Science of Limnology and Oceanography; Aquatic Sciences Meeting, San Juan, Puerto Rico, USA, February 2019. (Poster presentation)
4. AC2020; Arctic Science Conference, virtual meeting, December 2020. (Oral presentation)
5. ASLO; Association for Science of Limnology and Oceanography; Virtual meeting 2021, August 2021. (Oral presentation)
6. BSSC 2021, Baltic Sea Science Congress, Aarhus, Denmark, October 2021. (Oral presentation)

# 3

# Study areas

## 3.1 The selection of study sites

Estuaries are some of the most heavily exploited and threatened natural systems globally (Lotze et al., 2006; Worm et al., 2006; Halpern et al., 2008). Today, their deterioration due to human activities is intense and increasing. However, estuaries provide critical ecosystem services, such as valuable seafood, nursery habitats for many aquatic species and filtering and detoxification services provided by suspension feeders, submerged and emergent vegetation (Worm et al., 2006). These complex and heterogeneous transitional ecosystems represent natural gradients of salinity, sediment granulometry, OM or nutrient contents. Most estuaries are generally shallow, therefore benthic-pelagic coupling is crucial in whole ecosystem functioning. Also, estuarine sediments offer unique opportunities for these kind of studies for multiple reasons and represent natural laboratories where the effects, of the loss of species or of invasions, on the physical, chemical and biological level can be tackled (*see chapter 1.3*).

In this study, two coastal lagoons, the Curonian Lagoon (SE Baltic Sea) and the Sacca di Goro (NW Adriatic Sea) were investigated to define common reference points and benchmarks, useful in a generalized understanding on the relationship among macrofauna functional diversity, benthic functioning and nutrient cycling (Fig. 2). Besides common environmental features, such as shallowness and trophic state (hypereutrophic), many other environmental features, ranging from marine water intrusions

to nutrient and organic matter loads, select and shape the benthic biotic-community in the two systems. In this thesis, the Curonian Lagoon represented a model-system that allowed a multi-level analysis, from whole benthic ecosystem (macroscale) to the holobiont (microscale) analysis in nutrient cycling, whereas the Sacca di Goro was investigated only at the macroscale, by combining macrofauna diversity and a range of biogeochemical transformations measured along gradients of salinity and pressures (e.g., molluscs farming).

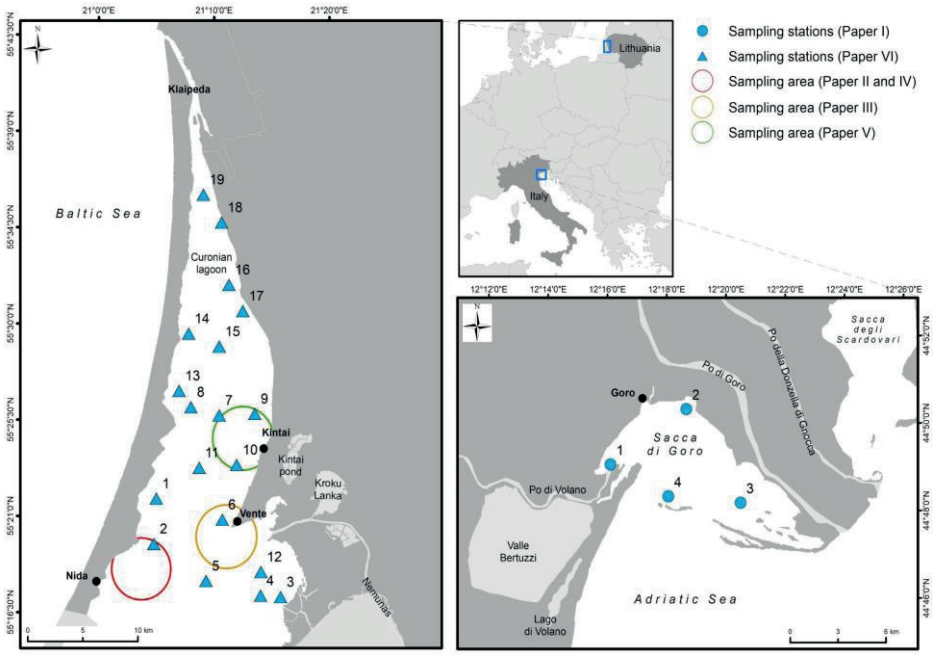



*Figure 2:* Maps of sampling stations in the Curonian Lagoon (Lithuania) and Sacca di Goro Lagoon (Italy). Blue triangles and dots refer to sampling stations used in the macro scale assessments of the lagoons (ecosystem level studies) (Paper I and Paper VI). Red circle - sampling area for Chironomids Larvae (Paper II and Paper IV). Orange circle - sampling area for *Dreissena polymorpha* (Paper III). Green circle - sampling area for *Pontogammarus robustoides* and Charophytes (Paper V). (Map created by G. Kilmonaite)

## 3.2 The Curonian Lagoon (SE Baltic Sea)

The Curonian Lagoon is a shallow (average depth – 3.8 m), oligohaline estuarine system. The lagoon is the largest coastal in Europe with area ~1600 km$^2$ (55.16–55.43°N, 21.5–21.25°E). It is located in the southeastern of the Baltic Sea, connected to the sea by the narrow Klaipėda Strait. The freshwater input is dominated by the Nemunas River, which is the largest river discharging in the lagoon (Jakimavičius & Kriaučiūnienė, 2013). In this lagoon, salty water intrusions are occasional and lead to an average increase in salinity of 1-2 (up to 7). Thus, the northern lagoon part is a rather transitional estuarine system that is flushed by freshwater and brackish water. The central-southern part is like a lacustrine system. The lagoon is characterized as a hypertrophic system with chlorophyll-a concentrations up to 400 µg Chl-a L$^{-1}$ and seasonally variable nutrient loads from the main freshwater inputs (Vybernaite-Lubiene et al., 2018, Vaičiūtė, et al. 2021). Large blooms of living cyanobacteria are associated with high $O_2$ demand in the water column and in the surface sediments, due to autotrophic and heterotrophic respiration, resulting in short-term $O_2$ depletion events in bottom waters (Zilius et al., 2014; Bartoli et al., 2021). The sedimentary environment of the lagoon is dominated from sand to silt, mud, and shell deposits (Trimonis et al., 2003), which distribution shapes benthic macrofaunal community composition and species abundance. The western-northern areas are characterized by a soft-bottom community, including oligochaetes, chironomids, and the invasive spionid *Marenzelleria viridis* (Olenin & Daunys, 2004). The central-eastern part is densely colonized by the invasive filter feeding zebra mussel *Dreissena polymorpha*, followed by oligochaetes and *Chironomidae* taxa (Zettler & Daunys, 2007).

## 3.3 The Sacca di Goro Lagoon (NW Adriatic Sea)

The Sacca di Goro Lagoon is a shallow (average depth – 1.5 m) microtidal lagoon (27 km$^2$) of the Po River Delta, North west of Adriatic Sea (44.78–44.83°N, 2.25–12.33°E). The lagoon is affected by freshwater inflow from the Po di Volano in the western area and in the central area by the Adriatic Sea (Viaroli et al., 2001). Therefore, salinity is highly variable from < 10 to 25, with the widest daily variations in the area near the sea mouth (Maicu et al., 2021). The lagoon presents a western portion influenced by the nutrient-rich freshwater inlet of the Po di Volano, a central portion with marine influence and an eastern, confined area. Nearly 40% of the lagoon surface, in particular the areas in the proximity of the opening with the sea, are cultivated with clams (*Ruditapes philippinarum*). A limited water circulation and a constant and high anthropogenic nutrient load from two branches of the Po River and from secondary channels have resulted in severe eutrophication processes and dystrophic events.

Diffuse runoff from agricultural activities within the Po River basin may lead to $NO_3^-$ concentrations up to 200 μM, sustaining frequent blooms of the seaweeds *Ulva sp., Gracilaria sp.,* and *Cladophora sp.,* especially in the easternmost shallow area, whilst phytoplankton blooms prevail in the deeper central zone. Sediment composition reflects a typical alluvial system: muds with high clay and silt contents in the northern and central zones and sand and sandy-muds bottom in the southern shoreline and eastern zone. The studies on composition and distribution of the macrobenthic community in the Sacca di Goro Lagoon resulted in the identification of 38 macrofauna taxa, representing 5 phyla. Gastropods, amphipods, and chironomid larvae dominate the macrofauna in terms of abundance, whereas bivalves represent a biomass dominant group of organisms. In this lagoon, macrofauna abundance undergoes considerable seasonal variations (Mistri et al., 2001).

# 4

# Methods

In this research, experimental activities were conducted at three levels of investigation (*Table 1*): 1) macro scale assessments (benthic ecosystem studies) of the benthic community and solute fluxes, 2) evaluation of the direct and indirect role of keystone species and, 3) a detailed assessment of microbes and invertebrates' interactions and their role in benthic ecosystem functioning.

Thus, part of this research (Paper I and VI) took into consideration the collective net effect of benthic community on the biogeochemical processes with the intention to identify relationships between biodiversity and benthic functioning using intact sediments cores collected along estuarine gradients. The second level of investigations (Papers II, IV and V) were carried out using reconstructed mesocosms, thus using simplified communities or individual key species, incubated in manipulated cores (i.e. by removing environmental heterogeneity by sediment sieving). At this level, animal respiration and excretion, macrophytes primary production and respiration were measured via individual incubations. A third group of studies (Papers II, III and IV) focused on the role of holobionts. Molecular and stable isotopes techniques were combined in incubations designed *ad-hoc* in order to measure specific processes of the N cycle, performed by microbial communities living on the exterior (e.g., shells) and/or interior (e.g., gut) of invertebrates.

*Table 1:* Summary of main experimental activities carried out in the Goro Lagoon and Curonian Lagoon in the 2019-2022 period.

| Paper | Scope | Studied effect | Main methods |
|---|---|---|---|
| **I** | - Community's role in nutrients dynamics<br>- N- Cycle | - Macroscale (Estuarine system)<br>- Inferential approach to link macrofaunal functional traits to benthic processes | - Intact cores incubation<br>- Isotope pair technique<br>- Multivariate analysis (RDA + RDA partitioning) |
| **II** | - Bioturbator: *C. plumosus*<br>- Holobiont<br>- N-Cycle | - Animal's functional role<br>- Hidden mechanism explaining specific biogeochemical dynamics at the larger scale | - Reconstructed mesocosms<br>- Individual animal incubation<br>- Molecular analysis |
| **III** | - Filter feeder: *D. polymorpha*<br>- Holobiont<br>- N-Cycle | - Animal's functional role<br>- Hidden mechanism explaining specific biogeochemical dynamics at the larger scale | - Intact sediments added with macrofauna clumps<br>-Individual animal incubations<br>- Molecular analysis |
| **IV** | - Bioturbator: *C. plumosus*<br>- Holobiont<br>- N-Cycle<br>- N export in midgets | - Interactions among macrofauna, sediments and microbial community | - Reconstructed mesocosms<br>- Individual animal incubations<br>- Molecular analysis |
| **V** | - P. producer: *C. contraria*<br>- Grazer: *P. robustoides* | - Interactions macrofauna-primary producers | - Intact cores incubation<br>- Individual animal incubation<br>- Primary production and respiration in macrophytes |
| **VI** | - The buffering role of macrofauna against anoxia | - Macroscale (Estuarine system)<br>- Inferential approach to link macrofaunal traits to processes | - Intact core incubations<br>- Multivariate analysis (DistLM + dbRDA) |

## 4.1 Cores incubations to study benthic functioning: Measures and experimental design

In a shallow estuarine environment, acquisition of representative soft-bottom samples is the fundamental prerequisite for research in aquatic ecology, geochemistry and environmental science. In order to be representative of the investigated benthic ecosystem and infer "if-then" statements or causal relationships, portions of the benthic

systems (mesocosms) were collected, including water, sediment and living micro- and macroorganisms. In this study, and specifically in Papers I, V and VI, intact sediment cores (transparent Plexiglass® liners; internal diameter of 8 cm) were randomly collected by hand corer or scuba diving at the most representative stations characterized by the dominant macrofauna assemblages. The collected intact cores underwent classical incubation to measure solute exchange at the sediment-water interface and microbial process measurements in temperature and light controlled conditions (Dalsgaard et al., 2000). Incubation time varied from study to study. During the incubations, dissolved $O_2$ was monitored in order to keep the $O_2$ concentration within 20 % of the initial concentration during the dark phase and avoid supersaturation in light conditions. In Paper I: up to 4 hours in dark conditions; Paper V: dark and light incubations of 4 and 3 hours, respectively; Paper VI: Prolonged dark incubation up to the onset of water column anoxia (dissolved $O_2$ values in the core water column below 50 µM; incubation's time ranged from 15 h to 24 h).

Measurements of solute exchange were standard, with initial and final samplings from each core's water phase. Measurements were based on changes of solutes (e.g., $NH_4^+$) and gas (e.g., $O_2$, $N_2$) concentration over time in the water phase, overlying the incubated sediment. The solute exchange (fluxes) were calculated according to Dalsgaard et al. (2000):

$$Flux = \frac{(C_f - C_i) \times V}{(A \times t)}$$

Where: solute flux at the sediment-water interface (µmol $m^{-2}$ $h^{-1}$), $C_i$ – concentration at time zero (µmol $L^{-1}$); $C_f$ – concentration at time final (µmol $L^{-1}$), $t$ – incubation time (hours), A – area of sediment surface in core ($cm^2$), $V$ – volume of water in core (L)

After flux measurement, denitrification was measured (Paper I, III) in intact cores using the isotope pairing technique (IPT; Nielsen, 1992). Briefly, stock solution of 15 µM $^{15}NO_3^-$ was added to the water phase of each studied intact core to trace rates of total denitrification (D14) and distinguish between the denitrification fueled by overlaying water $NO_3^-$ (Dw) and denitrification coupled with nitrification (Dn). To calculate enrichment, the natural $NO_3^-$ concentration was measured prior to the addition of $^{15}NO_3^-$. Then intact cores were closed and incubated under conditions as described for solutes flux measurements. After addition to the water column the $^{15}NO_3^-$ diffuses towards the denitrification zone and after a certain time the evolution rate of $^{15}N_2$ and $^{14}N_2$ can be estimated and denitrification rates can be calculated. Intact cores incubations and IPT, are accurately described in the Protocol handbook of the NICE

project (Nitrogen Cycling in Estuarie; Dalsgaard et al., 2000), in order to standardize sampling, analysis and calculations.

In most of the experiments, fluxes and denitrification measurements were carried out on a minimum of 4 cores per sampling sites or treatment. Whereas in Paper I, 8 intact cores per each site were collected randomly in 4 distinct areas of the Sacca di Goro Lagoon. Higher number of replicas were needed to cover larger variability and to better present a dominant benthic environment. In a few experiments (Paper III and V), intact cores were collected intentionally with and without epifaunal structures, such as mussel reefs, and charophyte stands. Such experimental design allowed us to assess whether the presence of biotic structure on the surface sediment may alter solute exchange and microbial N cycling pathways. Additional factors, such as illumination (light/dark) and $O_2$ conditions (oxic/anoxic), were included in studies dealing with charophyte stands or $O_2$ depletion effect on biogeochemical buffers.

After incubations, all cores were always carefully sieved (0.5 mm mesh size) in order to retrieve and analyze the macrofauna composition, abundance, and biomass and the dominant (higher biomass) macrofaunal taxa were then selected for further investigations.

To infer the relationship between measured fluxes/process rates (response variable) and macrofauna (explanatory variable), a multivariate analysis was applied (Paper I and VI) (Zuur et al., 2007; Legendre & Legendre, 2012). Multiple linear regressions were used to predict how biotic factors (species biomass) determine solute fluxes. In Paper I, denitrification rates were used as supplementary response variables (D14, Dw and Dn). A similar approach was applied in paper VI, using a distance-based linear models (distLM; Anderson et al., 2008) to explore the relationship between the biomass of dominant macrofauna taxa and sediment properties (explanatory variables) and the net solute benthic fluxes (response variables) measured in 76 intact cores of the Curonian Lagoon. In this study, two distinct models were built depending on the two $O_2$ treatments. The application of two models is advanced to highlight how benthic fluxes vary depending on biogeochemical buffers, which is hypothesized to be established via macrofauna activities (e.g., bioturbation and bioirrigation). The obtained results were visualized with distance-based redundancy analysis (db-RDA, Anderson et al., 2008) and the overlay of response and explanatory vectors in the graphical output was used to identify relationships among variables.

All statistical analyses were run using the software Brodgar 7.5.5 and Primer 6 & Permanova+ add-on (v.6, Primer-E Ltd.) packages.

## 4.2 Reconstructed mesocosms and direct measures of macrofaunal effects

The use of reconstructed microcosms instead of intact cores is sometimes necessary in order to remove the small-scale variability that characterize estuarine sediments and to isolate the factors that are tested (e.g., presence/absence of macrofauna, density dependent processes) (Heiri & Lotter, 2001). Within this thesis, reconstructed sediments were used to study the effect of chironomid larvae on microbial community and its functions, with respect to N cycle (Paper II and IV). Briefly, in the laboratory, collected sediment was sieved (0.5 mm mesh) to remove large debris, or occasional macrofauna, and mixed to slurries. Thereafter, mesocosms were filled with *in situ* water, added with different number of larvae and top submerged into tanks for preincubation. This period, lasting from 2 to 3 weeks, is required to allow the development of microbial communities and chemical gradients along the sediment column (Stocum & Plante, 2006). The main idea behind this approach was to generate microcosms with a determined number of functional traits, or with isolated organisms in order to gain information about their direct influence on benthic biogeochemical processes. In reconstructed mesocosms, dissolved solute flux and microbial $NO_3^-$ reduction were measured following the same protocol as described for the intact mesocosms in *chapter 3.1*.

In addition, single individuals, of the same species added in reconstructed mesocoms, were also incubated alone in filtered water. Such incubations targeted animal's metabolism (excretion and respiration), and thus allows to quantifying their direct contribution to benthic fluxes (Fig. 3.3). This approach was applied in Papers II, III, IV and V. Three key species of the Curonian Lagoon were incubated in microcosms: *Chironomus plumosus, Dreissena polymorpha* and *Pontogammarus robustoides*. Briefly, 22-mL glass microcosms containing filtered water were set up. The microcosms were sealed without leaving a headspace and incubated at *in situ* conditions. The concentrations of $O_2$ were monitored in all microcosms continuously with $O_2$ optodes. At the beginning and at the end of the incubation, water aliquot was collected from each vial and filtered for nutrients and $NH_4^+$ analyses. Rates of individual species were then calculated as a function of animals' dry weight (g) and then used to correct some of the above-mentioned processes, measured via intact core incubation and/or reconstructed mesocosms. This allowed us to quantify the contribution of organisms, via rate upscaling, to the whole ecosystem.

In Paper V, in order to investigate the interaction effect (grazers-charophyte) on benthic metabolism, the net primary production and respiration rates of *C. contraria* and its associated epiphytes was measured. Light-dark bottle incubations under laboratory–controlled conditions were prepared. In the laboratory, fragments of *C. contraria* were carefully washed and dissected with unfiltered lagoon water to remove attached epiphytes. Three different treatments were considered: 1) dissected *C. contraria* fragments alone, 2) epiphytes removed from *C. contraria*, and 3) unfiltered lagoon water

with a natural phytoplankton community (as a control). Six replicates (BOD glass bottles) were prepared for each treatment. Dissolved $O_2$ concentration was measured at the beginning and end of the incubations. Results from reconstructed microcosms and individual animal incubations were extremely predictive as the measured biogeochemical processes were directly or indirectly related to the natural biomass or abundance of the targeted species. This procedure was useful to disentangle the contribution of animals, sediments and primary producers to net ecosystems metabolism.

## 4.3 Biogeochemical measurements in holobionts

Advanced laboratory approaches based on the use of isotopic tracers allowed more accurate quantitative assessment of multiple microbial N transformations (i.e., ammonification, nitrification and nitrate reduction processes) and their regulation. Integrating biogeochemical approaches with novel molecular tools, such as the metabarcoding of microbial biodiversity or quantification of target functional genes, enabled the detailed exploration of macrofaunal–microbial interactions and of their wider role in benthic ecosystem functioning.

In Papers II, III and IV were investigated the role of *Chironomus plumosus* larvae and *Dreissena polymorpha* holobionts. Incubations of holobionts were carried out in small bottom-capped Plexiglas cylindrical microcosms filled with 0.2 μm filtered, aerated lagoon water and amended with different stable isotopes (e.g. $^{15}NO_3^-$, $^{15}NH_4^+$, $^{15}N\text{-}N_2$). Filtration was necessary to remove phytoplankton, spores, and free-living and particle-associated microbes and to target only microbial processes carried out by the animal's microbiota growing on the animal cuticle and in its digestive system. The revised IPT was used to assess $NO_3^-$ reduction processes associated with animals, including denitrification, DNRA, and Anammox (Nielsen, 1992; Thamdrup & Dalsgaard, 2002). Whereas, $N_2$ fixation rates in animals were measured using filtered water enriched with $^{15}N\text{-}N_2$ (N-fixation experiments in Paper III and IV) (Fig. 3.2).

In parallel to biogeochemical measurements in intact cores (Paper III) and reconstructed mesocosm (Paper II and IV), potential players involved in N cycling pathways were identified from isolated microbial DNA and RNA. In addition, functional marker genes (*nifH, nirS, nirK, nrfA* and *amoA*), encoding different N cycling pathways were quantified. This allowed to better understand whether animals affect the surrounding microbial microbiome (i.e., within the burrow walls) and whether their associated microbiome may contribute to nitrification and/or to $NO_3^-$ reduction. In Paper II, III and IV, nucleic acids were extracted from the soft tissue of *C. plumosus* and *D. polymorpha*. Quantitative PCR (qPCR) was used to quantify the abundance of functional genes and transcripts involved in N-cycling in holobionts targeting specific genes (e.g., *nifH* - microbial communities involved in nitrogen fixation; *nirS* -denitrifiers).

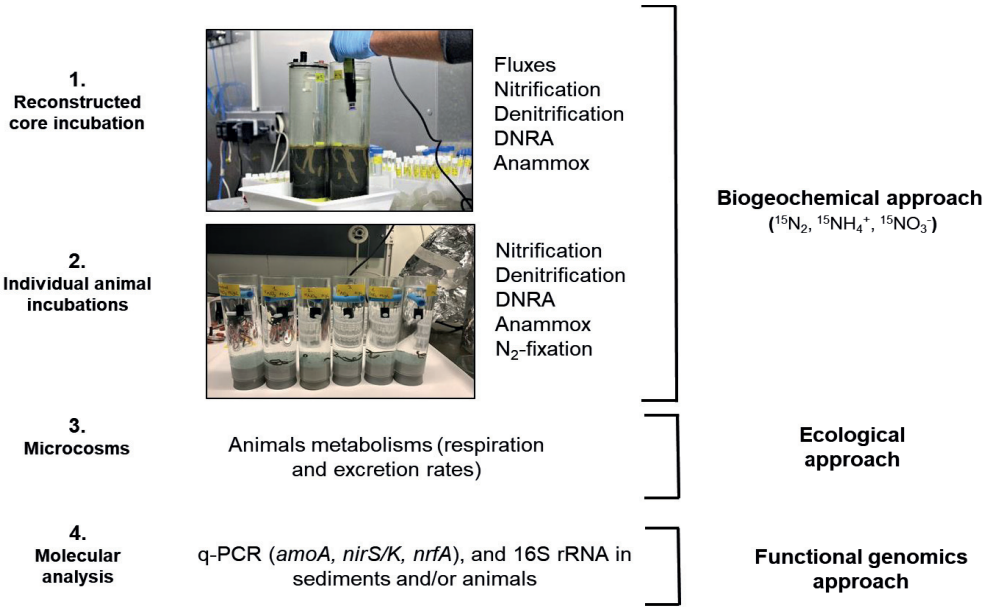



*Figure 3:* Summary of the methodological approach and main experimental activities targeting the direct and indirect effect of *Chironomus plumosus* and *Dreissena polymorpha* on N cycle (Paper II, III and IV). The reported approach can be generalized to any sedimentary environment hosting macrofauna and includes analysis carried out at different scales leading to the mechanistic interpretation of processes and the reconstruction of element cycles.

# 5

# Results and Discussion

## 5.1 Relationships between macrofauna diversity and benthic functioning

One of the tasks of this thesis is to investigate how macrofaunal communities may affect the processes that play important roles in ecosystem functioning. Among the hypotheses generated in this task we also expect that i) the benthic-pelagic exchange of particulate and dissolved nutrients is particularly important in lagoon functioning (Brady et al., 2013), ii) all micro- and macroorganisms are essential in benthic-pelagic coupling, and thus for the functioning of the ecosystem (Wrede et al., 2018), iii) all organisms interplay and produce combined effects on the benthic ecosystems (e.g., animal's nutrients excretion-plants uptake), and iv) complex and heterogeneous estuarine environments result in benthic community extremely variegate in terms of species richness and species composition. In light of these statements, Sacca di Goro Lagoon and Curonian Lagoon were investigated at the macroscale level. These two lagoons display common features: they are extremely productive and exhibit dramatic algal blooms during summer, coinciding with the lowest nutrient input from rivers. Primary producers are different, with cyanobacteria dominating in the Curonian Lagoon (Bartoli et al., 2018) and *Nitrophilous macroalgae* dominating in the Sacca di Goro (Viaroli et al., 2008). The investigation of the role of macrofauna in the two lagoons

was considered relevant in this study as macrofauna may play a key role in facilitating nutrient regeneration from sediments to the water column and increase the role of internal recycling to overcome missing inputs from outside (e.g., river input). Moreover, the accent of the two investigations was different as in the Curonian Lagoon a major biogeochemical focus was on phosphorus, which limits N-fixing cyanobacteria, whereas in the Sacca di Goro the main focus was on nitrogen, which limits macroalgal blooms. Results of Paper I and VI (macroscale assessment) are briefly presented and discussed in the following paragraphs.

### 5.1.1 Benthic community description at the two study areas

The Curonian Lagoon and the Sacca di Goro host a variety of sedimentary environments including sand deposits with limited OM content and fine, fluffy and organic sediments. However, this heterogeneity and zonations, which derives from flushing and water residence time in the Curonian lagoon, is particularly evident in sediments, sometimes more than in the water column, the salinity gradients for example are very smooth due to scarce water intrusion and to the low salinity of Batic Sea waters. In the Sacca di Goro on the contrary salinity gradients are quite sharp, with fully marine and freshwater end members mixing in the lagoon with tidal currents. Variable salinity leads to higher heterogeneity of macrofauna communities in the Sacca di Goro, with more marine and more freshwater assemblages than in the Curonian Lagoon. It is also important to stress that the Sacca di Goro has a vast surface (nearly 35%) with is cultivated with *Ruditapes philippinarum*; clams' densities are 2–3 orders of magnitude higher than natural densities and this has deep implications for the other macrofauna representatives (Bartoli et al., 2016). In the Curonian Lagoon there are no cultivated macrofauna species, however, this lagoon was invaded in the past by the opportunistic bivalve *Dreissena polymorpha*, that colonized large portion of the lagoon bottom with impressive densities of molluscs per square meter (56.000 ind. $m^{-2}$; Daunys et al., 2006; Zaiko et al., 2009; 2010).

The location of sampling stations in the two lagoons was decided in order to include all the different sedimentary environments, in turn reflecting different lagoon's hydrodynamics, water residence time, salinity, nutrient and OM gradients and as a consequence the dominating macrofauna assemblages. In the Sacca di Goro the 4 investigated macro areas revealed significantly different macrofauna communities reflecting strong environmental gradients despite the small total lagoon surface (25 $km^2$). In the Curonian Lagoon the 19 analyzed stations revealed much lower variability of macrofauna community, despite marked gradients of sediment granulometry and OM content (29 - 310 μm medium particle size and 0.4 - 22.9 %, respectively) and much larger explored surface (500 $km^2$).

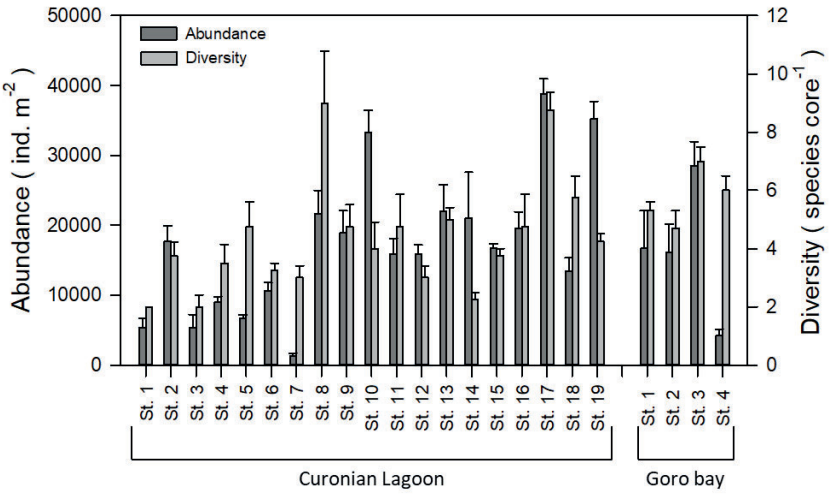



*Figure 4:* Total abundance of benthic macrofauna (ind. $m^{-2}$) and taxonomic diversity (no. of species per core) in incubated sediment cores from 19 stations in the Curonian Lagoon (n=4 replicates per station) and from 4 stations in Sacca di Goro (n=8 replicates per station) (error bars denote standard errors; some are not visible). (Redrawn from Paper I and VI)

Up to 17 different macrofaunal taxa species defined a clear community diversification among the 4 stations of Goro lagoon (average abundance of 82 ± 12 ind. $core^{-1}$, equivalent to ca. 23.700 ind. $m^{-2}$). Whereas the Curonian Lagoon, characterized by higher macrofauna diversity (23 species and 7 higher order taxa and an average of 87 ± 7 ind. $core^{-1}$, equivalent to ca. 25.100 ind. $m^{-2}$), showed homogeneous distribution of species among the 19 stations with no clear spatial pattern in their distribution (Fig. 4). In the Curonian Lagoon, sediment characteristics, in terms of grain size (µm), OM content, porosity and density, differed substantially among sampling stations; however, the invertebrate community structure (abundance and taxonomic diversity) did not diverge greatly among sites. A low number of recurring taxa was retrieved in the 76 cores and high variability among cores within the same site led to very poor grouping of cores (and stations) along the spatial gradient of the lagoon. In terms of biomass, oligochaetes and chironomids were dominant in two thirds of the cores characterized by fine sediments. Whereas in sandy sediments *D. polymorpha* was dominant in terms of biomass. Among the other species, in the Curonian Lagoon, stood out Gammarids, which reached high densities (up to 1000 ind. $m^{-2}$). However, they were

found to be biomass dominant only in a few stations characterized by sediments with low macrofauna diversity.

The four studied areas of the Sacca di Goro lagoon revealed to be distinct environments, here the macrofauna composition reflected the sediment zones identified in the literature, in turn dependent upon average salinity (Mistri et al., 2001; Viaroli et al., 2006). Spionids, oligochaetes, and *Monocorophium insidiosum* (that together accounted for 92% of the total macrofauna abundance) characterized the situation with a high sedimentation rate (clayish material from terrestrial origin) in proximity of the Po di Volano River. This station was, among the four sampled sites, relatively homogenous in the context of taxonomic composition. The station with the lowest taxonomic diversity was characterized by black sulfidic sediments rich in OM from decaying macroalgae. Here, *M. insidiosum*, *Chironomus salinarius* and Gammarids were the most abundant taxa. Sediments within clam's farming areas were rich in biodeposits and were characterized by large densities of macrofauna. *M. insidiosum* attained the highest abundance, however, the characteristic species for this station was *Ruditapes philippinarum*.

### 5.1.2 Benthic macrofauna as biological buffer during oxic-anoxic transitions

Whole benthic ecosystem analysis in the Curonian Lagoon consisted in statistical inference on the macrofauna community and sediment metabolism under specific redox conditions. In particular, the idea was to verify whether macrofauna can represent a biological-biogeochemical buffer preventing or contrasting the negative effects induced by oxic-anoxic transitions in sediments facing frequent short-term events of bottom water anoxia. An example, shown in Petkuviene et al. (2016), is related to the cyanobacteria bloom that may generate high reducing conditions through labile organic input to sediments, favoring sedimentary reactive P release. The latter may sustain a second phytoplankton bloom, and support the theory that cyanobacteria promote chemical conditions that keep unbalanced N:P ratios. It can be hypothesized that surface and deep burrowers (e.g. chironomids, that in some periods and stations are very abundant in the Curonian Lagoon) via intense bioirrigation activity may increase the oxidized iron and manganese pools and buffer P mobility (e.g. they can delay the short-term P release).

The overarching aim of this study (Paper VI) was to assess macrofauna contributions under those circumstances and how, more in general, macrofauna affect biogeochemical processes (e.g. nutrient or metals fluxes at the water-sediment interface). Thus, net fluxes were measured in intact cores hosting natural macrofauna communities. Benthic net fluxes of dissolved metals (DFe and DMn) and nutrients (DIN, DIP, DSi) displayed a large variability among stations and between the redox treatments (i.e. oxic and anoxic conditions, *chapter 3.1*). At the lagoon scale, measured net fluxes were mostly positive

and directed from the sediment to the near-bottom water. All investigated stations were net $NH_4^+$ and DSi sources to the water column, with higher rates measured in the oxic treatment. An opposite trend was registered for DIP fluxes. In the anoxic incubations, DIP effluxes increased significantly as compared to oxic incubations in 15 out of 19 stations. Across the redox treatments, higher DMn and DIP fluxes were also measured in the anoxic condition (both fluxes increased by 60–70% in the redox transition).

Multivariate analysis (DistLM) was applied to assess the effect of benthic invertebrates on microbial processes and measured solute fluxes. Macrofauna biomass and sedimentary features of each sampling station were used as explanatory variables whereas solute fluxes were used as response variables. Besides the macrofauna community biomass of 6 dominant macrofaunal groups, sediment properties such as density + medium sediment size and OM content were added to the multivariate analysis. Two distinct models were applied for the two incubation types - oxic and anoxic, an additional model was built using the same explanatory variables and the calculated difference between the anoxic and oxic benthic fluxes (Delta fluxes). The latter allowed us to evaluate the potential "buffering capacity" provided by the activity of macrofauna.

The best selected distLM models for the oxic and anoxic conditions had common results (Fig. 5). Flux dynamics were significantly influenced by the individual effects of sediment properties. OM content, granulometry and grain size accounted for major fractions (> 84%) of the total explained variation, whereas macrofauna cumulatively accounted for 10% and 15% in the anoxic and oxic model, respectively. The anoxic model (35%) and the model built on delta fluxes (41%) had lower power than the oxic model (49%) in predicting the nutrient fluxes. Against our hypothesis the role of burrowers (chironomids and oligochaetes) was of secondary importance or not statistically significant in the prediction of measured fluxes whereas other species (functional groups) such as the grazers Gammaridae and Gastropoda were recurrent and statistically significant. In the Curonian Lagoon, among the wide range of environmental factors that can influence benthic processes, OM content in sediments was the most important driver of variations in solute fluxes. Different studies stressed the main role of abiotic sedimentary features as regulators of benthic metabolism, nutrient and metal fluxes. Such regulation occurs through sediment grain size and porosity, OM pool and its macromolecular quality (Pusceddu et al., 2009; Belley, 2016 a, b; Bartoli et al., 2021). Organic carbon content in sediments has been proposed in these studies to be the principal driver of benthic remineralisation. Similarly, both distLM models applied to the Curonian Lagoon data highlight how complex hydrodynamics such as the water stagnation/renewal regulates the distribution of sediments and sets the risk of anoxia in the system.

In the DistLM, a minor fraction of the total variability, more under oxic conditions, but also under anoxia, was explained by the macrofauna communities. In the condition of normoxia macrofauna was to a certain extent statistically related with measured rates, whereas in the condition of $O_2$ shortage, sediment properties more than macrofauna activ-

ity can be related with the majority of measured fluxes. However, these results suggest that macrofauna may still play a role in the biogeochemical dynamics and in buffering negative effects of anoxia. This role may vary depending on the functional group and its tolerance to hypoxia. For example, burrowers such as chironomids are extremely tolerant to reductive and anoxic sediments, they frequently ventilate their burrows to keep the sediment oxidized and promote the presence of oxidized manganese pools that avoid iron reduction and DIP release (Carpintero-Moraes et al., 2018; Gautreau et al., 2020). These issues are important to address as bioturbation by burrowing deposit feeders supports biogeochemical services as particles reworking, mixing of new and old OM pools, facilitation of the heterotrophic microbial community, phosphate retention within the sediments, immobilization of sulfides into insoluble iron mono-sulfides or coupled ammonification-nitrification and nitrification-denitrification (de Wit et al., 2001; Stief et al., 2010; Sturdivant et al., 2012). The effects of other functional groups of macrofauna living in surface sediments or in the water column are much less intuitive. Generally, under $O_2$ shortage, macrofauna display different responses in terms of distribution, feeding mode and metabolism (Gibson & Atkinson, 2003; Vaquer-Sunyer & Suarte, 2008; Rakocinski & Menke, 2016). All these responses have biogeochemical implications. Lower respiration, excretion and bioturbation under severe hypoxia for example are expected to decrease rates of $O_2$ consumption and nutrient recycling (Pearson & Rosenberg, 1978; Riedel et al., 2008; Braeckman et al., 2010). In turn, decreased metabolic activity results in limited excretion and secretion rates of nutrients-rich wastes (i.e. mucus, $NH_4^+$, urea, fecal pellets).

The statistical analysis of the effects of macrofauna on measured metals and reactive phosphorus exchanges during oxic-anoxic transition suggested a minor importance of macrofauna as a driver of benthic fluxes. This outcome contrasts the working hypotheses of this experiment and states that abiotic factors (e.g. OM content, which is correlated with mean grain size) are stronger predictors of fluxes than macrofauna. In a previous work, Zilius et al. (2012) analyzed comparatively diffusive $O_2$ fluxes calculated from sedimentary microprofiles and total fluxes (diffusive + advective) from whole core incubations and concluded that macrofauna in the Curonian Lagoon has a seasonally variable contribution to sediment metabolism, which is minimum during summer. This is due to the build up of chemically reducing conditions in sediments and to the elevated respiratory quotient, >>1, suggesting the accumulation of anaerobic metabolism end-products in sediments, and high microbial respiration rates, sustained in turn by large OM inputs (Hopkinson, 1985). It is also true that laboratory experiments on reconstructed cores revealed large effects of chironomids on iron ($Fe^{2+}$), manganese ($Mn^{2+}$) and orthophosphate ($PO_4^{3-}$) concentrations in pore water (Benelli et al., 2018). In particular, in Benelli et al., 2018, vertical micro-profiles of $Fe^{2+}$, $Mn^{2+}$ and $PO_4^{3-}$ revealed marked reduction of their concentrations in treatments with low and high chironomid densities as compared to control sediments without macrofauna. Chironomids have therefore the potential to oxidize sediments via bioir-

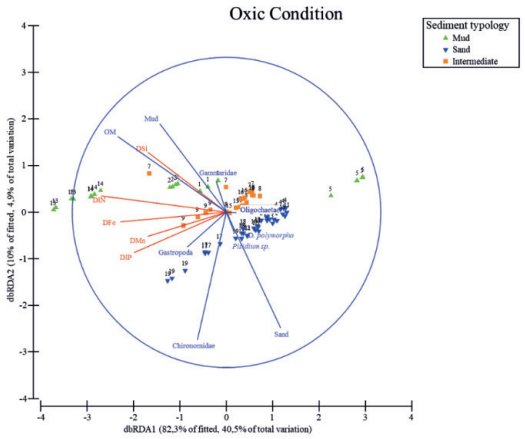
**Oxic condition (R2 = 0.49)**

**Marginal test**

| Exp. variables | Pseudo-F | P | % var. |
|---|---|---|---|
| OM | 20,0 | **0,0001** | 21,3 |
| Mud | 7,5 | **0,0013** | 9,2 |
| Sand | 6,2 | **0,0026** | 7,8 |
| Oligochaeta | 0,7 | 0,5228 | 0,9 |
| *Pisidium sp.* | 0,8 | 0,4755 | 1,0 |
| *D. polymorpha* | 0,6 | 0,5735 | 0,8 |
| Gammaridae | 1,7 | 0,1510 | 2,3 |
| Chironomidae | 4,0 | **0,0164** | 5,2 |
| Gastropoda | 4,6 | **0,0102** | 5,8 |

Sequential test

| Exp. variables | Pseudo-F | P | % var. | % var. (cum.) |
|---|---|---|---|---|
| OM | 20,01 | **0,0001** | 21,3 | 21,3 |
| Mud | 16,54 | **0,0001** | 14,5 | 35,8 |
| Chironomidae | 8,37 | **0,0002** | 6,7 | 42,5 |
| Gammaridae | 4,20 | **0,0294** | 3,2 | 45,7 |
| Gastropoda | 2,64 | **0,0417** | 2,0 | 47,7 |
| Sand | 2,08 | 0,0859 | 1,5 | 49,2 |




**Anoxic condition (R2 = 0.35)**

**Marginal test**

| Exp. variables | Pseudo-F | P | % var. |
|---|---|---|---|
| OM | 27,1 | **0,0001** | 26,8 |
| Mud | 19,4 | **0,0001** | 20,8 |
| Sand | 8,5 | **0,0001** | 10,3 |
| Oligochaeta | 2,3 | 0,0624 | 3,1 |
| *Pisidium sp.* | 0,8 | 0,5080 | 1,1 |
| *D. polymorpha* | 0,9 | 0,3954 | 1,2 |
| Gammaridae | 1,5 | 0,2164 | 1,9 |
| Chironomidae | 4,2 | **0,0068** | 5,4 |
| Gastropoda | 2,5 | **0,0470** | 3,2 |

Sequential test

| Exp. variables | Pseudo-F | P | % var. | % var. (cum.) |
|---|---|---|---|---|
| OM | 27,1 | **0,0001** | 26,8 | 26,8 |
| Mud | 3,7 | **0,0108** | 3,5 | 30,3 |
| Gastropoda | 3,0 | 0,0573 | 2,7 | 33,1 |
| Gammaridae | 2,2 | 0,0668 | 2,0 | 35,1 |

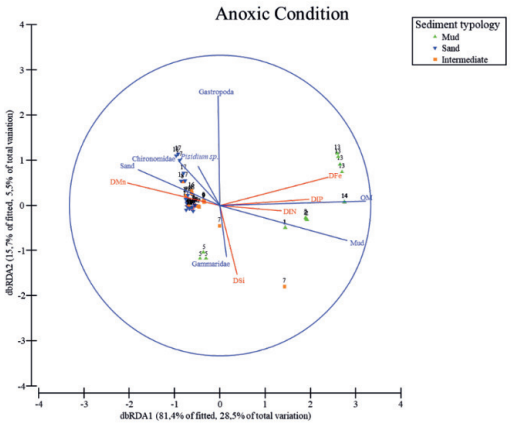



*Figure 5:* Distant-based linear model output and Distance-based triplot of redundancy analysis (db-RDA) for two models: oxic and anoxic conditions. Biological and environmental drivers were tested against solute fluxes of nutrients and metals at the sediment–water interface (n=76). In tables: Var – explained variance (%) in fluxes by explanatory variables. Significance level is < 0.05. In the DistLM triplots: vectors of solute fluxes (DIN, DIP, DSi, DFe, and DMn) are marked in red. whereas, vectors of macrofauna biomass ($g_{dw}$) (Chironomidae, Oligochaeta, Gastropoda, Gammaridae, *D. polymorpha* and Pisidium sp.) and vectors for the three sediment variables (OM – organic matter, Sand, and Mud) are marked in blue. Numbers indicate single cores collected at sampling stations. The projection of any sample onto arrows approximates the measured value in that sample. (from Paper VI)

rigation, but this is true in reconstructed cores and less evident in natural sediments (Paper VI shows that the role of bioturbators may be masked by other biotic and abiotic variables). A reason for such discrepancy might be due to the loss of the more reactive and labile pool of organic matter during sediment sieving, which slows the microbial activity in reconstructed cores and enhances the apparent effect of bioturbation (Bartoli et al., 2020). Under *in situ* conditions the large microbial heterotrophic activity masks the effects of macrofauna bioirrigation and prevents the increase of oxidized metal pools along burrow walls. As such, induced (or *in situ*) anoxia results in quick exhaustion of $Mn^{4+}$ and $Fe^{3+}$ and increases reactive P regeneration.

### 5.1.3 Macrofauna drives the equilibrium between regenerative and dissipative N paths

Whole benthic ecosystem analysis in the Sacca di Goro considered the large abundance of cultivated organisms (bivalves) that are natural reactors for particulate pelagic OM and facilitate their recycling as mineral nutrients (Bartoli et al., 2001). It was hypothesized that high densities of low mobile, surface burrowing bivalves may enhance, indirectly via biodeposits and directly via excretion, the $NH_4^+$ production and sediment-water solutes fluxes. It was also hypothesized that high densities of surface and deep burrowers may enhance the coupling between nitrification and denitrification, resulting in net N losses from the system. Last, but not least, it was hypothesized that the coexistence of filter feeders and burrowers, together may further stimulate N losses, due to large $NH_4^+$ availability fueling nitrification under oxic conditions and large $NO_3^-$ reduction in sediments with labile biodeposits. To test these hypotheses, net nutrient fluxes were measured via intact core incubations; additionally, the IPT was used in order to specifically analyze dissimilative $NO_3^-$ reduction pathways. Combining all fluxes and process rates it was possible to reconstruct a tentative N cycle, and infer how different macrofauna functional groups locally shape the cycle (Fig. 6). Results show that N cycling pathways considerably differed among four studied sites, indicating specific activity of dominant macrofaunal organisms.

At Station 1, located in the area influenced by the Po di Volano freshwater inflow, sediments were highly bioturbated and oxidized in the upper 3–5 cm horizon and displayed large and coupled rates of ammonification and nitrification. This is supported by measures of large $NO_3^-$ net effluxes and $NH_4^+$ uptake from sediments. A major percentage of the $NO_3^-$ produced via sediment nitrification accumulated in near-bottom water and only a small amount diffused to anoxic sediments where it was denitrified. At this station the macrofauna community is mainly composed by surface burrowers and sediment reworkers such as the abundant *M. insidiosum* and polychaetes. These organisms are able to create a dense network of burrows which extend the surface for solute exchange and the volume of oxic niches, stimulating aerobic microbial pro-

cesses such as nitrification. Spionids were also abundant at this station. They typically burrow deeper than *M. insidiosum*, enhancing $NH_4^+$ mobilization from deeper sediment layers to the surface horizons (Mermillod-Blondin et al., 2004; Quintana et al., 2011). Mobilized $NH_4^+$ was not released to the water column, rather it was oxidized to $NO_3^-$ in the upper layers, in the proximity of *M. insidiosum* burrows. As a result, nitrification largely prevailed over denitrification in this type of habitat.

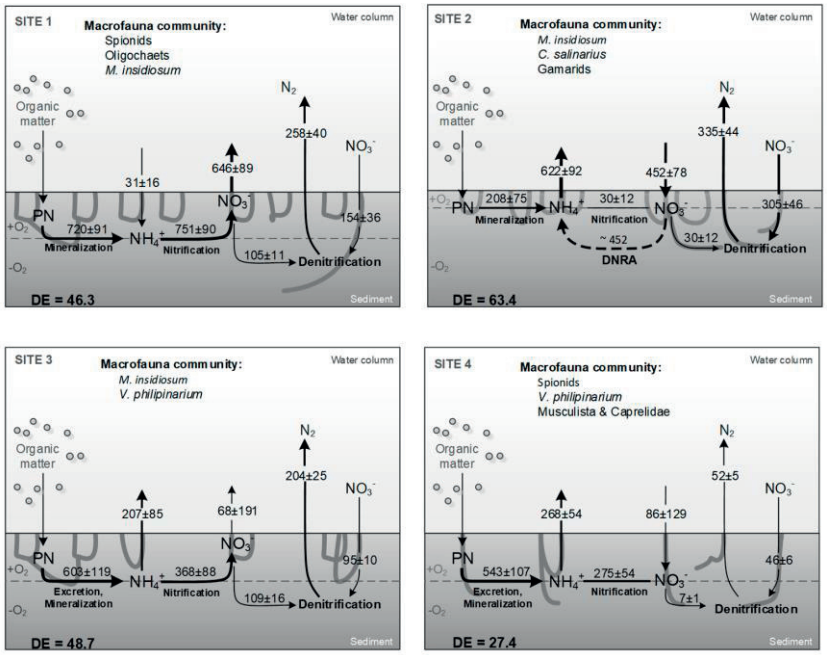



*Figure 6:* Flow scheme for benthic N pathways at four sites in the Sacca di Goro. N pathways, shaped by four different macrofaunal communities, were calculated from combinations of measured fluxes and process rates in intact cores. The mean rates (average ± st. error) are expressed on an hourly basis per unit of sediment surface (μmol $m^{-2}$ $h^{-1}$). Denitrification efficiency (DE) is the ratio between dinitrogen ($N_2$) flux and the sum of $N_2$ and dissolved inorganic N ($NH_4^+$+$NO_x^-$) effluxes. (From Paper I)

At station 2, the sedimentary environment was chemically reduced and characterized by sulfide accumulation in pore water, high $Mn^{2+}$ effluxes and very limited $O_2$ penetration depth. Under these circumstances the unfavorable chemical conditions limited the effect of macrofauna in terms of N oxidation and stimulated dissimilative nitrate removal, increasing denitrification efficiency (DE = 63%). At this station, a relatively large amount of regenerated $NH_4^+$ accumulated in bottom water due to negligible nitrification. The thin oxic sediment layer constrained the nitrification

process in the very upper sediment layer, and thus rates were uncoupled to those of ammonification. The limited $O_2$ penetration resulted in a short path for nitrate to get to the anoxic layer, and as a result denitrification was mainly fueled by water column $NO_3^-$. Under sulphidic conditions macrofauna was constrained by the toxicity of sediments and pore waters to the upper sediment layers, and consisted mainly in shallow burrowers such as *C. salinarius*, *M. insidiosum*, and gammarids that via ventilation of burrows may enhance exchange of microbial metabolism end-products ($NH_4^+$ sedimentary effluxes). Sulfidic sediments also stimulate nitrate ammonification more than denitrification. Under these circumstances, *M. insidiosum*, an organism abundant at both stations 1 and 2, via its burrowing activity is likely to produce contrasting effects depending on site-specific features, enhancing nitrification at site 1 and fueling N recycling via the dissimilative $NO_3^-$ reduction to $NH_4^+$ (DNRA) at station 2.

Site 3 is characterized by sandy bottom densely populated by clams (*R. philippinarum*). Clams deeply influence N-cycling: (i) directly by sediment bioturbation and (ii) indirectly by filtration and biodeposition of OM from the water column to bottom sediments. *R. philippinarum* can be considered as shallow-burrower rather than deep-burrower but its bioturbation activity is strongly correlated to the denitrification process. At this site, a large amount of sedimentary $NH_4^+$ is oxidized to $NO_3^-$ within the sediment and subsequently almost the entire pool is denitrified to $N_2$. Denitrification is mainly due to the coupled nitrification–denitrification process. The direct stimulation of N processes, in particular nitrification, is likely facilitated by the sandy environment and by additional habitat provided by burrow walls of *M. insidiosum*. Excretions produced by clams and microbial end-products might in fact be transported within burrows during its ventilation, thus a large part of $NH_4^+$ is nitrified while the remaining fraction is released to bottom water. The high abundance of *M. insidiosum* at station 3 supports nitrification and can be explained by the presence of clams, as these filter-feeders may provide high quality food for the amphipods. Station 4, where sediment is poor in OM and macrofauna, is characterized by low macrofauna biomass but by a high number of functional traits: filter-feeders, deep burrowers, and scrapers. Disentangling the individual role of different species was more difficult; however, in the organic-limited sandy environment, rates of ammonification were only partially coupled to nitrification, resulting in $NH_4^+$ efflux. The $NH_4^+$ fraction oxidized to $NO_3^-$ within the sediment was thereafter denitrified to $N_2$.

All these results were also supported by the multivariate analysis (RDA) applied to macrofauna biomass (explanatory variable) and fluxes and metabolic pathways (response variables). The multivariate analysis showed that the total amount of variation explained in the response variables (solute fluxes and N-related processes), by the biomass of species alone, equaled 19%. The deep burrower *C. salinarium* contributed most to the variation, followed by filter feeder *R. philippinarum* and Gammarids. The results from the RDA stressed the strong positive relationship among burrowing organism abundance and $NH_4^+$ and $NO_3^-$ fluxes at the muddy stations. The abundant *M.*

*insidiosum* and polychaetes populations are able to create a dense network of burrows which extend the surface for solute exchange and the volume of oxic niches, stimulating microbial activity. Furthermore, continuous ventilation of burrows by *M. insidiosum* is likely to support nitrification ($D_n$). RDA analysis highlighted also strong collinearities between macrofauna functional traits and fluxes of DIP, DMn and $NH_4^+$ and with N loss via denitrification (Dw) and supports the fact that animals can produce contrasting effects on sedimentary processes depending on site-specific features. An example is given by *M. insidiosum*, its presence was likely to determine an enhancement of denitrification rate in one station or drive the dissimilative DIN reduction to $NH_4^+$ (DNRA) rather than OM mineralization, in a second station with sulfidic sediments.

## 5.2 Narrowing the scale: individual functional traits as drivers of benthic functioning?

Multivariate analysis, performed on single intact cores, each with a specific community and metabolic rates, provided statistical evidence on how macrofaunal communities may affect benthic metabolism. However, this method does not allow a deep understanding of the mechanisms underlying the stimulatory effects of individual species on various microbial processes. The use of functional diversity or individual species traits, instead of the community, may increase our understanding of the relationship and the variability that characterize the link between benthic biodiversity and ecosystem functioning (Marmillod-Blodin et al., 2015). In this paragraph, three different studies (Papers II, IV, and V) extended the methodological approach to include manipulative experimental activities. Within the macrofaunal community of the Curonian Lagoon, two key functional species were selected and their ecological roles were analyzed in detail in reconstructed microcosms or in isolated, single macrofauna individuals in order to test specific hypotheses.

In the Curonian Lagoon, vegetated hotspots along the shoreline of the lagoon may host diversified benthic and planktonic invertebrate communities (Arbačiauskas & Gumuliauskaitė, 2007). The second investigated key species is the invasive amphipod *Pontogammarus robustoides*. Amphipods are widely used as test organisms for assessment of estuarine quality and represent an important link at the benthic-pelagic compartment (Postma et al., 2002; Chapman et al., 2013). Due to their habitat and feeding mode, close or above the sediment-water interface, strictly associated to macrophyte fronds, they contribute with direct and indirect effect to the benthic biogeochemical dynamics. In vegetated shallow areas of the Curonian Lagoon they reach high densities, up to 6000 ind. $m^{-2}$, and via grazing activity on the submerged vegetation, excretion and biodeposition, they represent an important link among the primary producers, the sediments and the water column.

Western part of the Curonian Lagoon, along spit coast, the benthic systems is characterized by organic-rich and chemically reduced sediments densely populated by chironomid larvae (*Chironomus plumosus* and *Chironomus balatonicus*) (Olenin & Daunys, 2004; Kornijow et al., 2019). These organisms may attain large densities (up to 11.000 ind. $m^{-2}$) and dominate the benthic community of many estuarine systems and eutrophic environments (McLachlan, 1977; Hölker et al., 2015). The multifaceted role of these organisms represents an interesting aspect for this thesis. These tube-dwelling organisms are considered ecosystems' engineers. Via active bioturbation of sediments, they create steep redox gradients across their burrows. They pump large volumes of $O_2$ and $NO_3^-$ rich water into sediments, supplying electron acceptors in deep sediment layers and therefore they actively stimulate various biogeochemical processes (Benelli et al., 2018; Hölker et al., 2015). An interesting aspect, which distinguishes chironomids from other bioturbating species, is their metamorphosis from burrowing larvae to flying adults. This process turns into a net organic N export that may be considered as an alternative to the N removal process via sedimentary denitrification or anaerobic ammonium oxidation (anammox).

### 5.2.1 Grazing–excretion loop contributes to benthic macroalgae primary production

In the Curonian Lagoon, *C. contraria* coexists with high densities of the amphipod *P. robustoides* in eutrophic settings. Characeans are generally sensitive to high nutrient availability and tend to disappear due to fast growth of epiphytes on their fronds, limiting their photosynthetic performances (Mulderij et al., 2003). *P. robustoides* is metabolically active and this allowed to hypothesize that intense grazing on epiphytic algae growing on *C. contraria* and nutrient excretion of this animal might support the growth and persistence of *C. contraria*, and thus potentially delay its senescence (van Donk & van de Bund, 2002). Benthic primary production, respiration, and inorganic N fluxes in a *C. contraria* stand were measured via light and dark intact core incubations, rates were contrasted with those measured in adjacent unvegetated sediments. Single individuals of *P. robustoides* and epiphytic algae were also incubated to calculate their contribution to benthic $O_2$ and nutrient dynamics.

Obtained results revealed that characeans and associated epiphytes photosynthetic activities result in wide daily variations of $O_2$ as compared to bare sediments, spanning from night hypoxia to day supersaturation (up to 140 %). They also revealed the large contribution of *P. robustoides* to N recycling, via grazing on particulate forms and excretion of reactive $NH_4^+$. Intact cores incubation revealed a relatively high $NH_4^+$ demand within charophyte stand (100–300 μmol $m^{-2}$ $h^{-1}$) indicating a large uptake by primary producers. This rate was comparable to that measured for other charophytes species at similar temperatures (Kufel & Kufel, 2002; Rodrigo et al., 2007). Results suggest the presence of other N sources, besides $NH_4^+$ sedimentary regeneration, in the benthic sys-

tem needed to meet such high algal demand when N is limiting in the water column of the Curonian Lagoon in summer periods (Zilius et al., 2018). Additionally, carried out individual amphipod incubations showed that a relevant amount of $NH_4^+$, excreted by *P. robustoides*, can be potentially provided to primary producers. The relatively high excretion activity by the abundant macrofauna (Fig. 7) within *C. contraria* supports nearly 40% of the macroalgae theoretical N uptake and cannot be ignored as a N source (e.g., Gammal et al., 2016). Additionally, the ratio between the $O_2$ respiration and $NH_4^+$ excretion by amphipods, close to 10, suggests that they feed on epiphytic and periphytic algae, that represent food with high nutritional quality (Dvorak & Best, 1982; Voigt, 2016).

| **Respiration rate** | | **Excretion rate** |
|---|---|---|
| -1.1 ± 0.2 mmol $O_2$ $m^{-2}$ $h^{-1}$ |  | +140.9 ± 21 µmol $NH_4^+$ $m^{-2}$ $h^{-1}$ |
| -78 ± 10 µmol $O_2$ $g_{dw}^{-1}$ $h^{-1}$ | | +12.5 ± 3 nmol $NH_4^+$ $g_{dw}^{-1}$ $h^{-1}$ |

*Figure 7:* Metabolic activity in *Pontogammarus robustoides*. Respiration and excretions rates were calculated from individual incubations in microcosms. The mean rates (average ± st. error) are expressed on an hourly basis per square meter (µmol $m.^{-2}$ $h^{-1}$) or grams of dry weight (nmol $g_{DW}^{-1}$ $h^{-1}$). Calculated rates were then upscaled for the average number of individuals retrieved in the vegetated intact cores. (Redraw, rates are recalculated on the basis of Paper V)

Therefore, dense coverage by periphyton and epiphytic algae growing on *C. contraria* at the study site could be an important food source for these grazers (Kotta et al., 2004; Bakker et al., 2016). Since charophyte stands suffer from heavy colonization by epiphytes in the Curonian Lagoon (Katarzyte et al., 2017), amphipods grazing might play an important role in the survival and growth of *C. contraria*. The incubations of individual amphipods, of charophytes' thalli and their associated epiphytes, allowed the identification of four main factors ruling the elevated benthic metabolism that characterize the Charophytes' stands. The first factor is a direct contribution by the charophytes and epiphyte respiration (46% of total $O_2$ demand). The second factor is the respiration of *P. robustoides* (20% of the total) (Fig. 7). The third factor is the increased inputs of labile OM via bio-deposits at the sediment surface, including feces and fractions of shredded vegetation. The last factor is indirectly related to dense charophyte stands that act as temporary traps for settling phytoplankton (Schulz et al., 2003 a, b). The third and fourth factors together justify the elevated benthic metabolism in the vegetated stand, as even small inputs of OM can stimulate microbial activity, in turn the increased benthic metabolism translates into higher nutrient turnover in the charophyte stand as compared to bare sediments (Kelly & Nixon, 1984). Additionally, $O_2$ production was measured in fragments of *C. contraria* and in the associated community of

epiphytes, whereas $O_2$ respiration and $NH_4^+$ excretion rates were measured in incubation of *P. robustoides* alone. The sequential incubation approach allowed to infer the contribution of different compartments of the benthic system to sustain the growth of *C. contraria* and to reconstruct in a conceptual scheme the benthic $O_2$ metabolism and N fluxes in charophyte stands and in adjacent bare sediment (Fig. 8).

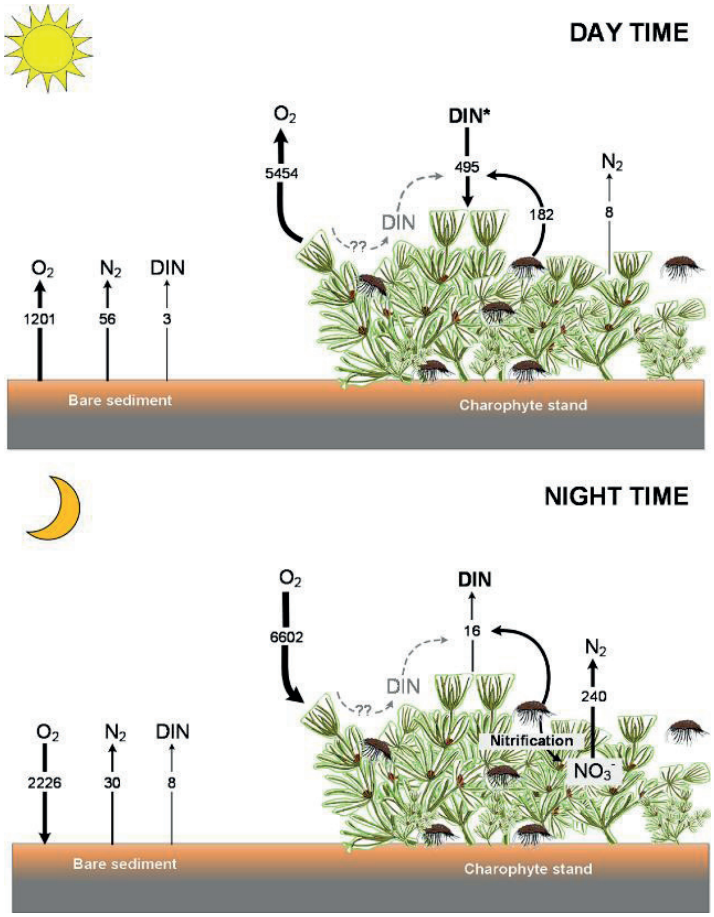



*Figure 8:* A conceptual scheme summarizing benthic $O_2$ metabolism and N fluxes in charophyte stand and in adjacent bare sediment (µmol $m^{-2}$ $h^{-1}$). In the light, amphipods excretion represents nearly 40% of calculated theoretical dissolved inorganic nitrogen (DIN*) uptake by the vegetated sediments. In the dark, excreted N is oxidized to nitrate and reduced to dinitrogen ($N_2$) or it undergoes dark assimilation. All fluxes were obtained combining data from incubations of whole core (sediment with and without vegetation) and individual (amphipods alone) incubations. Means are based on replicates (n = 6). (From Paper V)

### 5.2.2 Direct and indirect effects of bioturbation on benthic metabolism

Two studies, Papers II and IV, were conducted on larvae of *Chironomus plumosus*. The main hypothesis was the substantial stimulation of N cycling in sediments inhabited by these burrowers via alteration of sediment's physical structure and stimulation of a repertoire of microbial community that catalyze the N transformations (Pelegrí & Blackburn, 1996; Benelli et al., 2018). The export of N during the metamorphosis was considered as an additional pathway for the translocation of this nutrient.

In the first study (Paper II), the presence of 9 larvae per core, corresponding to a larvae density of 1800 ind. $m^{-2}$ (natural abundance estimated in the Curonian Lagoon; Zettler & Daunys, 2007) led to a stimulation of $O_2$ respiration 72% higher than in control sediment. In parallel to sediment core incubations, individual larvae incubations allowed the estimation of the $O_2$ demand associated with chironomids themselves that equaled 28% of the total respiration rates, and allow to extrapolate the total $O_2$ consumption directly taken up by the sediment surface ~24%, which leaves approximately 48% of the $O_2$ consumption related to newly oxidized burrow structures. Similar to $O_2$ consumption, net production of $N_2$ was higher in sediment populated by larvae, confirming their stimulatory effect on sedimentary denitrification and nitrification. It is well known that bioturbation (burrows construction) and burrow bioirrigation stimulate nitrifiers within the sediments (Welsh et al., 2003; Michaud et al., 2006). Since nitrifying microbes require $O_2$ for $NH_4^+$ oxidation, measured respiration ($O_2$ uptake) increased alongside nitrification and denitrification Unlike the experiment previously synthesized in Paper IV three different densities of larvae were applied: only sediment (0 ind. $m^{-2}$), low (600 ind. $m^{-2}$) and high (1,800 ind. $m^{-2}$) densities, by adding none, three, and nine larvae per core, respectively. Higher incubation's temperature and higher nutrient availability in the water column (10 vs 16 °C and low vs high $NO_3^-$, in Paper II and IV respectively) led to impaired results on the net effect of larvae. At lower temperatures, the net effect of bioturbation and burrow irrigation resulted in an overall increase of $NH_4^+$ and $N_2$ efflux and in a decrease of $NO_3^-$ efflux at the sediment-water interface, whereas in paper IV the effect of larvae varied among the different densities, and depended on measured solutes. For example, the net flux of $NH_4^+$ varied from −19.1 to 8.7 μmol N $m^{-2}$ $h^{-1}$ without a clear correlation with larvae density. Sediment shifted from a sink to a source of $NH_4^+$ only at the intermediate larvae density treatment, increasing the upward flux of $NH_4^+$ from deeper layers (Michaud et al., 2006; Carpintero-Moraes et al., 2018). There was no measurable $NH_4^+$ efflux in control sediments, with no chironomids, or at high densities, due to the absence of bioirrigation and solute transport from subsurface sediments or to nitrification, favored by large oxygen availability in highly bioturbated sediments. The assumed main source of $NH_4^+$ in deep sediment pore water was the mineralization of OM, as the excretion of $NH_4^+$ by chironomid larvae rarely exceeds 20% of the

immobilized ammonium pool (Henry & Santos; 2008). Rates of total denitrification tended to increase with the density of chironomid larvae, although such a trend was not statistically significant. In this study it was also demonstrated that a consistent fraction of total $NO_3^-$ reduction through denitrification and DNRA is attributed to processes occurring in burrow walls.

Paper IV explored the effect of different life stages (larvae and adults) of *Chironomus plumosus* on microbial N transformations. We hypothesized that chironomids, with their multifaceted ecological role, differentially affect N transformations. In this chapter part of the results achieved in the study will be briefly described. The present study aimed at better understanding how sediment-dwelling chironomid larvae facilitate not only solute transport but the net export of N associated to chironomids biomass, when these organisms leave sediments (Fig. 9). The complete metamorphosis from burrowing larvae to flying adult midges was revealed to act as a net OM transfer through different compartments of the aquatic systems (Gratton et al., 2008). In the late spring, millions of emerging midges leave the lagoon sediments, exporting organic N (Scharnweber et al., 2014). At densities of 1,800 ind. $m^{-2}$, adult midges emergence may result in the displacement of a consistent amount of particulate organic N (up to 47.8 mmol N $m^{-2}$) out of the sediment . This instantaneous loss is comparable to the amount of N removed via denitrification in bioturbated sediments over nearly 20 days and represents an important N export from the lagoon ecosystem, not accounted for in previous budgets (Zilius et al., 2018).

These two studies (Paper II and IV) reveal how detailed experimental studies allow disentangling the complex regulation of a cycle like that of N when a single macrofauna species is present in large densities. Chironomids amplify and change the equilibrium of microbial transformations and, interestingly, displace large amounts of N from the aquatic to the atmospheric compartment. The theoretical efflux of particulate N associated with flying insects is instantaneous and equivalent to 20 days of denitrification. However, predation effect on larvae, seasonal variability in larvae metamorphosis and the eggs deposition within the same aquatic environment at the final life's stage would inevitably alter this estimation. It is important to include these issues in future biogeochemical studies targeting organisms that do metamorphosis and leave sediments as it is important to remember that all the modification of N cycle induced by chironomids ends suddenly when they leave sediments. Last, but not least, in order to catch the ecological role of these abundant organisms in sediment biogeochemistry it is important to have a multielement approach (e.g. include the effects chironomids produce also on P and Si). Interestingly, chironomids leave sediments in July and the important role of sediment oxidation they perform ends in the warmest period and at the moment of highest OM inputs from the water column (Zilius et al., 2018). They leave the sediments to avoid difficult times and in doing so they stop to provide biogeochemical services supporting benthic ecosystem functioning.

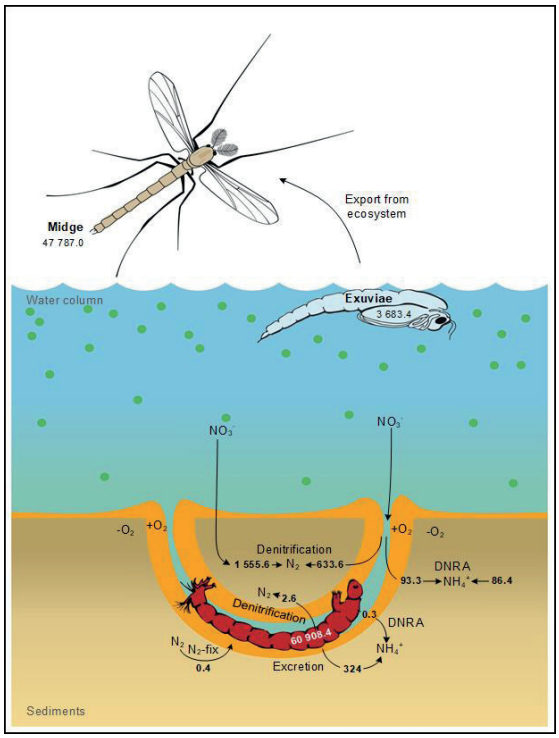



*Figure 9.* Flowchart of N-cycling associated with chironomid larvae activities. All fluxes were obtained combining data from incubations of whole core (sediment + larvae) and individual (larvae alone) incubations. Rates are reported for sediments inhabited by 1,800 larvae $m^{-2}$ and are expressed as µmol N $m^{-2}$ $day^{-1}$. The particulate organic N storage in each life stage is reported in µmol N $m^{-2}$ and refers to a population with density 1,800 ind. $m^{-2}$. (Drawing by V. Gasiūnaitė; from Paper IV).

## 5.3 Holobionts' potential role in benthic N cycling

Since microbes are ubiquitous and diverse they continuously interact with benthic invertebrates, forming unique ecological associations – holobionts. In this thesis, we show how a micro scale measurement at holobiont level can be beneficial to explain the role of microbes and invertebrates' interactions in benthic ecosystems. Nevertheless, recent progress in this field (i.e., Zilius et al., 2022), the role of invertebrates-associated microbiomes remains largely unexplored both in terms of metabolic repertoire and magnitude of the N transformations. These important and novel issues were explored within this thesis.

In Paper II, is shown how sediment reworking by chironomid larvae influence redox conditions and creates new niches for microbes (*see paragraph 4.2.1*). Application of high throughput sequencing of 16S rRNA gene and quantification functional marker genes allowed to better understand how invertebrates shape and affect microbial communities in their bodies and burrows. The 16S rRNA gene sequencing revealed prominent differences between the microbial community composition in samples of sediment and chironomid larvae tissues, showing that chironomid-associated microbial community was specific and with high genetic potential in N cycle. Specifically, in chironomid larvae, the microbial community was dominated by three phyla: Firmicutes (40.1%), Proteobacteria (27.6%) and Bacteroidetes (24.4%). These abundant and diverse groups include microbial taxa capable of multiple N transformations, including anammox, denitrification, DNRA and $N_2$ fixation. Although these microbes are ubiquitous, they may benefit from the association with chironomid larvae, due to the easy access to electron acceptors (e.g. $NO_3^-$) or organic substrate needed for energy acquisition (Chaston & Goodrich-Blair, 2010). The quantitative real-time PCR assays was used to quantify the abundance and transcriptional activity of the targeted functional genes associated with the oxidation of $NH_4^+$ to $NO_2^-$ (*amoA*) and its reduction to nitric oxide (NO; *nirS*/*nirK*) or to $NH_4^+$(*nrfA*). Results showed that microbial *amoA* genes were present in all the samples, with significant differences detected only for *nirS* and *nrfA* gene abundance between the anoxic sediment and chironomid larvae. These results show that the larvae-associated microbial community exhibited transcriptional activity of the *nrfA* gene, which encodes the DNRA. This process is strictly anaerobic and occurs in the anoxic larvae gut where active $NO_3^-$ respiring intestinal microbes may act as an active source of $NH_4^+$ (Stief & Eller, 2006).

Similar investigation was conducted in *D. polymorpha* holobiont (Paper III). Bivalves may alter benthic N cycling both directly and indirectly via stimulating microbial activity at the sediment level and through its microbiome. Active communities of diazotrophs were detected in mussel samples. Along with Tenericutes and Spirochetes, common of bivalves, *D. polymorpha* was characterized by high abundances of Betaproteobacteria (average, 12.8%), Gammaproteobacteria (6.5%), and Bacteroidetes (22.5%) that account for a considerable fraction of the microbial community. The diversity of active diazotrophs in zebra mussels, as characterized by *nifH* gene transcription analysis, also differed substantially from that of surrounding environment. The *nifH* transcript diversity of the mussels was dominated by Paenibacillus and other taxa closely related to Clostridia and Bacteroidetes. Such taxa have been previously described as diazotrophs, although, a few evidences have suggested – so far – their association with bivalves.

In this work the molecular approach was supported by biogeochemical measurements. Individual incubations of these animals and the use of revised IPT revealed that these organisms have capability for different N-related processes, such as $N_2$-

fixation, denitrification and DNRA (Fig. 10). Transcriptional activity of the *nrfA* gene in Chironomids larvae confirms whether $NH_4^+$ excretion rate, measured in individual larvae incubation, is solely a physiological process or if it could also be attributed to larvae–microbes associations (e.g., gut microbiomes). Results from the incubations suggest that $NH_4^+$ production via DNRA is of minor importance as compared to that produced through larval excretion (< 1% of total animal production). Incubations of animals using two different tracers (additions of $^{15}NH_4^+ + {}^{14}NO_3^-$) revealed measurable $N_2$ ($^{29}N_2$) production rates, suggesting the presence of putative anammox. However, this pathway contributed only 2% of the total $N_2$ production measured in microcosms. $N_2$ fixation accounted for 0.2 ± 0.1 nmol N ind.$^{-1}$ day$^{-1}$.

For the first time, rates of DNRA, $N_2$-fixation and denitrification were effectively detected and quantified in chironomid larvae' holobiont. (Fig. 10).

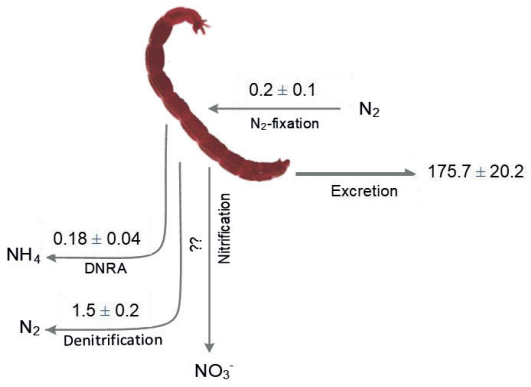



*Figure 10:* Nitrogen pathways associated with chironomid larvae holobionts. Process rates were calculated from individual incubations (larvae of *Chironomus plumosus* alone). The mean rates (average ± st. error) are expressed on a daily basis per larva (nmol ind.$^{-1}$ d$^{-1}$). Nitrification rates were not directly measured however, the finding of low *amoA* transcript numbers suggests a low nitrifying activity. (Redrawn from Paper IV)

This study is the first to report $N_2$ fixation associated with *D. polymorpha* holobionts (Fig. 11). At the lagoon level (macroscale studies), $N_2$ fixation has been traditionally attributed to pelagic cyanobaceria activity (Lesutienė et al., 2014; Bartoli et al., 2018; Zilius et al., 2020) whereas the holobiont's potential for this process was never accounted for in the estimations of the lagoon's N mass balance. Measures of $N_2$-fixation in individual mussel incubations have been upscaled to the average densities reported in the literature for the Curonian Lagoon (up to 57,000 individuals per square meter, Daunys et al., 2006). Results suggest that holobionts activity may account for

a consistent part of the total N fixed in the summer period in the Curonian Lagoon (up to 19.9 µmol of fixed N $m^{-2}$ $h^{-1}$). Additionally, the animal's gut, more than the surrounding sediment, resulted to be a favorable niche for DNRA activity. This finding was suggested by the comparison between denitrification and DNRA rates measured in intact core incubations (*D. polymorpha* + sediment) and the rates measured in individual animal incubations. The latter showed that holobionts contribution accounted only for 15% of the total denitrification rates (measured in the benthic-community incubations). This suggests that the impact of mussel on denitrification was mainly indirect (related to the altered sediment microbial activity), rather than via stimulation of $NO_3^-$ reduction in anoxic sections of the animal body. On the contrary, DNRA activity in the holobiont incubation accounted for a major fraction of the increment in DNRA measured in the benthic community incubation indicating the dominant effect of the mussels' microbiome in stimulating DNRA. The capability for zebra mussels to host bacteria that recycle or fix and do not dissipate N might be particularly advantageous to facilitate their establishment and spread in nutrient-poor environments or in environments with large seasonal variability in N abundance.

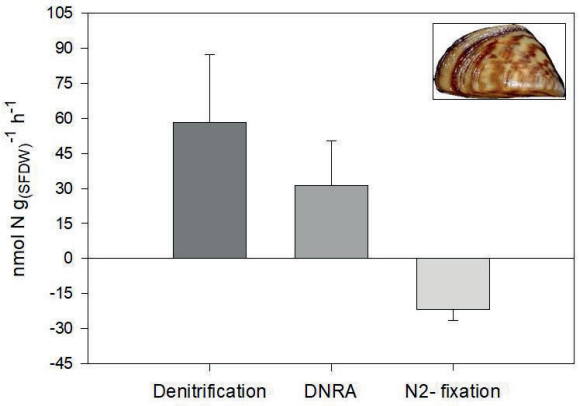



*Figure 11:* Nitrogen pathways associated with *D. polymorpha* holobionts. Process' rates were directly measured in individual incubations (mussels alone). The mean rates (average ± st. error) are expressed on an hourly basis per gram of dry weight shell free (nmol $g_{(SFDW)}^{-1}$ $h^{-1}$). (n = 10 for denitrification and DNRA; n = 4 for N-fixation). Anammox rates were not detected. (Redrawn from Paper III)

# 6

# Conclusions

1) In the Curonian Lagoon, experimental and multivariate analyses highlighted a marked shift towards higher metals and P mobilization under $O_2$ shortage. Such transition was strongly correlated with sediment characteristics more than with macrofauna presence or absence. Macrofauna is likely important in certain periods, such as the winter, the spring and the early summer but not when abiotic conditions (e.g., temperature, sediment redox) are too extreme (water temperature up to 26°C). Under these circumstances, sediment reworking by macrofauna has comparatively minor biogeochemical effects on sediment buffer capacity. In future, more experiments should be done to elucidate seasonal changes in sediment features and their interplay with macrofauna activity.

2) In the Sacca di Goro, macrofauna have contrasting but significant effects on microbial N cycling pathways, which largely cope with physical and abiotic factors. As a result, there are areas that net recycle large amounts of $NH_4^+$, due to the scarce capacity of macrofauna to oxidize sulfidic sediments with no nitrifying capacity and at the opposite there are areas that net recycle large amounts of $NO_3^-$, due to intense bioturbation promoting fast N mineralization and its oxidation. Other areas with large densities of filter feeders, including cultivated organims and burrowers display large N removal via denitrification. Finally, organic poor sediments have biodiverse macrofauna communities with low animal densities and low rates of N processing.

3) Results evidenced a strong association between charophytes stands and amphipods, likely indicating mutualistic relationships between primary producers and grazers. Such close association between primary producers and consumers represents an adaptation to the peculiar conditions of the Curonian Lagoon during summer, when charophytes are overgrowth by filamentous algae and amphipods feed on them. A continuous N recycling and reusing through the grazing–excretion–assimilation loop operated by amphipods and primary producers, constitutes a hidden N-dynamic. It suggests that a collapse of the amphipods population would result in enhanced colonization by epiphytes and negative feedback on *C. contraria*.

4) Sediment reworking by tube-dwelling chironomid larvae produced direct effects on the volume of oxidized sediments, creating new oxic niches. This, in turn stimulated nitrification, denitrification and $NH_4^+$ production, increasing N recycling, but reducing denitrification efficiency. Process rates are not always correlated with organism's density. With the metamorphosis form larvae to flying insects, the emerged midges contribute to reducing excess N loading in lagoon sediments by displacing particulate N to the atmosphere.

5) Chironomid larvae harbor a unique and active array of microbes compared to those found in the surrounding environment. The quantification of abundance and transcriptional activity of targeted functional genes (*amoA*, *nirS* and *nrfA* genes) revealed the presence of the microbial community involved in contrasting processes, affecting the N-cycle. These processes (DNRA, $N_2$-fixation and denitrification) were, for the first time, effectively detected and quantified. However, holobionts' measured rates are orders of magnitude lower than sediment rates, at least in the hypertrophic conditions of the Curonian Lagoon system.

6) In the Curonian Lagoon, *Dreissena polymorpha* favors the recycling of N via excretion of ammonium and by stimulating nitrification and DNRA in the sediment. Furthermore, this study revealed that mussel-associated microbiome was actively involved in N cycle. Among measured microbial N processes, $N_2$ fixation exhibited the highest rates. The capability to host diazotrophic microbes might be particularly advantageous for mussels, and facilitate their establishment and spread in nutrient-poor environments. It might therefore represent an important factor in determining their high invasiveness and adaptive capacity. Interestingly, mussel-associated diazotrophic community substantially diverged from the $N_2$-fixing community found in the surrounding sediments and water column. As the Curonian Lagoon is eutrophic, the mussels-microbes association is not lost even under conditions of high nutrient background.

# 7

# Final considerations

Ecological investigations on biodiversity and ecosystem functioning are generally carried out at the large scale or on a limited set of ecosystem attributes or processes. They miss sometimes to reveal the mechanisms by which different organisms support biogeochemical transformations. In this thesis macro and micro scale approaches were used to improve our understanding of benthic functioning. If in the macro scale approach multivariate statistical analyses were employed to contrast biodiversity/functioning datasets, whereas in the small-scale approach fine biogeochemical measurements coupled to molecular analysis of biodiversity and specific gene expression were adopted to understand mechanisms and the reasons of macrofauna-microbes associations.

In the Sacca di Goro experimental data and multivariate analyses allowed the reconstruction of benthic N cycle in different areas characterized by different macrofauna communities and to highlight how different functional groups drive specific microbial processes or help their coupling. In the Curonian Lagoon experimental data revealed large biogeochemical changes in P cycling during oxic anoxic transition but multivariate analyses did not reveal any significant role of macrofauna in such changes, that mostly depend on sedimentary features. The small-scale experimental approach was used to reveal the ecological role of three distinct macrofauna functional groups in the Curonian Lagoon. It aimed at understanding mechanisms underlying N

cycling in chironomid's and mussel's holobionts, net solutes exchange at the benthic level, the association between grazers and macrophytes and to speculate about the large invasive potential of a not native bivalve.

The sequence of incubations and the molecular approach described in this thesis can be applied to a variety of other macrofauna species or functional groups, forming intimate or casual associations with microbial communities. The idea of constructing a database of macrofauna-microbial associations seems very important in order to deepen our understanding of the interactions between macro and microorganisms, and of the reasons, need and regulation of such interactions by the physical environment. Such associations have been mostly studied in a few organisms (e.g., lucinids; Cardini et al., 2019) growing in tropical, nutrient-limited systems and not in eutrophic environments. Results from this thesis, despite initial and preliminary, suggest that similar associations are occurring also under not limiting nutrient conditions, in eutrophic lagoons. More studies are needed to expand our knowledge on this interesting topic.

# 8

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*The last four years have been the most informative, full of discoveries and powerful: a happy period of my life. As these doctoral studies come to an end I would like to acknowledge the support and encouragements and the faith in successes of my discoveries.*

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# 9

# Santrauka

## ĮVADAS

Šiame darbe pagrindinis dėmesys skiriamas dugno ekosistemai, kuri apima paviršines estuarijų nuosėdas. Seklių estuarijų ir lagūnų nuosėdų tyrimas yra idealus pavyzdys analizuojantant sąveiką tarp mikroorganizmų, meio- ir makrofaunos bei pirminių gamintojų bendrijų. Dugno ekosistemos yra heterogeniškos dėl jas stipriai formuojančių hidrodinaminių procesų. Šiame tyrime dugno ekosistemų funkcionavimas tiriamas analizuojant biogeocheminius procesus, apimančius visos sistemos metabolizmo pokyčius vykstant dujų ($O_2$, $CO_2$ ir $N_2$) ir maistinių medžiagų (N, P, Si) asimilaicijai ir produkcijai. Ypatingas dėmesys skirtas dugno nuosėdų N ciklui dėl jo virsmų kompleksiškumo, kuriuos lemia įvairūs mikroorganizmai bei makrofaunos ir pirminių gamintojų veikla bei sąveika. Biogeocheminiai šio elemento virsmai, iš tiesų, susideda iš skirtingų oksidacijos-redukcijos reakcijų, vykstančių tiek aerobinėmis, tiek ir anaerobinamis sąlygomis. Šias sąlygas ypač aktyviai keičia makrofaunos vykdoma veikla. Makrofauna gali pakeisti nuosėdų pasiskirstymą ir dalelių judrumą, cheminių salygų gradientą įsirausdama, maitindamasi ir judėdama. Dėl šios priežasties gali padidėti N junginių mobilumas ar sulaikymas nuosėdose, pernaša į priedugnio vandenį. Makrofaunos vaidmuo tai pat svarbus formuojant dugno nuosėdų savybes, skatinant organinės medžiagos skaidymą, vandenilio sulfido oksidavimą, fosforo junginių surišimą į oksidus, kurie tiesiogiai lemia visos ekosistemos funkcionavimą.

Būtina paminėti, kad dėl makrofaunos vykdomos veiklos keičiasi ir maistmedžiagių prieinamumas pirminiams gamintojams. Todėl makrofauna gali teigiamai arba neigiamai veikti pirminių gamintojų augimą ir jų vaidmenį ekosistemoje.

Sujungiant eksperimentinius ir modeliavimo metodus galima fundamentaliai suprasti netiesioginę ir nematomą saveiką tarp skirtingų funkcinių grupių bei ekosistemos komponentų ir pateikti realistiškus šios sąveikos scenarijus. Visos dugno ekosistemos detalus supratimas pasiekiamas apimant biogeocheminių procesų matavimus skirtingose skalėse – nuo mikro- iki makroskalės, taikant tarpdisciplininius metodus, įskaitant analitinius, biogeocheminius, molekulinius bei daugiamatę statistinę analizę. Duomenys apie biologinę įvairovę analizuojami kartu su ekosistemų funkcijomis – elementų virsmais, suteikia galimybę suprasti labiausiai tikėtinus priežastinius ryšius. Todėl sujungus skirtingus metodus skirtingose skalėse nuo pavienio individo iki dugno sistemos, galima aprašyti eksistemos funkcionavimą.

## TIKSLAS IR HIPOTEZĖ

Pagrindinis šio darbo tikslas – ištirti dugno bioįvairovės ir ekosistemos funkcionavimo ryšį estuarijų nuosėdose. Siekiant atskleisti sąveiką tarp mikrobų, bentoso makrofaunos ir pirminių gamintojų iškelti šie uždaviniai:

1) įvertinti, ar dugno makrofaunos bendrijos veikla suteikia išmatuojamą biogeocheminių salygų stabilumą nuosėdose, kuris lemia reaktyviojo fosforo judrumą trumpalaikėmis deguonies trūkumo sąlygomis eutrofinėje borealinėje lagūnoje;
2) išanalizuoti, kaip vyraujančios makrofaunos bendrijos, įskaitant kultivuojamus moliuskus, veikia dugno nuosėdų metabolizmą ir maistmedžiagių pernašą skirtingose Vidužemio jūros eutrofinės lagūnos makrozonose;
3) identifikuoti ir kiekybiškai įvertinti abipusį ryšį tarp makrofaunos (šoniplaukų) ir maurabragių sąžalynų per jų metabolizmo ciklą, skatinant maurabragių išlikimą eutrofinėse ekosistemose;
4) nustatyti chironomidų lervų funkcinį vaidmenį organinėse nuosėdose ir jų poveikį N ciklui;
5) taikant molekulinius ir biogeocheminius metodus ištirti pavienių chironomidinių lervų ir zebrinių midijų individų mikrobiomų įvairovę ir jos vaidmenį N virsmuose.

Darbe iškeltos hipotezės: įsirausianti makrofauna gali neutralizuoti trumpalaikį neigiamą anoksijos poveikį, padidindama nuosėdų vaidmenį reaktyviojo P sulaikymui surišdama elementą į metalų oksidus (1 uždavinys); antra, sąveika tarp skirtingų dominuojančių makrofaunos funkcinių grupių skirtingai paveikia N ciklą nuosėdose (2 uždavinys); trečia, šoniplaukos palaiko maurabragių išlikimą ir augimą eutrofinėje aplinkoje (3 uždavinys); ketvirta, makrofauna teigiamai veikia nuosėdose gyvenančių arba su jų kūnu susijusių mikrobinių bendrijų veiklą, o ši sąveika lemia kiekybiškai svarbias biogeochemines transformacijas (4 ir 5 uždaviniai).

## DARBO NAUJUMAS

Visų pirma darbo naujumas apima kelių skirtingų mastelių tyrimus: nuo makroskalės (lagūnos sistemų tyrimai) iki mikroskalės (pavieniai individai ir holobiontai). Nauja ir tai, kad eksperimentai buvo atliekami analizuojant mikroorganizmų filogenetinę įvairovę, funkcinius genus, koduojančius skirtingus N virsmus, tiek makrofaunoje, tiek ir ją supančioje aplinkoje. Tarpdisciplininis požiūris į tyrimus paskatino glaudų bendravimą su kolegomis iš kitų institutų ir sričių (mikrobiologinės ekologijos, molekulinės biologijos) bei leido praplėsti taikomų metodų galimybes. Pritaikius molekulinius metodus buvo atskleista neįtikėtina mikrooorganizmų įvairovė, gyvenančių makrofaunos išorėje ir viduje, aktyviai tarpininkaujančių elementų virsmuose ir greičiausiai pasinaudojančių makrofaunos judrumu nuosėdose ir vandens storymėje. Tai paskatino rekonstruoti mikrobinio N ciklo virsmus holobiontų lygmenyje (t. y. kiekybiškai įvertinti procesų greičius makrofaunos kūne), apskaičiuoti, kiek makrofaunoje vykstantys N virsmai prisideda prie visos dugno ekosistemos metabolizmo ir kaip tai priklauso nuo individų gausumo. Taip pat buvo sukurtas kelių pakopų matavimų metodas, kuris apėmė inkubacijų derinį, pradedant nuo nesuardytos struktūros dugno nuosėdų kolonėlių su natūralia makrofaunos bendrija analizės, išmatuojant tirpių medžiagų apykaitą ir toliau matuojant specifinius N ciklo srautus su žymėtu $N^{15}$ izotopu. Kitoje pakopoje atliktos pavienių individų inkubacijos tam, kad būtų galima atskirti makrofaunos vaidmenį dugno ekosistemoje, įskaitant nuosėdas, mikroorganizmus ir meiofauną. Tai leido atskirti individualų makrofaunos vaidmenį ekosistemos metabolizme (kvėpavimą ir šalinimą) ir specifiniuose N procesuose, susijusijuose su holobiontais. Galiausiai paskutinėje tyrimo pakopoje buvo atliktas mikrobų bendrijos identifikavimas ir jos genetinio potencialo, transformuojant N junginius, kiekybinis įvertinimas naudojant molekulinius įrankius.

# DUOMENYS IR METODAI

Kuršių marios (pietryčių Baltijos jūra) ir Sacca di Goro (šiaurės vakarų Adrijos jūra) buvo tirtos siekiant nustatyti bendrus atskaitos taškus ir ribas, būtinas norint suprasti makrofaunos funkcinės įvairovės, dugno ekosistemos funkcionavimo ir maistmedžiagų apykaitos ryšį. Šiame darbe eksperimentinė veikla vykdyta trimis lygmenimis: 1) natūralių dugno bendrijų ir elementų apykaitos matavimai (dugno ekosistemos tyrimai); 2) kertinių rūšių tiesioginio ir netiesioginio vaidmens vertinimas; 3) išsamus mikroorganizmų ir bestuburių sąveikos ir jų vaidmens dugno ekosistemos funkcionavime vertinimas.

Darbe iš dalies atsižvelgta ir į bendrą dugno bendrijos poveikį biogeocheminiams procesams ir elementų apykaitai, siekiant nustatyti ryšį tarp biologinės įvairovės ir dugno ekosistemos funkcionavimo. Pagrindiniai tyrimo metodai apėmė nesuardytos sturktūros dugno nuosėdų kolonėlių su natūralia makrofaunos bendrija surinkimas ir inkubacija šviesos ir tamsos sąlygomis. Inkubacijos metu buvo matuojama ištirpusių maistmedžiagų ir dujų apykaitos greičiai tarp dugno nuosėdų ir priedugnio vandens. Mikrobiologinių N virsmų (nitrifikacijos, denitrifikacijos) kiekybinis vertinimas atliktas taikant žymėto $^{15}N$ izotopo metodą. Siekiant nustatyti priežastinį ryšį tarp išmatuotų elementų apykaitos, virsmų greičių (priklausomi kintamieji) ir makrofaunos (nepriklausomas kintamasis) taikyta daugiamatė statistinė analizė.

Antrajame tyrimų lygmemyje dugno nuosėdų ekosistema buvo rekonstruota mikrokosmuose naudojant supaprastintas bendrijas arba atskiras kertines rūšis, vėliau juose išmatuojant elementų ir virsmų greičius. Šiame lygmenyje taip pat buvo vykdomas ir gyvūnų kvėpavimo ir ekskrecijos, makrofitų fotosintezės ir respiracijos greičių matavimai mažuose mikrokosmuose. Šie tyrimai atlikti su trimis kertinėmis Kuršių marių rūšimis: uodo trūklio lerva (*Chironomus plumosus*), dvigeldžiu moliusku (*Dreissena polymorpha*) ir mažuoju maurabragiu (*Chara contraria*).

Trečia tyrimų sritis, skirta holobiontų vaidmeniui nustatyti, apėmė išsamius biogeocheminių procesų matavimus kartu su mikrobų įvairovės identifikavimo ir jų genetinio potencialo vertinimu. Potencialūs mikroorganizmai dalyvaujantys N ciklo virsmuose, identifikuoti iš izoliuotų mikroorganizmų DNR ir RNR. Taip pat kiekybiškai įvertinti funkciniai žymenų genai (*nifH, nirS, nirK, nrfA* ir *amoA*), koduojantys skirtingus N ciklo transformacijas. Tai suteikė galimybę geriau suprasti, kaip makrofauna veikia aplinkinį mikrobiomą (t. y. per urvelių sieneles) ir jo vaidmenį N ciklo virsmuose.

# REZULTATAI IR DISKUSIJA

## 1. Ryšys tarp makrofaunos įvairovės ir bentinio funkcionavimo

Kuršių marioms ir Sacca di Goro būdinga sedimentacinės aplinkos heterogeniškumas, apimantis smėlio nuosėdas, pasižyminčias nedideliu organinių medžiagų kiekiu, ir smulkų, purų dumblą, prisotintą organinės medžiagos. Keturiose Goro lagūnos stotyse dominavo bendrijos, kurias sudarė iki 17 skirtingų makrofaunos taksonų (vidutinis gausumas 82 ± 12 ind. ėminyje$^{-1}$; ca. 23 700 ind. m$^{-2}$). Kuršių mariose, pasižyminčiose didesne makrofaunos įvairove (23 rūšys ir 7 aukštesnio rango taksonai, ir vidutiniškai 87 ± 7 ind. ėminyje$^{-1}$; ca. 25 100 ind. m$^{-2}$), rūšių pasiskirstymas tarp 19 stočių buvo vienalytis, neturintis aiškaus jų pasiskirstymo erdvinio dėsningumo.

Kuršių marios pasižymėjo didele ištirpusių metalų ir maistmedžiagų apykaitos greičių variacija tarp tirtų stočių. Daugiamatė analizė (DistLM) buvo panaudota siekiant įvertinti bentoso bestuburių poveikį mikrobiologiniams procesams ir tirpių medžiagų apykaitai tarp dugno nuosėdų ir priedugnio vandens. Makrofaunos bendrijos paaiškino nedidelę visos tirpiųjų medžiagų apykaitos kintamumo dalį (DistLM), labiau vyraujant oksinėms sąlygoms, nei esant anoksijai. Normoksijos sąlygomis makrofauna buvo statistiškai reikšmingai susijusi su išmatuotais pokyčiais, o esant deguonies trūkumui dauguma matuotų tirpiųjų medžiagų srautų pokyčių buvo daugiau susiję su nuosėdų savybėmis, nei su makrofaunos aktyvumu. Vis dėlto, makrofauna gali turėti įtakos biogeocheminių procesų greičiams, redoksinių-oksidacinių sąlygų stabilumui deguonies trūkumo sąlygomis. Priešingai iškeltai hipotezei, įsirausiančių organizmų vaidmuo prognozuojant tirpiųjų medžiagų apykaitos pokyčius buvo antraeilis arba statistiškai nereikšmingas, o kitų funkcinių grupių, tokių kaip paviršiuje besimaitinantys pirminiai vartotojai Gammaridae ir Gastropoda, vaidmuo, nuolat fiksuojamas eksperimentiniuose mezokosmuose, buvo statistiškai reikšmingas.

Keturios tirtos Sacca di Goro lagūnos sedimentacinės aplinkos skyrėsi pagal makrofaunos sudėtį ir sutapo su literatūros šaltiniuose aprašytomis nuosėdų pasiskirstymo tendencijomis. Daugiamatės analizės (RDA) rezultatai parodė stiprų teigiamą ryšį tarp įsirausiančių organizmų gausos ir ištirpusio neorganinio N pokyčių drumstumo zonose. Gausios *M. insidiosum* ir daugiašerių kirmėlių populiacijos sukuria tankų urvelių tinklą, kuris išplečia nuosėdų paviršių tirpių medžiagų apykaitai ir oksinių nišų tūrį, skatindamas mikroorganizmų aktyvumą. RDA analizė taip pat atskleidė stiprų kolineariškumą tarp makrofaunos funkcinių savybių ir DIP, DMn bei $NH_4^+$ pokyčių. Be to, patvirtino faktą, kad dėl denitrifikacijos metu pašalinamo N makrofauna gali sukelti kontrastingą poveikį mikrobiologiniams procesams nuosėdose, priklausomai nuo sedimentacinės aplinkos. Pavyzdžiui, tikėtina kad *M. insidiosum* buvimas lėmė

denitrifikacijos greičių padidėjimą vienoje iš aplinkų, tuo tarpu kitoje, kur vyrauja sulfidinės nuosėdos, paskatino disimiliacinę nitratų redukciją iki amonio (DNRA).

## 2. Individualios funkcinės savybės kaip bentinio funkcionavimo veiksnys

Analizuojant saveiką tarp makrofaunos ir pirminių gamintojų per matabolizmo ciklą, nustatyta, kad maurabragiai ir su jais susijusių epifitų fotosintezės veikla lemia didelius $O_2$ svyravimus, palyginus su augmenija nepadengtomis nuosėdomis. Taip pat paaiškėjo, kad maurabragiai intensyviai asimiliuoja santykinai didelį $NH_4^+$ kiekį (100–300 μmol $m^{-2}$ $h^{-1}$), nepaisant jo tūkumo supančioje aplinkoje. Tai leidžia manyti, kad dugno ekosistemoje yra nežinomas ištirpusio neorganinio azoto (DIN) šaltinis, palaikantis maurabragio augimą. Eksperimetų metu išmatuota gana intensyvi makrofaunos ekskrecija, kuri gali patenkinti beveik 40 % teorinės makrodumblių N asimiliacijos. Vadinasi, šoniplaukos gali būti svarbus N šaltinis. Kuršių mariose maurabragiai kenčia nuo intensyvios epifitų kolonizacijos, todėl šoniplaukų mityba jais gali turėti didelę reikšmę maurabragio *C. contraria* išlikimui ir augimui – šoniplaukoms nuėdant epifitus ir išskiriant N į aplinką.

Tiesioginis ir netiesioginis bioturbacijos poveikis dugno metabolizmui buvo tiriamas atliekant du eksperimentus su *C. plumosus* lervomis. Pirmo tyrimo metu nustatyta, kad dugno ekosistemos metabolizmo greičiai priklauso nuo lervų gausumo. Santykinai didžiausias metabiolizmo greitis, t.y. ištirpusio deguonies ($O_2$) suvartojimas, buvo, kai lervų tankis siekė – 1800 ind. $m^{-2}$. Tada $O_2$ suvartojimas buvo 72 % didesnis nei kontrolinėse nuosėdose. Rekonstruojant $O_2$ suvartojimo procesus, nustatyta, kad lerva iškvėpavo 28 % viso $O_2$ kiekio, tuo tarpu 24 % deguonies sunaudojama mikroorganizmų nuosėdų paviršiuje, o likęs kiekis (48 %) – lervos sukonstruotuose urveliuose. Dėl lervų vykdomo nuosėdų rausimo ir urvelių ventiliacijos padidėjo $NH_4^+$ asimiliacija ir $N_2$ išsiskyrimas dugno ekosistemoje, kas leidža manyti suintensyvėjusią nitratų redukciją denitrifikacijos metu. Be to, dėl didelio chironomidų tankio suaugusių vabzdžių išsiritimas metamarfozės metu gali lemti reikšmingą organinio N (iki 47 787 μmol N $m^{-2}$) pašalinimą iš nuosėdų į atmosferą. Šis momentinis srautas prilygsta denitrifikacijos būdu iš bioturbuotų nuosėdų pašalintam N kiekiui per beveik 20 dienų ir jis iliustruoja svarbų iš lagūnos ekosistemos vykstantį N eksportą, į kurį nebuvo atsižvelgta ankstesniuose vertinimuose.

Mezokosmuose lervų poveikis skirtingų maistmedžiagių apykaitai tarp nuosėdų ir priedugnio vandens kito priklausomai nuo jų tankio ir nuo koncentracijos. Pavyzdžiui, $NH_4^+$ apykaitos greitis svyravo nuo −19,1 iki 8,7 μmol N $m^{-2}$ $h^{-1}$, tačiau be aiškios koreliacijos su lervų tankiu. Tikėtina, kad pagrindinis $NH_4^+$ šaltinis buvo šių jonų pernaša iš gilesnių nuosėdų sluoksnio. Atlikti tyrimai (II ir IV straipsniai) atskleidė

kompleksinį azoto virsmų ir srautų reguliavimą, priklausantį ne vien nuo lervos gausumo, ypač esant dideliam tankiui. Akivaizdu, kad lervos sustiprina ir keičia mikrobiologinių medžiagų virsmų pusiausvyrą ir transportuoja didelius N kiekius iš vandens į atmosferą metamorfozės metu.

## 3. Potencialus holobiontų vaidmuo bentiniame N cikle

16S rRNR genų sekos nustatymas atskleidė ryškius mikroorganizmų bendrijos sudėties skirtumus nuosėdų ir chironomidų lervų mėginiuose, parodydamas, kad su chironomidais susijusi mikroorganizmų bendrija buvo specifinė ir turėjo didelį genetinį potencialą N cikle. Uodo trūklio lervose mikroorganizmų bendrijoje dominavo trys tipai: Firmicutes (40,1 %), Proteobacteria (27,6 %) ir Bacteroidetes (24,4 %). Šie tipai, pasižymintys didele įvairove, apima ir mikrobų taksonus, galinčius atlikti daugybę N transformacijų, įskaitant anamoksą, denitrifikaciją, DNRA ir $N_2$ fiksaciją. Nors šie mikroorganizmai paplitę visur, jiems gali būti naudinga gyventi asociacijoje su uodo trūklio lervomis dėl lengvai pasiekiamų elektronų akceptorių (pavyzdžiui, $O_2$, $NO_3^-$) arba organinio substrato, reikalingo energijai gauti.

Šis darbas yra pirmasis, kuriame pateikiami duomenys apie molekulinio azoto ($N_2$) fiksaciją, susijusią su *D. polymorpha* holobiontais. Iki tol buvo manoma, kad $N_2$ fiksacija vyksta tik vandens storymėje dėl heterocistinių melsvabakterių. Šis darbas patvirtina, kad $N_2$ fiksacija yra labiau paplitęs procesas ekosistemoje ir gali būti svarbus jos funkcionavimas. Atlikus $N_2$ fiksacijos matavimus moliuskų holobiontuose ir vėliau gautus greičius perskaičiavus bendram moliuskų gausumui Kuršių mariose (iki 57 000 ind. $m^{-2}$), nustatyta, kad į ekosistemą patenka ženkli dalis N vasaros periodu (iki 19,9 μmol fiksuoto N $m^{-2}$ $h^{-1}$).

# IŠVADOS

1) Kuršių mariose atliktų tyrimų rezultatai parodė padidėjusią redukuotų metalų apykaitą ir P judrumą susidarant $O_2$ trūkumo salygoms. Šie pokyčiai labiau koreliavo su nuosėdų savybėmis nei su makrofaunos biomase. Makrofauna yra labai svarbi tam tikrais periodais, pavyzdžiui, žiemą, pavasarį ir vasaros pradžioje, bet ne tada, kai lemiamas veiksnys yra su hidrodinaminiais procesais susijusios abiotinės sąlygos. Tokiomis aplinkybėmis makrofaunos vykdomas nuosėdų rausimas turi santykinai nedidelį biogeocheminį poveikį nuosėdų cheminių salygų stabilumui. Ateityje reikėtų atlikti daugiau eksperimentų, siekiant išsiaiškinti sezoninius nuosėdų savybių pokyčius ir jų sąveiką su makrofaunos veikla.
2) Sacca di Goro lagūnoje makrofauna turi kontrastingą, bet reikšmingą poveikį mikrobiologiniams N ciklo virsmams, kuriuos daugiausia veikia fiziniai ir

abiotiniai veiksniai. Tam tikrose sedimentacinėse aplinkose, kuriose dėl riboto makrofaunos gebėjimo oksiduoti sulfidines nuosėdas, su menku nitrifikuojančių bakterijų pajėgumu, kaupiasi dideli $NH_4^+$ kiekiai amonifikacijos metu. Tuo tarpu aplinkoje, kurioje makrofauna intensyviai rausia nuosėdas, vyksta greita N mineralizacija, $NH_4^+$ oksidacija iki $NO_3^-$ ir jo redukcija. Aplinkose, kuriose dominuoja filtruojantys moliuskai ir besirausiančios daugiašerės kirmelės, denitrifikacijos būdu pašalinamas didžiausias N kiekis. Galiausiai, smėlio nuosėdose su nedideliu organinės medžiagos kiekiu vyrauja nedidelė biologinė įvairovė ir makrofaunos tankis, o tai nulemia ir mažą N virsmų intensyvumą.

3) Rezultatai parodė stiprų ryšį tarp pavienių maurabragių ir šoniplaukų. Tikėtina, tai atskleidžia abipusius ryšius tarp pirminių gamintojų ir vartotojų. Toks glaudus pirminių gamintojų ir vartotojų ryšys parodo prisitaikymą prie išskirtinių Kuršių marių sąlygų vasarą, kai maurabragiai apauga siūliniais dumbliais, o šoniplaukos jais minta. Nuolatinis N perdirbimas ir pakartotinis naudojimas per metabolizmo (mitybos ir šalinimo) ciklą, kurį sudaro šoniplaukos ir pirminiai gamintojai, atskleidė paslėptą N pernašą tarp skirtingų ekosistemos komponentų. Galima spėti, kad šoniplaukų populiacijos išnykimas padidintų epifitų kolonizaciją ir neigiamą grįžtamąjį ryšį maurabragiui *C. contraria*.
4) Besirausdamos ir urvelius konstruodamos chironomidų lervos daro tiesioginį poveikį oksiduotų nuosėdų tūriui ir sukuria naujas oksines nišas mikroorganizmams. Tai paskatina nitrifikaciją, denitrifikaciją ir amonifikaciją, bet sumažina denitrifikacijos efektyvumą. Tačiau procesų greitis ne visada koreliuoja su organizmų tankiu. Kai lervos virsta suaugėliais, išskridę vabzdžiai prisideda prie perteklinio N kiekio mažinimo lagūnos nuosėdose, transportuodami kietąsias daleles į atmosferą.
5) Chironomidų lervose egzistuoja unikali ir aktyvi mikroorganizmų bendrija, palyginus su supančia aplinka. Funkcinių genų (*amoA*, *nirS* ir *nrfA*) gausumo ir transkripcinio aktyvumo kiekybinis vertinimas atskleidė mikroorganizmų bendrijos vaidmenį skirtinguose N ciklo virsmuose (DNRA, $N_2$ fiksacija ir denitrifikacija). Šie procesai pirmą kartą buvo dokumentuoti ir kiekybiškai įvertinti uodo trūklio lervoje. Visgi holobiontuose išmatuoti N virsmų greičiai yra ženkliai mažesnis nei nuosėdų, bent jau hipertrofinėmis Kuršių marių sistemos sąlygomis.
6) Šis darbas atskleidė, kad su *D. polymorpha* susijęs mikrobiomas aktyviai dalyvavo N ciklo virsmuose. Tarp išmatuotų virsmų dominavo $N_2$ fiksacija. Gebėjimas priimti diazotrofinius mikroorganizmus gali būti ypač naudingas patiems moliuskams, plengvinatis jų įsitvirtinimą bei plitimą oligotrofinėse sistemose. Tai gali būti svarbus veiksnys, lemiantis didelį jų invaziškumą ir prisitaikymo galimybes. Įdomu tai, kad su moliuskais susijusi diazotrofinė makroorganizmų bendrija buvo unikali ir iš esmės skyrėsi nuo pelaginės.

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# Publications

# PAPER I

Tobia Politi, Mindaugas Zilius, Giuseppe Castaldelli,
Marco Bartoli and Darius Daunys

Estuarine Macrofauna Aects Benthic Biogeochemistry
in a Hypertrophic Lagoon

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water

MDPI

*Article*

# Estuarine Macrofauna Affects Benthic Biogeochemistry in a Hypertrophic Lagoon

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**Abstract:** Coastal lagoons display a wide range of physico-chemical conditions that shape benthic macrofauna communities. In turn, benthic macrofauna affects a wide array of biogeochemical processes as a consequence of feeding, bioirrigation, ventilation, and excretion activities. In this work, we have measured benthic respiration and solute fluxes in intact sediment cores with natural macrofauna communities collected from four distinct areas within the Sacca di Goro Lagoon (NE Adriatic Sea). The macrofauna community was characterized at the end of the incubations. Redundancy analysis (RDA) was used to quantify and test the interactions between the dominant macrofauna species and solute fluxes. Moreover, the relevance of macrofauna as driver of benthic nitrogen (N) redundancy analysis revealed that up to 66% of the benthic fluxes and metabolism variance was explained by macrofauna microbial-mediated N processes. Nitrification was stimulated by the presence of shallow (corophiids) in combination with deep burrowers (spionids, oligochaetes) or ammonium-excreting clams. Deep burrowers and clams increase ammonium availability in burrows actively ventilated by corophiids, which creates optimal conditions to nitrifiers. However, the stimulatory effect of burrowing macrofauna on nitrification does not necessarily result in higher denitrification as processes are spatially separated.

**Keywords:** fluxes; denitrification; macrofauna; functional diversity; Sacca di Goro Lagoon

## 1. Introduction

Bioturbation by benthic macrofauna—which includes a wide set of different processes among which burrow construction, ventilation, bioirrigation, sediment reworking, and biodeposition—makes sedimentary processes variable and complex [1–5]. Macrofauna communities display different adaptations to live within or on the surface sediment and produce sometimes contrasting effects on microbial processes, depending upon functional traits and tolerance to environmental stress. Macrofauna activity may determine the fate of nutrients and their transfer rates among environmental compartments [6,7]. Depending on the species and their vital habitats, associated bacterial processes can be accelerated or slowed down (e.g., anaerobic ammonium oxidation—anammox) [8,9]. Bioturbating macrofauna communities are responsible for the rearrangement of the original microbial stratification within the sediment by creating and destroying the oxic and anoxic microenvironments in the sediment, and also by direct action on the physical properties of colonized substrates [4]. Complex and species-rich macrofaunal communities or, on the other hand, communities dominated by few key species govern the ecosystem functioning in various ways. Nevertheless, species that seem redundant under

natural conditions may be important for ecosystem functioning when ecosystems are disturbed [10]. The understanding of the biogeochemical dynamics in environments characterized by high biodiversity is strongly limited by the complex and multiple interactions among species [11]. In strongly anthropized environments, generally associated with a strong loss of biodiversity, identification of important ecological niches is even more difficult. Grouping the diversity of benthic macrofauna into functional groups, and no longer referring to single species, and identifying their single contribution to the benthic ecosystem can be a solution to the complexity of this type of study. Equally, with the appropriate functional attributions at different benthic groups, it will be easier to model the entire ecosystem [12].

A number of previous studies were targeting a heterogenous set of parameters including dissolved oxygen ($O_2$), carbon dioxide ($TCO_2$), various nitrogen (N), phosphorus, silica forms, chlorophyll, and functional genes [13–16]. A large body of scientific work has clearly defined, sometimes at the microscale, how burrowers via intermittent ventilation import $O_2$ into their burrows and temporally enhance microbial aerobic activity, or how filter-feeders increase sedimentary organic matter via feces and pseudofeces production [17,18]. Nevertheless, majority of these studies were built on laboratory experiments, with reconstructed sediments and a single macrofauna species [16,19–22]. While such approach enables to characterize target organisms and reduces background noise (sediment heterogeneities, presence of non-target macrofauna groups, etc.), the overall system layout is far from that observed in nature. For example, oversimplified communities (e.g., a single population) do not host multiple ecological interactions present among organisms (including predation, competition, facilitation, various host–microbe associations). Furthermore, sediment sieving removes reactive pools of organic matter, changing the physical and chemical gradients in sediments. Homogenization also alters the vertical distribution of the organic matter quality, redistributing and diluting high quality surface sediment organic matter along the sediment horizon. The addition of macrofauna in such sediments generally results in high stimulation of processes like nutrient regeneration. These effects may partly be the consequence of burrow construction, while in situ burrow environments are aged and well-structured in terms of microbial community composition [23]. Short-term experiments with reconstructed sediments therefore cannot fully reproduce what happens in situ, since the development of bacteria communities along burrows may take weeks and may undergo variations along the life cycle of burrowers [23]. To overcome such limitations, an alternative approach is to collect and incubate undisturbed cores with natural abundance and composition of macrofauna [24–26]. A large number of replicate cores can be incubated and sieved at the end of measurements in order to retrieve macrofauna and analyze relationships among macrofauna and biogeochemical processes a posteriori.

In estuarine systems, the understanding of the role of macrofauna communities on benthic N-cycling is a keystone, due to its large inputs from catchments and potentially large macrofauna-mediated microbial N losses [27–31]. It is well known that macrofauna actively contribute to the translocation and transformations of N within and among different compartments of aquatic ecosystems, stimulating microbial processes [32,33]. However, some species, more than others, have stronger effects on microbial dissimilative N paths [34,35]. The study of the effects of macrofauna communities on benthic N-cycling is challenging as macrofauna might produce contrasting effects on the multiple oxic and anoxic microbial N transformations.

In this work, intact sediment cores were randomly collected from four sites representative of different dominating areas within a hypertrophic coastal lagoon. The main aim was to compare key functional characteristics at the four sites and highlight how macrofauna shape microbial respiration and nutrient regeneration rates, with a special focus on benthic N-cycling. To this purpose, we used multiple approaches, including flux measurements, isotope pairing technique, characterization of macrofauna community, and multivariate analysis.

## 2. Materials and Methods

The Sacca di Goro lagoon is a shallow (average depth 1.5 m) water embayment (27 $km^2$) of the Po River Delta, situated in the norther part of the Adriatic Sea (Figure 1). This lagoon is a brackish system

with pronounced daily variations of salinity and nutrient concentrations resulting from microtidal forcing (tidal amplitudes vary up to 0.9 m) and freshwater inputs from the Po di Volano and Po di Goro rivers, and saline water input from the Adriatic Sea.

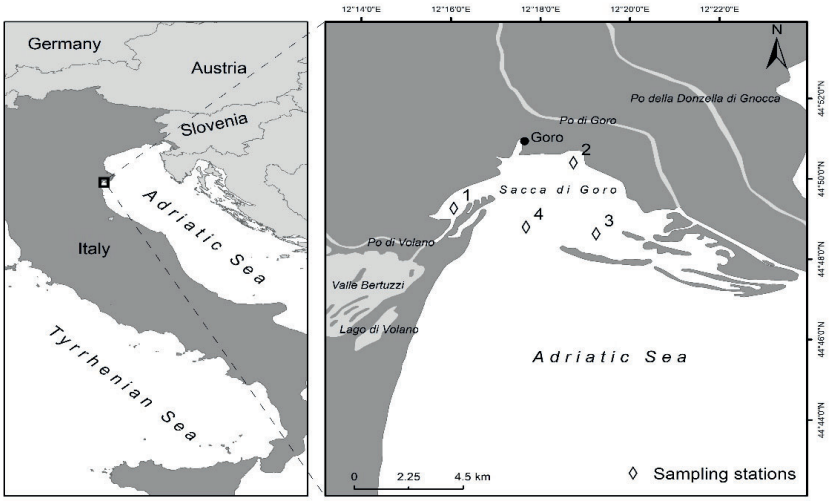


**Figure 1.** Map of the Sacca di Goro Lagoon and location of sampling sites.

The Sacca di Goro Lagoon has been intensively studied as one of the most economically important farming sites of the clam *Vanerupis philippinarum* in Europe, but also in a context of dystrophic events threated by this activity [36–38]. Studies mainly focused on the lagoon biogeochemistry [36–40], on the ecophysiology of blooming macroalgae [41], on meio- and macrofauna communities [42,43], and on ecosystem-level ecological processes (e.g., net ecosystem metabolism, sink-source functions [44]). Only a few studies have linked biogeochemical processes to macrofauna activity. However, these studies almost exclusively considered the introduced species *V. philippinarum*. To our knowledge, this is the first study addressing macrofauna biodiversity–benthic functioning relationship in the Sacca di Goro Lagoon.

The Sacca di Goro Lagoon is generally divided into three different zones: (1) the western part that is affected by freshwater inputs from the Po di Volano River, which leads to lower salinity and wider salinity fluctuations; (2) the central part that is connected directly to the Adriatic Sea via a 1 km wide mouth, therefore it is flushed by seawater; and (3) the eastern part (10 $km^2$), called the Valle di Gorino, which is separated from the sea by a sand barrier and receives freshwater inputs from the Po di Goro. This eastern zone is very shallow (maximum depth 1 m) but represents about half the surface of the entire lagoon. It is characterized by a relatively low salinity and higher water temperatures. The Sacca di Goro Lagoon sediment composition reflects a typical alluvial system: muds with high clay and silt contents in the northern and central zones and sand and sandy-muds bottom in the southern shore-line and eastern zone. A limited water circulation and a constant and high anthropogenic nutrients load from two rivers and secondary channels lead this lagoon to severe eutrophication processes and dystrophic events [37]. Diffuse runoff from agricultural activities within the Po river basin may lead to nitrate ($NO_3^-$) concentrations up to 200 µM, sustaining frequent blooms of the seaweeds *Ulva* sp., *Gracilaria* sp., and *Cladophora* sp., especially in the easternmost shallow area, whilst phytoplankton blooms prevail in the deeper central zone [45].

The studies on composition and distribution of the macrobenthic community in the Sacca di Goro Lagoon resulted in identification of 38 macrofauna taxa, representing 5 phyla [43,46–48]. Gastropods,

amphipods, and chironomid larvae dominate the macrofauna in terms of abundance, while bivalves represent biomass dominant group of organisms. Macrofauna abundance undergo considerable seasonal variations due to development of macroalgae and $O_2$ depletion in the near-bottom water layer and sediment.

### *2.1. Intact Core Collection and Benthic Flux Measurement*

At four sites within the lagoon, eight large (i.d. 8.4 cm, length 30 cm, for flux measurements) and three small cores (i.d. 4.6 cm, length 25 cm, for sediment characterization) were randomly collected in May 2013 by hand corer, covering dominating macrofauna assemblages along environmental gradients (Figure 1). Sediments and water height in the large cores were levelled to 15 and 10 cm, respectively, so that the water volume overlying sediments was nearly 0.5 L. In addition, 80 L of in situ water were collected from each station for core maintenance during transportation, pre-incubation, and incubation. Within 4 hours of sampling, all cores were transferred to the laboratory where they were maintained overnight submerged into four tanks containing in situ water (20 °C). Each core was provided with a Teflon-coated magnetic bar suspended 5 cm above the sediment–water interface and driven by an external magnet rotating at 40 rpm. The magnetic bars ensured water mixing within each core, avoiding sediment resuspension. The water in each tank was also stirred by aquarium pumps in order to maintain $O_2$ saturated conditions during pre-incubation period. The large cores were used to measure benthic metabolism ($O_2$, $TCO_2$ and manganous manganese ($Mn^{2+}$)) and net ammonium ($NH_4{}^+$), combined nitrate and nitrite ($NO_x{}^-$), and soluble reactive phosphorus (SRP) fluxes in the dark. After the overnight pre-incubation, a gas-tight top lid was placed on each core, without gas headspace, and the 4-hour incubation started. The incubation time was set in order to keep $O_2$ within 20% of initial concentration. Initial water samples were taken in triplicate from each tank, whereas final water samples were taken from the water phase of each core [49]. At the beginning and at the end of the incubation a 20 mL aliquot of water was collected from each core, transferred and flushed into 12 mL exetainer (Labco, UK), and fixed with 100 µL of 7 M $ZnCl_2$ for dissolved $O_2$ measurements. Thereafter, three more aliquots of 50 ml were immediately filtered (Whatman GF/F filters) and transferred into scintillation vials and exetainers for nutrient, $TCO_2$, and $Mn^{2+}$ analysis, respectively. Aliquots for $Mn^{2+}$ analyses were acidified with 50 µl of ultra-pure concentrated $HNO_3$. The solute exchange at the sediment–water interface were calculated according to the Equation (1):

$$F_x = \frac{(C_f - C_i) \times V}{A \times t} \quad (1)$$

where $F_x$ (µmol $m^{-2}$ $h^{-1}$) is the flux of the chemical species x, $C_i$ and $C_f$ (µmol $L^{-1}$) are concentrations of chemical species x at the beginning and at the end of incubation, respectively, V (L) is the water volume in the core, A ($m^2$) is the surface of the sediment, and t (h) is incubation time.

Small cores were used to measure sediment properties in the upper layer (5 cm). Sediments were extruded from each core, sliced, and homogenized. After homogenization, 5 mL of sediment subsample was dried at 60 °C for 48 h to determine bulk density and porosity. Thereafter, dried sediment subsamples were analyzed for organic carbon ($C_{org}$) and total nitrogen (TN).

### *2.2. Denitrification Measurement with Isotope Pairing Technique*

After flux measurements, the cores were submerged with the top open for 3 hours in in situ-aerated and well-mixed water. Thereafter, we performed a second incubation targeting the rates of denitrification measurements with isotope pairing technique [50]. This approach allows to measure total denitrification (D14) in and the contribution of denitrification supported by overlaying water $NO_3{}^-$ ($D_w$) and denitrification coupled with nitrification ($D_n$). Briefly, stock solution of 15 mM $^{15}NO_3{}^-$ (98% of $K^{15}NO_3$, Cambridge Isotope Laboratories, MA, USA) was added to the water column of each core to the final concentration of 50 µM. To calculate the isotopic enrichment, water samples

for $NO_3^-$ analysis was collected prior and after to the isotope addition. Thereafter, cores were closed and incubated for 4 hours in the dark as described for nutrient flux measurements. At the end of the incubation, the whole sediment column was carefully slurred and mixed with the water column as bioturbating animals can transport $^{15}NO_3^-$ downward and stimulate nitrification and denitrification in deep layers. A glass syringe containing 50 mL of slurries was transferred to 12 mL exetainers (Labco, UK), allowing abundant overflow and gas bubbles removal and fixed with 200 µL of 7 M $ZnCl_2$ to stop microbial activity. Immediately after the end of this second incubation, sediments from all cores were carefully sieved (0.5 mm mesh size) in order to retrieve and analyze the macrofauna composition, abundance, and biomass.

### *2.3. Laboratory Analysis*

Concentration of dissolved gas ($O_2$, $^{29}N_2$ and $^{30}N_2$) were measured within a week from collection with a membrane inlet mass spectrometer (MIMS, Bay instruments, MD, USA) at Ferrara University [51]. Dissolved inorganic N ($NH_4^+$, $NO_2^-$ and $NO_x^-$) and SRP were measured with a continuous flow analyzer ($San^{++}$, Skalar) using standard colorimetric methods [52]. $NO_3^-$ was calculated as the difference between $NO_x^-$ and $NO_2^-$. Dissolved $Mn^{2+}$ was measured with a Varian atomic absorption at Parma University. $C_{org}$ and TN were analyzed with an element analyzer (Thermo Electron Corporation FlashEA 1112, Thermo Fisher Scientific, Waltham, MA USA). Before measurement, samples were acidified with 1 N HCl in order to remove carbonates.

### *2.4. Multivariate Analysis*

Redundancy analysis (RDA) was used to quantify and test the interactions between the numerically dominant 7 species (explanatory variables), net solute fluxes (total $O_2$ uptake (TOU), $TCO_2$, $NH_4^+$, $NO_2^-$, $NO_x^-$, SRP and $Mn^{2+}$), and denitrification pathways (D14, Dw, and Dn) in the 32 intact cores, collected from the 4 sites. We completed this variation partitioning analysis using Partial-RDA to calculate the contribution of each site to the total variance unexplained by the first RDA approach [53]. According to [54], the total sum of canonical eigenvalues from five different RDA analyses have been used to explain the shared information, the pure effect of macrofauna presence and the pure effect of sites as a percentage of the total inertia. The significance of the environmental variables (axis) was tested against 9000 Monte Carlo permutations. Data on macrofauna communities were tested for normality assumption using the Kolmogorov–Smirnov test, while relationships between abiotic parameters and macroinvertebrate communities examined using linear regression [55].

A one-way analysis of variance (ANOVA) was used to test the significance of site in explaining variation in metabolism, net fluxes, and denitrification pathways. Validity of normality assumption and homogeneity of variance was checked using Shapiro–Wilcoxon and Cochran's test, respectively, and square root transformation was applied for data with significant heteroscedascity. A pair-wise comparison of means using the post-hoc Bonferroni test was performed for significant effects. Hierarchical cluster analysis and multidimensional-scaling (MDS) were performed on pairwise similarities between couples of samples using the Bray–Curtis similarity index in order to determine the macrofauna species complexity between and within sites [56].

All statistical analyses were performed with Brodgar 7.5.5 statistical software package.

## 3. Results

### *3.1. Bottom Water and Sediment Features at the Sampling Sites*

The concentrations of dissolved nutrients displayed strong spatial variability among studied sites and differed by up to one order of magnitude (Table 1). Peak concentrations of $NO_3^-$, SRP, and $TCO_2$ were observed at the brackish site 1 where river enters the lagoon. $NO_3^-$ was the dominating form of dissolved inorganic N at sites 1 and 4, whereas $NH_4^+$ concentrations were higher at sites 2 and 3. Salinity and nutrient content can vary dramatically on a daily basis at all sites due to tidal forcing.

At site 4, we found relatively low salinity and high $NO_3^-$ concentration despite its proximity to the open sea, likely due to the sampling performed at low tide when the station is influenced by freshwater inputs from the Po di Goro.

**Table 1.** Bottom water physico-chemical features and surface sediment (0–5 cm) characteristics at the sampling sites in the Sacca di Goro Lagoon. Averages ± standard error are reported (n = 3).

| PARAMETERS | SITE 1 | SITE 2 | SITE 3 | SITE 4 |
|---|---|---|---|---|
| **Water column** | | | | |
| Temperature (°C) | 21 | 21 | 19 | 19 |
| Salinity (PSU) | 5 | 12 | 12 | 7 |
| $TCO_2$ (mmol $L^{-1}$) | 5.2 ± 0.01 | 3.3 ± 0.01 | 2.6 ± 0.01 | 2.6 ± 0.01 |
| $NH_4^+$ (µmol $L^{-1}$) | 7.1 ± 0.12 | 32.1 ± 0.17 | 31.9 ± 0.17 | 19.1 ± 0.69 |
| $NO_x^-$ (µmol $L^{-1}$) | 114.7 ± 4.45 | 40.8 ± 1.67 | 56.5 ± 2.02 | 52.3 ± 3.93 |
| SRP (µmol $L^{-1}$) | 2.2 ± 0.02 | 0.4 ± 0.01 | 1.1 ± 0.01 | 0.5 ± 0.01 |
| **Sediment** | | | | |
| Type | Clayish mud | Detrital mud | Muddy sand | Fine sand |
| Porosity | 0.85 ± 0.02 | 0.89 ± 0.01 | 0.43 ± 0.01 | 0.50 ± 0.03 |
| Density (g $cm^{-3}$) | 1.16 ± 0.01 | 1.12 ± 0.02 | 1.83 ± 0.02 | 1.78 ± 0.02 |
| $C_{org}$ (%) | 4.02 ± 0.27 | 7.48 ± 0.26 | 1.29 ± 0.14 | 1.42 ± 0.14 |
| TN (%) | 0.34 ± 0.01 | 0.85 ± 0.05 | <0.01 | <0.01 |
| C:N (mass) | 11.8 | 8.8 | – | – |

Sediment characteristics differed substantially among sampling sites reflecting sedimentation of clayish material from terrestrial origin (site 1), organic matter from decaying macroalgae (site 2), biodeposits from clams farming (site 3), and strong flushing (site 4). As a result, sites 1 and 2 were mainly muddy, site 3 consisted of muddy sand, whereas site 4 was mainly sandy (Table 1). Sites 1 and 2 had highest $C_{org}$ and TN content. At these sites, high porosity and low density values suggest high sedimentation rates, limited export, and net accumulation of material. Sediments from sites 1 and 3 appeared heavily bioturbated, with light brown halos surrounding burrows in the upper 3–5 cm, sediments from site 2 appeared black, sulfide smelling, and poorly bioturbated, whereas sediments from site 4 appeared oxidized and without redox discontinuities along the vertical profile.

### *3.2. Benthic Macrofauna*

In total, 17 species or higher order taxa with an average abundance of 82 ± 12 ind. $core^{-1}$ were found after sieving incubated sediment (see the list of species in Electronic Supplementary Materials). Abundance and taxonomic diversity differed greatly among sites (Figure 2) and macrofauna structure was more similar among cores collected within the same site, than among sites (Figure 3).

In site 1 (1264 ± 407 ind. $m^{-2}$), spionids, oligochaetes, and *Monocorophium insidiosum* accounted for 92% of the total macrofauna abundance on average. The site was relatively homogenous in a context of taxonomic composition (Figure 3) and abundance (273–2986 ind. $m^{-2}$, 4–7 taxa $m^{-2}$).

Site 2 (1218 ± 311 ind. $m^{-2}$) had the lowest taxonomic diversity (8 taxa), but similar macrofauna characteristics for individual cores (561–2622 ind. $m^{-2}$, 3–7 taxa $m^{-2}$) compared to site 1. *M. insidiosum*, *Chironomus salinarius* and gammarids contributed to 79% of the total macrofauna (Figure 3), however with considerable variation among replicates (0–1546, 61–485, and 61–864 ind. $core^{-1}$, respectively). Only one replicate (2(6), see Figure 3) was extremely different from the rest in this site with exclusively high 62% relative abundance (909 ind. $core^{-1}$) of *Hydrobia* sp.

Site 3 (2160 ± 263 ind. $m^{-2}$) was relatively homogenous in a context of macrofauna composition and abundance (1410–2895 ind. $m^{-2}$, 5–8 taxa $m^{-2}$) with the highest average number of individuals per core (Figure 2). *M. insidiosum* attained the highest abundance (455–1758 ind. $m^{-2}$, with an average of 970 ± 192 ind. $m^{-2}$) in the context of studied sites (Figure 3) and alone accounted for 45% of the total macrofauna abundance. However, the characteristic species for this site was *V. philippinarum* with relatively consistent abundance of 258–1228 ind. $m^{-2}$ (601 ± 106 ind. $m^{-2}$ in average).

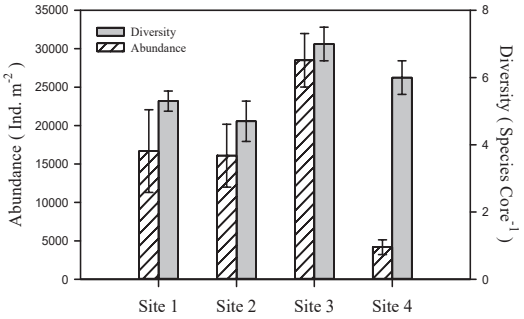


**Figure 2.** Taxonomic diversity (number of species per core) and total abundance of benthic macrofauna (ind. $m^{-2}$) in incubated cores from four study sites in the Sacca di Goro Lagoon (average ± st. error).

Site 4 (316 ± 73 ind. $m^{-2}$) had the lowest abundance of macrofauna individuals (Figure 2) varying between 91 and 667 ind. $m^{-2}$, but the highest overall taxonomic diversity (13 taxa). At the same time, consistency of dominant macrofauna among cores was low (Figure 2). The majority of cores was dominated by spionids (4(3)–4(7), 61–364 ind. $m^{-2}$; Figure 3), but other cores included *V. philippinarum* (4(2), 258 ind. $m^{-2}$), musculista (4(8), 91 ind. $m^{-2}$), and *Caprelidae* (4(1), 45 ind. $m^{-2}$).

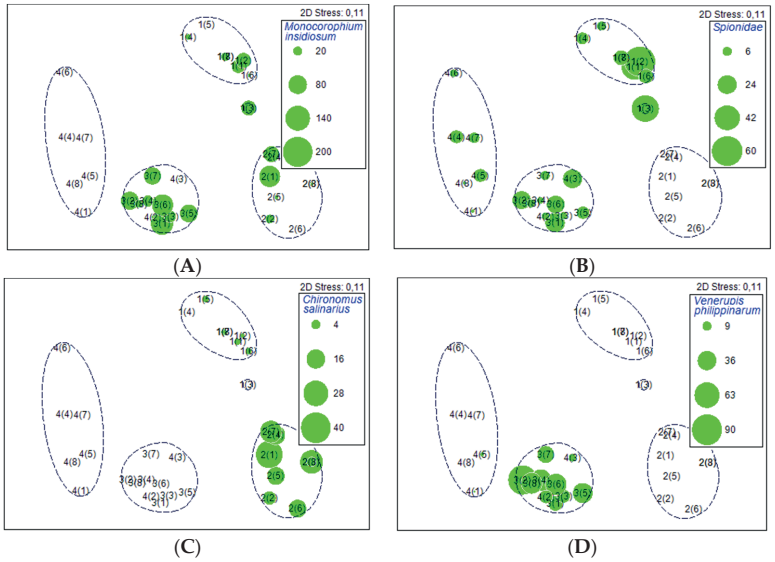


**Figure 3.** MDS plot according to taxonomic composition of dominating benthic macrofauna (presence/absence transformation) in incubated cores: *Monocorophium insidiosum* (**A**), *Spionidae* (**B**), *Chironomus salinarius* (**C**) and *Venerupis philipinarium* (**D**). Labels and brackets refer to site and replicate number correspondingly, and abundance of main macrofauna taxa (diameter of bubbles proportional to the abundance).

### *3.3. Benthic Metabolism and Respiration*

Total $CO_2$ production rates were similar in three out of four sites (3.2 mmol $m^{-2}$ $h^{-1}$ on average), with site 1 as only exception (One-way ANOVA, F = 20.80, P = 0.001) (Figure 4). There $TCO_2$ uptake

dominated likely due to sharp chemical gradients between the high carbonate content of near bottom and pore water (see Table 1). TOU ranged from 0.7 to 8.1 mmol $m^{-2}$ $h^{-1}$ with significant differences among sites (One-way ANOVA, F = 8.308, P = 0.001). Considerably (P < 0.001) higher TOU was measured at sites 2 and 3 in comparison to site 4. A net $Mn^{2+}$ efflux was measured at all sites; in three out of four sites, fluxes were similar with an average rate of 50 µmol $m^{-2}$ $h^{-1}$. Significantly (P < 0.001) higher efflux was found at site 2 (170.18 ± 33 µmol $m^{-2}$ $h^{-1}$). Calculated respiratory quotients (the ratios between $TCO_2$ and TOU) at sites 2, 3, and 4 were close to unity, suggesting the dominance of aerobic metabolism.

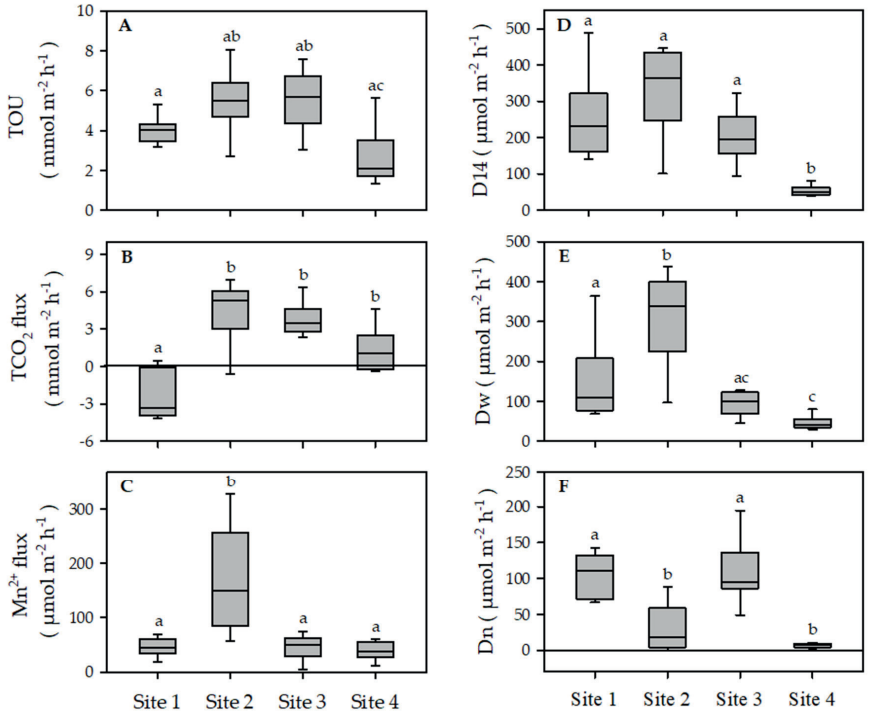


**Figure 4.** Total oxygen uptake (**A**), sediment–water fluxes of total inorganic carbon (**B**), manganous manganese (**C**), total denitrification (**D**) and denitrification of water column $NO_3^-$ (**E**), and coupled nitrification–denitrification (**F**) measured at four study sites in the Sacca di Goro Lagoon (median and percentiles, n = 8). Different letters indicate statistical differences among sites.

At sites 1, 2, and 3, total denitrification rates (D14) were elevated and sustained a relevant portion of total mineralization (10–20%). D14 ranged from 37.9 to 481.0 µmol $m^{-2}$ $h^{-1}$ and differed between sites (One-way ANOVA sqrt transformed, F = 20.12, P < 0.001) (Figure 4). Significantly (P < 0.05) lower rates of D14 (52.3 ± 14.4 µmol $m^{-2}$ $h^{-1}$) were observed at site 4. In other sites, rates of D14 were similar with an average of 263.5 ± 113.2 µmol $m^{-2}$ $h^{-1}$. The relative importance of Dw and Dn to the total rates of denitrification varied among sites (One-way ANOVA sqrt transformed, F = 20.31 and F = 32.03, respectively, P < 0.001), depending on availability of $NO_3^-$ in the water column. At sites 2 and 4, total denitrification was sustained mainly by Dw, which represented from 87% to 90% of $N_2$ production. The share of total denitrification supported by nitrification coupled denitrification was more important at sites 1 (46%) and 2 (40%). The rates of Dw were in the rage of 29.4–437.9 µmol $m^{-2}$ $h^{-1}$ at the studies sites. Significantly (P < 0.05) higher rates (305.1 ± 122.6 µmol $m^{-2}$ $h^{-1}$) of Dw were measured at site

2, while relatively lower rates (45.6 ± 16.5 μmol $m^{-2}$ $h^{-1}$) were measured at sandy sediment at site 4. Dn varied from to 0 to 194.7 μmol $m^{-2}$ $h^{-1}$ with significantly ($P < 0.001$) higher rates at sites 1 and 3 (106.9 ± 36.9 μmol $m^{-2}$ $h^{-1}$) as compared to sites 2 and 4 (18.3 ± 25.3 μmol $m^{-2}$ $h^{-1}$). Denitrification of water column $NO_3^-$ was calculated with the model proposed by Christensen et al. [57] and compared with measured rates. In three out of four sites, theoretical rates overestimate measured rates by a factor 5, while at site 2 predicted (≈430 μmol $m^{-2}$ $h^{-1}$) and measured (≈300 μmol $m^{-2}$ $h^{-1}$) rates were closer.

### *3.4. Benthic Nutrient Fluxes*

Net fluxes of $NH_4^+$ varied from −201.4 to 917.0 μmol $m^{-2}$ $h^{-1}$ and significantly differed among sites (One-way ANOVA, $F = 15.5$, $P = 0.001$) (Figure 5). The highest flux (641.8 ± 215.6 μmol N $m^{-2}$ $h^{-1}$) was measured at site 2 ($P < 0.001$). The negative net $NH_4^+$ fluxes were observed only at site 1 where it was significantly ($P < 0.05$) lower in comparison to sites 2 and 4. On the contrary, $NO_x$ fluxes were erratic without clear patterns among sites (One-way ANOVA, $F = 1.9$, $P = 0.147$). At site 1, it has been measured the higher efflux of $NO_x^-$ (562.8.3 ± 225 μmol N $m^{-2}$ $h^{-1}$) which coincided with uptake of $NH_4^+$ (−30.5 ± 44 μmol $m^{-2}$ $h^{-1}$), suggesting large nitrification rates. Denitrification efficiency (DE), which is the ratio between dinitrogen ($N_2$) flux and the sum of $N_2$ and dissolved inorganic N ($NH_4^+$ + $NO_x^-$) effluxes, varied between 27.4% (at site 4) and 63.4% (at site 2). Sites 1 and 3 had a comparable DE (≈46–49%). The flux of SRP was in the range of −26.6–57.9 μmol $m^{-2}$ $h^{-1}$ without any significant (One-way ANOVA, $P > 0.05$) difference among sites. Overall, sediments were net sources of SRP except sea exposed sand at site 4.

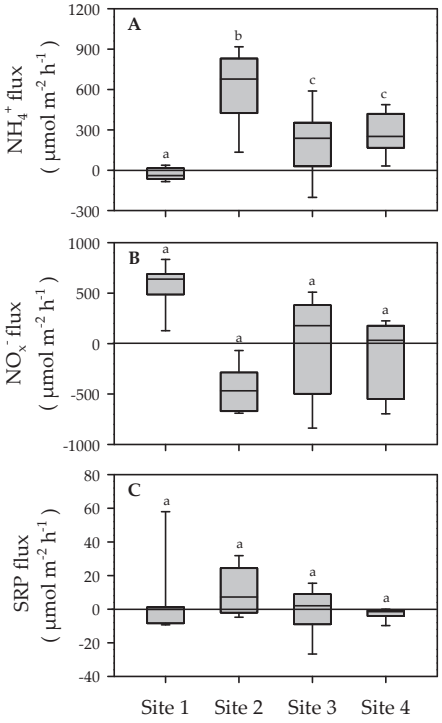


**Figure 5.** Sediment–water fluxes of ammonium (**A**), combined nitrite and nitrate (**B**), and soluble reactive phosphorus (**C**) measured at four study sites in the Sacca di Goro Lagoon (median and percentiles, n = 8). Different letters indicate statistical differences among sites.

*3.5. Macrofauna Community, Functional Traits and Benthic Ecosystem Functioning*

In order to assess the effect of benthic invertebrates on microbial processes and fluxes at the water–sediment interface, an RDA model was used. Macrofauna species were considered as explanatory variables whereas fluxes and metabolic pathways were used as response variables. The model was significant (Monte Carlo significance test of all canonical axes, F = 5.6780, P = 0.0001). The total amount of variation explained in the response variables equaled 66% (sum of all canonical eigenvalues). The first two axes (38.8% and 13.8%) were extracted as independent variables from the RDA. Taken together, they accounted for 79.5% of the total explained variance in biogeochemical parameters (response variables). *C. salinarium* contributed most to the variation (15.2%), followed by *V. philippinarum* (6.2%) and gammarids (2.4%). The first axis was mainly correlated to chironomid larvae, gammarids, and corophiids, and was strongly but negatively correlated to spionids. The second axis was positively correlated with spionids and oligochaetes and negatively correlated to caprelids. Most of found explanatory–response variable relationships were ecologically reasonable and with a direct ecological background (see the discussion section).

The triplot (Figure 6) allows to distinguish specific correlation, between site-specific macrofauna and particular metabolic pathways and fluxes measured in the four studied areas. Benthic net fluxes of $Mn^{2+}$, $NH_4^+$, and $TCO_2$ were strongly associated to the benthic activity of gammarids and *C. salinarius*. Denitrification (D14, Dw, and Dn) were best explained by a model when including *V. philippinarum*, *M. insidiosum*, and caprelids (with an opposite effect) presence. The presence of *V. philippinarum* and *M. insidiosum* were also positively correlated to SRP flux and TOU. Oligochaetes, *Neanthes*, and spionids negatively affected $TCO_2$ and $NH_4^+$ fluxes.

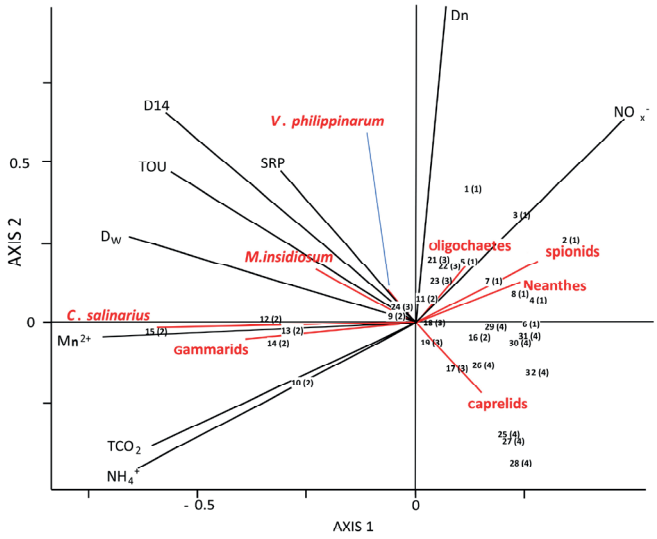


**Figure 6.** Distance triplot of redundancy analysis (RDA) on fluxes (TOU; $TCO_2$, $Mn^{2+}$, $NH_4^+$, $NO_X^-$, and SRP) and processes (denitrification—D14, Dw, and Dn) in the Sacca di Goro Lagoon, using the eight most representative benthic macrofauna as explanatory variables (*V. philippinarum*, *Neanthes*, spionids, oligochaetes, *C. salinarius*, *M. insidosum*, gammarids, and caprelids). Numbers (1–32) indicate single cores collected from the four sampling sites. The thick arrows are the vectors of the explanatory variables. The projection of any sample onto arrows approximates the measured value in that sample.

Partial-RDA analysis (triplot not shown) was used to disentangle the pure effect of the 8 species of macrofauna (X1) from the possible effect given from the four sites (X2) taken in consideration

(macrofauna-location portioning). Variation partitioning analysis of benthic processes explained by functional diversity and sites (as nominal explanatory variables) indicates a strong synergistic effect on the total variance explanation. The total amount of variation explained in the response variables equaled 44% (sum of all canonical eigenvalue) (Table 2). Variable species alone explained (X1 | X2) 19% of benthic flux variation, whereas sites differences (X2 | X1) alone explained only the 8%; the sum of effects explained the 47% of the variation.

**Table 2.** Summary table showing variation partitioning and calculation of benthic fluxes explained by macrofauna species, sites, and joined effect. Explained variance can be portioned in [A], [B], and [C]. [A] = percentage of variability merely explained by macrofauna; [C] = percentage of variability merely explained by location; [B] = percentage of variability explained by a synergistic effect.

| Predictors and Covariables | Df | Sum of All Canonical Eigenvalues (%) |
|---|---|---|
| (Species effect ∪ Site effect) = [A+B+C] | 9 | 74 |
| Species \| Site effect = [A] (Site effect as covariable) | 6 | 19 |
| Site effect \| species = [C] (Species as covariable) | 3 | 8 |
| Species effect ∩ Site effect = [(Species effect ∪ Site effect) – (Species effect \| Site effect) − (Site effect \| Species effect)] = [B] | 0 | 74 – (19) – (8) = 47 |
| Residuals = [Total inertia – (Species effect ∪ Site effect)] | 0 | 100 – 74 = 26 |

## 4. Discussion

While in most deep aquatic ecosystems, meiofauna and microbial communities are drivers of benthic processes, in coastal estuarine systems macrofauna play a major role in organic matter mineralization and nutrient cycling [8,58–60]. The analysis of macrofauna diversity, abundance, functional role, and distribution is therefore central to understand coastal lagoon functioning [16,24]. The latter can be defined as the capacity of sediments to process organic matter inputs, avoiding excess carbon accumulation and resulting in fast nutrient turnover. Excluding the autotrophic component, benthic functioning in an eutrophic ecosystem with large N excess can also be defined as the degree of coupling of microbial processes (e.g., ammonification, nitrification, and denitrification) that results in net N loss and limited regeneration to the water column [61,62]. In present study, we did not characterize organic matter input to sediments and we cannot calculate the balance between input and output terms; however, we can reconstruct some paths of benthic N cycle and speculate on the role of macrofauna as regulator of microbially mediated N-processes.

### *4.1. Physico-Chemical Zonation and Macrofauna Composition*

The four studied dominating areas of the lagoon revealed to be distinct environments, differing in sediment type, organic matter content, average salinity, and bottom water nutrient concentration (Table 1). Despite its small size, the Sacca di Goro Lagoon has a marked zonation that depends on the freshwater inputs, sea–lagoon water exchange, the extent of primary production, and deposition rates [43,44,47,48]. The observed difference in a number of bottom macrofauna species and functional traits (Figure 2) is most likely related to these environmental differences.

Freshwater input is the main driver of benthic ecosystem functioning in site 1, where nutrient loads affect rates and direction of the benthic solute fluxes. Intensive discharge transports sediment-bound nutrients (particularly phosphorus and silica), as well as dissolved inorganic nutrients such as $NO_3^-$, due to its high solubility and mobility. Due to shallow depths, most allochthonous particulate matter settles on surface sediment, where it is mineralized to inorganic nutrients. Dissolved nutrients from bottom water accumulate in pore water due to gradient driven diffusive transport, resulting in deep penetration of electron acceptors (e.g., $NO_3^-$) [40]. Due to tidal exchange and freshwater flushing from the Po di Volano River, a major portion of macrofauna biomass mainly consists of sediment dwelling

opportunistic species, such as spionids, oligochaetes, and *M. insidiosum*, tolerant to high organic matter content and lower $O_2$ concentration [63].

In the sheltered accumulation area (site 2), mainly autochthonous organic matter inputs (macroalgae and phytoplankton) are delivered by dominating hydrodynamic circulation. Such conditions favor the development of a thick layer of organic matter on the surface sediment and limits $O_2$ or $NO_3^-$ sediment penetration [38]. As a consequence, the dominant pathways of anaerobic respiration lead to the accumulation of reduced metabolites such as sulfides and $NH_4^+$, which are toxic for living macrofauna. This may explain the lack of macroinvertebrates other than *M. insidiosum* and *C. salinarius.*

Site 3 is located within the clams farming area. Our findings are consistent with results from previous surveys [48,64,65], which show that the most diverse benthic community is generally found in the central–western part of the lagoon. Clams farming operations may lead to a moderate or high disturbance of benthic community [36], setting to zero the competitive advantages of potentially dominant species. High densities of filter-feeders produce changes in sediment physico-chemical characteristics, as organic enrichment that may favor the proliferation of small-sized tolerant macrofauna [66,67]. High clams density results also in large stimulation of $O_2$ and $NH_4^+$ fluxes due to combination of respiration, labile particles mineralization, and direct excretion [29,46].

Site 4 is a sandy area exposed to tidal forcing and strong currents that prevent organic matter accumulation and restrict macrofauna distribution [68,69]. We speculate that macrofauna community composition at this marine site is shaped by hydrodynamic condition and sedimentary features. The limited organic pool in the sediments and the low concentrations of suspended matter in the water column result in a diversified benthic community but with low densities (Figure 2). Spionids are abundant at this site as this taxa prefers sandy substratum and tolerates wide salinity variations [65,70].

### *4.2. Macrofauna Affect Benthic Metabolim and N-Cyling across Sites*

In the Sacca di Goro Lagoon, as in other eutrophic estuarine systems, low $O_2$ levels and anoxic crises are frequent and may affect the whole system functioning [37,71]. Due to the shallowness of the lagoon, $O_2$ dynamics in the water column is primarily driven by benthic metabolism. Although rates of benthic $O_2$ uptake were similar at the sites 1, 2, and 3 (5 mmol $m^{-2}$ $h^{-1}$ on average), we speculate that mechanisms underlying oxygen consumption were different. At the first two sites, re-oxidation of anaerobic metabolism end-products (e.g., free sulfides, ferrous iron, or manganous manganese) was likely an important sink for $O_2$, whereas at the other sites, respiration by benthic organism was the dominant oxygen-consuming path.

We tentatively reconstructed the benthic N-cycling from combined measured fluxes and calculated processes at each site. Benthic N-cycling consists of multiple processes, mostly mediated by bacteria, but strongly influenced by the presence and the activity of macrofauna. We therefore tried to explain at each site how macrofauna community drives N paths (Figure 7).

At site 1, a high efflux of $NO_3^-$ suggests high rates of $NH_4^+$ oxidation via nitrification which is tightly coupled to ammonification. This idea is supported by large $NO_3^-$ efflux and negligible $NH_4^+$ release from sediments. A major percentage of $NO_3^-$ (86%) produced via sediment nitrification accumulates in near-bottom water and only a small amount diffuses to anoxic sediments where it is denitrified (12%). The results from the RDA stress the strong positive relationship among burrowing organism abundance at the site 1 and the measured $TCO_2$, $NH_4^+$, and $NO_x^-$ fluxes (Figure 5). Abundant *M. insidiosum* and polychaete populations are able to create a dense network of burrows which extend the surface for solute exchange and the volume of oxic niches, stimulating bacterial activity. Furthermore, continuous ventilation of burrows by *M. insidiosum* supports nitrification process that requires $O_2$ and $CO_2$ [35]. Therefore, in this type of sediments, $CO_2$ production via mineralization is likely offset by high nitrifiers assimilation. We also expect synergetic effect of spionids and *M. insidiosum* on nitrification. Spionids, which typically burrow deeper than *M. insidiosum*, enhance $NH_4^+$ mobilization from deeper sediment layers to the surface horizons [72,73]. Here, $NH_4^+$

is oxidized to $NO_3^-$ in burrows of *M. insidiosum*. Moares et al. [35] showed that the production of $NO_3^-$ is significantly correlated with the abundance of *M. insidiosum*. As a result, nitrification largely prevails over denitrification in this type of habitat. However, low denitrification efficiency suggests that nitrification and denitrification are spatially uncoupled processes because of active burrow ventilation and the thickness of the oxic zone. The diffusion path of $NO_3^-$ from the water column to the anoxic denitrification zone is so thick that Dw is significantly reduced, despite high water column $NO_3^-$ concentration. Modelled rates of Dw are in fact much higher than measured rates (829 versus 153 μmol $m^{-2}$ $h^{-1}$ [57]). We calculated that only 6% of the water column $NO_3^-$ pool (assuming 1 m depth) is denitrified per day.

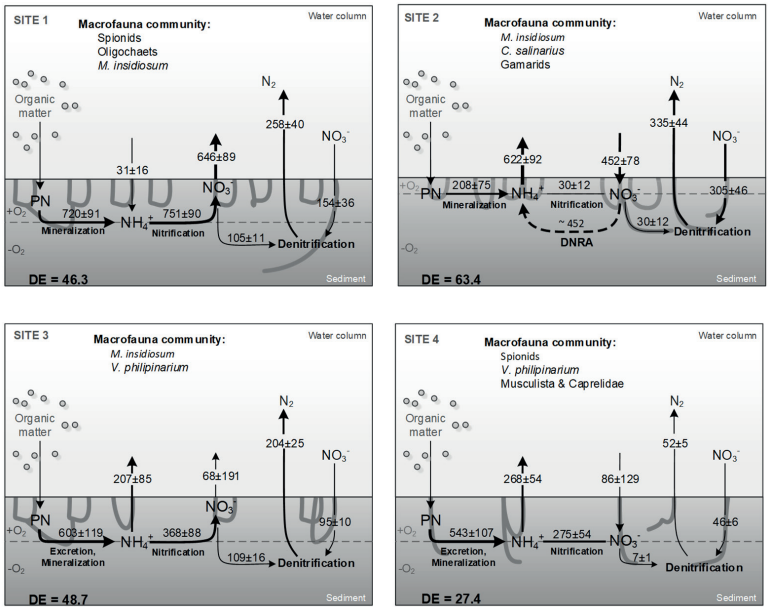


**Figure 7.** Flow scheme for benthic nitrogen (N) pathways for each site, which were calculated from combinations of measured fluxes and process rates. The mineralization of organic N was estimated as the sum of net $NH_4^+$ and $NO_3^-$ efflux and nitrification coupled to denitrification (Dn); nitrification rates were estimated as the sum of the net $NO_3^-$ efflux and Dn; net $NO_3^-$ efflux is the sum of measured $NO_3^-$ efflux and denitrification based on bottom water $NO_3^-$. The mean rates (average ± st. error) are expressed on an hourly basis per unit of sediment surface (μmol $m^{-2}$ $d^{-1}$). Dissimilative $NO_3^-$ reduction to $NH_4^+$ (DNRA) was not measured but is represented as a dotted line, a likely occurring path at site 2. Denitrification efficiency (DE) is the ratio between dinitrogen ($N_2$) flux and the sum of $N_2$ and dissolved inorganic N ($NH_4^+$+$NO_x^-$) effluxes.

At site 2, the sedimentary environment was chemically reduced as suggested by black color, smell of sulfides, and high $Mn^{2+}$ efflux. This indicates the dominance of anaerobic respiration (e.g., metal reduction) with subsequent accumulation of $Fe^{2+}$, $Mn^{2+}$ and $H_2S$ in bottom water. Measured high concentration of $Mn^{2+}$ suggests an intensive manganese hydrooxides recycling. In coastal sediments, manganese hydrooxides can be either reduced to $Mn^{2+}$ through microbial respiration or by the chemical oxidation of reduced iron and sulfur species [74,75]. In such $O_2$-poor sediments, the reduction of metal hydrooxides with subsequent SRP mobilization to pore and adjacent bottom water is frequently observed [76]. Macrofauna community in this part of the lagoon mostly consists of small-sized surface burrowers such as *C. salinarius*, *M. insidiosum*, and gammarids. Ventilation of burrows by these

specimens can also enhance exchange of microbial metabolism end-products. RDA analysis shows macrofauna functional traits being related to the SRP, $Mn^{2+}$, and $NH_4^+$ release from sediment and N loss via denitrification (Dw). Previous studies showed minor or negligible effect of these macrofauna taxa on nitrification and subsequently its coupling to denitrification in the burrow walls [77]. At site 2, ammonification is the dominant pathway within the N cycle. Relatively large amount of regenerated $NH_4^+$ (94%) accumulates in bottom water. The thin oxic sediment layer constrains nitrification process in the upper sediment layer, and thus rates are uncoupled to those of ammonification. We speculate that the limited $O_2$ penetration results in a short path to get to the anoxic layer, and as a result denitrification is mainly fueled by water column $NO_3^-$ (92% of $N_2$ production) which reduces up to 18% of the nitrate pool in water. We also speculate that at this site $NO_3^-$ reduction to $NH_4^+$ could play an important role as nitrate sink and ammonium source. The comparison between macrofauna effects at sites 1 and 2 reveals that *M. insidiosum* can produce contrasting effects depending on site-specific features. If a fraction of the $NH_4^+$ flux measured at the site 2 is driven by dissimilative $NO_3^-$ reduction to $NH_4^+$ (DNRA) rather than organic matter mineralization, part of the $NO_3^-$ flux to sediment could be recycled as $NH_4^+$ (Figure 7). The specific conditions of site 2 result in much higher denitrification efficiency.

We assume that at site 3, where high macrofauna biomass was observed, $O_2$ is respired by benthic macrofauna itself. According to [46], *V. philippinarum*, which is abundant at this site, contributes to a significant part of $O_2$ uptake and $TCO_2$ production. In addition, clams alone can excrete SRP and such direct excretion may account for up to 90% of the net flux measured during summer [39,78]. SRP fluxes can be sustained also by deposition and subsequent mineralization of feces [76]. Limited SRP fluxes in clams farming areas can be surprising, but the co-presence of clams and *M. insidiosum* may provide a reasonable explanation. SRP directly or indirectly produced by clams might in fact be transported within *M. insidosum* burrows during its ventilation and trapped through co-precipitation with metal hydrooxides. The same clams–amphipods association may result in coupled rates of ammonification, nitrification, and denitrification, promoting N-loss.

Our results suggest that clams also influence N-cycling in the followings ways: (i) directly by sediment bioturbation and (ii) indirectly by filtration and biodeposition of organic matter from water column to bottom sediments. *V. philippinarum* can be considered as shallow-burrower rather than deep-burrower but its bioturbation activity is strongly correlated to denitrification process (Dn in particular, see Figure 7). At this site, nearly 61% of sedimentary $NH_4^+$ is oxidized to $NO_3^-$ within the sediment and subsequently almost the entire pool is denitrified to $N_2$. Approximately 62% of the total denitrification comes from the coupled nitrification–denitrification process. The direct stimulation of N processes, in particular nitrification, is likely due to additional habitat provided by burrow walls. The syphons of *V. philippinarum* provide a microoxic environment within sediments, which may support the activity of nitrifiers [39]. In addition, clams can excrete relevant amount of $NH_4^+$, which accounts up to 80% of overall sediment N-regeneration [46]. A large part of $NH_4^+$ is nitrified while the remaining fraction is released to bottom water. The high abundance of *M. insidiosum* at site 3 can support also nitrification and can be explained by the presence of clams, as these filter-feeders may provide high quality food for the amphipod [79].

The lowest respiration with respect to $O_2$ uptake and $TCO_2$ production was found at site 4, where sediment is poor in organic matter and macrofauna is characterized by low biomass and by high number of functional traits: filter-feeders, deep burrowers, and scrapers. The balanced $O_2$ uptake and $TCO_2$ production suggest dominating aerobic respiration. In this hydrodynamic active area, sediment is always a sink for SRP due to high $O_2$ penetration, which enhance buffer capacity, and microphytobenthos uptake [80]. Continuous current exposure and sediment erosion prevent organic matter accumulation and enhance pore water advection. Hence, low mineralization and end-product oxidation as $NH_4^+$ via nitrification is expected. Surprisingly, we observed a relatively higher $NH_4^+$ efflux in comparison to site 3, where sediment also is poor in organic matter but host clams. At this site, nearly 98% of sedimentary $NH_4^+$ is released to the bottom water while negligible amount is oxidized to $NO_3^-$ within the sediment, which later is completely denitrified to $N_2$. Since sediment is poor in

organic matter, it is difficult to explain $NH_4^+$ efflux by mineralization. Closer inspection of each single core suggests that the highest $NH_4^+$ effluxes largely coincided with presence of musculista and partly of V. *philippinarum*, while spionids have a less evident effect. It seems likely that $NH_4^+$ excreted by musculista is not directly incorporated into the benthic microbial communities at such sites under high hydrodynamic activity. Approximately 88% of the total denitrification is fueled by $NO_3^-$ from overlaying water, however, the denitrification rates were the lowest as compared to the other sites. Due to high $NH_4^+$ efflux and low denitrification rates, denitrification efficiency was low.

### *4.3. Conclusions*

Predicting the effect of macrofauna diversity on benthic functioning can be critically important, given present threats to biological diversity such as habitat destruction, loss of species, overharvesting, and climate change. In the present work, we demonstrate that the relationships between biodiversity and benthic functioning can be tackled with multiple approaches on natural, undisturbed sediments collected along estuarine gradients. Multivariate analysis performed on single cores, each with a specific community and metabolic rates, provide a statistical evidence on how macrofaunal species drive sedimentary processes. The analysis of benthic N-cycling conducted at a larger scale, grouping cores collected from the same site, allows to analyze how the interactions among different macrofauna groups determine different net effects on multiple microbial process. The combination of results from the two approaches allows in turn to speculate on underlying, macrofauna-mediated processes. Our results suggest the occurrence of complex relationships among the physical environment, the microbial communities and the macrofauna groups, exemplified by very different, macrofauna-community-dependent N paths. Such complex relationships cannot be evaluated in reconstructed sediment with single species, which provide very partial understanding of what happens in nature. Surface and deep burrowing organisms provide key ecosystem services in eutrophic shallow lagoon as they favor the oxidation of anaerobic path end-products, maintain active geochemical buffers (e.g., against sulfides or SRP release), and prevent excess decrease of sediment redox, resulting in negative feedbacks for macrofauna diversity. Cultivated clams are generally considered as nutrient sources due to their elevated excretion rates and biodeposits, but our results suggest that the co-occurrence of bivalves and burrowing organisms may promote nitrate loss via denitrification coupled to nitrification. Our approach can be extended to manipulative studies in which different macrofauna species can be added or removed in order to test specific hypotheses.

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# PAPER II

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The Effect of Chironomid Larvae on Nitrogen Cycling and Microbial Communities in Soft Sediments

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water

MDPI

*Article*

# The Effect of Chironomid Larvae on Nitrogen Cycling and Microbial Communities in Soft Sediments

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**Abstract:** The combination of biogeochemical methods and molecular techniques has the potential to uncover the black-box of the nitrogen (N) cycle in bioturbated sediments. Advanced biogeochemical methods allow the quantification of the process rates of different microbial processes, whereas molecular tools allow the analysis of microbial diversity (16S rRNA metabarcoding) and activity (marker genes and transcripts) in biogeochemical hot-spots such as the burrow wall or macrofauna guts. By combining biogeochemical and molecular techniques, we analyzed the role of tube-dwelling *Chironomus plumosus* (Insecta, Diptera) larvae on nitrification and nitrate reduction processes in a laboratory experiment with reconstructed sediments. We hypothesized that chironomid larvae stimulate these processes and host bacteria actively involved in N-cycling. Our results suggest that chironomid larvae significantly enhance the recycling of ammonium (80.5 ± 48.7 μmol $m^{-2}$ $h^{-1}$) and the production of dinitrogen (420.2 ± 21.4 μmol $m^{-2}$ $h^{-1}$) via coupled nitrification–denitrification and the consumption of water column nitrates. Besides creating oxygen microniches in ammonium-rich subsurface sediments via burrow digging and ventilation, chironomid larvae serve as hot-spots of microbial communities involved in N-cycling. The quantification of functional genes showed a significantly higher potential for microbial denitrification and nitrate ammonification in larvae as compared to surrounding sediments. Future studies may further scrutinize N transformation rates associated with intimate macrofaunal–bacteria associations.

**Keywords:** chironomid larvae; nitrogen; microbial community; 16S rRNA; functional genes; denitrification; sediment

## 1. Introduction

Shallow estuarine systems are efficient coastal filters and biogeochemical reactors which regulate organic matter and nutrient loads from land to the sea [1,2]. Their elevated retention of organic sediment fuels high rates of benthic heterotrophic activity and primary production [3]. Such energy and matter flows have positive feedbacks for benthic biodiversity and for the network of ecological interactions that connect physical and biological compartments [3,4]. Thus, estuarine nitrogen (N) cycling is a paradigmatic example of a set of biogeochemical transformations connecting micro- and macro-organisms, modulated by physical environments and undergoing complex regulation and feedbacks [5–7]. Within the estuarine N cycle, permanent N removal via dissimilative processes such as denitrification is of particular interest, as well as undesired dissimilative nitrate reduction to ammonium (DNRA)–N recycling, which is favoured under eutrophic conditions [8,9]. Processes leading to permanent N removal counteract the excessive loads of reactive N to coastal areas, resulting from anthropogenic activities such as agriculture and animal farming and inducing eutrophication, loss of biodiversity and the deterioration of ecosystem health [10,11].

Microbially-mediated N transformations in estuarine sediments are supported and stimulated by a range of macrofauna-related processes, collectively defined as bioturbation [3,12,13]. The multiple paths by which metabolic or feeding strategies and behavioural features of macrofauna affect the physical and biological environment and N-cycling have been scrutinized in many experimental studies. These demonstrated that macrofauna may directly alter inorganic N concentrations as well as the quality and quantity of organic N via respiration, excretion and biodeposition activities [14–21]. Biodeposits from filter-feeding macrofauna, for example, may increase denitrification and ammonification rates [19,22]. Macrofauna are demonstrated to also produce indirect effects on N biogeochemistry via sediment reworking and burrow construction, ventilation or bioirrigation, which may stimulate coupled nitrification–denitrification [14,15,23,24].

Therefore, being involved in N-cycling, macrofauna may facilitate the growth of primary producers or smooth their competition with bacteria for N, resulting in simultaneous high uptake and high loss via denitrification [7,25,26]. Macrofauna may also locally affect microbial communities, creating specific niches such as shallow or deep burrow lining, where peculiar microbiomes develop along the chemical gradients [17,20,27]. Fascinating but less explored is the role of macrofauna as microbial community elevators or transporters, as macrofauna continuously migrate vertically and horizontally across sediments, exposing their associated microbiome to different chemical gradients (e.g., from oxygen ($O_2$) and nitrate ($NO_3^-$)-rich to anoxic, ammonium ($NH_4^+$)-rich). Even less is known about microbial activities occurring within the guts, gills or intestines of macrofauna [28,29]. Substantial densities of symbiotic microbes (e.g., N-fixers, sulphide oxidizers) were detected, for example, in the gills of lucinid bivalves [30]. Even though intimate microbe–macrofauna interactions are likely widespread, they are largely understudied due to methodological limitations or the oversimplification of experimental approaches. Therefore, the cumulative effects of macrofauna and their associated microbiomes are rarely accounted for in biogeochemical studies, and their actual magnitude may be underestimated when assessing ecosystem-wide processes.

The advances of in situ and laboratory approaches based on the use of isotopic tracers have allowed more accurate quantitative assessment of multiple microbial N transformations and their regulation, including ammonification, nitrification and nitrate reduction processes [7–9,16,21]. Integrating biogeochemical approaches with novel molecular tools (such as the metabarcoding of microbial biodiversity or quantification of target functional genes) enables the detailed exploration of macrofaunal–bacterial interactions and of their wider role in benthic ecosystem functioning [18,20,29].

In this study, we analysed the effects of sediment-dwelling *Chironomus plumosus* (Insecta, Diptera) larvae on benthic N transformations combining biogeochemical and functional genomic measurements under controlled conditions. We targeted chironomid larvae—an understudied group compared to other macrofauna—for multiple reasons. They may attain large densities and dominate in organic-rich, chemically reduced sediments, where they may create steep redox gradients across

their burrows [12]. They are suspension feeders and pump large volumes of $O_2$ and $NO_3^-$-rich water into sediments [31]. Therefore, we hypothesized a substantial stimulation of N-cycling in sediments inhabited by chironomid larvae, as they may alter the sediment's physical structure and host bacteria that catalyse N transformations. To test this hypothesis, we measured inorganic N fluxes in bioturbated sediments in combination with the 16S rRNA metabarcoding of bacterial communities isolated from (1) subsurface, anoxic sediments, (2) burrow wall sediments, and (3) chironomid larvae. We then quantified the representative marker genes involved in N-cycling and their transcripts to better understand whether chironomid larvae and their associated microbiome may contribute to nitrification and/or to $NO_3^-$ reduction.

## 2. Materials and Methods

### *2.1. Experimental Setup*

In July 2018, muddy sediments with high organic carbon and total N contents (12% and 1.8%, respectively; [2]), water, and chironomid larvae (*C. plumosus*) were collected in the Lithuanian part of the Curonian Lagoon (55°17′51.7″ N, 21°00′36.0″ E) at a water depth of 3 m. In the laboratory, approximately 15 L of sediment was sieved through a 0.5 mm mesh to remove large debris, chironomid larvae and other occasional macrofauna, and gently mixed to a slurry. The homogenized sediment (median grain size = 0.032 µm) was transferred into 10 bottom-capped Plexiglass liners (height = 30 cm, inner diameter = 8 cm) to reconstruct a 10 cm sediment layer in each microcosm. Thereafter, all microcosms were carefully filled with unfiltered water from the sampling site.

Two treatments (five replicates each) were applied to the microcosm cores: sediments without macrofauna (control) and sediments with nine added chironomid larvae per core (corresponding to 1800 ind. $m^{-2}$). All chironomid larvae added to the cores immediately burrowed in the sediment down to 3–5 cm depth. In each core, a magnetic bar was fixed 10 cm above the sediment surface to stir the water while avoiding sediment resuspension. Then, all the cores were submerged into a 200 L tank, filled with aerated and well-mixed lagoon water (salinity = 0.2, pH = 8.3, dissolved inorganic N conc. ~2 µmol $L^{-1}$) maintained at ambient temperature (16 ± 0.2 °C). The tank was equipped with two central magnets rotating at 40 rpm, driving the magnet bars inside the cores. Thus, water exchange with the tank was ensured to avoid water column stratification inside the cores and regulate oxygenation. The cores were pre–incubated in the dark for 14 days to attain (1) stable vertical and horizontal chemical gradients after sediment sieving and homogenisation and chironomid larvae addition, and (2) stable bacterial communities within the sediment and along the burrows' walls [32]. During the pre-incubation period, all microcosms were regularly checked for the development of light brown halos along chironomid larvae burrows. About 30% of the tank water was renewed every 2 days to maintain suspended matter, nutrient concentrations and chemical gradients across the sediment–water interface close to in situ conditions. A scheme of the experimental set up is provided in Figure 1.

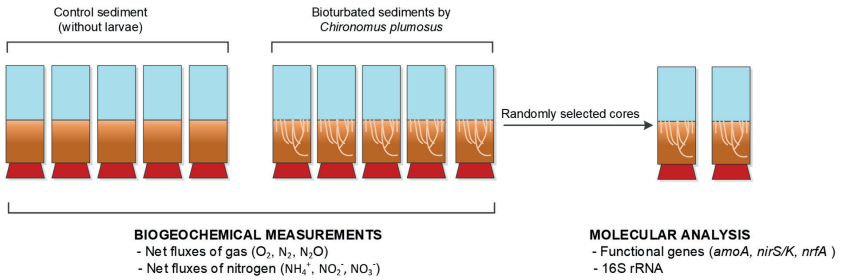


**Figure 1.** Scheme of the experimental set up.

### 2.2. Benthic Flux Measurement

After 14 days of pre-incubation, benthic fluxes of dissolved gas ($O_2$, $N_2$, $N_2O$) and inorganic N ($NH_4^+$, $NO_2^-$ and $NO_3^-$) were measured in all microcosms during 5 h incubation in dark [33]. Measurements were taken through a gas-tight top lid equipped with an optical sensor spots (PyroScience GmbH, Aachen, Germany) and a sampling port. The incubation was restricted to 5 h to keep oxygen within 20–30% of the initial concentration. At the beginning and at the end of the incubation, 40 mL water samples were collected, with two aliquots immediately transferred into 12 mL exetainers (Labco Limited, Lampeter, UK) and fixed with 7 M $ZnCl_2$ for $O_2$, $N_2$, and $N_2O$ measurements. A 10 mL aliquot was filtered (Frisanette GF/F filters), transferred into a plastic test tube and frozen immediately (−20 °C) for later inorganic N analyses. The solute exchange at the sediment–water interface was calculated as follows:

$$F_x = \frac{(C_f - C_i) \times V}{A \times t} \quad (1)$$

where $F_x$ (μmol $m^{-2}$ $h^{-1}$) is the flux of the chemical species $x$, $C_f$ and $C_i$ (μmol $L^{-1}$) are the final and initial concentrations of the chemical species $x$, respectively, $V$ (L) is the water volume in a core, $A$ ($m^2$) is the surface area of the core sediments, and $t$ (h) is the incubation time.

Dissolved $O_2$ and $N_2$ were measured as $O_2$:Ar and $N_2$:Ar ratios by membrane inlet mass spectrometry (MIMS, Bay Instruments, Maryland, USA) at Ferrara University, Ferrara, Italy [34]. Ratios were multiplied by theoretical Ar concentration at experimental water temperature and salinity. Nitrous oxide ($N_2O$) concentrations were determined by headspace analysis on a Thermo Scientific Gas Bench-Precon-IRMS system at UC DAVIS the Stable Isotope Facility, California, USA. Dissolved $NH_4^+$, $NO_2^-$ and $NO_3^-$ were measured with a continuous flow analyzer (San$^{++}$,Skalar Analitical B.V., Breda, The Netherlands ) using standard colorimetric methods [35].

### 2.3. Oxygen Consumption by Individual Chironomid Larvae

In parallel to sediment core incubations, individual chironomid larvae were incubated in 0.22 μm filtered water to assess the $O_2$ consumption associated with the animals alone. Briefly, three individuals collected from the same location as above were placed in a Plexiglass chamber (total volume 227 mL) which was partly filled (41 mL) with glass beads (diameter 200–300 μm), and the rest filled with water (186 mL). For this experiment, five chambers with animals plus one chamber with filtered water only (control) were set up.

The chambers were sealed without including any air bubbles and were incubated for approximately 16 h in the dark at in situ temperature. A stirring magnet was placed in each chamber, allowing continuous water mixing during incubation. The concentrations of $O_2$ in the water were monitored before and after incubation by a pre-calibrated Clark-type oxygen microelectrode (OX-50, Unisense A/S, Aarhus, Denmark). At the end of the incubation, larvae were recovered and weighed. In this experiment, the chironomid larvae wet weight was 0.030 ± 0.003 g (mean ± st. dev.). Oxygen consumption was then calculated as a function of animal wet weight (g WW), according to Bonaglia et al. [36]. The $O_2$ consumption rates in the experimental chambers with chironomid larvae were corrected for the minimal $O_2$ consumption in the chamber with filtered water only.

### 2.4. Nucleic Acid Extraction

At the end of the incubation, sediments for molecular analyses were subsampled from the two randomly selected bioturbated cores (see Figure 1) by collecting approximately 1.5–2 g of subsurface (3–5 cm depth) anoxic sediment and sediments along the burrow wall with a sterile spatula. Anoxic sediments and sediment around burrows were clearly distinguishable by their color, with a light-brown-to-black transition from the oxidized burrow to the outer, chemically reduced sediment. Then, 0.3 g (wet weight) of sediment from each zone was homogenized and equally split for DNA and RNA extractions. Chironomid larvae (n = 6) were retrieved for molecular analyses from sediments

and washed with sterile distilled water (three times). The three types of collected samples—(1) anoxic sediments, (2) oxidized burrow walls, and (3) chironomid larvae—were immediately processed for DNA and RNA extractions. Briefly, DNA was extracted and purified using the QIAamp Fast DNA Stool Mini Kit (Qiagen, Germantown, USA) following the manufacturer's protocol with an amended lysis temperature (temperature was increased to 90 °C to improve bacterial cell rupture).

RNA was extracted with the RNA easy Mini Kit (Qiagen, Germantown, USA) applying additional incubation with lysozyme (20 mg/mL) and mutanolysin (35 µL/1 mL for 90 min at 37 °C). After incubation, 1 mL of Trizol was added and samples was subjected to four cycles of bead beating with glass beads (for 2 min) and rested in the ice (for 3 min) followed by incubation at room temperature (for 5 min). Sediment and cell debris were pelleted by centrifugation at 12,000 rpm for 15 min at 4 °C. The supernatant was transferred to a fresh tube and fixed with 0.2 mL of chloroform, mixed by inversion and left at room temperature for 15 min prior to centrifugation, as described above. The upper aqueous layer containing the RNA was transferred to a new sterile 1.5 mL tube, and RNA cleaning was performed using the RNAeasy Mini Kit (Qiagen, Germantown, USA) according to protocol instructions. The purified DNA and RNA was stored at −80 °C.

### *2.5. Synthesis of cDNA*

Extracted RNA were treated with TURBO DNase (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions. To check whether the RNA sample was free of DNA, a control polymerase chain reaction (PCR) was carried out using universal bacterial primers of 16S rRNA [37]. PCR amplification was undertaken in a total volume of 22 µL using 11 µL of Platinum Green Hot Start 2X Master Mix (Invitrogen, Carlsbad, USA), 0.3 µM of each primer, 1.25 µg $\mu L^{-1}$ of bovine serum albumin (BSA) and 2 µL of template. Thermocycling conditions were 95 °C for 3 min, followed by 30 cycles of 95 °C for 30 s, 54 °C for 30 s, 72 °C for 45 s, and a final extension of 72 °C for 10 min.

Reverse transcription (RT) was performed with a SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, USA) following the manufacturer's instructions. Two negative controls lacking either reverse transcriptase or RNA were included. Control PCRs (same as above) were performed to confirm the transcription to complementary DNA (cDNA) and the negative controls using the product of the RT reaction as a template.

### *2.6. Amplification and Sequencing of 16S rRNA Gene and Bioinformatics*

Partial 16S rRNA gene sequences were amplified from extracted DNA using the primer pair Probio Uni/Probio Rev, targeting the V3 region of the 16S rRNA gene sequence [37]. The amplification of the 16S rRNA gene was verified as previously described by Milani et al. [37]. High-throughput sequencing was performed at the DNA sequencing facility of GenProbio srl (Parma University, Parma, Italy) on an Illumina™ MiSeq according to the protocol previously reported in [37]. Metabarcoding reads recovered by paired-end sequencing were merged using the Illumina MiSeq analysis software under the default settings.

Following sequencing, the fastq files were processed using a custom script based on the QIIME software suite [38]. Paired-end read pairs were assembled to reconstruct the complete Probio Uni/Probio_Rev amplicons. Quality filtering retained sequences had a length between 140 and 400 bp and a mean sequence quality score > 20, while sequences with homopolymers > 7 bp and mismatched primers were discarded. Operational taxonomic units (OTUs) were defined at ≥99% sequence homology using uclust [39], and OTUs with less than 10 sequences across datasets were filtered out. All reads were classified to the lowest possible taxonomic rank using QIIME [38] and a reference dataset from the SILVA 132 database [40].

### *2.7. Quantitative PCR Analyses*

Quantitative polymerase chain reactions (qPCR) were used to quantify the abundance and activity of functional genes involved in N-cycling: (1) genes of haem-containing nitrite reductase (*nirS*),

(2) Cu-containing nitrite reductase (*nirK*), (3) ammonia monooxygenase (*amoA*), and (4) cytochrome C nitrite reductase (*nrfA*) (Table 1).

**Table 1.** List of primers, strains and annealing temperatures used in this study. AOA—ammonia oxidizing archaea, AOB—ammonia oxidizing bacteria.

| Gene | Primer | Primer Sequence | Ann. Temp. | Reference Strain |
|---|---|---|---|---|
| *nirS* | F3nir<br>R4bcd | SCCGCACCCGGGBCGYGG<br>CGTTGAAYTTRCCGGTSGG | 60 °C | *Pseudomonas stutzeri* (DSM 4166) |
| *nirK* | F1aCu<br>R3Cu | ATCATGGTSCTGCCGCG<br>GCCTCGATCAGRTTGTGGTT | 60 °C | *Achromobcter* sp. (DSM 30128) |
| AOA-*amoA* | AOA-amoA-f<br>AOA-amoA-r | CTGAYTGGGCYTGGACATC<br>TTCTTCTTTGTTGCCCAGTA | 54–60 °C | *Nitrosopumilus maritimus* (NCIMB 15022) |
| AOB-*amoA* | amoA-1F<br>amoA-2R | GGGGHTTYTACTGGTGGT<br>CCCCTCKGSAAAGCCTTCTTC | 63 °C | *Nitrosomonas europaea* (DSM 28437) |
| *nrfA* | nrfA-F2aw<br>nrfA-R1 | CARTGYCAYGTBGARTA<br>TWNGGCATRTGRCARTC | 60 °C | *Citrobacter freundii* (DSM 30039) |

Genomic DNA from reference organisms was used to make standard curves and positive controls. Standard curves were constructed using PCR products of the *nirS/K*, *nrfA* and *amoA* genes from the corresponding reference strains (Table 1). For this, the PCR products were purified with the commercial kit (PureLink PCR Purification Kit, Invitrogen, Carlsbad, USA) and their concentration was measured by Qubit 3.0 (Invitrogen, Carlsbad, USA). Obtained products were sequenced at BaseClear B.V (Leiden, The Netherlands) to confirm their identity. Then, serial dilutions were applied to verified products within the range of $10^3$–$10^7$ copies of a gene per reaction and used to calibrate the quantification of target genes in samples.

Quantitative PCR was performed with the StepOnePlus Real Time PCR system (ABI 7900 HT Sequence Detection System, PE Biosystems, Waltham, USA) using optical grade 96-well plates. The PCR reaction was run in the final volume of 20 µL containing 10 µL of SYBR Green master mix, 0.2 µM of forward and reverse primers, 2 mM of $MgCl_2$ (25 mM) and 2 µL of DNA sample (diluted 1/10). The thermocycling conditions were as follows: 50° C for 2 min; initial denaturation at 94 °C for 10 min; 40 cycles at 94 °C (1 min), 60 °C (1 min), 72 °C (1.5 min); and final elongation at 72 °C (5 min). To assess the specificity of amplifications, a melting curve analysis was performed. Each sample was analyzed in triplicate. Triplicate no-template controls were included in each qPCR assay. The abundance and expression of target genes (DNA and RNA samples respectively) were recalculated to copies per g wet weight of a sample (sediment or chironomid larvae).

### *2.8. Statistical Analysis*

The D3 JavaScript library [41] was used to visualize the taxonomic composition of metabarcoding data. Venn diagrams were generated using R package VennDiagram [42] to visualize the proportion of overlapping and unique OTUs within each dataset (anoxic sediments, burrow wall sediments, and chironomid larvae).

Quantitative data (benthic fluxes and abundance and expression of target genes) were visualized using boxplots. Non-parametric Kruskal–Wallis tests were applied to determine the significant difference in benthic net fluxes derived from experimental treatments (control and chironomid larvae microcosms) as well as the difference in the abundance and expression of target genes in anoxic sediments, burrow wall sediments and chironomid larvae. Where relevant, the post-hoc pairwise comparisons were performed using Dunn's test with Bonferroni alpha-correction implemented in the Pairwise Multiple Comparison of Mean Ranks package (PMCMRC, [43]). All analyses were performed in R v3 software [44].

## 3. Results

### *3.1. Benthic Fluxes at the Sediment–Water Interface and Animal $O_2$ Consumption*

The presence of chironomid larvae significantly increased benthic metabolism (Kruskal–Wallis test, $p < 0.01$, for $O_2$ and $N_2$), reduced $NO_3^-$ efflux and stimulated $NH_4^+$ recycling (Figure 2). The uptake of $O_2$ varied from −45.3 to −3125 µmol $m^{-2}$ $h^{-1}$ with 72% higher respiration in the chironomid larvae treatment. The $O_2$ uptake associated with chironomid larvae was −269 ± 21 µmol $g^{-1}$ WW $d^{-1}$ (range between −329 and −216 µmol $g^{-1}$ WW $d^{-1}$). When extrapolated to a square meter of sediment containing 1800 individuals, this resulted in a chironomid–associated $O_2$ uptake of −598 µmol $m^{-2}$ $h^{-1}$.

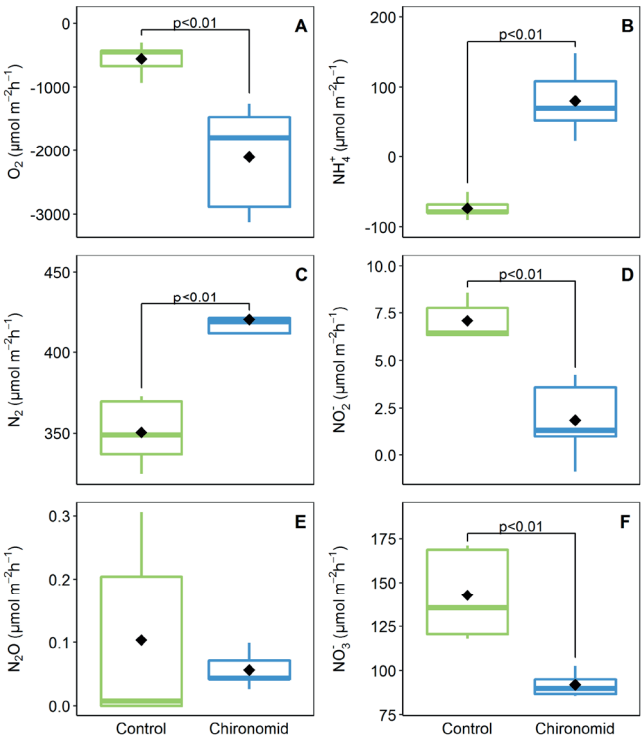


**Figure 2.** Benthic net fluxes of dissolved oxygen (**A**), ammonium (**B**), dinitrogen (**C**), nitrite (**D**), nitrous oxide (**E**) and nitrate (**F**) at the sediment–water interface measured in the control and the chironomid larvae microcosms. Each boxplot (n = 5) represents the data range (whiskers), upper and lower quartiles (edges), the median (horizontal line), and the mean (black diamond).

Similar to $O_2$, net production of $N_2$ was also higher in the chironomid treatment (420.2 ± 21.4 µmol $m^{-2}$ $h^{-1}$). We measured $N_2O$ efflux in both treatments, but due to high variability, the difference between the control and the chironomid treatment was not significant (Kruskal–Wallis test, $p > 0.05$; Figure 2). The net flux of $N_2O$ being two orders of magnitude lower than $N_2$ suggests that complete denitrification was the dominant process.

Chironomid larvae had a significant (Kruskal–Wallis test, $p < 0.01$) effect on nutrient exchange at the sediment–water interface (Figure 2). In the presence of larvae, sediments shifted from a sink (−73.5 ± 14.9 µmol $m^{-2}$ $h^{-1}$) to source of $NH_4^+$ (80.5 ± 48.7 µmol $m^{-2}$ $h^{-1}$). Conversely, the efflux of

the oxidized form of inorganic N decreased when sediments were bioturbated by chironomid larvae. The net fluxes of $NO_2^-$ and $NO_3^-$ were 4 and 2 times lower in the chironomid treatment compared to the control.

### *3.2. Bacterial Community Composition*

An overview of the bacterial community composition using 16S rRNA metabarcoding revealed a prominent difference between sediment samples and chironomid larvae (Figure 3). Of the 35 bacterial phyla detected across all samples, Proteobacteria was the most dominant in sediment samples, followed by Nitrospirae and Chloroflexi. In chironomid larvae, the bacterial community was dominated by three phyla: Firmicutes (40.1%), Proteobacteria (27.6%) and Bacteroidetes (24.4%). No Archea sequences were detected in the samples.

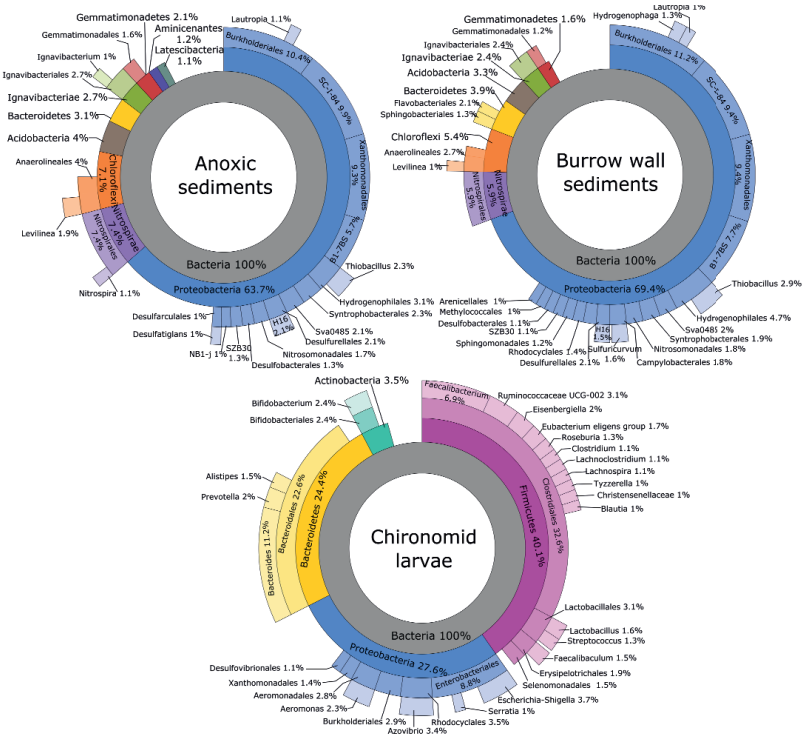


**Figure 3.** Overview of the bacterial community composition detected using the 16S rRNA marker gene in anoxic sediments, burrow wall sediments and chironomid larvae. The charts show the relative abundance of sequences at different taxonomic levels. To aid in visualization, taxa contributing <0.1% are not shown. The inner circle demonstrates the percentage of taxa assigned at the highest level (Bacteria).

Out of 4841 OTUs detected across three samples, 17.8% were shared between sediments (anoxic sediment and burrow walls) and larvae (Figure 4). These OTUs represented 187 families and 24 phyla. Most of the shared OTUs present in chironomid larvae belonged to Firmicutes (41.4%), Proteobacteria (27.6%), and Bacteroidetes (24.4%). The OTUs detected exclusively in chironomid samples (326 OTUs assigned to 14 families) were dominated by Tenericutes (59.3% of reads), followed by Firmicutes

(15.6%), Saccharibacteria (10.4%), Cyanobacteria and Elusimicrobia (3.8% each), Actinobacteria (3.6%) and Bacteroidetes (1.4%).

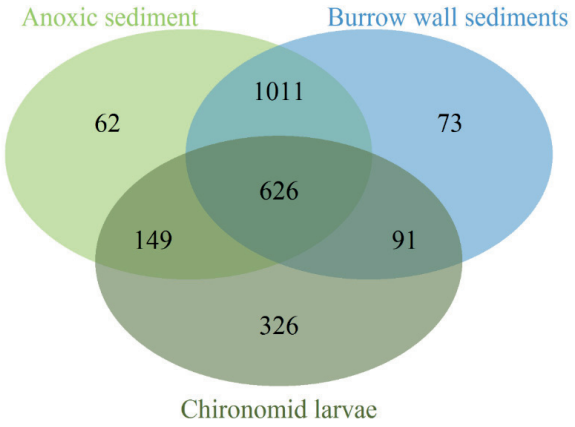


**Figure 4.** Venn diagram showing the numbers of unique and shared operational taxonomic units (OTUs) in anoxic sediment, burrow wall sediment and chironomid larvae samples (OTU numbers are indicated in the corresponding cross-sections of the diagram).

### *3.3. Abunadance and Activity of Nitrifying and Denitrifying Genes*

Quantitative PCR assays used to quantify the abundance and transcriptional activity of the targeted functional genes associated with the oxidation of $NH_4^+$ to $NO_2^-$ (*amoA*) and its reduction to nitric oxide (NO; *nirS*/*nirK*) or to $NH_4^+$ (*nrfA*) showed that bacterial *amoA* genes were present in all samples, whereas archaeal *amoA* genes were not detected. Significant differences ($p < 0.05$, Kruskal–Wallis test followed by pairwise Dunn's test) were detected only for *nirS* and *nrfA* gene abundance between the anoxic sediment and chironomid larvae samples (Figure 5A,D). Gene transcripts of nitrite reductase genes *nirS* and *nrfA* were detected in significantly lower copy numbers than their relative genes copy numbers ($p < 0.001$, Kruskal–Wallis test). Gene transcripts of bacterial *amoA* genes were detected and quantified from all types of samples at rather consistent rates, similar to DNA copy numbers detected for this gene (Figure 5C), whereas no *nirK* gene transcripts were detected in any type of samples (Figure 5B).

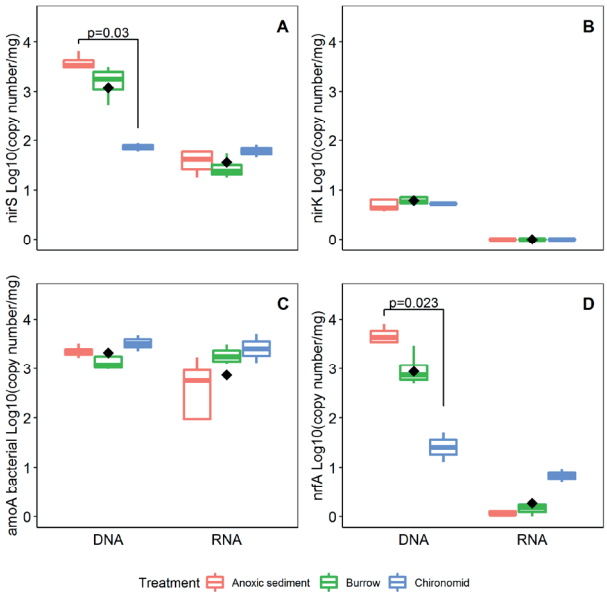


**Figure 5.** Abundance (DNA) and transcriptional activity (RNA) of the four analyzed target genes—*nirS* (**A**), *nirK* (**B**), *amoA* (**C**) and *nrfA* (**D**)—assessed in the anoxic sediment, burrow wall sediment and chironomid larvae samples. Each boxplot represents the data range (whiskers), upper and lower quartiles (edges), median (horizontal line) and group mean (black diamond). The square brackets indicate significant pairwise difference between treatments (Kruskal–Wallis test followed by pairwise Dunn's test).

## 4. Discussion

Benthic macrofauna affect the distribution and quality of organic matter and the availability of electron acceptors by burrowing, ventilating and feeding, thus altering microbial communities and their metabolic activity [23,45]. In soft-sediment estuarine environments, it is generally expected that the cumulative effect of burrowing infauna on solute fluxes results in an overall increase of $NH_4^+$ and $N_2$ efflux and a decrease of $NO_3^-$ efflux [12,16,29]. The present study aimed at a better understanding of how sediment-dwelling chironomid larvae facilitate solute transport, including electron acceptors ($O_2$ and $NO_3^-$), from the overlaying water column to the deep sediment, or vice versa. Furthermore, we were interested in how changes in solute transport in turn may stimulate microbial communities involved in nitrification and $NO_3^-$ reduction processes within the sediment.

Higher $N_2$ production in the presence of chironomid larvae confirms their stimulatory effect on denitrification, while $N_2O$ production did not change significantly between treatments and was considerably lower than $N_2$ production. Our results are in line with those of a previous study indicating that with high $O_2$ availability and low $NO_3^-$ concentrations in the water, the overall $N_2O$ flux from both bioturbated and non-bioturbated sediments is minimal [36]. Stief et al. [46] described higher sedimentary $N_2O$ fluxes associated with the activity of *C. plumosus*, resulting from incomplete microbial denitrification in the larval gut. It was suggested that these $N_2O$ emissions were mainly constrained by the temperature and $NO_3^-$ concentrations [47]. A recent study by Sun et al. [48] revealed that with the presence of nutritional food such as planktonic cyanobacteria, $N_2O$ production in the larval gut can significantly decrease. Cyanobacterial blooms, a common phenomenon in the Curonian Lagoon, may explain why we observed low $N_2O$ production in our study. Alternatively, a large portion of $N_2O$ possibly produced by the larvae [46,47] was likely consumed by sediment-denitrifying bacteria before

reaching the overlying water. However, to confirm these hypotheses, additional measures of $N_2O$ and analyses of functional genes encoding $N_2O$ reduction to $N_2$ (i.e., *nosZ*) are needed.

As indicated by metabarcoding, the diversity of the bacterial community (numbers of OTUs) was slightly higher in sediment samples (both anoxic and burrow walls) compared to chironomid larvae. The latter had a peculiar composition and was dominated by bacterial groups (Firmicutes/Clostridiales) typical for animals' intestinal microbiota. These bacterial taxa during their fermentative growth, using reduced nicotinamide adenine dinucleotide (NADH) and $NO_2^-$ or $NO_3^-$ as substrates [49], can contribute to the production of $NH_4^+$ [50]. However, some overlap was present in the composition of the microbial community associated with chironomid larvae and that of the surrounding sediments, which primarily comprised Proteobacteria. This abundant and diverse group includes microbial taxa capable of multiple N transformations, including anammox denitrification, DNRA and N-fixation [51].

Burrow ventilation, including the pumping of $NO_3^-$ through the burrow, is considered one of the main mechanisms by which denitrification is stimulated in sediments reworked by chironomid larvae [14,21,52,53]. However, the degree of stimulation depends on $NO_3^-$ concentration in the overlaying water [14]. In the Curonian Lagoon, $NO_3^-$ concentration varies seasonally, and therefore the effect of chironomid larvae can differ among the seasons. The current study was carried out in summertime, when $NO_3^-$ concentrations were generally low (1.4 μmol $L^{-1}$ on average). In a previous similar incubation experiment performed by Benelli et al. [12], $N_2$ production was 2.6-fold higher than reported here, likely due to higher dissolved $NO_3^-$ concentrations in spring (109 μmol $L^{-1}$).

Simple calculations, assuming that two moles of $NO_3^-$ are required to produce one mole of $N_2$, suggest that the increased uptake of $NO_3^-$ in bioturbated sediment could only explain 41% of the measured $N_2$ production. This indicates the potential relevance of coupled nitrification–denitrification in sediments bioturbated by chironomid larvae. By constructing burrows and pumping $O_2$ through them, chironomid larvae create new niches for nitrifiers within the sediments [6,10,15]. Since bacteria require $O_2$ for $NH_4^+$ oxidation, respiration ($O_2$ uptake) is expected to increase alongside nitrification. In the present study, chironomid larvae stimulated respiration by 3.8-fold in comparison to the controls—a stronger effect than that reported earlier by Benelli et al. [12] and by Svensson and Leonardsson [14]. Our extrapolations showed that approximately 28% of the total $O_2$ consumption was taken up directly by chironomid larvae and 24% by the sediment surface, which leaves approximately 48% of the $O_2$ consumption related to newly oxidized burrow structures (for example, see the substantial volume of oxidized sediment around larvae borrows in Figure 6). Our estimated 28% of oxygen consumption by larvae is consistent with that reported by Svensson and Leonardsson [14].

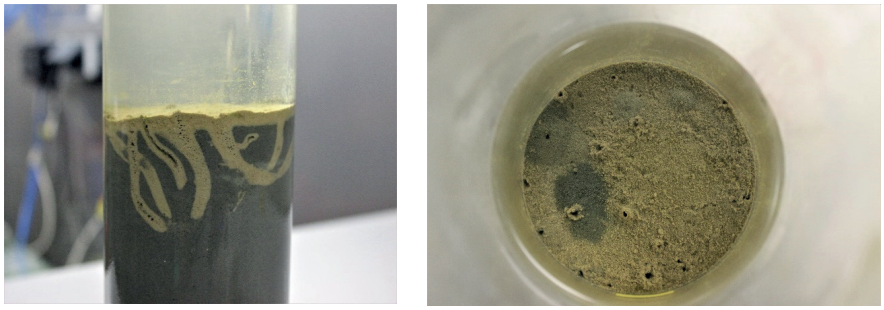

**Figure 6.** The bioturbation effects of chironomid larvae, evident in sharp contrast between the light-brown oxidized surface and burrow sediment, and black chemically reduced subsurface sediment (photo by A. Mačiūtė).

However, the degree of stimulation of nitrifiers might be species-specific, as chironomid larvae vary in size, bioturbation mode or grazing pressure on bacteria. Small chironomid species such

as *Chironomus riparius* construct burrows in the upper 1 cm layer where they directly (grazing) or indirectly (burrow ventilation) affect nitrification processes [15]. Larger species, such as *C. plumosus* or *Chironomus* sp., can construct burrows down to 10 cm, significantly extending the niche for nitrifiers and consequently enhancing N-cycling rates [21,54]. Therefore, our findings cannot be generalized for all chironomid species or other macrobenthic invertebrates.

Despite denitrification increasing with the presence of chironomid larvae, its efficiency, expressed as the ratio between the $N_2$ flux and the sum of $N_2$ + DIN fluxes, decreased by 20% in bioturbated sediments (from 84% to 65%). The increased $NH_4^+$ efflux in bioturbated sediments can explain this pattern. Deep-burrowing chironomids are able to increase the upward flux of $NH_4^+$ from deeper layers (where its pool is considerably higher due to the limited oxidation through nitrification) [12,21,55]. The assumed main source of $NH_4^+$ in deep sediment pore water is the mineralization of organic matter, as the excretion of $NH_4^+$ by chironomid larvae rarely exceeds 20% of the immobilized ammonium pool [11,14].

It has never been questioned whether $NH_4^+$ excretion by chironomid larvae is solely a physiological process or if it could also be attributed to larvae–bacteria associations (e.g., gut microbiomes). Poulsen et al. [29] showed that the *C. plumosus* gut can host $NO_3^-$ reducing microbes, but their targeted functional gene (*nar*) did not allow them to distinguish between nitrite denitrifiers or ammonifiers. Here, we show that the larvae-associated microbial community exhibited transcriptional activity of the *nrfA* gene, which encodes DNRA. This process is strictly anaerobic and may thus potentially occur in the anoxic larvae gut [28]. Therefore, active $NO_3^-$-respiring intestinal bacteria of infauna can act as an alternative source of $NH_4^+$ in soft-bottom environment.

This was also supported by our functional gene quantification that showed a higher potential for denitrification and DNRA in chironomid larvae rather than in the surrounding sediments. Although *nirS* and *nrfA* genes, encoding $NO_2^-$ reduction to NO and to $NH_4^+$, respectively, were abundant in anoxic sediment and on the burrow walls, their expression was comparatively low. This indicates that chironomid larvae can provide favourable conditions for bacteria harbouring these genes, facilitating their activity. The expression of *nirS* and *nrfA* genes can be affected by a number of environmental variables, including $O_2$, temperature, and organic matter availability [56]. This notwithstanding, there is numerous evidence that ingested bacteria can remain active in the larvae gut and carry out $NO_3^-$ reduction [29,46]. In addition, an anoxic gut environment might stimulate $NO_3^-$ reduction by facultative aerobic bacteria [28,29]. A higher *nirS* gene copy number points at denitrification as the dominating pathway of $NO_3^-$ reduction. The differences in the expression of *nirS* or *nrfA* genes can be explained by different bacterial affinity to labile carbon, $NO_3^-$ and sulphide concentration, and temperature [57,58].

The quantification of *amoA*, which encodes the ammonia monooxygenase for the oxidation of $NH_4^+$ to $NO_2^-$, revealed that its abundance and activity was primarily associated with ammonium oxidizing bacteria (AOB) in burrows and chironomid larvae. This is likely due to the simultaneous availability of $O_2$ and $NH_4^+$, which is favourable for AOB. Surprisingly, relatively high copy numbers of *amoA* were associated with the larval body. Since nitrification is an aerobic process and is unexpected in the anoxic environment of larvae gut and intestine, the active nitrifying bacteria might have been located on the external biofilm of the chironomid body, thus having direct access to $NH_4^+$ and $O_2$-enriched pore water, within the larvae-ventilated burrow.

## 5. Conclusions

The present study confirms that tube-dwelling invertebrates such as *C. plumosus* may have a considerable influence on N-cycling in estuarine sediments. Combining biogeochemical (flux measurements) and molecular (metabarcoding, functional gene analysis) approaches allowed the precise identification of N transformation hot-spots (burrow lining, macrofauna gut) and the quantification of their contribution to in-sediment N-cycling processes. Chironomid larvae stimulated nitrification, denitrification and $NH_4^+$ production, while increased N recycling reduced denitrification efficiency.

Chironomid larvae produced visible, direct effects on the volume of oxidized sediments, creating new suboxic niches via burrowing and ventilation. They harboured a unique and active array of bacteria compared to those found in the surrounding environment. Interestingly, active functional genes involved in contrasting processes such as nitrification and $NO_3^-$ reduction were detected both in sediments and the larvae microbiome, suggesting the co-occurrence of adjacent oxic and anoxic niches also within the larvae. Our study suggests that overlooked invertebrate–bacteria associations could be a significant component of N-cycling in benthic environments. Future studies should further scrutinize microbially mediated N processes in isolated macrofauna to partition nitrification and denitrification processes associated with intimate animal–bacteria interactions.

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# PAPER III

Ugo Marzocchi, Stefano Bonaglia, Anastasija Zaiko, Grazia M. Quero,
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# Zebra Mussel Holobionts Fix and Recycle Nitrogen in Lagoon Sediments

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Bivalves are ubiquitous filter-feeders able to alter ecosystems functions. Their impact on nitrogen (N) cycling is commonly related to their filter-feeding activity, biodeposition, and excretion. A so far understudied impact is linked to the metabolism of the associated microbiome that together with the host constitute the mussel's holobiont. Here we investigated how colonies of the invasive zebra mussel (*Dreissena polymorpha*) alter benthic N cycling in the shallow water sediment of the largest European lagoon (the Curonian Lagoon). A set of incubations was conducted to quantify the holobiont's impact and to quantitatively compare it with the indirect influence of the mussel on sedimentary N transformations. Zebra mussels primarily enhanced the recycling of N to the water column by releasing mineralized algal biomass in the form of ammonium and by stimulating dissimilatory nitrate reduction to ammonium (DNRA). Notably, however, not only denitrification and DNRA, but also dinitrogen ($N_2$) fixation was measured in association with the holobiont. The diazotrophic community of the holobiont diverged substantially from that of the water column, suggesting a unique niche for $N_2$ fixation associated with the mussels. At the densities reported in the lagoon, mussel-associated $N_2$ fixation may account for a substantial (and so far, overlooked) source of bioavailable N. Our findings contribute to improve our understanding on the ecosystem-level impact of zebra mussel, and potentially, of its ability to adapt to and colonize oligotrophic environments.

**Keywords:** ***Dreissena polymorpha*, nitrogen, denitrification, DNRA, nitrogen fixation, *nifH*, Curonian Lagoon**

## INTRODUCTION

Microbial symbionts may drive speciation and evolution (Shropshire and Bordenstein, 2016), but their relevance in organismal ecology has only recently gained widespread recognition (Dittami et al., 2020). Huge progress has been made in this research field thanks to rapidly advancing molecular tools (Petersen and Osvatic, 2018). However, molecular methods alone cannot

overcome the major challenge of understanding how host-microbe associations, otherwise known as holobionts (Bordenstein and Theis, 2015), contribute to the functioning of the ecosystems they inhabit (see nested ecosystem concept – Pita et al., 2018). Interdisciplinary approaches combining molecular and geochemical investigations are thus urgently needed to investigate the role of complex and diverse host-microbe associations *in natura* (Petersen and Osvatic, 2018; Beinart, 2019). Historically, most ecological research into biological invasions has focused on detrimental species interactions such as predation and competition. However, microbial associates may play an important role by facilitating niche adaptations and allowing their host to occupy otherwise inaccessible habitats (Shapira, 2016). Recent research shows that associations between bivalves and bacteria are paramount in regulating benthic biogeochemical processes (Smyth et al., 2013; Benelli et al., 2017; Bonaglia et al., 2017; Cardini et al., 2019), with microbes contributing to the metabolic potential and impact of the holobiont, in particular concerning carbon (C) and nitrogen (N) cycling (Petersen et al., 2016; Arfken et al., 2017; Konig et al., 2017). Still, little is known on microbiomes of invasive bivalve holobionts and their role in phenotypic plasticity and colonization potential of the invader, and ultimately its ecosystem-level impact (e.g., alteration of biogeochemical processes).

Zebra mussels (*Dreissena polymorpha*, Pallas 1771) are filter-feeding bivalves native to the Ponto-Caspian region, which successfully invaded several regions in Europe and North America, where they significantly altered community structure and ecosystem functioning (Strayer et al., 1999). Their rapid colonization rates together with proficient filter-feeding activity have been linked with the decline in chlorophyll-a, and increase in water transparency and total phosphorous (P) (Caraco et al., 1997), which may result in an overall shift of the trophic state of the colonized freshwater ecosystems (Kumar et al., 2016). The impact of zebra mussel on N cycling is manifold and includes enhanced release of ammonium ($NH_4^+$) from digested algal biomass (Lavrentyev et al., 2000), stimulation of benthic nitrification (Bruesewitz et al., 2008) and denitrification (Bruesewitz et al., 2006), and release of P to the water column (Benelli et al., 2019) potentially stimulating pelagic dinitrogen ($N_2$) fixation. The nature and extent of such impacts may however be seasonal (Bruesewitz et al., 2006) and depend upon intrinsic features of the water body such as morphometry (Higgins and Zanden, 2010) and sediment organic matter content. An additional level of complexity in unraveling the overall impact of zebra mussel on N cycling is the distinction between its ability to alter key microbial transformation indirectly (via stimulating the activity of pelagic and benthic communities) and directly, via the hosted microbiome (e.g., Svenningsen et al., 2012). Although the indirect impact has been extensively documented, the role of its microbiome remains largely unexplored both in terms of metabolic repertoire and magnitude of the N transformations. Unraveling the diverse impacts of zebra mussel on nutrient cycling is pivotal to achieve a comprehensive understanding of its invasiveness and role as ecosystem engineer.

In this study, a combination of biogeochemical and molecular approaches was employed to investigate the impact of zebra mussel on N cycling in the sediment of the largest European lagoon (Curonian Lagoon, SE Baltic Sea). Both a "benthic community" (i.e., intact sediment + zebra mussel colony) and a "holobiont" (i.e., zebra mussel alone) incubations were conducted to quantitatively assess the effect of the zebra mussel holobiont on N cycling and to discern it from its effect on sediment processes.

## MATERIALS AND METHODS

### Site Description and Samples Collection

Sediment and zebra mussel specimens were collected in June 2018 at a fine-sand site (median grain size 0.238 mm) from a shallow area (1.2 m depth) of the oligohaline Curonian Lagoon (55°20′25.9″N, 21°11′24.4″E). The Curonian Lagoon, is a micro-tidal, low-energy system, characterized by a reduced vertical mixing in particularly in the summer months when the discharge from tributaries and wind intensity are at minimum (Ferrarin et al., 2008; Mezine et al., 2019). At the time of sampling, water temperature was 22.5°C, salinity was 0.2, concentration of dissolved organic and inorganic nitrogen (i.e., DON and DIN) was 57.2 ± 0.7 (Mean ± SEM) and 1.8 ± 0.1 μM, respectively. Height intact cores were collected by hand using Plexiglas liners (i.d.: 8.4 cm, length: 30 cm). Four cores included sediments with an overlying colony of zebra mussels and four cores included bare sediments without mussels or other visible macrofauna. Each liner contained approximately 10 cm of sediment overlaid by 16 cm of water. Additional *in situ* water and zebra mussel specimens were collected for single animal incubations and molecular analyses (see details below). Within 1 h, the samples were transported to the laboratory in cool box filled with *in situ* water. At the laboratory, intact cores were submerged overnight in a temperature-controlled tank (23 ± 0.2°C, 200 L) containing unfiltered, aerated *in situ* water. Homogeneous water conditions were kept in each core via magnetic stirring bar (40 rpm). The following day, intact sediment cores with and without mussel colonies were incubated to assess the impact of zebra mussels on (i) sediment nutrients and oxygen fluxes, and subsequently on (ii) nitrate ($NO_3^-$) reduction processes (benthic community). A second set of incubations was conducted to assess the diversity and relevance of N transformations associated with zebra mussel specimens and their microbiome (holobiont incubations).

### Benthic Community Incubations

After a preincubation of 15 h, the water in the tank was partly renewed. Thereafter, the top of each core was sealed with a Plexiglas lid without leaving a head-space and net fluxes of $O_2$, DIN (i.e., $NH_4^+$, $NO_3^-$, $NO_2^-$), DON, and phosphate ($PO_4^{3-}$) between the benthic compartment and the water were measured in dark, while keeping the water stirring on, as described in Samuiloviene et al. (2019). Incubations lasted for less than 4 h to limit the change in water column $O_2$ concentration to ≤20% as this is a prerequisite to maintain a linear rate of change in nutrients concentration over time (Dalsgaard et al., 2000).

Oxygen concentration was monitored throughout the incubation with an optical $O_2$ meter (FireStingO2, PyroScience GmbH). At the start and at the end of each incubation, 30 mL of water were collected from each core, filtered (Frisenette GF/F filters) and stored into 12 mL Polyethylene vials for later determination of DIN. An additional 40 mL aliquot was filtered into a 20 mL glass vials for DON and $PO_4^{3-}$ determination. Water samples were stored frozen (−20°C) until analyses.

Following the flux measurements, microcosms were left submerged with the top open overnight before starting the $NO_3^-$ reduction [i.e., denitrification and dissimilatory nitrate reduction to ammonium (DNRA)] measurements via $^{15}NO_3^-$ tracer as described by Dalsgaard et al. (2000). Briefly, $^{15}NO_3^-$ was added to the water of each core from a stock solution (20 mM, 99 atom % $Na^{15}NO_3$; Sigma-Aldrich) to a final $^{15}N$ enrichment of approximately 60% ($^{14+15}NO_3^-$ concentration 6.9 μM). The cores were then closed and incubated for 1.5–3 h in the dark. At the end of the incubation, the mussels were removed, and the water and the sediment were gently mixed to a slurry. Thereafter, 20 mL aliquots of the slurry were transferred into 12 mL exetainers (Labco Ltd.) and 200 μL of 7 M $ZnCl_2$ were added to stop microbial activity. An additional 40 mL subsample was collected for $^{15}NH_4^+$ determination. Rates of total denitrification ($D_{tot}$) and its components i.e., denitrification of $NO_3^-$ from the water ($D_w$) and denitrification coupled to nitrification ($D_n$), were calculated from the fluxes of $^{29}N_2$ and $^{30}N_2$ according to Nielsen (1992). Overestimation of denitrification due to anaerobic ammonium oxidation (anammox) (Risgaard-Petersen et al., 2003) was assumed negligible, since anammox has been previously reported to be marginal in the lagoon sediment (Zilius, 2011). Rates of DNRA were calculated from the $^{15}NH_4^+$ production, $D_{tot}$, and denitrification of $^{15}NO_3^-$ as in Risgaard-Petersen and Rysgaard (1995). At the end of the incubation, sediment from all cores was carefully sieved (0.5 mm mesh-size) to assess the mussel density and to determine their shell-free dry weight (SFDW) after drying the soft tissue at 60°C to a constant weight.

Inorganic nutrient (i.e., $NO_x^-$, $NO_2^-$, $NH_4^+$, $PO_4^{3-}$) concentrations were measured with a 5-channel continuous flow analyzer ($San^{++}$, Skalar) using standard colorimetric methods (Grasshoff et al., 1983). Nitrate concentration was calculated as the difference between $NO_x^-$ and $NO_2^-$. Total dissolved nitrogen (TDN) was analyzed by the high temperature (680°C) combustion, catalytic oxidation/NDIR method using a Shimadzu TOC 5000 analyzer with a TN module. Dissolved organic nitrogen (DON) was calculated as difference between TDN and DIN. Samples for $^{29}N_2$ and $^{30}N_2$ were analyzed by gas chromatography-isotopic ratio mass spectrometry (GC-IRMS, Thermo Delta V Plus). Samples for $^{15}NH_4^+$ were analyzed by the same technique (GC-IRMS) after conversion of $NH_4^+$ to $N_2$ by the addition of alkaline hypobromite (Warembourg, 1993).

## Zebra Mussel Holobiont Incubations

To determine rates of N transformation (i.e., denitrification, DNRA, anammox, and $N_2$-fixation), associated with the zebra mussel holobiont, a series of $^{15}N$ isotope incubations were carried out with individual specimens in the absence of sediment. Prior to the incubation, the biofilm on the mussels' shell was carefully removed using a toothbrush and mussels were then rinsed in 0.2 μm double-filtered water. Incubations were performed in bottom-capped Plexiglas cylindrical microcosms (total volume 227 ± 3 mL). The microcosms were filled with 0.2 μm double-filtered aerated *in situ* water amended with $^{15}N$ tracers (see the details below). A stirring magnet allowed for continuous water mixing (40 rpm) during the incubation. Microcosms were capped with gas-tight lids provided with two sampling ports for sample collection and water replacement.

### Nitrate Reduction

Rates of denitrification, DNRA and anammox were estimated following the revised isotope-pairing technique (r-IPT) (Thamdrup and Dalsgaard, 2002; Risgaard-Petersen et al., 2003). Three treatments were prepared: (1) low $^{15}NO_3^-$ addition (final concentration 6.2 μM), (2) high $^{15}NO_3^-$ addition (final concentration 19.1 μM) and (3) $^{15}NH_4^+ + {}^{14}NO_3^-$ (final concentration 6.3 + 5.3 μM, respectively). Treatments 1 and 2 were used to measure rates of denitrification and DNRA. The different tracer concentrations in treatments 1 and 2 allowed to validate the main assumption of IPT, (i.e., tracer concentration-independency of rates). Treatment 3 allowed to measure anammox. Each treatment included five microcosms: four containing one mussel and one control with filtered water only. To calculate the degree of isotopic enrichment, water samples for $NH_4^+$ and $NO_3^-$ analysis were collected prior and after to the isotope addition. Microcosms were incubated in the dark for 8 h at 23 ± 0.3°C. Every 3 h aliquots were subsampled from each replicate/control, transferred into 12 mL exetainers (Labco, United Kingdom) and poisoned with 200 μL of 7 M $ZnCl_2$ for later $N_2$ and $NH_4^+$ isotopic determination as described above. Significance of the increase in 15N species (i.e., $^{29}N_2$, $^{30}N_2$, and $^{15}NH_4^+$) over time was tested via regression analysis using the whole datasets (including all data points) for denitrification ($p < 0.05$) and DNRA ($p < 0.10$), respectively. Production rates were calculated from single incubations (time series) and normalized per grams of biomass (SFDW).

### $N_2$ Fixation

To determine rates of $N_2$ fixation, a stock solution of $^{30}N_2$-enriched filtered water was prepared using a modified version of the protocol described in Klawonn et al. (2015) (see **Supplementary Material**). Before starting the incubation, the stock solution was gently transferred into four microcosms to minimize gas exchange with the atmosphere. After the mussels were added, the top lids were closed and incubated in the dark for 12 h. Four additional microcosms were prepared and incubated as above but with unlabeled water to serve as a control for isotopic contamination. At the end of the incubation, the mussels were collected and dissected for SFDW determination. Mussel tissues were then stored at −20°C for later $^{15}N$ incorporation analysis. In addition, ten non-incubated specimens were dissected and store as above for later determination of the natural $^{15}N/^{14}N$

ratios. Prior to the isotopic analysis, mussels' tissues were freeze-dried for 48 h, ground to fine powder and weighed into tin capsules. Samples were analyzed for N elemental composition (%) and isotope ratios ($\delta^{15}N$) by continuous flow isotope ratio mass spectrometry (IRMS, Isoprime, GV Instruments Ltd.) coupled with elemental analyzer (Costech Instruments). $^{15}N_2$ incorporation rates were calculated as in Montoya et al. (1996). $^{15}N_2$ incorporation was considered significant for those samples that showed an atom % excess that was higher than two times the standard deviation of the atom % of the unlabeled samples.

## Molecular Analyses of the Prokaryotic Communities

### Nucleic Acids Extraction and Sequencing

Analysis of 16S rRNA gene and of the *nifH* gene expression were conducted to characterize the $N_2$-fixing community in the mussel's microbiome and its possible relationship with the $N_2$-fixing community in the water via filter-feeding activity. Nucleic acids were extracted from the soft tissue of zebra mussels (from in the holobiont incubation) and from the suspended material from *in situ* water sample. Suspended material was size-fractioned in two size groups, i.e., >10 μm, and 0.22–10 μm (from here on referred to as *large* and *small* fraction, respectively) by step-wise filtration of the water as described in Zilius et al. (2020). All samples were collected and analyzed in triplicates. Samples was snap-frozen in liquid nitrogen and stored at −80°C until DNA and RNA extraction. DNA was extracted using the QIAamp Fast DNA Stool Mini Kit (QIAGEN) with increased lysis temperature to 90°C to improve the bacterial cell rupture. RNA was extracted using the RNAeasy Mini Kit (QIAGEN) as in Zilius et al. (2020) and treated with TURBO DNase (Invitrogen). Complementary DNA (cDNA) was synthesized using SuperScriptIII Reverse Transcriptase (Invitrogen), RNaseOUT Ribonuclease Inhibitor (Invitrogen) and random primers. Two negative controls without either reverse transcriptase or RNA were included to assess the potential contamination with residual DNA. Partial 16S rRNA gene sequences were amplified using primer pair Probio_Uni (5′-CCTACGGGRSGCAGCAG-3′) and Probio_Rev (5′-ATTACCGCGGCTGCT-3′), targeting the V3 region of the 16S rRNA gene sequence as described by Milani et al. (2013). High-throughput sequencing was performed at the DNA sequencing facility of GenProbio srl[1] on an Illumina™ MiSeq with the length of 250 × 2 bp, according to the protocol reported in Milani et al. (2013).

The cDNA-based amplification of *nifH* gene was performed using a nested PCR approach (Zehr and Turner, 2001) with nifH3 and nifH4 primers in the first PCR round followed by second amplification round with nifH1 and nifH2 primers with Illumina indices. Nested PCR conditions were set as in Zilius et al. (2020). Only single bands of appropriate size (359 bp) were detected after the second round of amplification. PCR products were purified from the gel (Thermo Scientific GeneJET Gel Extraction Kit), quantified (Qubit 3.0 Fluorometer) and the sequencing library was constructed following the two-step tailed PCR amplicon procedure, as described in Kozich et al. (2013). Paired-end sequences (2 × 250 bp) were generated on an Illumina MiSeq® instrument using the TruSeq® SBS kit. Sequence data were automatically demultiplexed using MiSeq Reporter (v2), and forward and reverse reads were assigned to samples. Raw sequence data for the 16S rRNA and *nifH* dataset were bioinformatically processed as described in Zilius et al. (2020). Briefly, primers from the raw sequence reads (with Illumina adapters removed by sequencing facility) were trimmed using cutadapt v2.10 (Martin, 2011), with no primer mismatch allowed. The bioinformatics pipeline was run using DADA2 package implemented in R (Callahan et al., 2016). Quality filtering and denoising of the trimmed fastq files were performed using the following parameters: "truncLen = c(150,150), maxEE = c(2,6), truncQ = 2, ndmaxN = 0." Singletons were discarded, and the remaining paired-end reads were merged with a minimum overlap of 65 bp and 1 mismatch allowed in the overlap region. Chimera removal was performed using the default (consensus) method and the resulting de-noised amplicon sequence variants (ASV) were used for taxonomic classification against the SILVA 132 database for 16S rRNA (Quast et al., 2013) and *nifH* Sequence Database (Gaby and Buckley, 2014). Sequences are available in the NCBI/SRA database under accession number PRJNA658818.

### Statistical Analyses on the Sequencing Data

The de-noised ASV tables and assigned taxonomy of *nifH* and 16S datasets were imported in RStudio (R Core Team, 2018), combined into two phyloseq objects and processed for data analysis (McMurdie and Holmes, 2013). Rarefaction curves were plotted for both 16S and *nifH* datasets using ggrare function in R (package *ranacapa*; Kandlikar et al., 2018). ASV tables for 16S and *nifH* were rarefied to the lowest number of reads (9,395 and 51,180 for the 16S and *nifH* dataset, respectively) (R package *phyloseq*). For 16S, alpha diversity indices (ASV richness, Shannon index, Simpson index and Pielou's evenness) were calculated using the R package *vegan* (Oksanen et al., 2019) and number of shared ASVs visualized with Venn diagram (package *venn*; Dusa, 2020). For *nifH*, only ASV richness was calculated. A Kruskal–Wallis test was used to assess differences in 16S and *nifH* ASV richness between water and zebra mussel samples (R package *phyloseq*). Differences in community composition were assessed using the Analysis of Similarity (ANOSIM) based on a Bray–Curtis similarity matrix implemented in *vegan*. PCoA was performed to explore and visualize similarities among the different samples, basing on the same Bray–Curtis similarity matrix, for both the 16S and *nifH* genes datasets. A heatmap with hierarchical clustering was plotted to visualize differences in the abundance of the top 70 16S rRNA gene ASVs (>0.01% across the dataset) using the R packages *Heatplus* (Ploner, 2020), *ggplot2* (Wickham, 2009), and *vegan*. Finally, to gain further information on the identity of unknown Bacteria and unknown Firmicutes identified in the *nifH* dataset for zebra mussel samples, blastn (search in nucleotide databases using a nucleotide query) and blastx (search in protein databases using a translated nucleotide query) (Altschul et al., 1990) analyses were performed against GenBank database (released version 237, May 2020).

[1]www.genprobio.com

## RESULTS

### Respiration and Nutrient Fluxes in Benthic Community Incubations

Mussel total biomass varied between 0.6 and 1.0 g $_{(SFDW)}$ per core, corresponding to an average areal biomass (±SD) of 134 ± 38 g $_{(SFDW)}$ $m^{-2}$ and a density of 30–64 mussels per colony. Mean benthic $O_2$ consumption was fivefold higher in the presence of the mussels (S + ZM) compared to the bare sediment (S) (**Figure 1A**). Bare sediment was a net sink for all the measured nutrients (**Figure 1B**). The presence of mussels reversed the fluxes resulting in the net efflux of all the analyzed species. Net $NH_4^+$ flux accounted for the largest share of the whole DIN efflux. For all measured parameters the difference between net fluxes in S + ZM and S was significant (Mann–Whitney U test, $p < 0.05$).

Rates of DNRA were significantly higher (+72%) in cores with mussels compared to the bare sediment (Mann–Whitney U test, $p < 0.03$) (**Figure 2**). $D_w$ tended to be higher in the presence of the mussels compared to the bare sediment (Mann–Whitney U test, $p = 0.06$). $D_n$ showed an opposite trend with lower rates in the presence of mussels, although the difference was not significant (Mann–Whitney U test, $p = 0.23$). The $D_n$:$D_w$ ratio was lower with the mussels compared to the bare sediment (Mann–Whitney U test, $p < 0.02$). Overall denitrification ($D_n + D_w$) was unaltered by the presence of the mussel (Mann–Whitney U test, $p = 0.66$).

### N Cycling Associated With the Zebra Mussel Holobiont

The average biomass of the incubated specimens was 37 ± 10 (SD) mg $_{(SFDW)}$. Regression analysis showed a significant increase in $^{15}NH_4^+$ and $^{15}N$-$N_2$ (i.e., $^{29}N_2$, $^{30}N_2$) in the DNRA and denitrification incubations, respectively (**Supplementary Table 1**). Biomass-normalized rates of DNRA spanned between zero and 192 nmol N g $_{(SFDW)}^{-1}$ $h^{-1}$ (average ± SEM, 31.2 ± 19.3 nmol N g $_{(SFDW)}^{-1}$ $h^{-1}$) (**Figure 3**). Rates of denitrification ranged between zero and 260 nmol N g $_{(SFDW)}^{-1}$ $h^{-1}$ (average ± SEM, 58.4 ± 28.9 nmol N g $_{(SFDW)}^{-1}$ $h^{-1}$). No anammox activity was detected within the timespan of the incubation (results not shown). $N_2$ fixation was detected in all tested animals (**Supplementary Table 2**) at rates ranging between 7.8 and 30 nmol N-$N_2$ g $_{(SFDW)}^{-1}$ $h^{-1}$ (average ± SEM, 21.9 ± 4.5 nmol N g $_{(SFDW)}^{-1}$ $h^{-1}$). On average, under our experimental conditions, $N_2$ fixation was equal to 37% of the denitrification rate.

### Water Column and Mussel-Associated Microbial Communities

After denoising and eukaryote sequence removal, the complete 16S rDNA dataset comprised 447,071 good quality sequence reads from the nine analyzed samples representing 2,705 bacterial ASVs. Rarefaction curves (**Supplementary Figure 1**) evidenced that the sequencing effort was sufficient to describe bacterial diversity. The normalized ASV richness (after rarefying the sequences at an even depth of 9,395) was significantly higher in zebra mussel compared to both size fractions of the water samples (Kruskal–Wallis, $p < 0.01$) (**Supplementary Figure 2**). Shannon and Simpson diversity and Pielou's evenness indices showed significantly higher values in the large compared to the small fraction of water samples. Shannon diversity tended to be higher in zebra mussel samples, although no significant difference with water samples was observed ($p < 0.05$). Zebra mussel and water

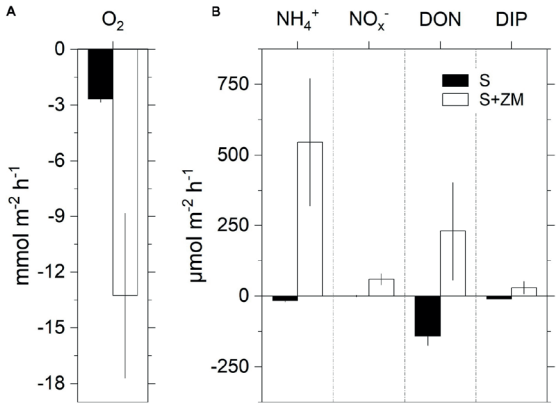


**FIGURE 1 |** Net fluxes of oxygen **(A)** and nutrients **(B)** at the sediment-water interface during the incubation of intact sediment cores with colonies of zebra mussel (S + ZM) and bare sediments (S). Data are shown as mean values ± standard error ($n$ = 3–5).

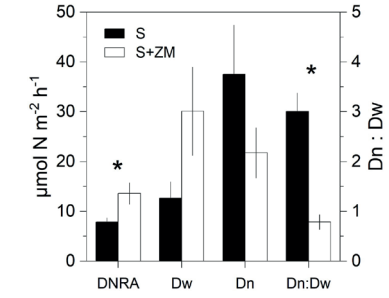


**FIGURE 2 |** Rates of DNRA and denitrification (showed as partitioned in its two components $D_w$ and $D_n$ and their ratio) in incubations of intact sediment cores with colonies of zebra mussel (S + ZM) and bare sediments (S). Data are shown as mean values ± standard error ($n$ = 4). Asterisks indicate significant differences ($p < 0.05$, Mann–Whitney U test, $n$ = 8).

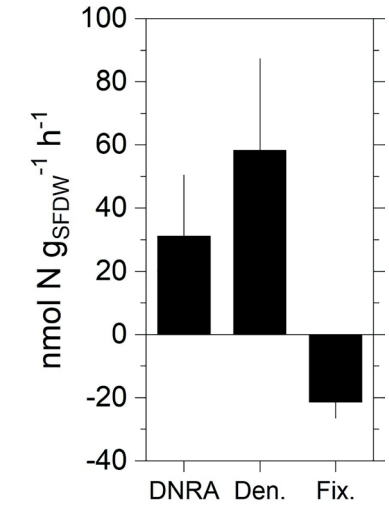


**FIGURE 3 |** Rates of DNRA, total denitrification, and $N_2$ fixation measured in the holobiont incubations with specimens of zebra mussel. Data shown are mean values ± standard error ($n$ = 10 for DNRA and denitrification; $n$ = 4 for fixation).

samples (small and large fractions) shared 13.3% of the detected ASVs ($n$ = 359), while 1,074 ASVs (39.7%) were exclusively associated with zebra mussels (**Supplementary Figure 3**).

The three types of samples showed distinct relative abundances of major prokaryotic taxa (**Figure 4A**) (ANOSIM, global $R = 0.88$; $p < 0.01$) and grouped separately when analyzed by PCoA (**Supplementary Figure 4A**). Zebra mussels were characterized by high abundances of Tenericutes (average, 25%), that were basically undetectable in water samples. Beta- (average, 12.8%) and Gammaproteobacteria (average, 6.5%), and Bacteroidetes (average, 22.5%) accounted for a considerable fraction in zebra mussel samples, while a general lower presence of Cyanobacteria (average, 4.3%) and Actinobacteria (average, 3.8%) was observed in comparison to both types of water samples. The Tenericutes phylum was almost entirely represented by members of the genus *Mycoplasma*, with relative abundances up to 40% of the overall community. In the large fraction of the water samples, Cyanobacteria clearly dominated the community (average, 67.8%), while in the small fraction, a more even community structure was observed, represented by Cyanobacteria (average, 24.7%), Bacteroidetes (average, 20.6%), and Actinobacteria (average, 16.9%), Alpha- (12.6%) and Betaproteobacteria (7.8%).

The heatmap visualization of the most abundant (>0.1%) ASVs, supported the taxonomic differentiation of the microbial communities between two water fractions and zebra mussel samples (**Figure 4B**). In particular, the zebra mussel microbial community was characterized by a pool of taxa mainly including *Mycoplasma* and Mycoplasmataceae (eight ASVs, average, 3% of zebra mussel reads), Spirochaetaceae (three ASVs, average 0.5%), Burkholderiaceae (two ASVs, average 0.6%), *Lacihabitans* (1), Sphingomonadaceae (1), *Dechloromonas* (1), *Hydrogenophaga* (1), *Flavobacterium* (1), and three unidentified Proteobacteria. Water samples were characterized by the presence of numerous Cyanobacteria and freshwater taxa (e.g., *Limnohabitans*, *Polynucleobacter*).

## Water Column and Mussel-Associated Active Diazotrophic Communities

The *nifH* dataset comprised a total of 2,045,435 good quality reads (on average, 227,270 reads per sample, ranging from 51,180 in a zebra mussel sample to 389,159 in a large fraction water sample), representing 360 *nifH* ASVs. Rarefaction curves for *nifH* (**Supplementary Figure 5**) confirmed the adequate diversity coverage at the attained sequencing depth. After rarefying at 51,180 sequence depth, a total of 344 ASVs were retained for the downstream analyses. No statistically significant difference in ASV richness was observed between the two water fractions and zebra mussel samples (Kruskal–Wallis rank sum test, $p = 0.707$) (**Supplementary Figure 6**). Diazotrophic communities significantly differed among the three type of samples (**Figure 5**; ANOSIM, global $R = 0.543$; $p = 0.006$) and grouped separately when analyzed by PCoA (**Supplementary Figure 4B**). In zebra mussel samples, the diazotrophic community was dominated by a large fraction of unknown Bacteria (54.4%), followed by *Paenibacillus* (33.2%) and a smaller fraction of unknown Firmicutes (12.2%). Interestingly, none of these groups were detected in water samples, which were almost completely dominated by Nostocales (83.4%) and *Zoogloea* (16.6%). More

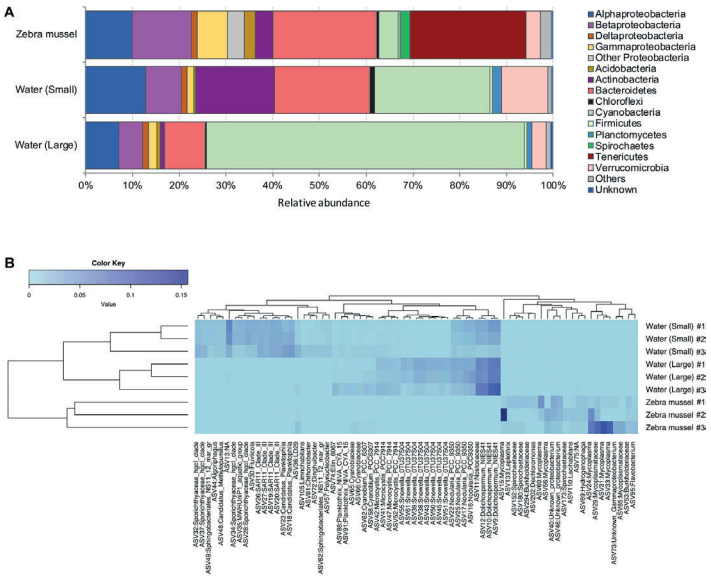


**FIGURE 4 | (A)** Bacterial community composition (based on the 16S rRNA gene) at the phylum and class (only for Proteobacteria) levels in zebra mussels and water column particulate samples of large (>10 μm) and small (0.22–10 μm) size fractions. Phyla and classes with an average relative abundance <0.5% across all samples were aggregated and reported as "Others." "Unknown" includes all those reads not matching any known bacterial taxonomy. **(B)** Heatmap summarizing the relative abundances of the top 71 ASVs in the analyzed samples.

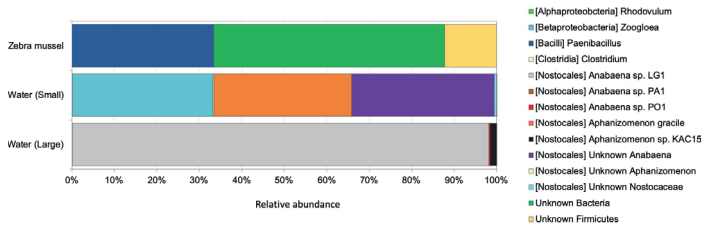


**FIGURE 5 |** Diazotrophic community composition based on *nifH* transcripts diversity in zebra mussel and water column particulate samples of large (>10 μm) and small (0.22–10 μm) size fractions. Data are shown as average of three replicates.

specifically, the small fraction of the water samples was dominated by *Anabaena* (66.2%) and *Zoogloea* (33.1%), while the large fraction of water samples by *Anabaena* (98.4%).

After blastn analyses, all ASV sequences identified as unknown Firmicutes matched with *nifH* sequences belonging to Clostridia, although weakly (<79% of similarity, 99% query coverage). Protein sequence similarity search analyses based on translated proteins (i.e., blastx) also indicated, that such ASVs were loosely related to Clostridia (90–92% of similarity, 99% query coverage). Blastn analyses performed on ASV identified as unknown Bacteria matched, although at low similarities (75–82%), mostly with *Azotobacter*, *Paenibacillus*, and Clostridia. Results from

blastx indicated that most of the ASVs identified as unknown Bacteria were highly related to Clostridia and *Paenibacillus* (80.5–93.5%). Some of the ASVs, showed similarities up to 91.5% with queries belonging to the phylum Bacteroidetes.

## DISCUSSION

### Impact of Zebra Mussel on Benthic N Cycling

Although it is well documented that benthic macroinvertebrates alter vital sediment processes such as N turnover through irrigation and bioturbation (e.g., Stief, 2013), animal-bacterial associations and their role on biogeochemical cycling remain largely unresolved. Here, we quantitatively assessed how a model invasive bivalve (the zebra mussel) alters benthic N cycling both directly (e.g., via excretion of DIN and DON) and indirectly via stimulating microbial activity both at the sediment level and through its microbiome. **Figure 6** summarizes rates of N fluxes measured in the benthic community incubations, and the biomass-specific rates measured in the holobiont incubations after extrapolation using the densities from the benthic community experiment. As evidenced, the presence of zebra mussels turned the benthic compartment from a sink to a source of all measured nutrients. In particular, $NH_4^+$ was the most prominent dissolved N species released into the water. Enhanced benthic release of $NH_4^+$ has been consistently reported in the presence of zebra mussels (James et al., 1997; Lavrentyev et al., 2000; Conroy et al., 2005; Ruginis et al., 2014) and other filter feeders (Mazouni et al., 1996; Bartoli et al., 2003; Nizzoli et al., 2006). The increase in $NH_4^+$ efflux can be sustained by three mechanisms: (i) mineralized algal biomass from filter-feeding activity of the mussel, (ii) stimulation of DNRA activity, and (iii) inhibition of nitrification due to the colony physical presence and consequent limitation of the $O_2$ transport into the sediment (Zaiko et al., 2010). The proximity of the $NH_4^+$: DIP ratio (i.e., 18) to the Redfield ratio calculated from the fluxes in the benthic-community incubations suggests that $NH_4^+$ most likely originates from mineralization of algae biomass by zebra mussel. Accordingly, enhanced rates of DNRA measured in the benthic-community incubations with the mussels could only contribute 1.0% of the enhanced $NH_4^+$ efflux. Net fluxes of $NO_3^-$ in the whole-community incubations suggest a stimulation of nitrification by zebra mussel rather than its inhibition. Assuming that the drop in $D_n$ measured in the presence of zebra mussel is caused by the suppression of nitrification, the resulting release of $NH_4^+$ would, however, only contribute to 4% of the overall sediment efflux of $NH_4^+$. Our data therefore indicate that the most prominent impact of zebra mussel on DIN dynamics is via the recycling of fixed N through mineralization of pelagic algae and other particulate organic matter either being egested as biodeposits or retained within the mussels' colony.

The net release of DON in the presence of the mussel might be sustained by the mussels' egestion/excretion or derive from exudates by settled phytoplankton aggregates within the colony. Recently, it has been shown that other dreissenids excrete dissolved organic matter with relatively low C to N ratio indicating high proportion of organic N compounds (DeVilbiss and Guo, 2017). The biochemical mechanisms at the basis of such DON release, the conditions that promote it, and its environmental relevance remain, however, poorly understood in filter-feeders.

Contrary to what previously reported from the upper Mississippi River (Bruesewitz et al., 2006, 2008) and from a freshwater Lithuanian lake (Ruginis et al., 2014), zebra mussels did not increase overall benthic denitrification in our incubations. Rather, the presence of the mussels altered

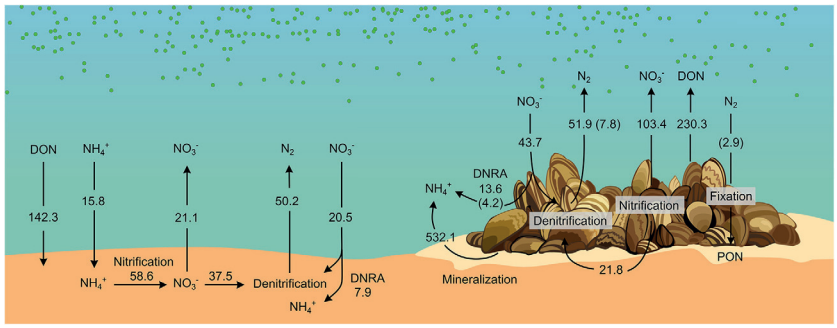


**FIGURE 6 |** Diagram of N cycling in bare sediments and sediment with zebra mussel colonies. Fluxes (μmol $m^{-2}$ $h^{-1}$) were obtained from benthic-community incubations. Contributions from Zebra mussels' microbiome (displayed within brackets) were obtained from biomass-specific fluxes measured in the holobionts incubation and scaled to the biomass present in the benthic-community incubations. See details on the calculation in **Supplementary Material** section "Calculations for the Build-Up of the N Diagram Showed in **Figure 6**." Drawing by V. Gasiūnaitė.

the balance between $D_w$ and $D_n$ favoring the former over the latter. Denitrification was previously reported from zebra mussels holobiont incubations, possibly occurring in the gut (Svenningsen et al., 2012). However, our holobiont incubations showed that such contribution accounted only for 15% of the denitrification rates measured in the benthic-community incubations (**Figure 6**), suggesting that the impact of zebra mussel on denitrification was mainly indirect (related to the altered sediment microbial activity), rather than via stimulation of $NO_3^-$ reduction in anoxic sections of the animal body.

On the contrary, DNRA activity in the holobiont incubation accounted for a major fraction (i.e., 74% = 4.2 μmol $m^{-2}$ $h^{-1}$, **Figure 6**) of the increment in DNRA measured in the benthic-community incubation in the presence of the mussels (i.e., +5.7 μmol $m^{-2}$ $h^{-1}$, **Figures 2**, **6**), indicating a dominant effect of the mussels' microbiome in stimulating DNRA. DNRA bacteria have a competitive advantage over denitrifiers when the organic carbon to $NO_3^-$ ratio is high (Tiedje, 1988). Such conditions are plausibly met in the anoxic section of the mussels' gut. Accordingly, DNRA to denitrification ratio was higher in holobiont incubations (i.e., 0.58) compared to the benthic-community incubation (i.e., 0.16–0.26), suggesting a relatively more favorable niche for DNRA activity in the animal's gut compared to the surrounding sedimentary environment.

Increase in $NO_3^-$ efflux and $D_w$ and simultaneous decrease in $D_n$ are compatible with the thinning of the sediment oxic layer as due to the accumulation of labile phytodetritus (Marzocchi et al., 2018). Similarly, the biodeposition of labile organic carbon by zebra mussel (in the form of feces and pseudo-feces) has been shown to enhance benthic respiration causing the thinning of the sediment oxic layer (Bruesewitz et al., 2008). Such a decrease of the $O_2$ penetration depth shortens the diffusional path for water-$NO_3^-$ to reach the denitrification zone, hence, enhancing $D_w$. At the same time, the contraction of the oxic portion of the sediment diminishes the sediment volume suitable for nitrification, favoring the diffusion of sediment $NH_4^+$ to the water and thus partially decoupling nitrification and denitrification. Moreover, nitrification activity occurring at shallower depths is expected to favor the diffusion of $NO_3^-$ to the water, further contributing to the decrease in $D_n$. Assuming that the drop in $D_n$ is caused by a preferential release of $NO_3^-$ to the water, the so generated $NO_3^-$ would account for 33% of the measured net $NO_x^-$ effluxes in the benthic-community incubation. The accumulation of biodeposits by zebra mussel was visually observed during our incubations, providing a plausible, additional, mechanism by which zebra mussel impacts benthic N dynamics via altering the architecture of the habitat.

## $N_2$ Fixation by Zebra Mussel Holobionts

This study is the first, to our knowledge, to report $N_2$ fixation associated with zebra mussel holobionts. Our incubations show that if unaccounted, this process can lead to 6 and ∼60% overestimation of the benthic community and holobiont-associated net $N_2$ fluxes, respectively. At the lagoon level, $N_2$ fixation has been traditionally attributed to pelagic cyanobacterial activity (Lesutienė et al., 2014; Bartoli et al., 2018; Zilius et al., 2020) and reported to occur seasonally (spring and winter) in undisturbed sediments (Zilius et al., 2018). Dinitrogen fixation associated with the zebra mussel (and more in general in mussel-colonized sediment) has not been accounted so far in estimations of the lagoon's N mass balance (Zilius et al., 2018). The zebra mussel is a dominant benthic organism in the Curonian Lagoon sediment where it has been reported at densities ranging between 40 and 57,000 individuals per square meter (median 12,600) (Daunys et al., 2006). Scaled-up to these abundances, $N_2$ fixation rates derived from our incubations can account for 0.01 to 19.9 μmol of fixed N $m^{-2}$ $h^{-1}$ (median 4.4 μmol N $m^{-2}$ $h^{-1}$), respectively. In summer, cyanobacterial-driven $N_2$ fixation has been reported at rates between 0.9 and 209.4 μmol $m^{-2}$ $h^{-1}$ (median 33.7 $\mu mol^{-2}$ $h^{-1}$; Zilius et al., 2018). Thus, zebra mussel holobionts could possibly contribute a substantial (and so far disregarded) input of N to the lagoon, offsetting the attenuation of the N load via denitrification, and therefore mitigating summer N limitation of the lagoonal system (Vybernaite-Lubiene et al., 2018). Considering maximum densities of 100,000 individuals per square meters reported from zebra mussel-colonized riverine sediments (Svenningsen et al., 2012), its impact could potentially alter N pathways at a scale significantly exceeding that assumed from our experiments and calculations. Further studies are needed to assess the overall relevance of $N_2$ fixation driven by zebra mussels holobionts, its variation under diverse environmental conditions and its seasonal patterns.

The analysis of the microbiome associated with zebra mussels showed that comparatively few ASVs were shared with the waterborne microbial community. The detected high diversity of mussel-associated assemblages (with many taxa not observed in water samples) is consistent with previous findings of specific and diverse bacterial communities associated with bivalves (Lokmer et al., 2016; Cleary and Polonia, 2018; Vezzulli et al., 2018; Mathai et al., 2020). Tenericutes, and more specifically *Mycoplasma*, abundant in the zebra mussel samples, are typical constituents of the core bivalve gut microbiome (Pierce, 2016; Aceves et al., 2018; Pierce and Ward, 2018, 2019), including zebra mussels (Mathai et al., 2020). These obligate cell-associated bacteria are commonly found within a number of eukaryotic hosts and, although previously considered as parasites or even a sign of infection, are now assumed to be involved in mutually beneficial interactions with the host (Fraune and Zimmer, 2008; Holm and Heidelberg, 2016; van de Water et al., 2018). However, no diazotrophic activity has been attributed to any taxa of the Tenericutes phylum (Dos Santos et al., 2012; Albright et al., 2019). On the other hand, the *Flavobacterium* genus, commonly identified in or isolated from bivalves (Pujalte et al., 1999; Aceves et al., 2018; Pierce and Ward, 2019), and fairly abundant in zebra mussel samples from our incubations, includes some species carrying nitrogenase genes. Several studies confirmed the ability of *Flavobacterium* isolates to perform $N_2$ fixation, although this has been demonstrated mainly in plants (Giri and Pati, 2004; Kampfer et al., 2015). A number of other potential diazotrophs were detected in zebra mussel samples in our study. Along with Tenericutes, Spirochetes are well-documented common members of bivalve

gut microbiome (Margulis and Hinkle, 1992). This taxon dominates the microbiome of other eukaryotic organisms in different environments (Lilburn et al., 2001; van de Water et al., 2016) and has been shown to exhibit diazotrophic activity. Burkholderiaceae are among the most well-known $N_2$-fixing bacterial groups in plants (Sprent et al., 2017). Species of this family occupy diverse ecological niches and can be found in soil and water, and in association – even symbiosis – with plants, animals, and fungi (Coenye, 2014). Species of the genus *Leptothrix*, belonging to the Burkholderiaceae family, are commonly found in lakes, lagoons, and swamps, and species of this genus have been studied and isolated as root endophytes in plants (López-López et al., 2010; Li et al., 2011). Finally, members of both *Hydrogenophaga* (i.e., *Hydrogenophaga pseudoflava*) and *Dechloromonas* have shown the ability to fix $N_2$ (Willems et al., 1989; Salinero et al., 2009), although this has been observed so far only in plants, and to our knowledge no indications of diazotrophic activity carried out by these taxa have been reported in bivalves.

The diversity of active diazotrophs in zebra mussels, as characterized by *nifH* gene transcription analysis, also differed substantially from that of water samples. Unlike pelagic diazotrophs, which were mainly represented by Cyanobacteria, *nifH* transcript diversity of the mussels was dominated by *Paenibacillus* and other taxa closely related to Clostridia and Bacteroidetes. Such taxa have been previously described as diazotrophs, although, a few evidences have suggested – so far – their association with bivalves. *Paenibacillus* (phylum Firmicutes) is a genus widely known to include $N_2$-fixing species in soil, and recent studies highlighted its frequent detection and potential role in $N_2$ fixation in aquatic environments (Yu et al., 2018; Pang et al., 2019; Tang et al., 2019). However, to our knowledge, this is the first study reporting its association with benthic invertebrates. On the contrary, Clostridiales have been described as the most frequently detected sequences in the microbiome of Unionidae mussels (Weingarten et al., 2019), which co-exist with zebra mussels in the Curonian Lagoon (Benelli et al., 2019). Besides, Clostridiales are common in the gut microbiome of vertebrates (Colston and Jackson, 2016). In addition, several members of the Clostridiales are euryhaline, may thus perform $N_2$ fixation in the wide range of conditions as those found in estuarine environments (Herbert, 1975). Finally, many Bacteroidetes bacteria possess nitrogenase genes, and are thus capable of $N_2$ fixation (Inoue et al., 2015); however, to the best of our knowledge, studies reporting associations between bivalves and members of Bacteroidetes and/or describing the role of this taxon in $N_2$ fixation in aquatic invertebrates are missing.

## CONCLUSION

Our results show that zebra mussels favor the recycling of N via algal mineralization and by stimulating DNRA activity both in the sediment and via its microbiome. In addition, the mussels mediate a so far overlooked input of nitrogen via $N_2$ fixation. Diazotrophic activity is likely sustained by a unique mussel-associated microbial community, which differs substantially from the $N_2$-fixing community in the water column. Further investigations are needed to assess whether the association of zebra mussels with diazotrophs is a transient interaction or a stable symbiosis, as well as potential fluxes of energy and matter between the microbiome and the host. The capability to host diazotrophic bacteria might be particularly advantageous for zebra mussels to facilitate their establishment and spread in nutrient-poor environments and might therefore represent an important factor in determining their high invasiveness and adaptive capacity. It may also provide an advantage in eutrophic estuaries such as the Curonian Lagoon, which typically display pronounced seasonal variations in inorganic N availability.

## DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, PRJNA658818.

## AUTHOR CONTRIBUTIONS

SB, MB, MZ, and UC conceived and designed the study. UM contributed to the concept. SB, IV-L, TP, AS, MZ, MB, and UC performed the experiments. UM, SB, IV-L, TP, and AS conducted the chemical analyses. UM, SB, and MZ analyzed the geochemical data. AS carried out the nucleic acid extraction and amplicon sequencing. AZ and GQ performed the bioinformatic and statistical analysis on the sequencing data. UM, GQ, and UC wrote the first draft of the manuscript. All authors contributed to draft revisions, read, and approved the final version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.610269/full#supplementary-material

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# PAPER IV

Tobia Politi, Rūta Barisevičiūte, Marco Bartoli, Stefano Bonaglia, Ulisse Cardini, Giuseppe Castaldelli, Akvilė Kančauskaitė, Ugo Marzocchi, Jolita Petkuviene, Aurelija Samuiloviene, Irma Vybernaite-Lubiene, Anastasija Zaiko, Mindaugas Zilius

A bioturbator, a holobiont, and a vector: The multifaceted role of Chironomus plumosus in shaping N-cycling

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# PAPER V

Tobia Politi, Mindaugas Zilius, Marco Bartoli, Martynas Bučas

Amphipods' grazing and excretion loop facilitates Chara contraria persistence in a eutrophic lagoon

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# PAPER VI

Tobia Politi, Mindaugas Zilius, Paola Forni, Anastasija Zaiko,
Darius Daunys, Marco Bartoli

Biogeochemical Buffers in Eutrophic Coastal Lagoon Along an Oxic-Anoxic Transition

# BIOGEOCHEMICAL BUFFERS IN EUTROPHIC COASTAL LAGOON ALONG AN OXIC-ANOXIC TRANSITION

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**Abstract**

The effects of bottom water oxic-anoxic transition on estuarine benthic ecosystems have been extensively studied due to their impacts on sediment biogeochemical functioning, often crashing due to sulphide accumulation and toxicity. Comparatively, the role of benthic macrofauna in modulating such effects is understudied. Macrofauna may increase the resilience of sediments to critical oxygen ($O_2$) transitions via direct and indirect mechanisms acting upon organic matter and solid-phase metal pools that represent internal geochemical buffers for sediments. In this study we analysed whether and how macrofaunal community may facilitate sediment resilience against short-term anoxic events. Thus, net fluxes of reduced metals and inorganic nutrients were measured under oxic conditions and induced anoxia in intact cores representing most of sedimentary environments of the eutrophic Curonian Lagoon. Afterwards, distance-based linear models (distLM) were applied to explore the relationship between the dominant macrofauna taxa, sediment properties and the net fluxes. The results show a mosaic of sedimentary environments including organic matter (OM) poor (0.3%) sandy areas, organic rich (23%) muddy areas and variable combinations of these extremes. The variability of the macrofauna community was comparatively much lower, with oligochaetes and chironomids representing the most abundant taxa at most sampling sites. Along with the induced redox transition metal fluxes tended to increase, together with dissolved inorganic phosphorous release, whereas dissolved inorganic nitrogen and silica tended to decrease. The distLM model evidenced that sediment properties accounted for 84.2% of the total flux variability whereas Chironomidae, Gammaridae and Gastropods cumulatively accounted for 15.8% of the total explained variation in fluxes under oxic conditions. Under anoxic condition macrofauna explained a measurable but minor fraction of flux variability whereas OM and granulometry were best predictors. In the Curonian Lagoon macrofauna has a minor role in regulating fluxes and in providing biogeochemical services as compared to abiotic factors. As such, the benthic system appears vulnerable to short-term events of $O_2$ shortage.

**Keywords**: Benthic fluxes, Redox gradients, Macrofauna, Biogeochemical buffers, Curonian Lagoon

# 1 Introduction

Elevated inputs of organic matter (OM) and nutrients, algal blooms, increasing air and water temperatures and water residence time facilitate the onset of hypoxia/anoxia events in estuarine and coastal areas (Diaz and Rosenberg, 2008; Gilbert et al., 2010; Carstensen et al., 2014). In shallow lagoons, the duration of hypoxic and anoxic events may last hours (diel cycles), days or weeks (Park et al., 2007; Tyler et al., 2009). Long-term (chronic) oxygen ($O_2$) depletion events in shallow systems are unlikely as they imply long periods of persistent and peculiar weather conditions (i.e., high temperatures, no wind, well-established stratification), whereas short-term events are more common and represent a pulse-like disturbance for benthic communities. Although the effects of such disturbances have been well studied at the level of individual species and communities, the potential of such disturbances in the regulation of biogeochemical processes and feedbacks remains poorly documented (Diaz and Rosembergh, 1995; Sousa, 2001; Villnas et al., 2012; Braeckman et al., 2014; Gammal et al., 2019).

Coastal lagoons are complex, heterogeneous ecosystems, at the interface between freshwater and seawater environments. Different environmental gradients, from sediment grain size and depth to the temperature and salinity regimes allow the co-occurrence of a variety of ecological niches and specialised macrofaunal communities (Thrush et al., 2013; Pratt et al., 2015; Karlson et al., 2016). Sedimentary processes and biogeochemical transformations are strongly regulated by the activity of benthic macrofauna via reworking, biodeposition, bioirrigation and the extension of interfaces and oxidised sediment volumes (Solan et al., 2004, Mermillod-Blondin and Rosenberg, 2006, Braeckman et al., 2010, 2014; Josefson et al., 2012; Allgeier et al., 2017). For example, the high density of ventilated burrows in the chemically-reduced sediment extend oxic-anoxic interfaces and promotes nitrogen (N) removal via coupled nitrification-denitrification processes (Mermillod-Blondin and Rosenberg, 2006; Braeckman et al., 2010). Macrofauna also increase the amount of oxidized chemical species, with feedbacks to sediment redox, precipitation of sulphides ($H_2S$) and dissolved inorganic phosphorus (DIP). On the other hand, filter-feeders may increase sedimentary OM by biodepositing faeces and pseudofeces, and enhance dissolved nutrient recycling via increased heterotrophic activity and direct excretion (Prins and Smaal, 1994; Mosley et al., 2015; Zilius et al., 2021). Simultaneously, by reworking sediments, macrofauna can prime the mineralization of more labile and reactive OM, preventing its accumulation (Braeckman et al., 2014). Such macrofauna activities have feedbacks for the pelagic environment, for primary producers (e.g. facilitation for rooted phanerogams), potentially affecting the resilience of the benthic compartment to biogeochemical stressors (e.g., anoxia).

Eutrophication of estuarine systems and related hypoxia events deeply affect macrofaunal communities, starting from the taxa highly sensitive to hypoxic conditions, surviving only a few hours after $O_2$ in bottom water. Depending on duration, $O_2$ depletion can modify the diversity of functional groups, induce loss of species, and decrease in macrofaunal biomass (Josefson and Widbom, 1988; Braeckman et al., 2014; Gammal et al., 2019). Typically, this is associated with altered biogeochemical cycling and increased energy flow through microbes, the lack of bioturbation will determine on oxidation processes (e.g., accumulation of free $H_2S$, sediment toxicity and large release of solutes to the water column) (Mermillod-Blondin et al., 2004). In

the case of eutrophication, the response of benthic fauna to $O_2$ depletion depends on the biological traits of the organisms. Different taxa have different activity patterns and the importance of faunal activities for ecosystem maintenance and geochemical buffers regeneration is closely related to individual functional traits (Papageorgiou et al., 2009; Bartoli et al., 2009).

The biogeochemical buffers in a coastal lagoon ecosystem depend on many biotic and abiotic factors, and their interplay. Among abiotic factors, the geochemical constituents, such as oxidized compounds, can trap or immobilize nutrients or potentially toxic compounds. The ferric iron ($Fe^{3+}$) or manganic manganese ($Mn^{3+}$), for example, affects $H_2S$ toxicity and immobilization of DIP (Kristensen et al., 2003, Giles at al., 2016). Among biotic factors macrofauna represent an important biogeochemical buffer by interfering with structural and chemical properties of the sediments. Macrofauna provide a potentially important mechanism for the re-oxidation of reduced sediment and the transfer and dilution to the water column of toxic compounds which accumulated in the pore water. At the sediment–water interface, more tolerant burrowers via irrigation and burrow ventilation may provide further buffering against toxic compounds thus they may preserve the system from the dystrophic crises. On the other hand, the benthic environment shapes the composition of macrofaunal community through multiple ecosystem's spatial-temporal gradients, including granulometry, OM content, flushing time and $O_2$ level (Gammal et al., 2019).

To address the existing knowledge gap on the functional role of dominant macrofaunal communities in the microbial metabolism and nutrient regeneration under variable $O_2$ conditions we carried out a study in the largest European lagoon, the Curonian Lagoon, SE Baltic Sea. The lagoon is characterized by different sedimentary environments (from coarse sand to silt), dramatic short-term variation in physical-chemical conditions, a relatively low diversity of macrofaunal taxa and reoccurring $O_2$ depletion episodes (Zettler and Daunys, 2007; Zilius et al., 2014). We hypothesized that the response of biogeochemical processes to the $O_2$ depletion depend on the dominant macrofaunal taxa or the functional community structure, characterized by different feeding modes, burrowing activities or resilience to $O_2$ shortage. The overarching aim of this study was to assess how macrofauna's direct or indirect regulation of biogeochemical processes (e.g. nutrient or metals release into the water column) changes in response to the depletion of $O_2$ in collected intact sediment cores from multiple stations across the lagoon, representative of the varying sedimentary environments. The present study goes beyond the identification of the biotic and abiotic variables related to biogeochemical functioning but examines the potential importance of the macrofaunal role in lagoon ecosystems.

## 2 Methods

### 2.1 Study Site

The Curonian Lagoon is a large (1584 $km^2$), shallow (mean depth = 3.8 m) freshwater to oligohaline system situated along the southeast coast of the Baltic Sea. The lagoon is connected to the sea through a narrow strait that limits brackish water intrusions, causing only temporal salinity fluctuations (typically by 1–2, maximum by 7) in the northern part of the lagoon (Zemlys et al., 2013). The Nemunas River is the principal tributary located in the central part of the lagoon. The river inflow affects water circulation patterns and results in

different water renewal times between the northern (annual mean = 76 days; seasonal range =50–100 days) and the southern part of the lagoon (annual mean = 190 days; seasonal range = 100–250 days, Umgiesser et al., 2016). Thus, the northern part is a transitional, estuarine system that is flushed by freshwater with occasional brackish water inflows. The central-southern part is functioning as a lacustrine-like system. The lagoon is characterized as hypertrophic with summer chlorophyll-a concentrations reaching 400 µg Chl-a $L^{-1}$ (Vaičiūtė et al., 2021). Massive seasonal blooms of cyanobacteria are associated with high $O_2$ demand in the water column, due to autotrophic and heterotrophic respiration, resulting in short-term hypoxic/anoxic events in near-bottom water layer (Zilius et al., 2014).

The sedimentary environment of the lagoon is dominated by sand, silt, mud, and shell deposits (Trimonis et al., 2003), which distribution shapes benthic macrofaunal community composition and species abundance (Zaiko et al., 2009). The soft-bottom communities are characterized by oligochaetes, chironomids, and the invasive spionid *Marenzelleria neglecta* (Olenin and Daunys, 2004). The central part of the lagoon is colonized by the invasive filter-feeding zebra mussel *Dreissena polymorpha* and their shell deposits (Zettler and Daunys, 2007).

### 2.2 Sampling and pre-experimental activities

In the period between the July 18th and August 10th, 2018, intact sediment cores for flux measurements and sediment characterization were collected by scuba diving at 19 stations within the northern-central part of the Curonian Lagoon (Fig. 1; Table S1). At each station, four large cores (i.d. 8 cm, length 30 cm) and four small cores (i.d. 4.6 cm, length 20 cm) were manually retrieved by gently pushing liners into sediments. Only undisturbed cores with visually clear overlaying water were used for subsequent analysis. In addition, ~70 L of lagoon water were collected from each station for sediment core maintenance during transportation, pre-incubation, and incubation. In the laboratory, cores intended for flux and benthic respiration measurements were transferred to a temperature-controlled room (24 °C), submerged with the top open into the incubation tanks, containing unfiltered aerated and well-stirred lagoon water for the overnight pre-incubation. A stirring bar, driven by an external magnet at 40 rpm, was inserted in each core approximately 15 cm above the sediment interface to maintain the water column mixed while avoiding sediment resuspension.

After the pre-incubation, two sequential incubations were carried out in the oxic and anoxic regimes. Briefly, the cores were sealed on the top with gas tight lids, equipped with sampling ports, and incubated in the dark for ~2 h (*oxic incubation*). At the beginning and end of each incubation, a 20 mL water aliquot was collected from each core, immediately filtered (using Whatman GF/F filters) to 12 mL PE test tubes for dissolved nutrient (DIN [$NH_4^+$ + $NO_2^-$ + $NO_3^-$], DIP, and DSi) analysis. In addition, 6 mL aliquots were collected, transferred to 5.9 mL exetainer (Labco, Ltd), containing 50 µL of concentrated ultrapure $HNO_3$, for later dissolved metals (Fe and Mn) measurements. Afterwards, the sampled volume was replaced by lagoon water, and cores were left closed for 15–24 h to attain anoxic conditions (< 50 µmol $O_2$ $L^{-1}$) in the water column. Dissolved $O_2$ concentration in the water column was monitored and when the concentration dropped to the desired level, a second short-term (~2 h) *anoxic incubation* was initiated following the previously

165 described procedure. After the second incubation, sediments from all cores were carefully sieved (0.5 mm
166 mesh size) to retrieve macrofaunal organisms for further taxonomic identification and determination of
167 abundance and biomass. Macrofauna was identified to the possible lowest taxonomic level. The biomass was
168 determined as dry weight (DW) after the desiccation at 70°C until constant weight.
169 Small sediment cores were sliced (top 0–5 cm sediment layer) and analyzed for porosity, median grain
170 size (Md) and OM content. Briefly, sediment slices were homogenised, subsampled (5 mL) and dried at 70 °C
171 for 48 h until constant weight. OM was estimated as percentage of weight loss on ignition (450 °C, 2 h) from
172 dried and powdered sediments. Md was determined with laser particle size analyzer (Analysette 22 MicroTec
173 plus, Fritsch GmbH).
174
175 **2.3 Chemical analyses**

176 Dissolved $O_2$ was monitored during the incubations with Clark-type oxygen microelectrode (OX-50, 50 µm
177 sensing tip, 90% response time in < 5s; Unisense A/S) connected to a picoamperometer (PA2000, Unisense,
178 A/S). Dissolved inorganic nutrient concentrations (DIN, DIP, and DSi) were measured with a continuous flow
179 analyzer ($San^{++}$, Skalar; sensitivity 0.3 µM) using standard colorimetric methods (Grasshoff et al., 1983).
180 Dissolved manganese (DMn) and iron (DFe) concentrations were determined by atomic absorption (AA240FS,
181 Varian; sensitivity 0.5 µM).
182
183 **2.4 Statistical analysis**

184 Non-parametric Mann-Whitney tests were applied to determine the effect of redox conditions (oxic vs
185 anoxic) on net fluxes of dissolved nutrient and metals (DIN, DIP, DSi, DMn, and DFe). A one-way analysis
186 of variance (ANOVA) was used to test the differences in macrofauna composition and biomass among stations.
187 Holm-Sidak method was applied as pairwise multiple comparison procedures. The assumptions of normality
188 and homogeneity of variance were checked using Shapiro-Wilk and Cochran's tests, respectively. In the case
189 of heteroscedasticity, biomass data were log(x+1)-transformed. The significance level was set at $\alpha = 0.05$.
190 Non-metric multidimensional scaling (MDS) ordinations based on taxa composition (Bray-Curtis similarity of
191 presence-absence data) were used to identify patterns of similarities among stations.
192 Distance-based linear models (distLM; Anderson et al., 2008) were applied to explore the relationship
193 between the biomass of dominant macrofauna taxa and sediment properties (explanatory variables) and the net
194 solute benthic fluxes (response variables) in the 76 intact sediment cores. Distinct models were applied for
195 each incubation type – oxic and anoxic, respectively. In order to better explain and infer whether macrofauna
196 smooth the short-term effects of anoxia (e.g., increased inorganic N, P or metal fluxes), an additional distLM
197 model was built using the same explanatory variables and the calculated difference between the anoxic and
198 oxic benthic fluxes (Delta fluxes). The models were constructed employing 9999 permutations with a stepwise
199 selection based on AICc values. Explanatory variables were selected following the collinearity assessment
200 using the Draftman plot, and variables with $|r| > 0.7$ were excluded from the models (i.e., porosity). Clustering
201 analysis, using two abiotic parameters (silt content (%) and medium grain size), allowed to group samples into

3 different sedimentary types (muddy, sandy and stations with intermediate characteristics). Using dummy values, two abiotic factors (mud and sand) were created *ex-novo* and included in distLM models as explanatory variables. The biomass ($g_{DW}$ $m^{-2}$) of the six dominant macrofauna taxa (> 97% of total biomass; 122.5 ± 15 $g_{DW}$ $m^{-2}$) was included in the models after the collinearity test. Resemblance matrix for the response variables was generated using Euclidean distance after normalization. Confidence level of 0.05 was accepted for statistical tests. The obtained results were visualized with distance-based redundancy analysis (db-RDA, Anderson et al., 2008), and vectors overlay functions was used to analyse predictor variables relationship with response vectors, Pearson correlation type. The analysis was performed with the software PRIMER 6 & PERMANOVA+ add-on (v.6, Primer-E Ltd.; Clarke and Gorley, 2006).

## 3 Results

### 3.1 Spatial variability in sediment properties, macrofauna assemblages, and solute fluxes

Sediment characteristics reflected the lagoon zonation previously described by Ferrarin et al. (2008), based on water residence time. Sand (65< μm <2000) was the predominant deposit fraction among the studied stations (Fig. 2A; Table 2S). Sandy deposits (~72% of size grain fractions) were primarily located in the hydrodynamically active zones of the lagoon, in the proximity of river inflow (st. 3, 4, 6, and 12) and at lagoon outflow (st. 16–19). The sediment porosity ranged from 0.33 to 0.80. At stations located along the west coast of the lagoon (st. 1, 2, 5, 13, and 14), where water residence time is generally longer, the proportion of silt (< 5 μm) and clay (5 < μm < 63) was substantially higher as compared to the other stations. At sites with silty sediments, Md was ~30 μm, and sediments were characterized by high porosity (≥ 0.90). OM content showed high variability among stations, varying from 0.3 to 23%, and could be predicted by the particles' Md ($R^2$ = 0.24, $p < 0.001$).

Macrofauna composition at the study sites comprised 23 species and 7 higher order taxa (Table S3), their diversity varied from 1 to 13 (average 4.3±0.5) taxa per core (Fig. 2B; Fig. 3). Oligochaetes and chironomids were the only taxa with the occurrence higher than 95%, whereas 21 taxa occurred in less than 10% of cores. Such low number of regularly occurring taxa resulted in a very poor grouping of cores (and sites) along the spatial gradient of the lagoon using MDS ordination and relatively high stress value (0.17).

Abundance of macrofauna organisms in the collected cores was highly variable and ranged from 599 to 43,756 ind. $m^{-2}$ (17,064 ± 1,260 ind. $m^{-2}$ on average) (Fig. 3). Oligochaetes and chironomids were numerically dominant (7,360 ± 616 and 7,475 ± 832 ind. $m^{-2}$ on average, respectively) and together or individually accounted for more than 60% of the total macrofauna organisms in all cores except two from station 13 (relative abundance of 39–40%). In terms of biomass, oligochaetes and chironomids were dominant (>50%) in two thirds of the cores.

The biomass of macrofauna peaked in a few cores (n=6) collected from sandy sediments and dominated by *D. polymorpha* (st. 8, 9 and 11, average biomass 622.1 ± 178.1 $g_{DW}$ $m^{-2}$, range 143.8< x <1359.5 $g_{DW}$ $m^{-2}$) (Fig. 2B). Such average biomass was considerably higher than that measured in another large group of cores (n = 21) distributed among the half of the studied sites, primarily represented by gastropod molluscs, such as *Valvata piscinalis* and *Bithynia tentaculata*. These two species contributed with 72% to the total macrofauna

biomass (30.5 to 298.6 $g_{DW}$ $m^{-2}$, 113.4 ± 16.4 $g_{DW}$ $m^{-2}$ on average). Although gammarids were represented by four species, their biomass was dominant only in two cores collected from station 7, which was mostly represented by *Dikerogammarus vilosus* and had low macrofauna diversity and biomass (2–4 taxa, 11.3–41.4 $g_{DW}$ $m^{-2}$). In spite of uncommon macrofauna structure and low abundance of this group of cores, they were used in further analysis due to highly simplified macrofauna structure, variability and numbers.

The net benthic fluxes of dissolved metals and inorganic nutrients displayed a large variability among stations and between the redox treatments (Fig. 4). At the lagoon scale, measured net fluxes were mostly positive, directed from the sediment to the near-bottom water. Across the redox treatment, higher DMn and DIP fluxes were reported in the anoxic condition (both fluxes increased by 60–70% in the shift oxic-anoxic condition; Mann-Whitney rank test, U = 883 and U = 1844, respectively, p = 0.01). Although net DFe fluxes tended to increase, in the shift from oxic to anoxic condition, differences were not significant (Mann-Whitney rank test, U = 5468, p > 0.05). Similarly, DIN (mostly sustained by $NH_4^+$) and DSi fluxes tended to decrease in the anoxic as compared to the oxic condition but differences were not significant (Mann-Whitney rank test, U = 6314 and U = 5998, respectively, p > 0.05).

### 3.2 Metal and nutrient fluxes in the redox treatments and driving factors

For the oxic incubation, the best selected distLM model explained 49.2% of the total flux variation, and included six explanatory variables: OM, Mud, Sand, Chironomidae, Gastropoda, and Gammaridae (Table 1). The sediment properties accounted for 84.2% whereas the three macrofaunal taxa cumulatively accounted for 15.8% of the total explained variation. The OM content in sediments was the most important contributor to variation in solute fluxes followed by a second abiotic vector (Mud). Oligochaeta, *Pisidium* sp. and *D. polymorpha*, were excluded from the best model's development due to their negligible contribution to total variance.

The distLM identified a significant role of Chironomidae, Gammaridae and Gastropods in structuring the biogeochemical dynamics (oxic fluxes). The results of sequential test showed that Chironomidae biomass explained up to 7% of the total variation in the oxic fluxes, Gammaridae and Gastropoda explained 3 and 2%, respectively. However, in the associated db-RDA, only Chironomidae vector had a strong and significative correlation (r = -0.8; P = 0.002) with the axes (i.e., dbRDA2) (Fig. 5A). The first two axes, alone, captured more than 92% of the explained variation and 45% of the total variation.

The sediment types clearly created three discrete groups: i) mud, ii) sand, and iii) muddy-sand, following a similar gradient ruled by the sedimentary vectors (Mud and Sand) (Fig. 5A). Sandy and muddy-sand stations were mainly associated with the vectors DIP and DMn, whereas the muddy stations likely ordinated the DIN vector (representing high net $NH_4^+$ effluxes). The highest variability in the net fluxes was mainly attributed to the muddy stations, distributed along the first axes (dbRDA1). The Chironomidae vector did not show any strong collinearity with the response vectors and had no obvious collinearity with other explanatory variables. Abiotic vectors (Mud, Sand, and OM) had a strong correlation (r > 0.5) with dbRDA2. Although the variables excluded from the distLM (i.e., Oligochaetes, *D. polymorpha*, and *Pisidium* sp.) were included in the graphical output (db-RDA), however they did not significantly correlated to any RDA axes.

The best model for the anoxic conditions indicated only 4 variables to have a significant effect on the net flux's dynamics: OM, Mud, Gammaridae and Gastropoda. OM contributed alone with 27% and Mud with 3.5% to the best model (Table 1). Fluxes dynamics were significantly influenced by the individual effects of sediment properties (OM, Mud, and Sand). The sediment properties accounted for 89.4% whereas the three macrofaunal taxa cumulatively accounted for less than 10% of the total explained variation. The first two axes together captured more than 97% of the fitted variation and 34% of the total variation. In this case, more biotic variables than in the oxic model were excluded from the best model: Chironomidae, Oligochaeta, *Pisidium* sp., and *D. polymorpha*.

The db-RDA triplot (Fig. 5B) depicted a visible clustering of sand and muddy-sand stations, with few outliers from st.7, suggesting close similarities in benthic fluxes. This net separation of stations and fluxes dynamics in two groups was ordinated along the first axes (dbRDA1). The vectors' overlay shows how the first db-RDA axis (dbRDA1) was strongly correlated to OM ($r > 0.8$), Mud ($r > 0.7$) and Sand ($r > -0.5$). The second dbRDA axis (dbRDA2) was mainly related to Gammaridae' biomass ($r > 0.7$) and to a less extent with Gastropoda ($r > 0.3$). Anoxic fluxes, in sandy and muddy-sand stations, poor in OM, created a data cloud clearly isolated from the muddy, OM-rich stations. Higher effluxes of DFe, DIN and DIP were related to the OM and Mud variables. DSi was positively related to Gammaridae and negatively related to Gastropoda's biomass.

The best model for the oxic-anoxic transition indicated OM, Mud, Chironomidae, and Gammaridae to have a significant effect on the delta flux's dynamics (Table 2). Similarly to the anoxic distLM model, OM contributed alone with 28% and Mud with 7% to the total explained variability of the best model. Sediment properties accounted for 87% whereas the two macrofaunal taxa cumulatively accounted for less than 14% of the total explained variation, a slightly higher contribution than in the anoxic model. The first two axes, alone, captured more than 95.5% of the explained variation and 38.1% of the total variation. The overlay vectors in the oxic-anoxic triplot (Fig. 6) showed correlations among the abiotic vectors and both db-RDA axes similar to the correlations evidenced in the anoxic model. Chironomidae was the biotic variable with the highest explanatory power in the distLM model, and the vector with higher correlation in the dbRDA triplot (axes dbRDA2). However, as it was found in the triplot built on the oxic model, this vector had no strong links with other predictor variables nor with response variables. A strong collinearity was found among vectors of DIP delta and delta metals and the sedimentary characteristics.

# 4 Discussion

## 4.1 Macrofauna functional groups and their potential role as biogeochemical buffers

In the present study, we analysed the role of sedimentary variables together with benthic macrofauna in buffering the release of dissolved metals and nutrients during a simulated short-term bottom anoxia event. Different studies have analysed macrofauna density-dependent effects on various processes, such as $O_2$ or $NO_3^-$ respiration or nutrient regeneration, whereas a limited number of studies have considered the role of macrofauna in providing or increasing the buffer capacity against extreme events (Gammal et al., 2017; Norkko

et al., 2019). Different functional groups may buffer environmental changes in specific ways (Pelegrí and Blackburn, 1995; Møller and Riisgård, 2006; Weissberger et al., 2009; Murphy et al., 2018; Norkko et al., 2019). Scrapers for examples can maintain macrophytes healthy also during the onset of eutrophic conditions, by actively removing epiphytic organisms; as such they buffer the negative effects of nutrient availability (Politi et al., 2021). Similar effects are expected by filter feeders, that may buffer phytoplankton blooms via particles removal. Deposit-feeders are likely to produce biogeochemical buffer capacity by increasing for example the volume of sediments containing oxidized minerals, preventing the release of DIP or toxic compounds as sulphides (Bonaglia et al., 2019). Besides buffering DIP release, surface or deep deposit feeders may prevent the toxic effect of $H_2S$ on nitrifiers and denitrifiers and changes in dominant $NO_3^-$ reduction pathways, from denitrification to dissimilative nitrate reduction to $NH_4^+$, therefore buffering the N regeneration to the water column.

In the Curonian Lagoon, the shift from oxic to anoxic condition can be a relatively fast phenomenon (day-night) generally coupled to cyanobacterial hyperblooms, with considerable implications on pelagic and benthic functioning (Zilius et al., 2014; Bartoli et al., 2018). We hypothesised that the presence of macrofauna may significantly smooth redox-dependent variation of net benthic fluxes due to bioturbation and maintenance of large pools of oxidized compounds within sediments (Kristensen and Blackburn, 1987; de Wit et al., 2001; Conley et al., 2009; Weissberger et al., 2009). This is supported by studies on the high tolerance of specific macrofauna groups to suboxic conditions (e.g., burrowing Chironomidae and Oligochaeta) (Hamburger et al., 1995; Riedel et al., 2012; Villnas et al., 2012; Zou et al., 2019). In sediments bioturbated by such organisms, therefore, a large pool of oxidized metals can build up and may delay the onset of strongly negative redox potential values in pore water (Benelli et al., 2019; Bartoli et al., 2021).

Our investigation, carried out at 19 sampling stations covered the full range of sedimentary environments in the Curonian Lagoon. A large proportion of the studied stations are characterized by low sediment density and high OM content due to dominating hydrodynamic factors, such as a long residence time and low shear stress, that favour sedimentation versus material erosion and export (Umgiesser et al., 2016; Mėžinė et al., 2019). This affects the trophic status of the lagoon, leading to hypertrophic condition and constraining benthic macrofauna abundance and functional diversity (Merrit and Cummins, 1996).

In the open Curonian Lagoon, macrofaunal communities mostly include opportunistic and resilient species, whereas in the littoral zone more biodiverse communities establish (Olenin and Daunys, 2004). In the present study, retrieved macrofaunal species could be grouped in those living in a more coarse sedimentary environment (sand flat, dominating MD fractions 65 < μm < 2000.) and in those living in soft, organic sediments (muddy areas, dominating MD fraction 65 <μm). We classified the retrieved species according to Gerino et al. (2003) and grouped them according to their feeding behaviour and burrowing mode. We therefore recognised 4 main functional groups including surface feeders (Gastropoda and Pisidiusm sp.), grazers-shredders (Gammaridae) and filter-feeders (*D. polymorpha*), that were abundant in sandy environments, and deposit-feeders (Oligochaeta and Chironomidae) that were widespread but primarily found in muddy environment. In low hydrodynamic areas, the sedimentation of phytoplankton over muddy sediments increases the secondary productivity of burrowing species such as Chironomidae, that have developed specific

353 adaptations to live under $O_2$-shortage (Jones et al., 2008). Chironomidae were demonstrated to condition the
354 sediment by increasing its redox and stimulating the retention of metals via precipitation with iron hydrooxides
355 (Benelli et al., 2017, Politi et al., 2021). Another dominant functional group, represented mainly by *D.*
356 *polymorpha*, has been demonstrated to have a large impact on N-cycling. Mussels enhance DIN efflux via
357 $NH_4^+$ excretion (Lavrentyev et al., 2000), stimulate benthic nitrification and denitrification (Bruesewitz et al.,
358 2006; 2008), and the release of DIP to the water column (Benelli et al., 2019), thus potentially stimulating
359 pelagic dinitrogen ($N_2$) fixation (Marzocchi et al., 2021).
360
361 **4.2 Abiotic and biotic regulation of benthic fluxes**

362 Different studies have stressed the main role of abiotic sedimentary features as regulators of benthic
363 metabolism, nutrient and metal fluxes and recycling under contrasting redox conditions. Such regulation
364 occurs through sediment grain size and porosity, OM pool and its macromolecular quality (Relexans et al.,
365 1992; Pusceddu et al., 2009; de Vicente et al., 2010; Belley, 2016 a, b; Bartoli et al., 2021). Similarly, both
366 distLM models applied to the Curonian Lagoon data highlight the sediments characteristics as main predictors
367 of benthic biogeochemical processes under oxic and anoxic conditions.
368 In the Curonian Lagoon, the OM content was the driving factor of dissolved nutrient fluxes, explaining
369 most of flux variations under anoxia. Although the onset of anoxia is expected to enhance DIN sedimentary
370 release in most of muddy and sandy stations, this seldom happened in the Curonian Lagoon sediments. In most
371 of the examined stations, DIN net fluxes tended to decrease, likely due to lower efficiency of ammonification
372 under oxygen shortage or lower activity and excretion by macrofauna. OM content in sediments was also a
373 driving factor of DIP fluxes. DistLM model for the anoxic condition depicted a strong collinearity among DIP
374 fluxes and metals. Negative linear collinearity between DIP and DMn and positive collinearity between DIP
375 and DFe suggest that DIP becomes more mobile when the oxidized pools of Mn within sediments are
376 exhausted. This may suggest the importance of Mn pools in maintaining oxidized pool of $Fe^{2+}$ and regulating
377 DIP release from sediments under anoxic conditions in the Curonian Lagoon (Bartoli et al., 2021).
378 However, a fraction of the total variability, more under oxic conditions, but also under anoxia, was
379 explained by the macrofauna communities. According to the distLM, biomass of few macrofaunal species
380 (biotic explanatory vectors) were statistically related with measured rates in the oxic conditions. At the
381 opposite, under anoxia, sediment characteristics more than macrofauna had strong links with the majority of
382 the measured fluxes. This issue may not be true in different sedimentary environments of more oligotrophic
383 systems where environmental gradients (e.g., the OM content of sediments) are not so pronounced and where
384 the role of macrofauna can be more important (Bracken et al., 2008; Norkko et al , 2015; Allgeier et al., 2017).
385 Macrofauna is expected to be less involved in the ecosystem functioning under anoxia. Under $O_2$ shortage
386 macrofauna may display different responses in terms of distribution, feeding mode and metabolism (Diaz and
387 Rosenberg, 1995; Gibson and Atkinson, 2003; Vaquer-Sunyer and Suarte, 2008; Rakocinski and Menke,
388 2016). All these responses have biogeochemical implications. Lower respiration, excretion and bioturbation
389 under severe hypoxia for example are expected to decrease rates of $O_2$ consumption and nutrient recycling
390 (Pearson and Rosenberg, 1978; Riedel et al., 2008; Braeckman et al., 2010). Decreased metabolic activity

391 results in a limited excretion rates of nutrients-rich wastes (i.e. mucus, $NH_4^+$, faecal pellets). These issues are
392 important to address as bioturbation by burrowing deposit feeders supports biogeochemical services as
393 particles reworking, mixing of new and old OM pools, facilitation of the heterotrophic microbial community,
394 phosphate retention within the sediments, immobilisation of $H_2S$ into insoluble iron mono-sulphides or coupled
395 ammonification-nitrification-denitrification (Stief et al., 2010; Sturdivant et al., 2012). Moreover, macrofauna
396 bioturbation stimulates advective solutes transport whereas, in the absence of macrofauna activity, diffusion
397 regulates the mobility of dissolved substances across the sediment–water interface (Meile and Van Cappellen,
398 2003).
399 In light of these considerations, our DistLM results for the oxic model are consistent with findings of Benelli
400 et al. (2017) and Politi et al. (2021), showing that at increasing burrowing animals' density (Chironomidae
401 biomass), solutes fluxes (e.g. DIN and DIP) may be substantially affected. Via biodiffusion and biorrigation
402 these organisms contrast nutrient effluxes stimulating downward transport of available electron acceptors as
403 $O_2$ and $NO_3^-$, augmenting the sedimentary pools of oxidized minerals and providing geochemical buffer
404 capacity also during hypoxic condition (Svensson et al., 2001; Samuiloviene et al., 2019, Politi et al., 2021).
405 However, this result was not clearly visible in the graphical output provided in the dbRDA triplot for the oxic
406 condition (Fig. 5A), as the vector Chironomids, although major component of the second RDA axes, is not
407 related to any sedimentary fluxes. The dbRDA ordination method presented as triplot did not highlight the
408 potential role of Chironomids and other shallow burrowers in regulating the direction and intensity of dissolved
409 nutrient fluxes. Experiments in reconstructed sediments revealed clear and measurable effects of Chironomids
410 in driving biogeochemical N and P cycling, and effecting their fluxes and pore water concentrations.
411 Reconstructed sediments remove a large fraction of natural variability, including the interactions of tested
412 macrofauna species with other organisms. As such they might artificially enhance the role of single macrofauna
413 species. Alternatively, we speculate that under the specific conditions of the present activity, the sedimentary,
414 abiotic features represented much stronger driver of processes than macrofauna. Experiments described in the
415 present study were carried out in the summer, at peak water temperature, that may represent a stress condition
416 for macrofauna due to low oxygen solubility and high OM inputs from the water column. They could be
417 repeated in other periods of the year as the spring, when water temperatures and microbial heterotrophic
418 activity are lower and macrofauna more abundant and less stressed.
419 Grazers-shredders (*Gammaridae*) presence and activity showed to be related with DSi fluxes in both the
420 models. Biogenic silica within the lagoon derives mostly from diatom frustules accumulated on surface
421 sediment during the spring, successively degraded and recycled to the water column during the summer period
422 (Pilkaitytė and Razinkovas, 2007). *Gammaridae* likely contribute to DSi recycling by the continuous feeding
423 activity on diatoms, active reworking of upper sediment microlayers and excretion in the water column,
424 (Quigley and Vanderploeg, 1991; Tuominen et al., 1999). Also, the abundant *D. polymorpha* and Pisidium sp.
425 were expected to contribute significantly to benthic pelagic coupling. Many studies highlighted the strong
426 contribution to N and P dynamics of *D. polymorpha* (Marzocchi et al., 2021 and references therein) whereas
427 less studies focused on the role of Pisidium sp. The presence of these two organisms, in oxic and anoxic
428 condition produced no appreciable variation once others variables are fitted. This might be due to coupled

429 animal‘s excretion and microbial uptake that results in a net and null effect in nutrients fluxes in the model
430 (Bartoli et al., 2018). For example, a high $NH_4^+$ excretion associated with animals (i.e. high chironomids'
431 biomass), may be visible in the triplot and related to higher DIN fluxes. However, the oxic condition coupled
432 with burrowing activity results in a direct oxidation of the produced $NH_4^+$ and in no visible correlation between
433 animals and DIN fluxes (Bartoli et al., 2018).
434 Gastropoda have low tolerance to chemically reduced sediments (Eden et al., 2003), however in both our
435 db-RDA models *Bithynia tentaculata* was associated to OM rich substrates, recycling large Si amounts
436 probably due to *Bithynia* multiple feeding habits (i.e., algae collector, filter-feeder or grazer). Some species of
437 Gastropoda (e.g., *Cerithium vulgatum)* play an important role in metal cycling in shallow water marine
438 ecosystems (Nicolaidou and Nott, 1999), they may retain metals by incorporating them into insoluble granules
439 formed in the digestive glands and excrete them as durable faecal pellets that are immobilized within the
440 sediment. The fate of metals in granules released from the faecal pellets depends on the properties of the metals
441 and the characteristics of the sediment, especially the oxidation status (Jokinen, 1992).
442 The multivariate model applied to the delta of the measured fluxes along with the redox transition reports
443 which biotic and abiotic factors are major determinants of the observed changes in fluxes. Along such a
444 transition, fluxes may decrease, increase or remain stable. The calculated difference between the anoxic and
445 oxic benthic fluxes (Delta fluxes) may be negative when macrofaunal bioturbation activity decreases, due to
446 inefficient mineralization under $O_2$ shortage and to inhibition of microbial activity due to the accumulation of
447 toxic metabolites (Braeckman et al. 2010; Bartoli et al., 2021). Positive delta fluxes, in particular of DIN, DIP
448 and metals, could be the consequence of nitrification inhibition and reduction of oxidized metal pools, in
449 particular $Fe^{3+}$. Similar fluxes under oxic and anoxic conditions (e.g. Delta fluxes = 0) may result as net effect
450 of opposite responses, buffering each other (e.g. N less excretion compensated by decreased nitrification and
451 increased $NH_4^+$ efflux). These alterations in the ecosystem functioning depend to a large extent on the degree
452 of anoxia disturbance on the benthic community performances. Anoxia induced disturbance results in a non-
453 random pattern and in a specific change of community structure and behaviour (altered community
454 performance) (Villnas et al., 2012).
455 The triplot (Fig. 6) built on transition between oxic and anoxic conditions showed how at muddy and
456 sandy stations, large and small variations of of DIP and DFe fluxes were measured, respectively. At both sites
457 biotic vectors were clearly second order player for such different responses. Interestingly, the biomass of
458 Chironomidae and Gammaridae was statistically important, at least when variables are considered alone.
459 Chironomidae likely contributed to delayed DIP release as previously explained, by increasing oxidized metal
460 pools under oxic conditions. This is in agreement with a previous study that showed how in the Curonian
461 Lagoon, a prolonged period of anoxia is needed (more than one day), to exhaust sedimentary geochemical
462 buffer in muddy sediments with burrowing chironomids (Zilius et al., 2015).
463 Contrarily to Chironomidae, Gammaridae amplified the variations in sediment effluxes of DIP and DIN
464 net fluxes. In natural conditions, mobile amphipods can migrate before the onset of anoxia thus abandoning
465 habitats. In our experimental set-up, containing isolated sediments, amphipods are directly facing anoxic
466 conditions, stress conditions and anomaly in their behaviours may be related to the significant role emerged in

our ordination model. However, under $O_2$ shortage conditions, these amphipods are likely to decrease their metabolic activity and interrupt surficial sediment reworking. In this scenario fluxes of DIN and DIP, directly correlated to mobile macrofauna metabolic activity, may be replaced and overtaken by higher rates of ammonification and P de-sorption and subsequent sediment efflux. Results of the present study show that the presence of abundant bivalves does not seem to have correlation with the capacity of sediment to buffer the variations in DIN, DIP or DSi, however their incomplete closing of the valves may introduce $O_2$ - and $NO_3^-$ rich water within sediments, with a net reoxidation of chemically reduced compounds (i.e., $Fe^{2+}$ and $Mn^{2+}$ pools).

## 5 Conclusions

In the summer period, benthic fluxes in the Curonian Lagoon are mostly regulated by abiotic factors as granulometry and organic matter content, and much less by macrofauna abundance and diversity. Sedimentary features vary considerably among lagoon areas, spanning from silty organic-rich sediments to sandy, organic poos areas and therefore including extremely different settings. Comparatively, macrofauna communities are more homogeneous and mostly represented by a few, widespread species of tolerant organisms.

The simplified macrofauna community is likely the result of multiple stressors acting upon the benthic system. Turbidity limits benthic primary production whereas large pelagic production results in elevated precipitation of labile particles enhancing the request of electron acceptors. The latter, associated to long water residence time and scarce gas solubility under summer temperatures affect oxygen availability in the benthic compartment.

The minor role of macrofauna as drivers of benthic fluxes might be true for the Curonian Lagoon during summer but not in other seasons or in other lagoons that have more diverse and structured macrofauna communities. Indeed, in pristine, oligotrophic ecosystems macrofauna facilitate nutrient recycling from the sediment (a nutrient-rich compartment) to the water column (a nutrient-poor one), and support recycling and reuse of N and P by primary producers. In the Curonian Lagoon the nutrient level can be high in both sediments and water column and the role of macrofauna in benthic-pelagic coupling is not central or necessary. We speculated that some functional groups (e.g., burrowers) provide in such hypertrophic system some alternative services, as those related to sediment oxygenation, to prevent or smooth the negative effects of anoxia on sediment toxicity and loss of sink function. This is a key service in heavily OM loaded systems that may experience short-term anoxia due to blooms and rapid $O_2$ consumption, also as consequence of interacting eutrophication and heath waves. Results indicate something different: macrofauna plays a little role also in increasing sediment resilience to anoxia. Chironomids, that are abundant and tolerant organisms in the Curonian Lagoon, become flying midges in the beginning of summer and leave sediments during the most critical period for the sediments. An improvement of macrofaunal community in the Curonian lagoon, and an increase of the biogeochemical services that macrofauna provide, can be expected only if a substantial reduction of nutrient and organic matter loads is achieved in this system.

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510 **Availability of data and material**

511 Data can be accessed upon request to the corresponding author.

512 **Conflict of Interest Statement**

513 The authors declare that they have no conflict of interest.

514 **Contributions**

515 TP, MB designed the study; MZ, TP, MB sampled in the field; MB, MZ, TP, PF worked in the laboratory and
516 characterized macrofauna; TP, AZ analysed data; TP, MB wrote the manuscript. All authors read and approved
517 the final manuscript.

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780 FIGURES

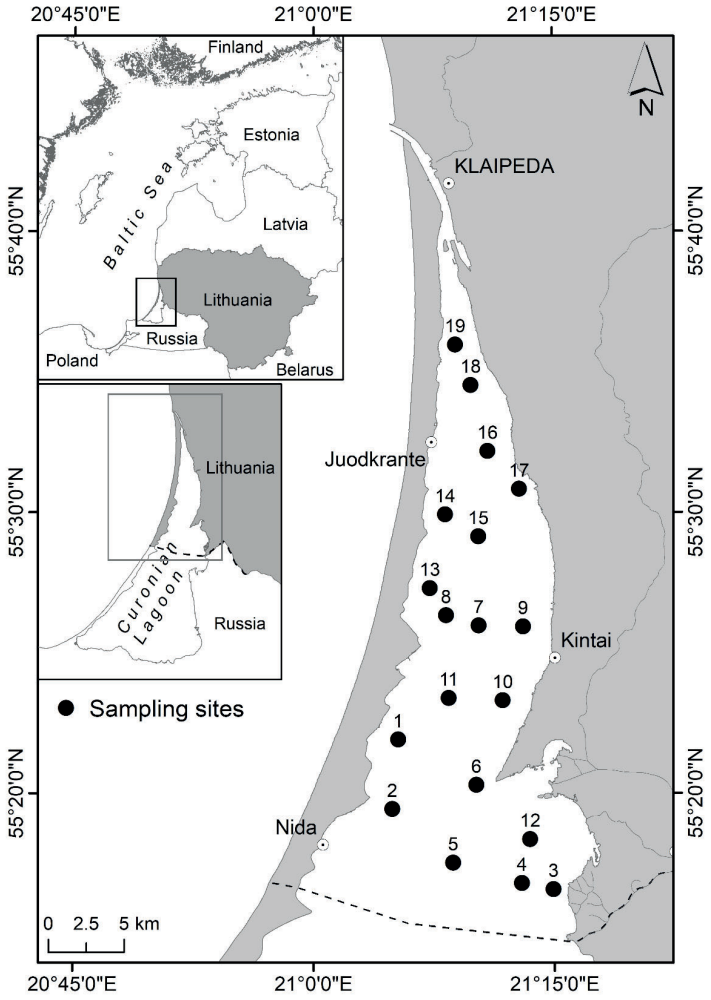


**Fig. 1** Map of the Curonian Lagoon with indicated network of sampling sites

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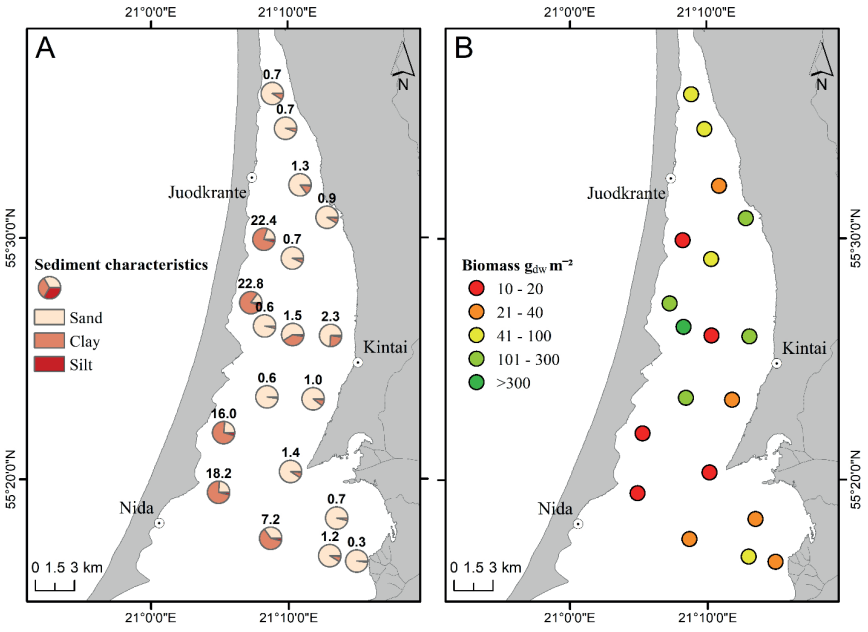


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787 **Fig. 2** Spatial patterns of sediment characteristics, including dominant fractions of deposits (pie–
788 chart) and organic matter content (%, on top of each chart) (A), and ranges of macrofaunal biomass
789 at each site ($g_{dw}$ $m^{-2}$) (B)

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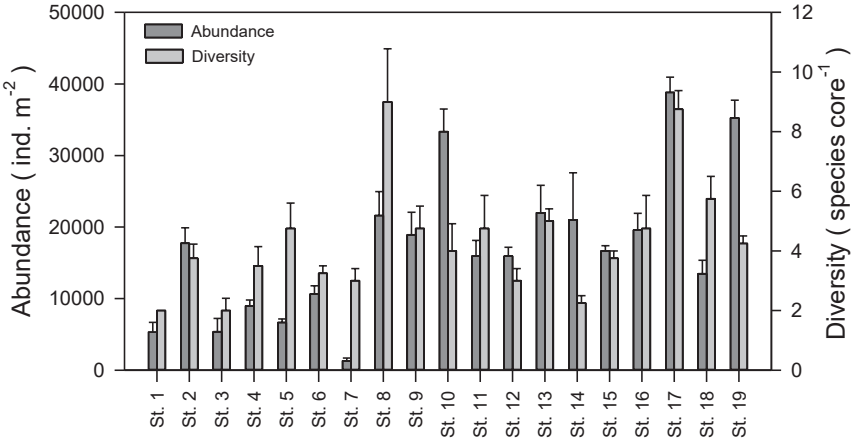


**Fig. 3** Total abundance of benthic macrofauna (ind. m−2) and taxonomic diversity (no. of species per core) in incubated sediment cores from 19 stations in the Curonian Lagoon (n=4 replicates per station) (error bars denote standard errors; some are not visible)

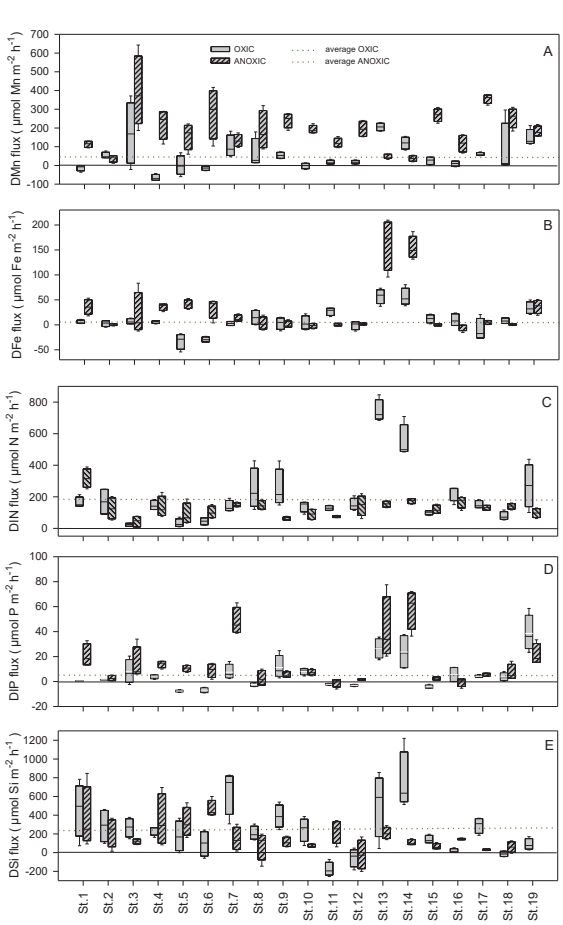


**Fig. 4** Sediment–water fluxes of dissolved manganese (A), iron (B), inorganic nitrogen (C), phosphorus (D), and silica (E) in oxic (grey boxes) and anoxic conditions (stripped boxes) from 19 stations in the Curonian Lagoon. Data range (whiskers), upper and lower quartiles (edges), and the median (horizontal line) are represented for n = 4 replicates. Dotted lines indicate mean fluxes in oxic (blue) and anoxic (red) conditions (n=76)

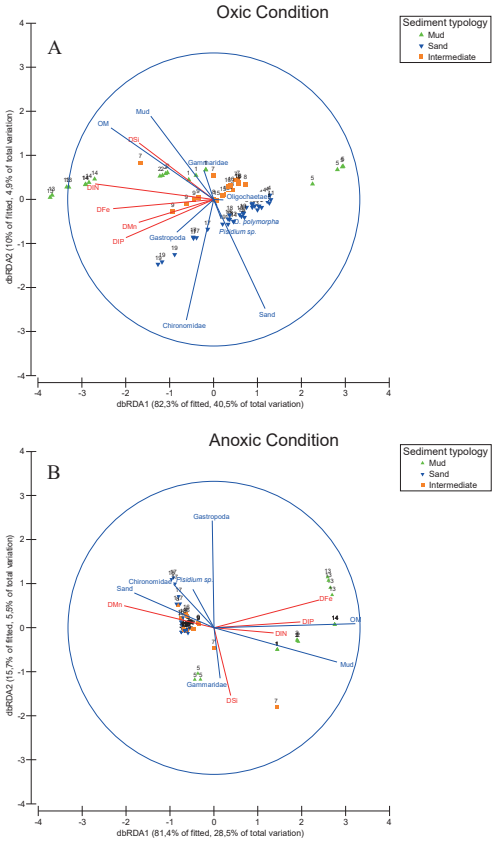


**Fig. 5** Distance based triplots of redundancy analysis (db-RDA) on solute fluxes (DIN, DIP, DSi, DFe, and DMn) in oxic (A) and anoxic conditions (B) using macrofauna biomass (Chironomidae, Oligochaeta, Gastropoda, Gammaridae, *D. polymorpha*, and Pisidium sp.) and sediment characteristics (OM – organic matter, Mud and Sand) as explanatory variables. Numbers indicate single cores collected at sampling stations. The projection of any sample onto arrows approximates the measured value in that sample

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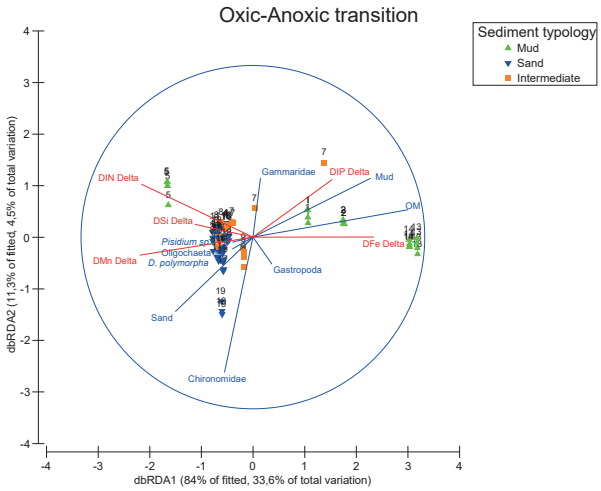


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821 **Fig. 6** Distance based triplot of redundancy analysis (db-RDA) on solute fluxes (DIN, DIP, DSi, DFe,
822 and DMn) recalculated as the difference between the oxic and the anoxic fluxes. The macrofaunal
823 biomass (Chironomidae, Oligochaeta, Gastropoda, Gammaridae, *D. polymorpha*, and Pisidium sp.)
824 and sediment characteristics (OM – organic matter, Mud and Sand) as explanatory variables.
825 Numbers indicate single cores collected at sampling stations. The projection of any sample onto
826 arrows approximates the measured value in that sample

827

828 TABLES

829

830 **Table 1** Distant-based linear model output for the two models (oxic and anoxic conditions) of
831 biological and environmental drivers against solute fluxes at the sediment–water interface (n=76).
832 Var – explained variance (%) in fluxes by explanatory variables. Significance level is < 0.05

833

| **Oxic condition ($R^2$ = 0.49)** | | | | | **Anoxic condition ($R^2$ = 0.35)** | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Marginal test** | | | | | **Marginal test** | | | | |
| Exp. variables | Pseudo-F | P | % var. | | Exp. variables | Pseudo-F | P | % var. | |
| OM | 20,0 | **0,0001** | 21,3 | | OM | 27,1 | **0,0001** | 26,8 | |
| Mud | 7,5 | **0,0013** | 9,2 | | Mud | 19,4 | **0,0001** | 20,8 | |
| Sand | 6,2 | **0,0026** | 7,8 | | Sand | 8,5 | **0,0001** | 10,3 | |
| Oligochaeta | 0,7 | 0,5228 | 0,9 | | Oligochaeta | 2,3 | 0,0624 | 3,1 | |
| *Pisidium sp.* | 0,8 | 0,4755 | 1,0 | | *Pisidium sp.* | 0,8 | 0,5080 | 1,1 | |
| *D. polymorpha* | 0,6 | 0,5735 | 0,8 | | *D. polymorpha* | 0,9 | 0,3954 | 1,2 | |
| Gammaridae | 1,7 | 0,1510 | 2,3 | | Gammaridae | 1,5 | 0,2164 | 1,9 | |
| Chironomidae | 4,0 | **0,0164** | 5,2 | | Chironomidae | 4,2 | **0,0068** | 5,4 | |
| Gastropoda | 4,6 | **0,0102** | 5,8 | | Gastropoda | 2,5 | **0,0470** | 3,2 | |
| Sequential test | | | | | Sequential test | | | | |
| Exp. variables | Pseudo-F | P | % var. | % var. (cum.) | Exp. variables | Pseudo-F | P | % var. | % var. (cum.) |
| OM | 20,01 | **0,0001** | 21,3 | 21,3 | OM | 27,1 | **0,0001** | 26,8 | 26,8 |
| Mud | 16,54 | **0,0001** | 14,5 | 35,8 | Mud | 3,7 | **0,0108** | 3,5 | 30,3 |
| Chironomidae | 8,37 | **0,0002** | 6,7 | 42,5 | Gastropoda | 3,0 | 0,0573 | 2,7 | 33,1 |
| Gammaridae | 4,20 | **0,0294** | 3,2 | 45,7 | Gammaridae | 2,2 | 0,0668 | 2,0 | 35,1 |
| Gastropoda | 2,64 | **0,0417** | 2,0 | 47,7 | | | | | |
| Sand | 2,08 | 0,0859 | 1,5 | 49,2 | | | | | |

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836 **Table 2** Distant-based linear model output for the model oxic-anoxic Transition (delta fluxes) of
837 biological and environmental drivers against solute fluxes at the sediment–water interface (n=76).
838 Var – explained variance (%) in fluxes by explanatory variables. Significance level is $P < 0.05$

839

**OX-ANOX Transition condition** ($R^2 = 0.40$)

Marginal test

| Exp. variables | Pseudo-F | P | % var. |
|---|---|---|---|
| OM | 28,3 | **0,0001** | 27,7 |
| Mud | 14,8 | **0,0001** | 16,7 |
| Sand | 7,2 | **0,0001** | 8,9 |
| Oligochaeta | 2,1 | 0,0967 | 2,7 |
| *Pisidium sp.* | 0,4 | 0,7884 | 0,5 |
| *D. polymorpha* | 1,0 | 0,3078 | 1,3 |
| Gammaridae | 1,5 | 0,1832 | 2,0 |
| Chironomidae | 3,9 | **0,0144** | 5,0 |
| Gastropoda | 2,4 | 0,0563 | 3,1 |

Sequential test

| Exp. variables | Pseudo-F | P | % var. | % var. (cum.) |
|---|---|---|---|---|
| OM | 28,3 | **0,0001** | 27,7 | 27,7 |
| Mud | 7,8 | **0,0001** | 7,0 | 34,7 |
| Chironomidae | 3,4 | **0,0122** | 3,0 | 37,6 |
| Gammaridae | 2,7 | 0,0637 | 2,3 | 39,9 |

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842 SUPPLEMENTARY MATERIAL

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844 **Table S1** Coordinates and map of the sampling stations in the Curonian Lagoon, with a qualitative
845 indication of water residence time (WRT).

| Stations | Latitude | Longitude | WRT |
|---|---|---|---|
| 1 | 55° 19,7004 | 21° 4,717 | High |
| 2 | 55° 17,206 | 21° 15,583 | High |
| 3 | 55° 17,279 | 21° 13,858 | Low |
| 4 | 55° 17,976 | 21° 9,179 | Low |
| 5 | 55° 20,832 | 21° 10,601 | Low |
| 6 | 55° 25,708 | 21° 10,352 | Low |
| 7 | 55° 26,089 | 21° 7,920 | Low |
| 8 | 55° 25,769 | 21° 13,415 | High |
| 9 | 55° 23,386 | 21° 11,837 | Low |
| 10 | 55° 23,259 | 21° 8,616 | Low |
| 11 | 55°18,382 | 21° 13,893 | Low |
| 12 | 55° 26,856 | 21° 6,903 | Low |
| 13 | 55° 29,527 | 21° 7,727 | High |
| 14 | 55° 28,92 | 21° 10,359 | High |
| 15 | 55° 31,884 | 21° 11,597 | High |
| 16 | 55° 30,728 | 21° 13,286 | Low |
| 17 | 55° 34,709 | 21° 10,594 | Low |
| 18 | 55° 36,017 | 21° 9,036 | Low |
| 19 | 55° 21,8234 | 21° 4,922 | Low |

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849 **Table S2** Mean sediment features of the upper 10 cm measured at 19 sampling stations in the
850 Curonian Lagoon (Lithuanian part). MD – median grain size, OM – organic matter. Averages ±
851 standard errors are reported based on 4 replicates

852

| Station | Porosity | Md (μm) | <63 μm (%) | OM (%) |
|---|---|---|---|---|
| 1 | 0.9 ± 0.0 | 35.9 ± 5.0 | 4.7 | 15.9 ± 2.4 |
| 2 | 0.9 ± 0.0 | 37.6 ± 2.6 | 4.9 | 18.2 ± 2.0 |
| 3 | 0.3 ± 0.0 | 239.4 ± 3.9 | 0.5 | 0.3 ± 0.1 |
| 4 | 0.4 ± 0.0 | 310.9 ± 5.5 | 1.3 | 1.2 ± 0.1 |
| 5 | 0.8 ± 0.1 | 45.2 ± 3.7 | 4.0 | 7.2 ± 0.1 |
| 6 | 0.4 ± 0.0 | 252.5 ± 16.1 | 1.5 | 1.4 ± 0.4 |
| 7 | 0.5 ± 0.1 | 277.2 ± 18.3 | 2.9 | 1.5 ± 0.4 |
| 8 | 0.3 ± 0.0 | 242.8 ± 3.2 | 0.9 | 0.6 ± 0.1 |
| 9 | 0.6 ± 0.2 | 159.7 ± 61.7 | 2.4 | 2.3 ± 1.4 |
| 10 | 0.4 ± 0.1 | 187.6 ± 14.3 | 1.5 | 0.9 ± 0.2 |
| 11 | 0.4 ± 0.0 | 226.2 ± 4.4 | 0.7 | 0.6 ± 0.1 |
| 12 | 0.4 ± 0.0 | 214.1 ± 10.7 | 0.9 | 0.7 ± 0.1 |
| 13 | 0.9 ± 0.0 | 29.6 ± 2.0 | 5.2 | 22.8 ± 4.0 |
| 14 | 0.9 ± 0.0 | 34.4 ± 1.7 | 4.2 | 22.4 ± 2.5 |
| 15 | 0.4 ± 0.1 | 209.8 ± 3.8 | 1.2 | 0.7 ± 0.1 |
| 16 | 0.6 ± 0.3 | 158.2 ± 23.0 | 1.9 | 1.3 ± 0.5 |
| 17 | 0.4 ± 0.1 | 179.8 ± 6.8 | 1.4 | 0.9 ± 0.1 |
| 18 | 0.4 ± 0.1 | 246.9 ± 5.7 | 0.9 | 0.7 ± 0.1 |
| 19 | 0.4 ± 0.1 | 292.1 ± 11.6 | 1.0 | 0.7 ± 0.2 |

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858 **Table S3** Checklist of macrofauna taxa recovered from incubated intact sediment cores

| Taxaa | Taxa | Stations | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 |
| Anellidae | *Oligochaeta* | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x |
| Anellidae | *Glossiphonia complanata* | | | | | | | | | | x | x | | | | | | | | |
| Anellidae | *Helobdella stagnalis* | | | | | | | | x | x | x | | | | | | x | | | |
| Anellidae | *Alboglossiphonia heteroclita* | | | | | | | | x | | x | x | x | | | | | x | | |
| Anellidae | *Erpobdella nigricollis* | | | | | | | x | x | | | x | | | | | | | | |
| Aracnida | *Hydracarina undet* | | | | | | | | | | | | | | x | x | | x | | |
| Bivalvia | *Pisidium casertanum* | | x | x | | x | x | | x | x | x | | x | x | | | x | x | | |
| Bivalvia | *Pisidium supinum* | | | | | x | | | x | | x | | | | | | | | | |
| Bivalvia | *Pisidium nitidum* | | | | x | x | | | x | | | | | x | | | | x | x | |
| Bivalvia | *Sphaerium solidum* | | | | | x | | | | | | | | | | | | | | |
| Bivalvia | *Sphaerium rivicola* | | | | | | | | | | x | | | | | | | | | |
| Bivalvia | *Dreissena polymorpha* | | | | | | | | x | x | | x | | x | | | | x | | |
| Bivalvia | *Unio pictorium* | | | | | | | | x | | | | | | | | | | | |
| Crustacea | *Ostracoda* | | x | | | | | | | | x | | | | x | | x | x | x | x |
| Crustacea | *Obesogammarus crassus* | | | | | | | x | | | | | | | | | | | x | x |
| Crustacea | *Pontogammarus robustoides* | | | | | | | x | | | | | | | | | | | x | |
| Crustacea | *Echinogammarus warpachowskyi* | | x | | x | | | | x | | | | x | | | | x | | | |
| Crustacea | *Gammaridae undet (juvenile)* | | x | | | | | | | | | | | | | | | x | x | |
| Crustacea | *Amphibalanus improvisus* | | | | | | | | | | | | | | | | | x | | |
| Crustacea | *Copepoda* | | | | | | | | | | | | | x | | | | | | |
| Crustacea | *Dikerogammarus villosus* | | | | | | | x | | | | | | | | | | | | |
| Diptera | *Chironomidae* | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x |
| Gasteropoda | *Bithynia tentaculata* | | x | | | | | | x | x | | x | | x | | x | x | x | x | |
| Gasteropoda | *Radix balthica* | | | | | | | | | | | | | | | | | x | x | |
| Gasteropoda | *Valvata piscinalis* | | | | x | | | | x | | | x | | x | | x | x | x | x | |

| | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Gasteropoda | *Potamopyrgus antipodarum* | | | | | | | x | | | | | x | | | | x | | | |
| Gasteropoda | *Lithoglyphus naticoides* | | | | | x | | | | | | | | | | | | | | |
| Gasteropoda | *Radix ampla* | | | | | | | | x | | | | | | | | | | | |
| Lepidoptera | *Lepidoptera undet* | | | | | | | | | | | | | | | | | | x | |
| Nematomorpha | *Gordius aquaticus* | | x | | | x | x | x | x | x | | x | x | | | x | | | | x |
| Trichoptera | *Limnephilidae undet* | | | | x | | x | | x | | | | | | | | | x | | |

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Klaipėdos universiteto leidykla

Tobia Politi
BIOGEOCHEMICAL INTERACTIONS AMONG BENTHIC MACROFAUNA, MICROBIAL COMMUNITIES AND MACROPHYTES IN EUTROPHIC COASTAL LAGOONS
*Doctoral dissertation*

SĄVEIKA TARP DUGNO BESTUBURIŲ, MAKROFITŲ IR MIKROORGANIZMŲ IR JOS VAIDMUO EUTROFINĖSE LAGŪNOSE
*Daktaro disertacija*

Klaipėda, 2022

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