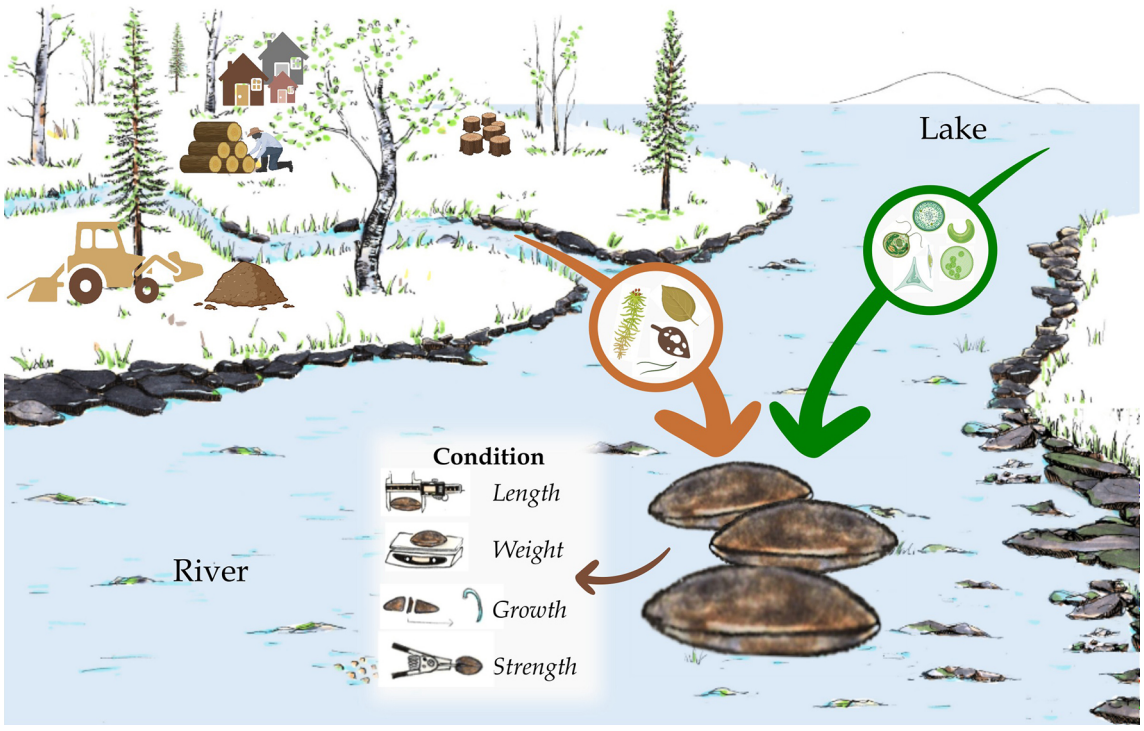
JYU DISSERTATIONS 789

Mahsa Hajisafarali

Effect of Catchment Characteristics on Dietary Resource Use and Condition of Freshwater Pearl Mussel (*Margaritifera margaritifera*)



UNIVERSITY OF JYVÄSKYLÄ
FACULTY OF MATHEMATICS
AND SCIENCE

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ABSTRACT

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Effect of catchment characteristics on dietary resource use and condition of freshwater pearl mussel (*Margaritifera margaritifera*)
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Diss.

Anthropogenic pressures can increase nutrient load in rivers and affect the availability and quality of food sources for filter feeders, such as the freshwater pearl mussel (FPM), a long-lived and critically endangered species. FPM inhabits oligotrophic headwater rivers, which receive large inputs of terrestrial organic matter (t-OM). Fatty acid (FA) and stable isotope (SI) analyses, as well as growth rate and strength of adductor muscle of FPM were used to study how catchment characteristics and water quality affected the availability of food sources, dietary habits, and condition of FPM in 29 rivers across northern Finland. Environmental variables (26 in total) mirroring the anthropogenic pressure, for example, chlorophyll-a, nutrient concentrations, forestry intensity, proportion of ditched area, proportion of conservation area and latitude were determined. FPM selectively fed on phytoplankton (i.e., diatoms); phytoplankton-derived FAs dominated both the selected food (stomach content) and the available food (seston) of FPM. With increasing anthropogenic pressures, 1) the phytoplankton FAs increased in seston as terrestrial contribution decreased, and 2) reliance on phytoplankton FAs increased in stomach content while dependence on bacterial sources decreased. FPM retained some polyunsaturated fatty acids (PUFAs) in their muscle, likely to fulfil their nutritional requirements. SI results showed t-OM to be the major energy source of FPM, indicating a strong connection of FPM to terrestrial environment. However, the observed reduction in terrestrial contribution in SI results with increasing anthropogenic pressure signaled breakage of terrestrial connectivity due to environmental degradation. Furthermore, shell growth rate and strength of adductor muscles of FPM decreased with increasing anthropogenic pressure. These results highlight the importance of pristine environments, aquatic-terrestrial connectivity, and mitigation of anthropogenic impacts in conservation and management of FPM.

Keywords: Accumulation index; allochthony; fatty acids; hydrogen; land use activities; stable isotopes; Unionida.

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TIIVISTELMÄ

Hajisafarali, Mahsa
Valuma-alueen ominaisuuksien vaikutus jokihelmisimpukan (*Margaritifera margaritifera*) ravinnonkäyttöön ja kuntoon
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Diss.

Valuma-alueen ominaisuudet yhdessä maankäytön kanssa voivat vaikuttaa jokien ravinnekuormitukseen ja suodattajien ravinnon laatuun ja saatavuuteen. Uhanalainen jokihelmisimpukka elää vähäravinteisissa latvajoissa, joihin päätyy tyypillisesti paljon terrestristä orgaanista ainesta. Tutkin väitöskirjassani rasvahappojen, vakaiden isotooppien, kuoren kasvun ja kuorensulkijalihasten voimakkuuden avulla, miten valuma-alueen ominaisuudet ja vedenlaatu vaikuttivat jokihelmisimpukan ravinnonlähteisiin, ravinnonvalintaan ja kuntoon 29 pohjoissuomalaisessa joessa. Joista ja valuma-alueilta määritettiin 26 erilaista ympäristömuuttujaa; esimerkiksi klorofylli, ravinnepitoisuus, metsätalouden intensiteetti, ojitetun maan osuus, suojellun alueen osuus ja leveysaste. Jokihelmisimpukka valikoi aktiivisesti ravinnokseen kasviplanktonia (esimerkiksi piileviä); kasviplanktonrasvahapot hallitsivat sekä valitussa ravinnossa (vatsan sisältö) että saatavilla olevassa ravinnossa (seston). Ihmisvaikutuksen kasvaessa 1) sestonin kasviplanktonrasvahappojen osuus lisääntyi terrestristen rasvahappojen osuuden vähentyessä; ja 2) kasviplanktonrasvahappojen osuus vatsan sisällössä lisääntyi bakteeriperäisten rasvahappojen osuuden vähentyessä. Jokihelmisimpukat näyttivät valikoivan välttämättömiä monityydyttymättömiä rasvahappoja kudoksiinsa. Vakaiden isotooppien perusteella terrestrinen orgaaninen aines oli simpukoiden tärkein energian lähde. Tämä viittaa siihen, että raakulla on vahva yhteys ympäröivään terrestriseen ekosysteemiin. Isotooppitulokset osoittivat, että tämä yhteys kuitenkin heikentyy ihmistoiminnan lisääntyessä. Myös kuoren kasvu ja sulkijalihasten voimakkuus heikkenivät ihmistoiminnan lisääntyessä valuma-alueella. Tulokset korostavat luonnontilaisen ympäristön ja jokea ympäröivän terrestrisen ekosysteemin merkitystä sekä ihmistoiminnan vaikutusten minimoimisen tärkeyttä jokihelmisimpukan suojelussa.

Avainsanat: Alloktonia; kertymäindeksi; maankäyttötoimet; rasvahapot; Unionida; vakaat isotoopit; vety.

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LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following original papers, which will be referred to in the text by their Roman numerals I–III.

I Hajisafarali M., Calderini M.L., Kiljunen M., Moser N., Nykänen S., Litmanen J., Taipale S. & Taskinen J. 2024. Environmental effects on the selective feeding and fatty acid retention of freshwater pearl mussel. Submitted manuscript.

II Hajisafarali M., Taskinen J., Eloranta A. P. & Kiljunen M. 2023. Ethanol preservation effects on stable carbon, nitrogen and hydrogen isotopes in the freshwater pearl mussel. *Hydrobiologia* 850: 1885–1895.

III Hajisafarali M., Nykänen S., Taskinen J., Cobain M., Kuha J., Oulasvirta P., Rautiainen K., Vaso A., & Kiljunen M. 2024. Environmental factors impact terrestrial-aquatic ecosystem connectivity and affect the condition of critically endangered freshwater pearl mussels in the field. Submitted manuscript.

Author contributions in the original papers:

	I	II	III
Planning	JT, MH, MK	JT, MH, MK	JT, MH, MK, SN
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Laboratory work	JK, JL, MH, MLC, ST	MH, MK	JK, MH, MK, SN
Catchment data	-	-	AV, SN
Data analysis	MH, NM	MH, AE	MC, MH, SN
First version of manuscript	MH	MH	MH, SN
Manuscript revision	AA	AA	AA
Correspondence	MH	MH	MH

Author abbreviations: AA: All author, AE = Antti Eloranta, AV = Asta Vaso, JK = Jonna Kuha, JL = Jaakko Litmanen, JT = Jouni Taskinen, KR = Katariina Rautiainen, MC = Mattew Cobain, MH = Mahsa Hajisafarali, MK = Mikko Kiljunen, MLC = Marco L. Calderini, NM = Niklas Moser, PO = Panu Oulasvirta, SN = Sabrina Nykänen, ST = Sami Taipale.

ABBREVIATIONS

FPM	Freshwater pearl mussel
t-OM	Terrestrial organic matter
FA	Fatty acids
PUFA	Poly unsaturated fatty acid
OM	Organic matter
SIA	Stable isotope analysis
DOM	Dissolved organic matter
DOC	Dissolved organic carbon
POM	Particulate organic matter
ALA	α-linolenic acid
LA	Linoleic acid
EPA	Eicosapentaenoic acid
DHA	Docosahexaenoic acid
ARA	Arachidonic acid
PC	Principal component

1 INTRODUCTION

1.1 Aquatic and terrestrial organic matter in freshwater ecosystems

In lotic systems, organic matter (OM) originates from either the photosynthetic production that occurs within the river, from the lake above, or from the adjacent terrestrial ecosystems (Dillon and Molot 1997, Allan and Castillo 2007). Significant in situ sources of 'autochtonous' aquatic production include periphyton, macrophytes, phytoplankton and microbial production while the 'allochtonous' terrestrial contributions consist of plant litter, suspended particulates and dissolved organic matter (DOM) that is derived from surrounding soils (Allan and Castillo 2007). Generally, there is an increase in autochthonous production when moving down the river continuum from smaller to high order rivers (Vannote *et al.* 1980). Thus, particularly the forested headwater rivers receive significant inputs of terrestrial organic matter (t-OM) that constitute the majority of a stream's total OM input (Cole et al. 2007). This linkage of aquatic to terrestrial ecosystem is particularly important in light-limited riparian lotic ecosystems where the in situ primary production can be limited (Hill and Dimick 2002, Warren *et al*. 2017). Amount and quality of OM may vary among streams and rivers with different terrestrial vegetation and anthropogenic impacts (Allan and Castillo 2007, Carpenter *et al*. 2011). Human activities (e.g., agriculture, forestry and ditching) can directly or indirectly affect the dynamics of DOM and nutrient transport from terrestrial to aquatic ecosystems (Graeber *et al*. 2012, Schelker *et al*. 2012). Forestry practices have been found to increase dissolved organic carbon (DOC) concentrations and total nitrogen (TN) in stream waters (Graeber *et al*. 2012, Schelker *et al*. 2012). This potentially enhances the degree of connectivity between ecosystems (Schelker *et al*. 2012) and results in spatial variation in food availability and quality which

have an effect on aquatic consumers (Xiong *et al.* 2019, Zhao *et al.* 2019, Lubanga *et al.* 2021).

In oligotrophic headwater rivers and other less productive aquatic ecosystems, OM from different sources plays an important role to sustain food webs (Berggren *et al*. 2010). Phytoplankton production in these systems is often constrained due to limited availability of light and inorganic carbon (Brett *et al*. 2017). This deficiency is offset by the incorporation of terrestrial DOM (t-DOM), primarily metabolized by aquatic bacteria. Bacterial production can be also supported by aquatic sources (phytoplankton origin). However, bacteria play a crucial role by affecting the flux of energy and material and make up a substantial part of the biomass (20-80 %) at higher trophic levels through the conversion of terrestrial carbon (Jansson *et al*. 2007, Reynolds 2008, Karlsson *et al*. 2015). Thus, secondary production in freshwaters is not only based on primary production but is also supported by t-DOM through microbial pathway (t-DOM-bacteria-protozoa) or even directly by terrestrial particulate organic matter (t-POM) (Taipale *et al*. 2009). Bacteria in freshwater systems potentially transfer aquatic and terrestrial energy and matter to upper trophic levels and in systems with higher input of t-OM a large part of basal production consists of heterotrophic bacteria growing on t-OM (Ask *et al*. 2009).

Also phytoplankton can enhance their photosynthetic efficiency by utilizing heterotrophic mechanisms, including osmotrophy, to directly use carbon rich t-DOM (Tittel *et al*. 2009). This so-called mixotrophy is a key strategy employed by certain phytoplankton, such cryptophytes, to effectively adapt to the above mentioned light and inorganic carbon constraints (López *et al*. 2019). Mixotrophy refers to the ability of phytoplankton to supplement their photosynthetic activity with external organic carbon sources to maintain or enhance their fitness in response to fluctuating environmental factors (Raven 1997). The ability to adapt to different carbon sources is vital for phytoplankton and this incorporates aquatic and terrestrial carbon into the food web (Taipale *et al*. 2023). Mixotrophic phytoplankton have an important function in converting basic compounds into biomolecules, such as fatty acids (FAs) which are essential for higher trophic level organisms (Taipale *et al*. 2023). Terrestrial organic carbon provides the backbone for these essential biomolecules, emphasizing the interdependence of terrestrial and aquatic systems (Taipale *et al*. 2023).

1.2 Rivers in the landscape: the impact of anthropogenic activities on river ecosystems

Streams and rivers are recognized as the most endangered freshwater ecosystems in the world, facing significant decreases in biodiversity, and many of the species living in this ecosystem are considered particularly vulnerable in global scale (Lydeard *et al*. 2004, Dudgeon *et al*. 2006, Geist 2010). Global environmental change, particularly human-induced activities, is expected to have substantial

and diverse ecological effects and is principal threat to the ecological integrity of riverine ecosystems (Allan 2004, Abbass *et al*. 2022). The combined effects of human activities, such as overexploitation of natural resources, pollution, destruction and fragmentation of habitats, and climate change, have resulted in changes to hydrological processes, ecological degradation, population decrease, and loss of biodiversity (Malmqvist and Rundle 2002, Strayer and Dudgeon 2010, Paredes del Puerto *et al*. 2022). These modifications influence the flow of energy and matter in food webs, which in turn affect the structure and functioning of ecosystems (e.g., Sintayehu 2018). Run-off from land-use practices is associated with increased concentrations of nutrients and OM in aquatic ecosystems. This can lead to eutrophication and sedimentation, which deteriorate the water quality of riverine ecosystems (Arbuckle and Downing 2001, Allan 2004, Lucas *et al*. 2022).

1.3 Freshwater mussels as a link between aquatic and terrestrial ecosystems

Freshwater mussels (Unionida) play a critical role as ecosystem engineers in freshwater biomes (Vaughn 2018). Mussels serve as a key link between terrestrial and aquatic ecosystems by filtering and feeding activities which integrate OM and nutrients into fluvial food webs (Nichols *et al.* 2005, Vaughn *et al.* 2008, Atkinson and Vaughn 2015). In many freshwater ecosystems, the response of organisms to the input of t-OM is significantly influenced by mussels (Smith *et al.* 2017). Freshwater mussels can exert a significant influence on the aquatic ecosystems, as they can comprise more than 90 % of the bottom fauna biomass and have a standing crop that is twice as high as that of fish (Negus 1966, Strayer *et al.* 1999). Mussels alter habitat physical conditions, which in turn affects the variety and organization of the fluvial communities (Vaughn *et al.* 2008). More specifically, aquatic ecosystems characterized by a larger abundance of freshwater mussels exhibit a greater variety of macroinvertebrates (Aldridge *et al.* 2007, Chowdhury *et al.* 2016). Freshwater mussels link multiple trophic levels through filtering activity by transferring nutrients from the water column to the sediment (Vaughn and Hakenkamp 2001, Howard and Cuffey 2006, Spooner and Vaughn 2006). Mussel species that reside buried in the substrate affect their immediate environment by moving and digging, a process known as bioturbation, which stirs up the sediment and releases vital nutrients like nitrogen and phosphorus (Vaughn and Hakenkamp 2001). Moreover, mussels impact the constitution of the sediment by depositing faeces and pseudofaeces (i.e., compounds expelled without being consumed) (Strayer *et al.* 1999, Vaughn and Hakenkamp 2001). Freshwater mussels also affect food webs by consuming vast amounts of phytoplankton, zooplankton, and t-OM from the water and the sediments through filtering and deposit-feeding activities (Nichols *et al.* 2005, Vaughn *et al.* 2008).

Freshwater mussels feed on variety of food sources and their diet is dependent on food availability and habitat (Kasai and Nakata 2005, Xu and Yang 2007, Vaughn *et al*. 2008). Phytoplankton dominate their diet in large productive rivers (Thorp *et al*. 1998), whereas diets are a mixture of phytoplankton, bacteria, and t-OM in smaller headwater rivers (Nichols and Garling 2000, Raikow and Hamilton 2001, Brauns *et al*. 2021). Although, phytoplankton is considered as the main dietary source for bivalves (Thorp *et al*. 1998, Gosling 2008, Vaughn *et al*. 2008), earlier studies have shown the significance of t-OM as the dietary source for, e.g., the freshwater pearl mussel (*Margaritifera margaritifera* L. 1758) (Geist *et al*. 2005, Brauns *et al*. 2021). The quality and quantity of t-OM derived from the catchments have been directly linked with the nutritional quality of food items filtered by freshwater bivalves (Madon *et al*. 2011, Ladeiro *et al*. 2017). In response to these dynamic dietary environments, freshwater mussels have evolved selective feeding strategies (Lopes-Lima *et al*. 2014). Selective feeding in freshwater mussels can vary based on the availability of food sources, environmental conditions and life stage (Lopes-Lima *et al*. 2014, Rosa *et al*. 2018, Jones *et al*. 2020). Some freshwater mussels can even actively select among phytoplankton species (Lopes-Lima *et al*. 2014) by utilizing chemical cues (Zhang *et al*. 2017).

Among Unionida, freshwater pearl mussel (hereafter FPM) is a long-lived, critically endangered bivalve found in cool oligotrophic headwater rivers of northern and western Europe and northeastern North America (Lopes-Lima *et al*. 2017, Aldridge *et al*. 2022). European populations had declined by 90 % by 1990s because of human activity, a trend that has persisted into the 21st century (Lopes-Lima *et al*. 2017). Decline of FPM is attributed most notably to anthropogenic impacts such as pearl fishing, habitat degradation, fragmentation, decline in host fish populations and particularly siltation of riverbeds (Geist 2010). The most densely populated mussel beds can inhabit one thousand FPM individuals m^{-2}, with size of FPM up to 16-18 cm in length (Oulasvirta *et al*. 2015). Thus, due to the declining mussel populations, ecosystem services they provide are diminishing. FPM mainly relies on t-OM due to its habitat in headwater oligotrophic rivers, which receive significant inputs of t-OM (Geist *et al*. 2005, Brauns *et al*. 2021), establishing a potential link between terrestrial and aquatic ecosystems.

1.4 Fatty acids in freshwater ecosystems: dietary needs of mussels

Anthropogenic activities and increasing water temperature due to climate change in freshwater ecosystems may enhance total production while simultaneously reducing the nutritional quality of food web components (Taipale *et al*. 2022). Changing environmental conditions influence phytoplankton communities from high to low nutritional quality for consumers and result in the dominance of undesired species such as cyanobacteria. Elevated nutrient loadings have been demonstrated to reduce the availability of

micronutrient such as physiologically essential omega-3 FAs in phytoplankton (Müller-Navarra *et al*. 2004). A shift in phytoplankton community would impact the growth and reproduction of higher trophic level (Müller-Navarra *et al.* 2004, Wang *et al.* 2021). The growth and reproduction of aquatic animals such as freshwater mussels strongly depend on the nutritional quality measured in terms of polyunsaturated FAs (PUFA) contents of their diet (Müller-Navarra *et al.* 2000, Wacker and Elert 2003, Brett *et al.* 2009a, Grunicke *et al.* 2023).

In aquatic food webs, the FAs are synthesized by phytoplankton and bacteria, which are directly consumed and transferred via zooplankton to higher trophic levels (Arts *et al*. 2001). Some PUFAs are essential for animals (i.e., mollusks, crustaceans, and fish, as well as humans) because animals lack the enzymes to synthesize these molecules de novo (Arts *et al*. 2001, Parrish 2009). Phytoplankton produce PUFA by de novo synthesis of palmitic acid and further enzymatic elongation and desaturation processes (Harwood and Guschina 2009, Cagliari *et al*. 2011). PUFAs are primarily produced by certain taxa of phytoplankton, including diatoms and cryptophytes (Taipale *et al*. 2013). These PUFAs can be directly taken up from the diet or converted from dietary shorter-chain omega-3 and omega-6 PUFA [i.e., α-linolenic acid (ALA, 18:3ω3) and linoleic acid (LA, 18:2ω6)] (Murray *et al.* 2014), which are abundant in green algae and terrestrial plants (Guo *et al*. 2016). As stream invertebrates have a limited ability to synthesize PUFAs, they must directly obtain them from phytoplankton, and can have a PUFA compositions that largely reflects the PUFA compositions of their diet (Cook and McMaster 2004, Newton *et al*. 2013). Omega-3 and omega-6 PUFAs have physiologically essential functions in aquatic animals (Arts *et al.* 2001). Long chain PUFA, eicosapentaenoic acid (EPA, 20:5ω3), docosahexaenoic acid (DHA, 22:6ω3), and arachidonic acid (ARA, 20:4ω6) are physiologically most important for consumers, and may call physiologically essential FAs and support growth and reproduction of aquatic consumers (Guo *et al*. 2017). Short-chain omega-3 and omega-6 PUFAs (ALA and LA) can be sourced from both phytoplankton and t-OM, are commonly regarded as essential FAs and known to affect the fitness of freshwater invertebrates (Brett and Müller-Navarra 1997, Peltomaa *et al.* 2017).

The supply of long chain PUFAs in streams and rivers is affected by altered light availability, increased nutrient input and other anthropogenic activities, and any decrease in phytoplankton long chain PUFA production will negatively affect the consumers that are nutritionally dependent on these molecules (Guo *et al*. 2017). The PUFA availability in food web is also affected by temperature. In cold environment, EPA and DHA are needed for maintaining cell membrane fluidity, which is an adaptation beneficial to both phytoplankton and animals (Arts and Kohler 2009) and has also anti-inflammatory effects (von Elert and Fink 2018). Diatoms are highly nutritious because of high content of omega-3 PUFA such as EPA (Nichols and Garling 2000, Torres-Ruiz *et al*. 2007). Green algae are regarded as medium-quality food due to low content of EPA and DHA, but high content of ALA and LA. Cyanobacteria typically lack EPA and DHA, containing only trace amounts of ALA and LA, and are therefore considered poor-quality food for aquatic invertebrates (Brett *et al*. 2006, 2009a).

PUFAs, especially EPA, are important components determining food quality for freshwater bivalves (Wacker & von Elert 2001, 2002, 2003), improving the growth and reproduction (Stoeckmann and Garton 2001, Wacker *et al*. 2002, Wacker and von Elert 2003) and growth of juvenile FPM (Grunicke *et al*. 2023). FA compositions of aquatic consumers largely mirror the FA profile of their diet. However, certain FAs are selectively retained to serve structural, physiological or anabolic functions (Galloway *et al*. 2015, Guo *et al*. 2017). The retention of key nutrients is crucial for the optimum physiological performance of consumers in aquatic food webs and facilitates more efficient dietary trophic transfer (Müller-Navarra *et al*. 2000, Kainz *et al*. 2004). The retention of EPA, DHA and ARA in aquatic animals (e.g., fish and bivalves) may maintain important physiological functions such as growth and reproduction (Arts and Kohler 2009, Parrish 2009, Gladyshev *et al.* 2011, Ezgeta-Balić *et al.* 2012, Kelly and Scheibling 2012, Bartsch *et al.* 2017).

1.5 Biotracers in feeding ecology of aquatic animals

Variety of biotracers are frequently employed in food web studies to estimate consumer dietary preferences and are increasingly used in tandem to understand consumer resource use and more complicated diet mixtures (Pickett *et al.* 2024). Biotracers reflect the assimilated component of the diet within varying temporal ranges (from days to years, depending on the biotracer and tissue types) (Pickett *et al.* 2024). FA compositions and stable isotope analysis (SIA) have been extensively used in both freshwater and marine food web research to unravel flow of matter and energy (Brett *et al.* 2009b). FAs, serving as source-specific biomarkers, enable effective quantifications of phytoplankton, terrestrial and bacterial-derived FA compounds (Kainz and Fisk 2009). In turn, SIA is commonly used to investigate the structure and function of food webs and the assimilated energy sources to organisms (Umbricht *et al.* 2018). This can provide information about basal material flow (McInerney *et al.* 2020). The stable isotope ratios of carbon ($^{13}C/^{12}C$; denoted as $\delta^{13}C$) are traditionally used to determine the origins of energy and matter in food webs, whereas nitrogen isotopes ($^{15}N/^{14}N$; denoted as $\delta^{15}N$) have been employed to evaluate the sources of nitrogen and the trophic levels of the organisms. In recent years, hydrogen isotopes ($^{2}H/^{1}H$; denoted as $\delta^{2}H$) have proven to be useful in differentiation of terrestrial and aquatic inputs as $\delta^{2}H$ values vary substantially between terrestrial and aquatic primary producers (Doucett *et al*. 2007). The use of SIA has been crucial in the field of ecology for more than four decades and the method is conceptually sound. However, there are several sources of uncertainty in the methodology which are still partly unsolved and need further investigation. For example, deeper understanding of how handling and preservation of samples affects isotope ratios is still lacking (Lau *et al*. 2012). Although fresh samples are preferred, preservation is frequently required for later analysis, which can impact the results (Jesus *et al*. 2015). Isotope ratios in animal tissues can be affected by

preservation methods and duration, which can reduce the accuracy of diet estimations in isotope models (Javornik *et al*. 2019). In the worst case, this may lead to misinterpretation of the results. To ensure precision of the models employed in dietary evaluations, a correction can be carried out to account for possible shifts in isotope values caused by preservation. If properly used, the combination of SIA and FA biomarker techniques can provide a robust method for studying energy pathways and estimating the dietary preferences of animals in fluvial ecosystems (Perga *et al*. 2006).

1.6 Main aims of the thesis

The main objectives of this thesis (Fig. 1) were: 1) to identify the primary dietary FA sources (phytoplankton, bacteria, t-OM) preferred by FPMs in their natural habitats, and to determine how catchment characteristics affect the availability, uptake and retention of high nutritional quality diet in FPM (I); 2) to evaluate the extent to which terrestrial food sources contribute to the diet of FPMs along an environmental gradient based on water quality and catchment characteristics of rivers (III); and 3) to investigate how preservation method and time affect the stable isotope ratios in tissues of FPM (II). Detailed hypotheses were:

Previous studies indicate that phytoplankton is a major food source for bivalves (Thorp *et al*. 1998, Gosling 2008, Vaughn *et al*. 2008). I hypothesized that phytoplankton, when available, is a dominant food source for FPM and they selectively feed on different FA-derived food sources (e.g., phytoplankton, bacteria, t-OM) and retain physiologically essential FAs based on their availability, nutritional value, and the physiological needs (I) (Fujibayashi *et al*. 2016, Komulaynen 2021, Grunicke *et al*. 2023). Given the significance of catchment characteristics in determining the availability of various food sources (Crapart *et al*. 2023), I anticipated that the food sources of FPM vary along the environmental gradient, which may affect the uptake and retention of physiologically essential FAs (i.e., EPA, DHA and ARA). Finally, I hypothesized that the physiologically essential FAs in FPM tissue are closely linked to FPM diet, and that they selectively retain certain essential FA from seston to meet their physiological needs (I) (Bartsch *et al*. 2017).

Ethanol preservation has been shown to affect carbon isotopes values and C:N ratios due to leaching of lipids (DeNiro and Epstein 1978, Kiljunen *et al*. 2006). Since lipids are also rich in hydrogen, I expected analogous, but larger preservation effect in δ^2H values and shifts in isotope values that should increase with preservation time (II) (Javornik *et al*. 2019). I assumed that these changes were predictable, which could enable the construction of simple models to correct for preservation effect in FPM.

I expected that t-OM constitutes a substantial component of assimilated energy in adult FPM (III) (Brauns *et al*. 2021). Given the effect of catchment characteristics in determining the availability of OM in freshwater systems (Carpenter *et al*. 2011, Crapart *et al*. 2023), combined with the filter feeding

behavior of FPM, I hypothesized that the contribution of t-OM to FPM diet will vary along the environmental gradient. Finally, I assumed that the condition and growth of FPM individuals and average condition of populations varies with the environmental gradients and contribution of t-OM (III).

Acknowledging that FPM is critically endangered, this thesis not only enhances our overall understanding of the FPM diet and its reliance on terrestrial ecosystems, but also provides valuable insight for conservation management practices to aid recovery of this highly vulnerable species.

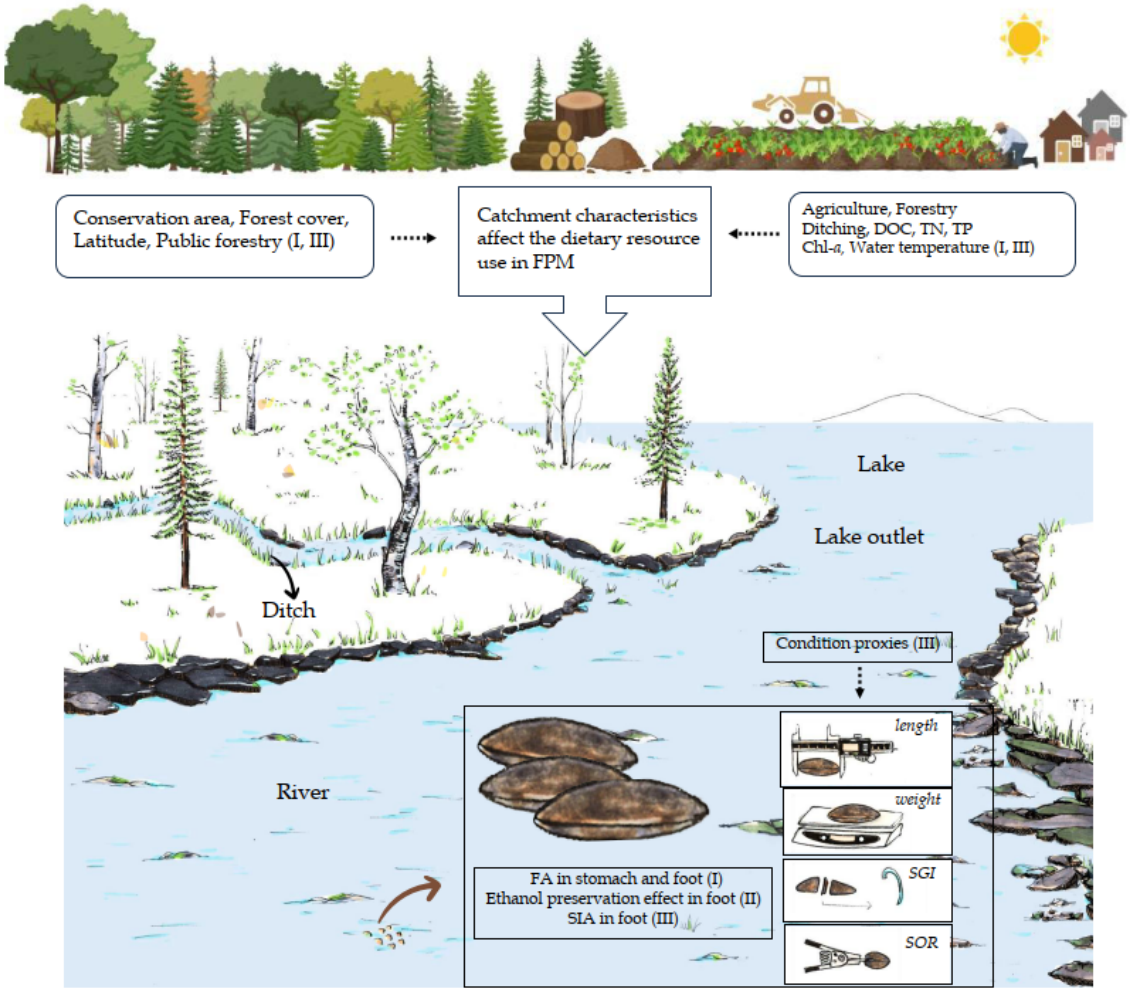

FIGURE 1 Graphical illustration of the main components of this PhD thesis, depicting the study system where the freshwater pearl mussel (*Margaritifera margaritifera*), water sample and various food sources were collected from lake outlet, ditch and river. The environmental gradient was illustrated using BioRender (BioRender.com), and study system was drawn by Xiaoxuan Hu.

2 MATERIALS AND METHODS

2.1 Study area

A total of 29 rivers harboring resident FPM populations were sampled over the summer in 2019-2021 in four distinct geographical regions in northern Finland (Fig. 2). The sampling period spanned from June to September and was conducted as part of the EU ENI Kolarctic CBC project Salmus. The FPM samples collected in 2019 were not included in the FA analysis (I) due to the ethanol preservation, which made them incompatible for the study. However, after modification to account for isotope shifts in δ^2H and C:N ratios (II) these specimens were used in III.

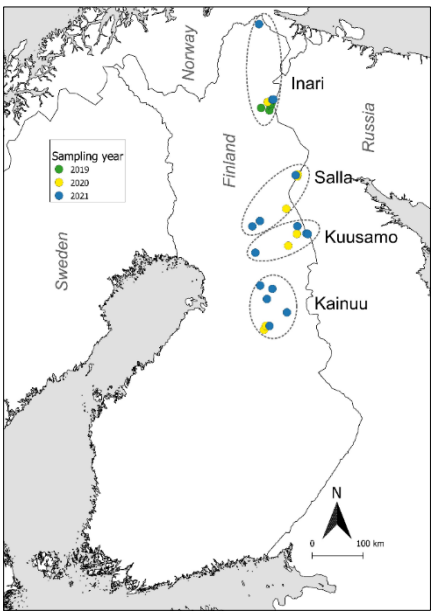

FIGURE 2 Map of the study regions. The color points indicate the year of sampling, and dashed circles indicate the study regions: Inari, Salla, Kuusamo, and Kainuu (north

to south, respectively). Study regions are not always defined by province or catchment boundaries. Credits for the background map: EuroGeographics (2020).

2.2 Data collection – Field work

From 29 rivers, 30 FPM individuals (842 in total) were randomly collected by hand using snorkeling. Special permissions were issued to gather living FPM, due to their endangered status, from the regional Centres of Economic Development, Transport, and Environment in Kainuu (KAIELY/296/2019 and 357/2019), Lapland (LAPELY/1929/2019 and 2252/2019), and North Ostrobothnia (POPELY/1276/2019 and 1490/2019). Live specimens were only collected from locations where estimated population size exceeded 1000 individuals. In situ estimates of FPM density (individuals m^{-2}) were performed using established population survey procedures (Oulasvirta *et al*. 2017). More specifically, mussel densities were determined from counts of living mussels inside transects. The transect lengths in small rivers were 20 meters and spanned the entire width of the river. In contrast, transects in larger rivers were placed perpendicular to the direction of flow and had a width of 2 meters (III). FPM density can be used as a measure of the mussel response to local conditions. Ideal conditions should lead to high densities e.g., due to better reproduction and survival and vice versa. The shell opening resistance (SOR) of FPM was assessed using methods adopted from Moorkens and Killeen (2018). SOR measures the strength of mussel adductor muscles, which may deteriorate due to poor feeding conditions, serving as an indicator of mussel condition (Moorkens and Killeen 2018). Out of the 30 individuals that were measured, 27 were released alive to their original locations while the three largest individuals were kept for additional laboratory analyses [69 individuals (I) and 84 individuals (III)]. The FPM was individually sealed in zip lock bags, instantly placed on ice, and transported to the laboratory at the Department of Biological and Environmental Science, University of Jyväskylä, Finland. However, 16 specimens collected in 2019 were stored in ethanol for one month before undergoing additional analyses. The collected FPM individuals were utilized for various purposes (e.g., age determination, trace element and genetic analyses) within the Salmus project.

For each study site, potential food sources were collected from three different locations: within the river, the lake upstream, and the ditches that flow into the river (or tiny tributaries in the absence of ditches) (III, Fig.3). Food sources derived from the river were collected slightly upstream of the mussel bed. Lake water samples were collected upstream from mussel beds at lake outlet. Sampling from ditches was conducted as close as possible to the upstream of the mussel bed, or close to the mussel sample site when that was more feasible. Various potential food and primary energy sources were collected, such as moss (from rivers and lakes), filamentous algae (rivers and lakes), DOM (rivers, lakes, and ditches), particulate organic matter [POM (rivers, lakes, and ditches)], along with forest soil, needles and leaves found near the river. Seston samples,

indicating potential food sources for FPM, were collected slightly upstream of mussel bed for FA analysis (I). Initially, a 100 µm filter funnel was used to filter 20 l of river water on-site, to remove larger particles and macrofauna. Subsequently, in the laboratory, additional filtration was conducted on water samples of volume up to 2 l utilizing pre-burned and pre-weighed GF/F filters (Whatman). These filters were then stored at -80 °C prior the analysis (I). To collect DOM and POM samples, 20 l of water was collected from lake outlet, river and ditch and filtered twice. The first filtration was performed in the field and in the same manner as seston samples filtration. The second filtration was conducted in the field laboratory using a tangential-flow filtration apparatus (a Millipore Durapore cassette with a pore size of 0.22 µm) to extract 1 l of filtrate. The DOM present in this filtrate was then collected and analyzed (III). Retentate POM from the cassette was collected into 50 ml falcon tubes using distilled water. The food source samples were promptly stored at -20 °C after being collected (III).

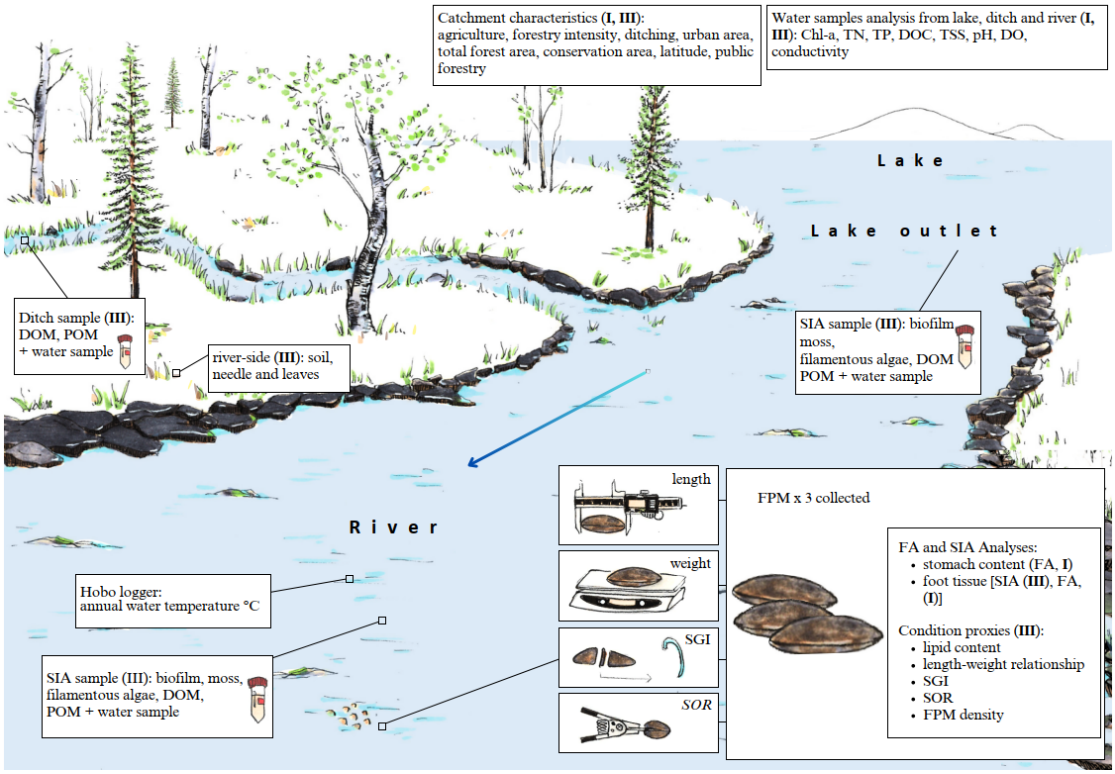

FIGURE 3 Graphical illustration of study system includes lake outlet, a ditch and a river. Water samples and potential food sources were collected from the lake outlet, ditch and river, upstream from the mussel bed. Freshwater pearl mussels (*Margaritifera margaritifera*) were collected from river. Figure created by Xiaoxuan Hu.

Additional water samples were collected (a total of approximately 5 l per site) for chlorophyll-*a* (Chl-*a*), total suspended solids (TSS), DOC, TN and total phosphorous (TP) analyses. For Chl-*a* analysis, a maximum of 2 l of water was filtered using GF/C filters (Whatman) and thereafter stored at -20 °C. TSS samples, with a maximum volume of 2 l, followed filtration using the pre-

combusted and pre-weighed GF/C. In the field, DOC and TN were pre-filtered using a mesh size of 100 µm. In addition, conductivity (temperature-adjusted) and pH levels were determined using a Radiometer CDM2e conductivity meter (Copenhagen, Denmark, and pH Meter 744, Metrohm AG, Herisau, Switzerland, respectively), with samples collected in 100 ml grinding-stopper glass bottle filled to the top with no air. For the TP analysis, sample collection was performed using 50 ml tubes that were pre-filled with 500 µl of 4 mol^{-1} sulfuric acid for sample preservation. Dissolved oxygen concentration (%) was measured on site using a pre-calibrated Pro ODO optical portable sensor (YSI Inc. / Xylem Inc., Yellow Springs, OH/USA). Finally, HOBO temperature loggers were installed to constantly monitor river water temperatures for at least one year after collecting FPM samples.

2.3 Laboratory analysis

2.3.1 Fatty acid and stable isotope samples

The FPM individuals were transported alive to the laboratory on ice, where they were measured for length and weight. The dissection was conducted during a timeframe of 1-10 days, during which the foot muscle and stomach content were extracted. The foot samples were split into two parts: one was stored in Eppendorf tubes at -80 °C along with the stomach content samples in separate tubes for FA analysis (I), while the other was stored at -20 °C for SIA (III). The Frozen FPM foot muscle and stomach content samples underwent freeze-drying using the Christ ALPHA 1-4 LD Plus until they reached a constant weight (I, III). The freeze-dried samples were ground into a fine powder using a pestle and mortar. If needed, tough foot muscles were exposed to liquid nitrogen (-193 °C) to aid in the grinding process (III). Approximately 3.6 ± 0.4 mg of freeze-dried foot muscle, 3.9 ± 0.6 stomach content, and 0.9 ± 0.5 of seston samples were measured and placed in glass test tubes and then stored in 3 ml chloroform-methanol solution (2:1 vol) at -20 °C for FA analysis. Filtered seston samples were freeze-dried and re-weighed to obtain seston biomass and were stored in 3 ml chloroform-methanol solution (2:1 vol) at -20 °C. For SIA (III) the food source samples were inspected and were thoroughly cleansed of macroinvertebrates, soil residuals, and other external substances, before being subjected to freeze-drying.

Following dissection, the mean standardized growth index (SGI) was estimated by determining the age of mussel and measuring annual shell growth increments. These increments were corrected for ontogenetic changes in the individual FPM shells (Dunca and Mutvei 2001, Dunca *et al*. 2011) in five years preceding the sampling. Therefore, the mean SGI represents shell growth in the past five years compared to the entire lifespan of the mussel. SGI was employed as a condition proxy to evaluate the impact of the different food sources (aquatic *vs*. terrestrial) and environmental changes on the FPM condition (III).

To determine the age of the mussels, a thin section was made from one of half of the shell (Fig. 4). One valve of each specimen was cut from umbonal part to the ventral margin, perpendicularly to the winter lines. Thick sections (three mm in thickness) were made from this valve with a high-speed saw. The thin sections were fixed to glass slides, subsequently smoothed using grinding paper and then polished with a diamond paste. Following this, all the thin sections were cleaned in an ultrasonic bath containing 95 % ethanol and were then left to dry in the air. The polished specimens were submerged in Mutvei's solution to enhance the visibility of the winter lines and improve the accuracy of age determination. Immediately after coloring the thin sections, they were promptly rinsed with demineralized water and air-dried. The growth pattern of samples was observed using a reflective light microscope equipped with a camera. The annual growth increments were determined by measuring the shortest vertical distance between two winter lines, specially between the prismatic layer and the nacreous layer, close to the border line to the nacreous layer (Fig. 4). A growth curve was then established using the shell length and age of the mussels (Dunca *et al*. 2011). Thin sectioning and age determination were done in Swedish Museum of Natural History, Stockholm, Sweden.

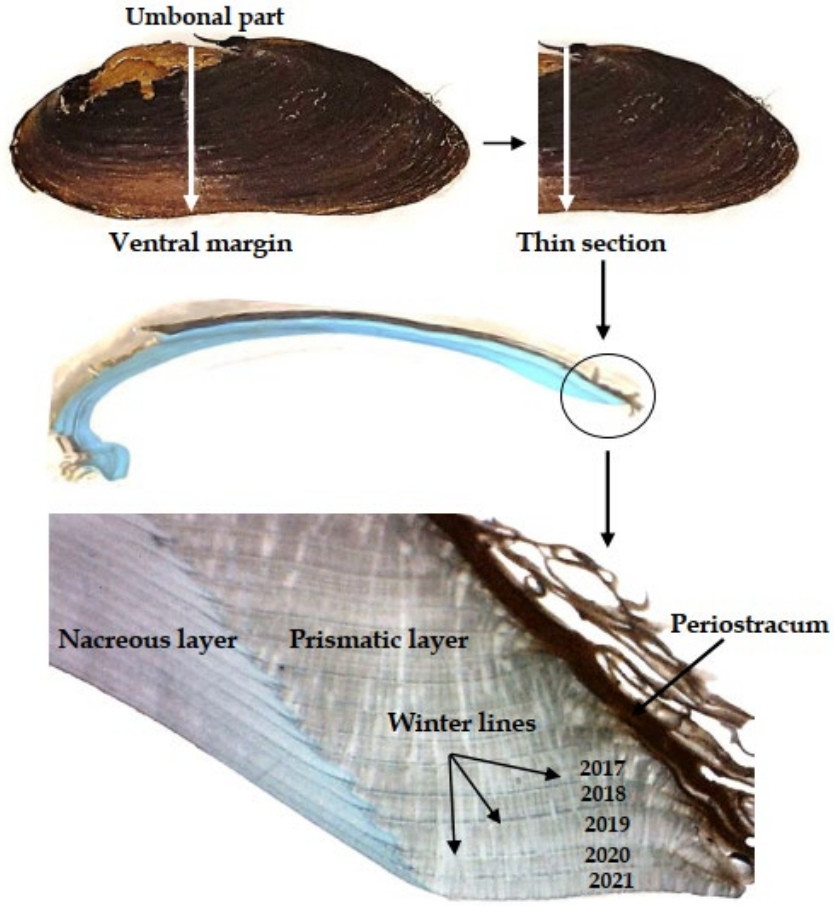

FIGURE 4 Thin section of freshwater pearl mussel (*Margaritifera margaritifera*) shell after being treated with Mutvei's solution, imaged using a light microscope.

Water quality analysis (I, III) followed to the protocol established by the Finnish Standards Association, as shown by SFS code (https://sfs.fi). Chl-*a* concentration (μg^{-1}) was calculated using the hot ethanol extraction method (SFS-5772) and measured spectrophotometrically with the Shimadzu UV-1800 spectrophotometer (Shimadzu Co., Kyoto, Japan). TSS measurement was conducted by filtering with GF/C (SFS-872). DOC and TN concentrations (mg^{-1}) were determined with Shimadzu TOC-V CSN Total Organic Carbon Analyzer (Shimadzu Co., Kyoto, Japan) from 20 ml of the sample passed through a 0.45 µm CA syringe filter. TP analysis was performed using spectrophotometry, specifically following the SFS–EN ISO 6878 method.

2.3.2 Lipid extraction and fatty acid analysis

Lipids were extracted from seston (filtered samples) and FPM (foot muscle and stomach content). A total of 23 seston samples and 138 FPM samples (69 muscle and 69 stomach content samples) were analysed from the 23 studied rivers. The process of lipids extraction was carried out by utilizing a method according to Folch *et al*. (1957) (I).

The accumulation index, which indicates the retention of ALA, EPA, DHA, LA, and ARA in the FPM foot muscle, relative to their diet (seston) and stomach content, was computed using the equation provided by Hessen and Leu (2006), referred to as the accumulation factor:

$$\text{Accumulation index} = (FA_{consumer}/FA_{diet}) - 1 \quad \text{(I) (1)}$$

where $FA_{consumer}$ refers to the content of the ALA, EPA, DHA, LA, and ARA (measured in µg mg^{-1} dry weight) in the foot muscle, and FA_{diet} represents the content of these FAs in the seston samples and the stomach content of FPM **(I).**

2.3.3 Ethanol treatment

Twenty-four FPM individuals, three individuals per river, were collected from eight rivers in Northern Finland during August–September 2020 (II). To investigate the effect of ethanol preservation and preservation time (1, 2, 4, and 6 months) on δ^2H values and the C:N ratio, dissected foot samples from each FPM individual were divided into two sections. One section was preserved overnight at –18 °C (referred to as "unpreserved tissue"/time 0) before being freeze-dried. The other section ("preserved") was preserved in 99.5 % ethanol and stored at the room temperature (~18 °C). Preserved foot muscle samples were extracted from ethanol, dissected for subsamples after 1, 2, 4, and 6 months, rinsed with ultrapure water, and then freeze-dried. Mortar and pestle were used to grind unpreserved and preserved freeze-dried samples to a fine powder. To investigate the effect of preservation time, the relative differences (Δ) in carbon, nitrogen and hydrogen isotope values of preserved samples (time points 1 to 6 months) to the values of unpreserved samples (time 0) were measured.

2.3.4 Stable isotope analysis

To determine the C:N ratios of biological tissues, 500–700 µg of freeze-dried material was weighed using a microbalance (Sartorius CP2P, 1-5 µg) and sealed in tin cups (II, III). To measure hydrogen stable isotope, 350 µg of freeze-dried sample was placed into silver capsules. The samples, along with the laboratory standards, were exposed to the laboratory environment for a minimum of three days before being sealed. This approach aimed to ensure equilibrium in the exchange of hydrogen between the samples and laboratory air (Wassenaar and Hobson 2003) (II, III).

Hydrogen stable isotope values and C:N ratios of tissues were determined at the Stable Isotope Laboratory in the University of Jyväskylä. The C:N ratios were measured using the FlashEA 1112 elemental analyzer (Thermo Fisher Scientific, Bremen, Germany) by weight. δ^2H values were measured using an Isoprime 100 isotope ratio spectrometer (Isoprime Ltd, Stockport, U.K) connected to an Elementar vario PYRO cube elemental analyzer (Elementar Analysensysteme GmbH, Hanau, Germany). Stable isotope ratios were compared to the international standard [Vienna Standard Mean Ocean Water (VSMOW)] and presented in delta (δ) per mille (‰) notation (as described in Fry 2006). Water samples collected from rivers were measured for δ^2H separately using laser cavity ring-down spectroscopy (CRDS) analyzers, Picarro L2130-i and Picarro L2140-i (Picarro Inc., Santa Clara, CA, U.S.A.), at the University of Oulu, Finland (II, III).

In each run, several replicates of two reference materials (caribou hoof [CBS] and kudu horn [KHS]) were analyzed relative to VSMOW (Standard Mean Ocean Water) for δ^2H measurements, following the method described by Hobson (1999). The standard deviation of the delta-value of replicate reference materials was always below 2.41 ‰ (II) and 3.59 ‰ (III) for δ^2H among subsequent runs. Lipid content of the FPM foot muscles was determined by estimating the C:N ratios, following the method described by Kiljunen *et al*. (2006). The δ^2H values and C:N ratios of the ethanol-preserved individual were adjusted using the methodology described in I. The δ^2H values of foot muscle samples were corrected for the contribution of dietary water using the following equation provided by Bartels *et al*. (2018):

$$\delta^2H_{corr} = (\delta^2H_{cons} - \omega \times \delta^2H_{wat}) \,/\, (1 - \omega) \qquad \text{(III) (2)}$$

where δ^2H_{wat} represents the water isotope ratio in the diet, obtained from river water samples, and ω is the dietary water contribution to δ^2H_{cons}. Following Wilkinson *et al*. (2015), ω was set to 0.22.

2.3.5 Catchment data collection

The river and catchment characteristics were computed using the Quantum Geographic Information System (QGIS) desktop geographical information system and the Feature Manipulation Engine (FME) data integration platform (I,

III). The river sampling sites served as discharge points for specified catchment areas. Land use types, including urban areas, agricultural areas, forests in peatlands, forests in mineral soils, total forest area, wetlands, and water bodies in the catchments, were determined using CORINE 2018 land cover raster with a pixel size of 20 by 20 m (https://syke.fi/avointieto). Conservation areas were sourced from the dataset of nature protected areas and wilderness reserves, provided by the Finnish Environment Institute (https://syke.fi/avointieto). Spatially explicit assessment of forestry management actions and history in catchment areas were determined using forest use notifications (valid for a three-year duration) collected by the Finnish Forest Centre (FFC). The data were used only from year 2000 onwards to capture recent trends in forest management. In Finland, forest owners are obligated to notify the FFC before to carrying out any forest management activities. However, the implementation of these reported measures is not always ensured. Thus, the forest use notifications were employed as a proxy of forestry actions inside the study areas. The indices of the overall intensity of forestry activities, including all forest management operations and the intensity of regeneration felling, specifically clear-cutting, were determined in relation to the total area of forestry. The proportion of privately-owned forest areas was obtained from the FFC database, which records privately-owned forests. Ditch areas were derived from the Topographic Database provided by the National Land Survey of Finland (< 2 m waterways, https://asiointi.maanmittauslaitos.fi/karttapaikka/tiedostopalvelu), and a buffer of 25 m was added to estimate the area covered by the ditches. All the areas are expressed as a percentage relative to the catchment size. Six of the catchments extended to the territory of Russian Federation where spatial data are not easily accessible (2 out of 6 were located > 50 % in Russia). After visually examining aerial maps of the catchments, it was assumed that data from the Finnish territory within these catchments could represent the entire catchment area for these rivers. This assumption was based on the similarity of land use and forestry practices on both sides of the national border.

2.3.6 Statistical analysis and modelling

Principal Component Analysis (PCA) was performed to reduce the dimensionality of the environmental variables and to account for the multi-collinearity between the variables (I, III). This included standardizing the water chemistry variables, land-use categories, forestry related parameters, and other spatial data using z-scores, which were then applied in the PCA.

PC1 indicates "productivity-latitude-anthropogenic pressure" gradient, but in different directions (I, III). In I, rivers with more negative PC1 loadings are more productive, more southern, warmer rivers having more ditching and agriculture activities in the catchments. Conversely, rivers with more positive PC1 loadings are more northern, oligotrophic, colder rivers and have less agriculture practices and ditching activities, but on average more conservation areas and forests within their catchments. In III, PC1 shows a similar pattern to that in I, but in different direction. PC2 represented "forestry intensity-catchment

size" gradient in I and could be defined as "conductivity-clear cut-catchment size" gradient in III.

The concentration of FA in the FPM stomach content and seston samples were normalized to form FA profiles (I). These profiles were used to estimate the composition of both FPM stomach content and the seston (%). In the estimation, Quantitative Fatty Acid Signature Analysis in R (QFASAR) was used (Iverson *et al*. 2004, Bromaghin 2017) with the 2-distance measure (Stewart *et al*. 2014). The method was employed with a previously determined seston FA profile library (Litmanen *et al.* in prep), which allowed estimation of the composition of FPM stomach content and seston, including diatoms, green algae, cyanobacteria, t-POM, and t-OM consumed by microbes (mt-POM). The effect of environmental variables (PC1) on the FA sources in seston and stomach content (phytoplankton, terrestrial and bacterial FAs) was examined. The Dirichlet regression (see e.g., Douma and Weedon 2019) with PC1 as an explanatory variable was conducted to model the proportional data on phytoplankton, terrestrial and bacteria using the DirichletReg R-package (Maier 2021). The model structure was constructed in a systematic model selection (ANOVA), which revealed that introducing PC2 as an explanatory variable did not improve the model explained variance ($p = 0.75$ for seston; $p = 0.37$ for stomach content, Table S2 in I).

Generalized Linear Mixed Effect Models (GLMM) were applied to test for the effects of ethanol preservation (unpreserved samples at time point 0 and preserved samples at time point 6; Preservation) and preservation time (preservation for 1, 2, 4, and 6 months; Time) on the δ^2H values and C:N ratios of FPM foot muscle (Tissue) (II). The lme function was employed in the nlme package with default settings for the GLMMs (Pinheiro *et al*. 2021). When differences were statistically significant ($p < 0.05$), pairwise post hoc comparisons were performed using Tukey-tests and the emmeans package in R (Lenth *et al*. 2022). The comparisons of δ^2H values and C:N ratios between different time points were performed using emmeans package.

Visual comparison of the stable isotope values of the potential food sources revealed that using DOM (river, lake, ditch) and filamentous algae (river, lake) as two food sources representing initial terrestrial and aquatic sources, respectively, was appropriate (Fig. S1 in III). As previously demonstrated, DOM reflected qualitatively the terrestrial sources (Kritzberg *et al*. 2004, Wilkinson *et al*. 2013). Collecting significant quantities of pure phytoplankton, the optimal aquatic food source, was not possible due to the complex nature of the sampling method (Keva *et al*. 2022). While FPM may not have direct access to filamentous algae, it was deemed to be a suitable δ^2H proxy of aquatic production (Keva *et al*. 2022). Bayesian mixing model, MixSIAR package in R (Stock *et al*. 2018), was used to estimate the contribution of initial aquatic and terrestrial energy sources to the diet of FPM (III).

Linear regression model was applied to analyze the effect of environmental variables (PC1) on the accumulation index and condition proxies, respectively (I, III). Multiple aspects of mussel physiology were considered and used as FPM condition proxies: SOR (individual and mean of 30 FPM from the same river), relative growth (SGI [individual and mean of 30 FPM from the same river]),

energy storage (lipid content [%]; length-weight relationship [i.e., weight divided by length, g mm^{-1}]), and population density (FPM density [individuals m^{-2}]) (III). Moreover, in logit regression models, the posterior estimates (3000 per mussel individual or river) of the proportional contribution of the terrestrial source to the FPM diet were derived from the output of the mixing model and utilized to determine the relationship between the assimilated diet and mussel condition. All statistical analyses and models were conducted using R statistical software through RStudio (R Core Team 2023).

3 RESULTS AND DISCUSSION

3.1 Diet of FPM and selective feeding

Diet of freshwater mussels depends on the habitat and food availability. In small and less productive rivers their diet primarily consists of phytoplankton, with a mixture of t-OM and bacteria, whereas in productive rivers phytoplankton dominate their diet (Kasai and Nakata 2005, Xu and Yang 2007, Vaughn *et al.* 2008). The QFASA analysis revealed distinct patterns in the proportion of FAs in seston and stomach content with phytoplankton FAs being the predominant component in both samples (Fig. 3A & B in I). In seston samples, phytoplankton FAs was dominant with an average proportion of 63 % across all regions. The results indicated that the lipid composition in the diet of adult FPM populations is largely derived from phytoplankton (91 %), with a lower contribution from terrestrial and bacterial FAs, 4 % and 5 %, respectively. The highest proportion of phytoplankton FAs in seston, which may result from its availability in the water specifically during summer, is mirrored in the FA profile of stomach content of FPM (Fig. 3B in I). This supports the hypothesis that FPM prefers high nutritional quality phytoplankton when it is available to facilitates reproduction and growth (Fujibayashi *et al*. 2016, Grunicke *et al*. 2023, Komulaynen 2021). Ezgeta-Balić *et al*. (2012) showed that mussels largely ingested phytoplankton when it is dominating in the water. Results of this thesis also showed variation in phytoplankton FA sources in the stomach of FPM, with diatoms being the most dominant (66 %), followed by green algae and cyanobacteria (20 % and 4 %, respectively) (Fig. 3D in I). However, the seston samples exhibited a different pattern where the cyanobacteria and diatoms had almost the same proportion, while the proportion of green algae was negligible (Fig. 3C in I). FPM lives in oligotrophic rivers with low production, and the present findings provide an insight into its feeding behavior during summer, when production is at its peak. This result shows that FPM may consume high-quality phytoplankton during

this time to compensate for a lack of key micronutrients in less productive seasons, crucial for the survival of FPM.

Phytoplankton species with high EPA concentration (e.g., diatoms) are high-quality food sources, essential for the growth and reproduction of aquatic animals (Torres-Ruiz *et al*. 2007, Wang *et al*. 2015, Grunicke *et al*. 2023, Liénart *et al*. 2023). Diatoms were the largest group of phytoplankton in the stomach of FPM, followed by green algae and cyanobacteria (I). Diets with low contents of EPA and DHA (e.g., green algae) are considered as medium-quality, whereas diets low in omega-3 FAs, such as cyanobacteria, are considered biochemically low-quality resources for animals (Guo *et al*. 2017). Thus, present results suggest that FPM feed selectively on high quality phytoplankton probably to meet its physiological needs (I). Recent research supports these findings by identifying diatoms as the primary food source (58 %) in microscopical stomach content analysis of adult FPM (Komulaynen 2021). The findings of Fujibayashi *et al*. (2016) also show that unionid mussels rely on diverse dietary sources, with diatoms as the dominant food, along with green algae and cyanobacteria. These results are consistent with the findings of the thesis. Further studies conducted by Biandolino *et al*. (2008) and Irisarri *et al*. (2014) demonstrated that diatoms were preferred as the primary food source for adult bivalves, with bacteria serving as a supplementary diet. Juvenile freshwater mussels have also been shown selectively feed on diatoms (Fung and Ackerman 2020). FPM probably selects diatoms, which are rich in EPA, to maintain membrane fluidity in low-temperature river habitats where average annual water temperature range between 5-7 °C (I). Phytoplankton contains a high concentration of PUFAs (Torres-Ruiz *et al*. 2007, Guo *et al*. 2016). In contrast, terrestrially derived seston, which is largely degraded and primarily composed of indigestible lignin and cellulose—contains only trace amounts of PUFAs (Taipale *et al*. 2016).

The analysis of FPM stomach content indicated that FPM is not only passively filtering their food, but actively selecting specific sources of phytoplankton, with a preference for diatoms followed by green algae and cyanobacteria (Fig. 3D in I). Previous research has demonstrated that freshwater mussels can selectively capture particles based on size and nutritional value, consuming the more nutritious and expelling the less desirable ones as pseudofaeces (Berg *et al*. 1996, Baker and Levinton 2003, Beck and Neves 2003, Wang *et al*. 2015, Rosa *et al*. 2018, Vaughn 2018). This selective behavior promotes the absorption of nutritious particles, enhancing the energy uptake of the mussels (Jones *et al*. 2020). However, the exact mechanism by which mussels uptake higher quality particles while rejecting others remains largely unknown (Evan Ward and Shumway 2004). Some studies have shown that the selective feeding may happen based on size or the chemical cues employed by mussels (Wong and Cheung 1999, Pales Espinosa *et al*. 2016, Rosa *et al*. 2018, Jones *et al*. 2020). Indeed, previous FA studies indicate that *Limnoperna fortunei* exhibits a preference for phytoplankton and bacteria. This happens through gill sorting mechanism to differentiate between suitable and unsuitable particles, guided by chemical cues rather than particle size (Rojas Molina *et al*. 2010, Zhao *et al*. 2013, Zhang *et al*. 2017). Similarly, Beck and Neves (2003) showed the importance of phytoplankton

as primary food source for juvenile unionid mussel *Villosa iris* through selective feeding based on cell characteristics (e.g., size), a trait also observed in adult unionids (Paterson 1984). Bacteria have also shown to serve as a dietary source in freshwater mussels (Newton *et al*. 2013, Fujibayashi *et al*. 2016, Hajisafarali *et al*. 2022). However, bacteria alone are probably not a sufficient diet for freshwater mussels as they lack long chain PUFAs (i.e., EPA and DHA), sterols, and certain amino acids. Bacteria play a major role in breaking down less accessible organic particles and consequently enhance the ingestion and assimilation process of essential nutrients by mussels (Newton *et al*. 2013).

3.2 Effect of ethanol preservation on isotope ratios

Logistical challenges often require preservation of organisms for later analyses (Jesus et al., 2015). However, preservation can substantially affect the analytical results as shown with stable isotopes (Javornik *et al*. 2019). Some of the samples in this thesis were preserved in ethanol, which made them incomparable with other specimen (III), requiring further examination of preservation effects on isotope values and C:N ratios. Indeed, results indicated that ethanol preservation can affect the δ^2H values and the C:N ratios of FPM foot muscle. The C:N ratios changes were generally small, and the largest shifts were found in the δ^2H values (ca. 7 ‰ increase) (II), substantial enough to cause bias in isotope mixing model estimates (III).

The reasons for preservation-induced changes in isotope values are unknown, but they may be related to leaching of lipids (Syväranta *et al*. 2008), exchange of heavy and light isotopes between samples and preservatives (Hobson *et al*. 1997, Edwards *et al*. 2002), or protein breakdown during the preservation process (Sarakinos *et al*. 2002, Lecea *et al*. 2011). Lipids are depleted in ^{13}C and ^{2}H compared to proteins and carbohydrates (Park and Epstein 1961, DeNiro and Epstein 1978, Hobson *et al*. 1997). Several studies have shown that extracting lipids from samples can increase δ^2H values and decrease C:N ratios because lipids are isotopically lighter and contain lots of carbon (DeNiro and Epstein 1978, McConnaughey and McRoy 1979, Gloutney and Hobson 1998, Kiljunen *et al*. 2006). Ethanol preservation effects can result from lipid hydrolysis (Gloutney and Hobson 1998, Bosley and Wainright 1999, Hobson *et al*. 1997) or uptake of ethanol into the tissue (Sarakinos *et al*. 2002). Observed increase in δ^2H values of preserved FPM foot muscle may be explained by ethanol-induced lipid extraction as indicated by the decreased C:N ratios (Kaehler and Pakhomov 2001, Arrington and Winemiller 2002, Sweeting *et al*. 2004). C:N ratios in animals, especially aquatic species, are good predictors of tissue lipid content. C:N ratios > 4 often suggest lipid-rich tissues, and C:N ratios < 4 indicate lipid-poor tissues (Post *et al*. 2007). Here, foot muscle of FPM showed a minor shift in C:N ratios, whereas the shift in δ^2H values was greater (II). If preservation results in a shift that exceeds the trophic discrimination factor (TDF), it might lead to misinterpretations in food web research (DeNiro and

Epstein 1978, Bosley and Wainright 1999, Bugoni *et al*. 2008). The TDFs in δ^2H have not been extensively investigated. However, there are significant differences in δ^2H values between terrestrial and aquatic primary producers, with an average difference of approximately 100 ‰ (Doucett *et al*. 2007). Due to relatively large shift in δ^2H values of FPM foot caused by ethanol, the δ^2H values and C:N ratios of all foot tissue samples were modified according to the changes observed after one month of ethanol preservation (II). These adjustments were made prior to running the Bayesian mixing model to investigate the contribution of aquatic and terrestrial food sources to FPM (III). This ensured that the values of the samples collected in 2019 and stored in ethanol for one month were comparable to those collected fresh in 2020 and 2021.

3.3 Proportion of terrestrial food sources in the FPM diet

FPM is predominantly found in oligotrophic headwater rivers and streams that exhibit low nutrient concentrations and clear water in undisturbed landscapes (Geist and Auerswald 2007, Bolland *et al*. 2010). All 29 FPM populations predominantly relied on terrestrial food sources, accounting for approximately 60-90 % of their assimilated material (Fig. 5A & B in III). This confirms the hypothesis that t-OM has a major contribution in the diet of FPM. This result aligns with findings of Brauns *et al.* (2021), which demonstrated the dominance of terrestrial resources in the diet of juvenile and semi-adult FPM, and with Geist *et al*. (2005), who proposed that FPM mainly feed on terrestrial food sources due to limited productivity in streams. Indeed, experimental feeding studies on juvenile FPM indicated that terrestrial detritus has a significant role in their growth and survival (Gum *et al*. 2011, Eybe *et al*. 2013). Some studies have demonstrated that the assimilated diet of freshwater mussels is influenced by both habitat and food availability (Kasai and Nakata 2005, Xu and Yang 2007, Vaughn *et al*. 2008).

The amount of t-OM in aquatic consumers is influenced by the export of t-OM from surrounding catchments and the amount of in situ primary production, which are generally associated with the trophic state of the ecosystem and catchment characteristics (e.g, forest cover, agriculture and forestry) (Tanentzap *et al*. 2017). In headwater and oligotrophic rivers characterized by low nutrient levels and/or limited light availability, it stands to reason that mixotrophic phytoplankton would dominate the aquatic flora (Pålsson and Granéli 2004). Bacterial production in aquatic ecosystems is supported by both aquatic OM (phytoplankton origin) and t-DOM. Therefore, it is plausible that secondary production in these rivers is also supported by a microbial pathway (DOM–bacteria–protozoa) or even directly by terrestrial particulate organic carbon (Taipale *et al*. 2009, 2014). In aquatic ecosystems with high input of terrestrial organic carbon, a large part of the basal production consists of heterotrophic bacteria growing on the terrestrial organic carbon (Karlsson *et al*. 2015). T-OM supplies carbon as an energy source for mixotrophic phytoplankton and

heterotrophic organisms (Pålsson and Granéli 2004), contributing to the overall resource pool within freshwater ecosystems (Kesti *et al*. 2022). Bacterial utilization of t-DOM, followed by bacterial consumption by protozoan and grazers, represents as alternative material and energy flow pathways to higher trophic levels in aquatic ecosystems (Taipale *et al*. 2007).

The results of FA analysis indicated that FAs in the stomach content of FPM were primarily derived from phytoplankton, suggesting selective feeding on phytoplankton by FPM (I). However, the SIA results indicated that the terrestrial energy sources represented most of the assimilated material in FPM, with a higher contribution than the aquatic food sources (III). The difference can arise from the different components of nutrition that these two methods capture. Isotope analysis measures the entire bulk tissue of organisms and the total POM, which includes all organic material. However, only a small portion of this total POM is accessible to the mussels. This method does not isolate individual dietary components such as FAs but reflects the overall signals from all food sources assimilated over long time. On the other hand, FA analysis focuses on the origins of FAs within an organism, providing extensive insights into the type of fats consumed and, as a result, identifying specific dietary sources (Gao *et al*. 2006, Twining *et al*. 2020). Moreover, along the environmental gradient towards the northern latitudes, characterized by lower anthropogenic activities, more conservation areas and forest cover, the proportion of phytoplankton FAs significantly decreased. However, the bacterial FA in stomach of FPM significantly increased (I). This may indicate that bacteria can serve as supplementary dietary source when phytoplankton availability decreased. This is aligned with the SIA results indicating that the contribution of terrestrial sources in assimilated food increased. The assimilated terrestrial sources may also include indirect assimilation of t-OM either through mixotrophic phytoplankton or heterotrophic bacteria using t-DOM, or by direct exploitation of t-OM. In addition, there is no information available about the turnover rate in FPM tissue, but the observed low multiplicative error term (ξ) strongly supports the idea that the FPM foot tissue has a very slow turnover rate (III) (Stock and Semmens 2016, Stock *et al*. 2018). The assimilated terrestrial sources in FPM tissue may reflect their diet during the previous winter or autumn when the primary production within river is seasonally lowest and the input of t-DOM and plant detritus from the catchment is significant.

3.4 Fatty acid retention

The positive accumulation index of FAs in foot muscle of FPM shows that FPM selectively retains long chain PUFAs (omega-3 and omega-6 FA) from seston (I). Notably, ARA demonstrated the highest average accumulation index across all regions (647 ± 396) in foot relative to the seston. This was followed, in descending order, by ALA, LA, EPA, and DHA (I). These findings indicates the selective retention of PUFAs in FPM, which should promote FPM growth and

reproduction (Bartsch *et al*. 2017). Bivalves, including freshwater mussels, exhibit a remarkable capacity to selectively retain FAs, which are vital for their growth and reproduction (Ezgeta-Balić *et al*. 2012, Bartsch *et al*. 2017). The highest retention of ARA is possibly due to its limited availability in the seston. This may indicate the biological need of FPM for ARA, which is vital for producing eicosanoids (i.e., bioactive compounds with hormone-like properties), that are crucial for inflammatory responses and reproduction in aquatic animals (Bell and Sargent 2003). It has been demonstrated that retention of ARA affects the reproductive processes and stimulate muscle contraction to release egg during spawning in bivalves (Soudant *et al*. 1999, Palacios *et al*. 2005, Peharda *et al*. 2006, Mladineo *et al*. 2007, Ezgeta-Balić *et al*. 2012). Even though the riverine systems receive large amount of t-OM from the catchments, river food webs are rich in omega-3 long chain PUFAs, EPA and DHA in particular (Allan 2004). Bivalves have absolute require for specific FAs including EPA, DHA and ARA (Budge *et al*. 2001) and the relatively low retention of EPA and DHA may indicate that these FAs are enough to meet the physiological needs of FPM.

Bivalves may have limited ability to synthesize long chain PUFAs and they need to get these essential nutrients from their diet (Albentosa *et al.* 1996, Delaporte *et al.* 2005, Alkanani *et al.* 2007). The PUFA composition in mussels is mostly determined by the PUFA profile of their food, which highlights the significance of dietary sources in fulfilling the nutritional requirements of freshwater mussels (Cook and McMaster 2004, Newton *et al*. 2013). Preferably, organisms such as freshwater mussels obtain long chain PUFAs from diet rather than synthesizing them de novo (Cook and McMaster 2004, Liénart *et al*. 2023). The negative retention of all PUFAs in FPM foot muscle, in relation to FAs in their stomach content (I), suggests that FPM diet may sufficiently fulfil its requirements for these FAs.

3.5 Environmental factors affect FPM diet, terrestrial contribution and accumulation index

Anthropogenic activities significantly alter the watershed characteristics, leading to potential impacts on the quantity and quality of nutrients and OM into streams and rivers. This has consequences on the food webs and ecosystem services (Li *et al*. 2023). There was a significant correlation between phytoplankton and terrestrial FAs in seston along the environmental gradient (PC1, I). The proportion of phytoplankton FAs in seston reduced significantly towards northern latitudes, which have lower anthropogenic activity, and higher forest cover and proportion of conserved area. Whereas terrestrial FAs increased significantly (I). In contrast, bacterial FAs in the seston did not show any clear pattern along the environmental gradient (PC1) (Fig. 5C in I). The proportion of phytoplankton FAs in the stomach content corresponded to seston, showing a significant decrease along the PC1 (Fig. 5 D in I). However, the terrestrial FAs did

not exhibit a distinct pattern, although the bacterial FAs increased towards the north (Table 1 in I, Fig. 5E, F in I). The availability and composition of OM in freshwater ecosystems is ascertained by the local climate and catchment characteristics (Carpenter *et al*. 1998, Crapart *et al*. 2023). Human activities, like forestry, agriculture and ditching, can directly and indirectly affect the dynamics of DOM and nutrient export from the terrestrial to aquatic ecosystems and result in increase in the DOC inputs and TN in streams and rivers (Graeber *et al*. 2012, Schelker *et al*. 2012). Webster *et al*. (1990) demonstrated that the loading of POM and DOM into the streams significantly increased after forestry activities. Consequently, in the present study, the proportion of phytoplankton FAs in seston and stomach content increased towards the southern regions. In northern latitudes, the reduction in phytoplankton FAs in stomach content was followed by significant increase in the bacterial FAs. It is important to note that bacterial production can also be significant source of nutrition for consumers, particularly in streams with high inputs of DOM from the catchment (Hall and Meyer 1998).

Increasing terrestrial FAs in seston along the PC1 gradient was not reflected into the terrestrial FAs in the stomach content of FPM (I). This indicates that phytoplankton is the most preferred source of FAs when it is available (I). In addition, the assimilated t-OM in FPM also showed variation along the PC1 gradient (III), with the higher proportion of terrestrial sources in the northern latitudes (Fig. 5A, B in III). Increasing proportion of aquatic food sources in the diet of FPM towards to southern regions likely results from the forestry intensity, ditching, and agriculture in these regions that may increase t-DOM and nutrient loadings to the recipient water bodies. This could result in higher level of primary production and accumulation of in situ derived organic material (Schelker *et al*. 2012). Indeed, the environmental gradient (PC1) was associated with productivity of the rivers (I).

The significant increase in the retention of LA and ARA in northern regions might be attributed to their limited availability in the food sources accessible to FPM, resulting in the selective retention of these FAs in tissues (I). Specifically, the green algae are rich in LA, and cyanobacteria have trace amounts of LA (Shanab *et al*. 2018, Taipale *et al*. 2020). The decrease in the ratio of green algae in the stomach content of FPM may potentially account for the elevated retention of these FAs. This suggests that there may be a compensation mechanism in place to deal with dietary deficiencies which enhance the availability of these FAs in the tissue of FPM (I).

3.6 Condition of FPM

The proportion of dietary sources to FPM and the physiological responses of FPM (SOR and SGI) exhibited variation along the environmental gradient (III). These, in turn, may affect the viability of the population. Positive association between relative growth (SGI in the past 5 years) and terrestrial dietary sources in FPM, indicated a higher recent growth in populations with a greater proportion of

assimilated t-OM. Grunicke *et al*. (2023) reported higher growth rates in juvenile FPM that were fed with high-quality stream detritus (i.e. mixture of processed riparian detritus and aquatic OM) compared to those fed only with riparian detritus (predominantly t-OM). However, t-OM is generally considered low-quality food source, lacking physiologically essential FAs, compared e.g., to diatoms (Brett *et al*. 2009a, Grieve and Lau 2018). Thus, FPM may obtain very small proportion of required micronutrients from t-OM. The high-quality aquatic food sources and low-quality terrestrial dietary components in tandem can have synergistic effects on FPM condition. The condition of mussels is not only related to food quality or quantity, but also to how different dietary components are assimilated and used for different physiological activities (Grieve and Lau 2018). This is likely due to the catabolic and anabolic processes led by terrestrial and aquatic OM. FPM may use less nutritious t-OM for catabolic metabolism, while using higher quality dietary sources for vital anabolic functions like growth and reproduction (Grieve and Lau 2018). Even though mussels showed better SGI towards to the northern regions, where there was a higher proportion of assimilated terrestrial OM compared to aquatic OM the SGI could also be influenced by the simultaneous availability of more nutritious aquatic OM (e.g., phytoplankton).

The negative association between SOR and PC1 suggests that FPM in northern latitudes were in better condition. These northern watersheds are characterized by lower productivity and nutrient concentrations. Furthermore, these regions experience lower human-induced activities such as forestry and agriculture, and they feature more forest cover and conservation areas. These factors likely contribute to the better condition of FPM in these areas. SOR assesses the strength of the adductor muscles, which indicates the feeding conditions experienced by the mussel over a longer period (Moorken and Killeen 2018). In this study, the other condition proxies, such as energy storage (quantified by lipid content), length-weight relationship, and FPM density (proxy of mussels' response to local conditions), did not show any statistically significant correlation with the environmental gradients. Previous studies have reported a connection between environmental factors and the physical condition of FPM. For instance, elevated nutrition levels can lead to higher metabolic activity and oxygen consumption and have harmful consequences for FPM, ultimately resulting in increased mortality (Bauer 1988). Land use activities and habitat degradation can lead to increased turbidity and sedimentation, which can adversely affect the growth of adult FPM (Österling *et al*. 2010). Additionally, laboratory experiments have demonstrated that elevating TSS concentrations can induce both physiological effects, such as changes in metabolic rate, and behavioral responses in FPM (Lummer *et al*. 2016, Curley *et al*. 2021).

4 CONCLUSIONS

This study revealed how catchment characteristics and environmental changes impacted the dietary habits of FPM and its reliance on t-OM. Results emphasized the significant effect of environmental alterations on FPM, which play a key role in connecting aquatic and terrestrial ecosystems. Based on FA results, FPM displayed a selective feeding, so that they primarily fed on phytoplankton (i.e., diatoms) rather than terrestrial and bacterial food sources. This preference was not constant, and it shifted across the environmental gradient. In rivers affected by human activities, where aquatic primary production was high, phytoplankton was dominant in FPM diet, whereas, in the pristine northern regions, with more conservation areas and less forestry activity, their diet included higher proportion of bacteria. Results indicated active selection in their food intake to meet their nutritional requirements, particularly retention of some essential PUFAs, such as ARA, crucial for their growth and reproduction. Present study was performed during summer when aquatic productivity is high. Therefore, variation in their diet during other seasons is possible, suggesting a need for further FA study to investigate if FPM shift their diet towards terrestrial sources during low productive seasons. The discovery that ethanol preservation can affect δ^2H values in FPM foot tissue indicated the importance of careful interpretation of stable isotope data, especially when utilizing the preserved samples to estimate the dietary proportions and track environmental changes. Samples preserved in short-term provided encouraging results, exhibiting only minor shifts in isotope values and C:N ratios, further research is needed to determine if archived samples of endangered and long-lived FPM can be effectively used to investigate long-term alterations in freshwater ecosystems.

SIA results revealed a strong connection in FPM to terrestrial ecosystem in terms of their energy intake as FPM substantially relied on energy derived from t-OM. Furthermore, the contribution of t-OM to FPM was affected by human-induced environmental changes. In areas with higher anthropogenic pressure, there was noticeable reduction in contribution of t-OM to FPM. These environmental alterations not only disrupted the FPM connection with terrestrial ecosystems, but also adversely affected their condition as indicated by lower

growth rate and weaker adductor muscles. Thus, the results highlight the importance of pristine environments and aquatic-terrestrial connectivity for FPM. In addition, the results emphasize the mitigation of anthropogenic impacts and the role of conservation areas in the protection and management of FPM. The highest contribution of t-OM to FPM, possibly derived from indirect assimilation of t-OM through mixotrophic phytoplankton or heterotrophic bacteria, align with river continuum concept or direct assimilation of t-OM. This emphasizes the deep interconnection between freshwater and terrestrial ecosystems.

This research provides essential insights for environmental management, stressing the importance of reduction in nutrient run-off from terrestrial landscape into rivers. Achieving this goal is possible through sustainable and effective management of catchments and implementation of regulatory frameworks. By implementing these measures, the protection of FPM as a keystone species in river ecosystems is ensured, and, in turn, the critical ecosystem services it performs are preserved. FPM is regarded as an umbrella species whose protection also safeguards a number of other (associated) species. Since many of the present human impact variables affecting FPM were related to forestry (e.g., forestry intensity index, diched area), mitigating especially the effects of forestry actions would be important in maintaining the integrity and balance of aquatic ecosystems and supporting the organisms within.

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YHTEENVETO (RÉSUMÉ IN FINNISH)

Valuma-alueen ominaisuuksien vaikutus jokihelmisimpukan (*Margaritifera margaritifera*) ravinnonkäyttöön ja kuntoon

Ilmastonmuutoksen ja maankäytön, kuten kaupungistumisen, metsätalouden ja maatalouden aiheuttamat ympäristömuutokset vaikuttavat merkittävästi ravinteiden ja energian kulkeutumiseen valuma-alueilta jokiin. Tämä näkyy virtavesissä muun muassa ravinteiden, sedimentoitumisen ja haitallisten aineiden lisääntymisenä, sekä muutoksena liuenneen orgaanisen aineen määrässä ja laadussa. Ravinteiden kulkeutuminen jokiin asutusalueilta sekä maa- ja metsätalousalueilta voi johtaa myös rehevöitymiseen ja sitä kautta muutoksiin kasviplanktonin määrässä ja laadussa. Rehevöitymisen seurauksena kasviplanktonyhteisön lajikoostumus voi muuttua sinilevävaltaiseksi ja haitalliset leväkukinnot lisääntyvät. Tällaisilla muutoksilla voi olla laajoja vaikutuksia jokiekosysteemin rakenteeseen ja toimintaan. Lisäksi ilmastonmuutos tuo mukanaan muutoksia vesieliöiden aineenvaihduntaan, kasvuun ja lisääntymiseen, asettaen haasteita etenkin mataliin lämpötiloihin sopeutuneille lajeille. Nämä tekijät voivat johtaa populaatioiden pienenemiseen tai katoamiseen ja siten heikentää luonnon monimuotoisuutta ja muuttaa ekosysteemien toimintaa lajien sopeutuessa uusiin olosuhteisiin. Edellä mainittujen ympäristömuutosten mahdolliset seuraukset korostavat kokonaisvaltaisten hoito- ja suojelustrategioiden toteuttamista, jotta esimerkiksi virtavesien biologinen monimuotoisuus säilyy ja muutosten aiheuttamia haitallisia vaikutuksia virtavesiyhteisöihin voidaan minimoida.

Ympäristössä tapahtuvat muutokset voivat heikentää eliöille tarjolla olevan ravinnon laatua ja koostumusta. Vedestä ravintonsa suodattavat simpukat ovat erityisen herkkiä tällaisille muutoksille, koska ne eivät kykene liikkumaan vapaasti ja ovat siksi riippuvaisia virran mukana tuomasta ravinnosta, kuten kasviplanktonista ja hajonneesta orgaanisesta aineesta. Useiden makeanveden simpukoiden (Unionida) populaatiot ovat maailmanlaajuisesti uhattuna elinympäristöjen muuttumisen, pirstaloitumisen ja isäntäkalojen vähenemisen tai puuttumisen takia. Makeanveden simpukoilla on tärkeä rooli vesiekosysteemeissä, koska ne muokkaavat elinympäristöään ja tästä syystä niitä usein kutsutaankin ”ekosysteemi-insinööreiksi”. Suodattaessaan hiukkasia ja orgaanista ainesta vesipatsaasta ja kaivautuessaan pohjaan, simpukat siirtävät tärkeitä ravinteita muun pohjaeläinyhteisön käytettäväksi. Vaikka makeanveden simpukoiden tiedetään valikoivan erilaisia ravintolähteitä, niiden ravintokoostumus riippuu pääosin ravintopartikkelien suhteellisesta osuudesta vedessä. Ihmisen aiheuttamat muutokset valuma-alueella vaikuttavat vedestä peräisin olevan, kasviplanktonin yhteyttämisen tuloksena syntyneen orgaanisen aineksen ja maalta valuntana tulevan, maakasvien tuottaman aineksen runsaussuhteisiin ja ravitsemukselliseen laatuun. Maalta peräisin olevan orgaaninen aineksen on yleisesti oletettu olevan ravitsemuksellisesti heikkolaatuista ravintoa vesieliöille, samoin kuin tiettyjen kasviplanktonlajien, jotka voivat sisältää vähemmän ravitsevia yhdisteitä tai jopa myrkkyjä. Siksi kasviplanktonin lajikoostumuksen muutokset tai

maalta tulevan orgaanisen aineksen lisääntyminen vedessä voivat vaikuttaa simpukoiden ravinnon laatuun ja sitä kautta yksilöiden kuntoon ja simpukoiden populaatiokokoon.

Jokihelmisimpukka eli raakku (*Margaritifera margaritifera*) on huomattavan pitkäikäinen, kookas simpukkalaji – vanhimmat yksilöt voivat elää jopa 250-vuotiaksi. Jokihelmisimpukalla voi olla runsaana esiintyessään (maksimissaan jopa 1000 yksilöä neliömetrillä) tärkeä ekologinen rooli vesistöalueiden yläosien viileissä, vähäravinteisissa sivu-uomissa, joihin päätyy merkittäviä määriä maaperän orgaanista aineista ympäröiviltä valuma-alueilta.

Väitöskirjatyöni tavoitteena oli 1) selvittää uhanalaisen jokihelmisimpukan saatavilla olevaa ravintoa ja ravinnon valintaa rasvahappoanalyysien perusteella ja 2) vertailla vedestä ja maalta peräisin olevan orgaanisen aineksen osuutta ja merkitystä jokihelmisimpukan energialähteenä vakaiden isotooppien avulla. Lisäksi tavoitteena oli 3) tutkia, miten ihmistoimintaan kytkeytyvät valuma-alueen maankäytön ja joen vedenlaadun muutokset vaikuttavat jokihelmisimpukan ravinnon saatavuuteen ja ruokavalioon sekä vedestä/maalta peräisin olevan orgaanisen aineksen rooliin jokihelmisimpukan elämässä.

Pohjois-Suomesta kerätty kenttäaineisto kattoi laajan joukon eritasoisen ihmistoiminnan alla olevia valuma-alueita, joista osa sijaitsi lähes kokonaan suojelualueilla ja osassa harjoitettiin vaihtelevasti metsä- ja maataloutta. Yhteensä 29 joen 30 simpukasta (842 yksilöä) tehtiin kustakin niiden kuntoa kuvaavia mittauksia ja joesta sekä valuma-alueelta kerättiin simpukoiden mahdollisia ravintokohteita sekä mitattiin ympäristöä kuvaavia muuttujia kuten vesikemiaa ja vuotuista jokien lämpötilaa vuosien 2019 ja 2021 välisenä aikana. Joet sijaitsivat Kainuun/Pohjois-Pohjanmaan, Kuusamon, Sallan ja Inarin alueilla. Maankäyttöä kuvaavat valuma-aluemuuttujat, kuten metsä– ja maatalouden, ojien ja suojeltujen alueiden suhteelliset pinta-alat, kerättiin sähköisistä aineistoista. Kustakin joesta kerättiin kolme yksilöä tarkempia laboratoriomittauksia varten (Ikä, C:N, morfologia). Kunkin simpukkayksilön kudoksista, vatsansisällöstä ja simpukoiden mahdollisista ravintokohteista (joen seston, rihmalevät ja liuennut orgaaninen aines) mitattiin rasvahappojen sekä vedyn vakaiden isotooppien suhteelliset osuudet. Näiden biologisten merkkiaineiden ja kerättyjen ympäristömuuttujien avulla pyrittiin arvioimaan miten valuma-alueiden ominaisuudet ja maankäyttö vaikuttavat jokihelmisimpukan ravinnonlähteiden saatavuuteen, ravintoon ja maalta tulevan energian osuuteen ravinnossa. Lisäksi arvioitiin näiden tekijöiden vaikutusta yksilön kuntoa ja populaatiota kuvaaviin muuttujiin (kasvunopeus, kuoren avautumisvastus, rasvapitoisuus, pituus-painosuhde ja populaatiotiheys).

Tutkimuksessa pystyttiin laboratorioanalyysien, automaattisten mittareiden ja tietokantojen avulla määrittämään yhteensä 26 valuma-alueiden maankäyttöä, maantieteellistä sijaintia, veden laatua ja ympäristön tilaa kuvaavaa selittävää muuttujaa: veden klorofylli-a pitoisuus, saostunut kiintoaines, veden liuenneen orgaanisen hiilen pitoisuus, kokonaistyppipitoisuus, kokonaisfosforipitoisuus, veteen liuenneen hapen osuus, veden pH, veden johtokyky, veden vuotuinen keskilämpötila, näytteenottopaikan etäisyys yläpuolella olevasta järvestä, maatalousmaan osuus valuma-alueesta, mineraalimailla olevan metsän

osuus valuma-alueesta, turvemailla olevien metsien osuus valuma-alueesta, urbaanin alueen osuus valuma-alueesta, vesistöjen osuus valuma-alueesta, metsien osuus valuma-alueesta, leveysaste, suojellun alueen osuus valuma-alueesta, valuma-alueen koko, metsätalousmaan osuus valuma-alueesta, julkisessa omistuksessa olevan metsän osuus metsätalousmaasta, avohakkuiden määrää kuvaava indeksi, metsätalouden intensiteettiä kuvaava indeksi ja ojitetun alueen osuus valuma-alueesta. Muuttujajoukon keskinäisiä suhteita selvitettiin pääkomponenttianalyysillä, mikä redusoi taustamuuttujissa olevan vaihtelun kahteen pääkomponenttiin, joista suurin selityspotentiaali oli ”tuottavuus-leveysaste-ihmistoiminnan vaikutus” pääkomponentilla. Sen saamaan arvoon vaikuttivat positiivisesti useat ihmistoiminnan vaikutukseen kytkeytyvät muuttujat: klorofylli-a, turvemailla olevien metsien osuus, maatalousmaan osuus, veden vuotuisen keskilämpötilan osuus, liuenneen orgaanisen hiilen määrä, metsätalouden intensiteetti ja ojitetun alueen osuus valuma-alueesta. Kyseiseen ”tuottavuus-leveysaste-ihmistoiminnan vaikutus” -pääkomponenttiin negatiivisesti vaikuttavista muuttujista osa oli puolestaan kytköksissä alhaiseen ihmistoiminnan vaikutukseen tai suojelualueisiin: mineraalimailla olevien metsien osuus, julkisessa omistuksessa olevien metsien osuus, leveysaste, metsien osuus valuma-alueesta ja suojellun alueen osuus valuma-alueesta. ”Tuottavuus-leveysaste-ihmistoiminnan vaikutus” -pääkomponenttia käytettiin muuttujana tilastollisissa malleissa kuvaamaan ihmistoimintaan kytköksissä olevaa vaihtelua valuma-alueen maankäytössä ja veden laadussa.

Tutkimuksen tulokset osoittivat, että valuma-alueiden ominaisuuksilla ja maankäytöllä oli merkittävä vaikutus jokihelmisimpukan ravinnon saatavuuteen, ravinnonkäyttöön ja yksilöiden kuntoon. Tutkimusalueen eteläisimmillä joilla vesi oli lämpimämpää ja maatalous-, ojitus- ja metsätaloustoiminta yleisempää. Maankäyttöön ja lämpimämmän ilmaston seurauksena jokiin päätyy enemmän ravinteita, joka johtaa tuottavuuden kasvuun. Rasvahappomittaukset osoittivat, että jokihelmisimpukat valitsevat ravinnokseen kasviplanktonia. Lisäksi rasvahappotulokset viittasivat siihen, että mitä etelämpänä populaatio sijaitsi, sitä enemmän simpukoiden ravinnossa oli kasviplanktonista peräisin olevia rasvahappoja suhteessa muihin ravintokohteisiin (bakteerit, maalta peräisin oleva orgaaninen aines). Vastaavasti pohjoisilla leveysasteilla, jossa valuma-alueella oli vähemmän ihmistoimintaa ja enemmän suojelualueita, maakasvillisuudesta peräisin olevat rasvahapot tulivat hallitsevammiksi ravinnon lähteissä ja valitussa ravinnossa. Vaikka näiden rasvahappojen osuus ravinnosta lisääntyi pohjoista kohti, simpukat näyttivät suosivan myös bakteereista peräisin olevia rasvahappoja, kun kasviplanktonin saatavuus väheni. Tämä johtui todennäköisesti bakteerien korkeammasta ravintoarvosta suhteessa maakasvillisuusperäiseen ravintoon. Rasvahapoille lasketut kertymisindeksit viittasivat myös siihen, että jokihelmisimpukka pystyy tarvittaessa valikoimaan ravitsevampia lähteitä ja pidättämään fysiologisesti välttämättömiä rasvahappoja ensisijaisesti kasviplanktonista, täyttääkseen ravitsemukselliset tarpeensa.

Vakaitten isotooppien analyysit puolestaan osoittivat, että maalta peräisin oleva orgaaninen aines on tärkeä osa jokihelmisimpukoiden energian saantia viitaten siihen, että jokihelmisimpukalla on kiinteä yhteys ympäröivään

maaekosysteemiin. Väitöskirjassa päädyttiin tutkimaan myös näytteiden säilönnän vaikutusta isotooppiarvoihin ja alkuainekoostumuksiin, koska osa näytteistä oli säilötty etanoliin. Säilönnän havaittiin vaikuttavan analyysituloksiin, mutta tämä muutos pystyttiin ottamaan huomioon isotooppimalleja rakennettaessa. Maalta peräisin olevan orgaanisen aineksen osuus pieneni ja vesistöstä peräisin olevan aineksen osuus kasvoi, kun ihmistoiminta valuma-alueella lisääntyi. Isotooppimallien tulosten perusteella valumamuutokset näyttivät häiritsevän vesi- ja maaekosysteemien välistä yhteyttä ja vaikuttavan negatiivisesti jokihelmisimpukoihin, mistä oli osoituksena jokihelmisimpukoiden kasvunopeuden hidastuminen ja kuorensulkijalihasten heikentyminen ihmistoiminnan vaikutuksen lisääntyessä. On mahdollista, että maalta peräisin oleva orgaaninen energia päätyi simpukoihin mixotrofisen kasviplanktonin tai heterotrofisten bakteerien kautta, mutta orgaanisen aineen suoraa assimilaatiokaan ei voida sulkea pois.

Tämän väitöskirjan tulokset osoittavat kuinka ihmistoiminta voi muuttaa aineen ja energian kuten liuenneen orgaanisen aineksen virtausta valuma-alueelta jokiin sekä kuinka tämä muutos vaikuttaa vesieliöiden ravinnonkäyttöön ja sitä kautta niiden kuntoon. Väitöskirjan tulokset tähdentävät uhanalaisten jokihelmisimpukkapopulaatioiden suojelua jokea laajemmalla, valuma- tai vesistöaluetasolla, mikä on mahdollista saavuttaa kestävämmällä metsä- ja maataloudella ja tunnistamalla virtausvälitteisiä yhteyksiä koko valuma-alueella. Lisäksi tulokset korostavat koskemattoman elinympäristön ja ympäröivän maaekosysteemin tärkeyttä jokihelmisimpukan suojelussa.

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ORIGINAL PAPERS

I

ENVIRONMENTAL EFFECTS ON THE SELECTIVE FEEDING AND FATTY ACID RETENTION OF FRESHWATER PEARL MUSSEL

by

Mahsa Hajisafarali, Marco L. Calderini, Mikko Kiljunen, Niklas Moser, Sabrina Nykänen, Jaakko Litmanen, Sami Taipale & Jouni Taskinen

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II

ETHANOL PRESERVATION EFFECTS ON STABLE CARBON, NITROGEN AND HYDROGEN ISOTOPES IN THE FRESHWATER PEARL MUSSEL

by

Mahsa Hajisafarali, Jouni Taskinen, Antti P. Eloranta & Mikko Kiljunen
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Ethanol preservation effects on stable carbon, nitrogen and hydrogen isotopes in the freshwater pearl mussel

Mahsa Hajisafarali · Jouni Taskinen · Antti P. Eloranta · Mikko Kiljunen

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Abstract Chemical preservatives can alter stable isotope ratios in animal tissues. The effects of preservation on δ^{13}C and δ^{15}N values have been investigated in a variety of species, but not on δ^{2}H values or on the freshwater pearl mussel (FPM, *Margaritifera margaritifera*) tissues. We evaluated the effect of ethanol preservation (unpreserved vs preserved tissues) over 6 months on the δ^{13}C, δ^{15}N and δ^{2}H values of FPM foot and gonad tissues. Ethanol preservation significantly increased δ^{13}C values (foot 0.4 ‰; gonad 0.3 ‰), whereas it did not significantly affect δ^{15}N values (foot 0.2 ‰; gonad −0.1 ‰). The positive effect of ethanol preservation on δ^{2}H values (foot 7.1 ‰; gonad 14.5 ‰) and the negative effect on C:N ratios (foot −0.1; gonad −0.5) depended on the tissue type, with larger effects found on the lipid-rich gonad. Overall, ethanol preservation affected δ^{2}H values more than the δ^{13}C, δ^{15}N or C:N ratios of FPM tissues. After 1 month of preservation, the isotope values remained rather stable, and significant changes were only observed in δ^{15}N values. The results imply that ethanol-preserved FPM samples can be used if potential shifts in isotopic and elemental ratios are accounted for prior running mixing models for estimating dietary proportions.

Keywords C:N ratio · Deuterium · *Margaritifera margaritifera* · Stable isotope analysis · Ethanol storage · Lipids

Introduction

The use of stable isotope analysis (SIA), such as the ratios of naturally occurring stable isotopes of carbon (^{13}C/^{12}C; hereafter denoted as δ^{13}C), nitrogen (^{15}N/^{14}N; δ^{15}N) and hydrogen (^{2}H/^{1}H; δ^{2}H), has improved our understanding of how aquatic ecosystems work (Fry, 2006). Stable isotope ratios are routinely used to study the structure and function of food webs and the energy sources of organisms within (Umbricht et al., 2018). Because primary producers vary in their isotopic composition, δ^{13}C is generally used to determine the origins of carbon in food webs or to identify feeding areas (DeNiro & Epstein, 1978). As organisms are generally enriched in ^{15}N relative to their diet, they show an increase in δ^{15}N values with each trophic step in the food web. δ^{15}N can also be used to assess nitrogen sources (Deniro & Epstein, 1981). δ^{2}H values have been applied to differentiate terrestrial from aquatic inputs because δ^{2}H varies between terrestrial and aquatic primary producers (Doucett et al., 2007) or to study dispersal of animals (Hobson & Wassenaar, 2008). C:N ratios in

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animals, including aquatic species, represent the overall macronutrient content in any given tissue and have been used to estimate lipid content of the tissue (Post et al., 2007).

Despite SIA being used successfully in ecological studies for over 3 decades, there are still many unknowns about the effects of sample treatment on isotope values (Lau et al., 2012). For SIA, fresh samples are preferred, but logistics often require the preservation of organisms for later analyses (Jesus et al., 2015). Preserved samples are also often found from museums or research institutes and SIA of such archived tissue samples may provide the only record about long-term ecosystem changes (Syväranta et al., 2008). Stable isotope values measured in animal tissues represent values generated by their diet during a given period (DeNiro & Epstein, 1978). However, many factors, including the preservation method and time, can affect the stable isotope ratios in animal tissues and therefore also the results of isotope mixing models used for estimation of consumer diets (Javornik et al., 2019). Since the isotope ratios are sensitive to even subtle variations, meticulous care must be taken to preserve samples (Yurkowski et al., 2017).

Numerous studies have examined preservation effects on stable isotope ratios of carbon and nitrogen, with inconsistent results across taxa, environments (e.g., marine vs freshwater), tissue types, preservation methods and duration of preservation (Vizza et al., 2013; Kiszka et al., 2014; Stallings et al., 2015; Hogsden & McHugh, 2017). Chemical preservatives can alter stable isotope ratios through a variety of mechani2004sms: they can either add carbon or nitrogen to tissues or they can leach macromolecules consisting of these elements (Sarakinos et al., 2002; Sweeting et al., ; Barrow et al., 2008; Ruiz-Cooley et al., 2011). Ethanol is often used as a preservative to store animal tissues. Since ethanol does not contain nitrogen, it cannot add nitrogen to samples, but it may affect stable isotope ratios by breaking bonds with nitrogen atoms in tissues (Hetherington et al., 2019). Some studies reported that ethanol storage did not affect the δ^{15}N values in sea stars, freshwater invertebrates and fish fin (Vizza et al., 2013; Hogsden & McHugh, 2017; Le Bourg et al., 2020). In contrast, other studies indicate that ethanol can increase δ^{15}N values in bivalves and ray fishes (Syväranta et al., 2011; Burgess & Bennett, 2017; Umbricht et al., 2018) but decrease δ^{15}N values in some other fishes (Olin et al., 2014). Carbon content of tissues and their δ^{13}C values may also be affected by ethanol but the magnitude and direction of changes varies among studies. For instance, previous studies of marine and freshwater bivalves, invertebrates, fishes and mammals indicate that ethanol preservation can either have no effect (Lau et al., 2012; Kiszka et al., 2014; Burgess & Bennett, 2017) or lead to higher δ^{13}C values of consumer tissues (Syväranta et al., 2011; Stallings et al., 2015; Hogsden & McHugh, 2017; Umbricht et al., 2018). Lipids are depleted in ^{13}C and ^{2}H as compared to proteins and carbohydrates (DeNiro & Epstein, 1978; Hobson et al., 1999). Thus, if ethanol extracts lipids from samples, a decrease in C:N ratio as well as an increase in the δ^{13}C and δ^{2}H values of preserved samples would be expected.

Freshwater bivalves are commonly used in biomonitoring (Farris & Van Hassel, 2007). They can be important components in freshwater food webs, provide valuable ecosystem services and have a large impact on ecosystem function, including nutrient cycling (Vaughn et al., 2008; Atkinson & Vaughn, 2014; Vaughn, 2018). Studies on the effect of ethanol preservation on stable isotope values of freshwater bivalves, Asiatic clams apart (Sarakinos et al., 2002; Syväranta et al., 2011), are practically non-existent. However, ethanol-preserved and archived samples of endangered freshwater mussels are indispensable sources of material for isotope analysis because they can be used to reconstruct historical energy and nutrient sources. Moreover, to the best of our knowledge, no studies have examined the effect of chemical preservation on δ^{2}H values of animal tissues. Hydrogen stable isotopes are increasingly used to study aquatic food webs and animal migrations, reinforcing the need to understand how different storage and laboratory preparation methods affect δ^{2}H values (Hobson & Wassenaar, 2008; Soto et al., 2013).

This study investigates the effect of ethanol preservation and its duration on δ^{13}C, δ^{15}N and δ^{2}H values and C:N ratios of the endangered freshwater pearl mussel [FPM, *Margaritifera margaritifera* (Linnaeus, 1758)]. The obtained results will allow subsequent correction for potential preservation-induced changes in FPM isotope values prior to comparing or using data from preserved and non-preserved samples in isotope mixing models. We prepared FPM foot and gonad tissues for SIA to evaluate if the ethanol

preservation effects varied among tissue types, with lipid-rich gonads expected to show larger changes. Specifically, we addressed the following questions: (1) How do isotope values and C:N ratios differ between unpreserved and ethanol-preserved tissues? (2) How does preservation time affect differences in isotope values and C:N ratios in FPM tissues? Based on previous studies, we hypothesized that ethanol preservation may increase $\delta^{13}C$ and $\delta^{2}H$ values, decrease C:N ratios of preserved samples and have no or minor effect on $\delta^{15}N$ values. Further, we assumed that ethanol preservation would have a larger effect on the lipid-rich gonads (Jokela et al., 1993) than on the foot tissue of FPM. Moreover, we predicted that shifts in isotope values should increase with preservation time.

Materials and methods

Mussel collection and preparation

Twenty-four FPM individuals were collected from eight rivers, three individuals per river, in Northern Finland (64–68 °N, 27–29 °E) during August–September 2020. FPMs were collected under permission (KAIELY/296/2019 and 357/2019, LAPELY/1929/2019 and 2252/2019, POPELY/1276/2019 and 1490/2019) granted from the Centers for Economic Development, Transport and the Environment of Kainuu, Lapland and North Osthrobothnia, Finland, respectively. Upon collection, the mussels were packed in individual plastic bags, stored immediately in ice, transported to the laboratory and kept in a cool box under ice until dissection. Mussels were dissected and tissues (foot and gonad) were extracted. In previous studies (Syväranta et al., 2011; Xu et al., 2011; Liu et al., 2013), the ethanol preservation effects on $\delta^{13}C$ and $\delta^{15}N$ values have been shown to stabilize after 6 months preservation. Therefore, we decided to conduct this experiment for only 6 months. To examine the effects of ethanol preservation and preservation time (1, 2, 4, and 6 months) on $\delta^{13}C$, $\delta^{15}N$, $\delta^{2}H$ values and C:N ratio, the foot and gonad samples dissected from each FPM individual were split in two parts; one stored at − 18 °C ('unpreserved tissue'/time 0) overnight prior to freeze-drying, and another stored in 99.5% ethanol and kept at room temperature (~ 18 °C) ('preserved'). The preserved samples of FPM foot and gonad tissues were removed from ethanol and dissected for subsamples after 1, 2, 4 and 6 months, rinsed with ultra-pure water and then freeze-dried. The unpreserved and preserved freeze-dried samples were ground to a fine powder with a mortar and pestle. Prior to final SIA, 0.500–0.700 mg of sample was weighed into a tin cup for $\delta^{13}C$ and $\delta^{15}N$ analyses and 0.350 mg of sample into a silver cup for $\delta^{2}H$ analysis. Prior to folding, the silver cups were stored open in laboratory atmosphere with laboratory standards for at least five days to allow hydrogen exchange between the samples and laboratory air (Wassenaar & Hobson, 2003). To examine the effect of preservation time, the relative differences (Δ) in isotope values of preserved samples (time points 1–6 months) to the values of unpreserved samples (time 0) were calculated.

Stable isotope analysis

We measured $\delta^{13}C$, $\delta^{15}N$, $\delta^{2}H$ values and C:N ratios at the Stable Isotope Laboratory at the University of Jyväskylä, Finland. We report stable isotope ratios in δ notation relative to Vienna Pee Dee belemnite (VPDB), atmospheric nitrogen (N_2) and Vienna Standard Mean Ocean Water (VSMOW) for carbon, nitrogen, and hydrogen, respectively. $\delta^{13}C$ and $\delta^{15}N$ values and C:N ratios (by mass) were measured by continuous-flow stable isotope ratio mass spectrometer (CF-SIRMS) coupled with a FlashEA 1112 elemental analyzer (Thermo Electron Corporation, Waltham, MA, U.S.A.). $\delta^{2}H$ values were measured using an Isoprime 100 CF-SIRMS (Isoprime Ltd, Stockport, U.K.) coupled with Elementar vario PYRO cube elemental analyzer (Elementar Analysensysteme GmbH, Hanau, Germany). In each run, multiple samples of two reference materials (caribou hoof [CBS], kudu horn [KHS]) were analyzed relative to VSMOW (Standard Mean Ocean Water) (Soto et al., 2017). Carbon, nitrogen and hydrogen stable isotope ratios are expressed as per mil (‰) and calculated following the equation:

$$\delta X = \left[\left(\frac{R_{sample}}{R_{reference}} \right) - 1 \right] \times 1000 \quad (1)$$

where R_{sample} and $R_{reference}$ are the $^{13}C/^{12}C$, $^{15}N/^{14}N$ and $^{2}H/^{1}H$ ratios for the sample and international standards, respectively. X represents the heavy isotopes of ^{13}C, ^{15}N or ^{2}H. Positive δ values indicate that the sample is isotopically enriched, meaning it

contains a greater proportion of the heavy stable isotope (^{13}C, ^{15}N or ^{2}H). The standard deviations of the delta-values of replicate reference materials in each run were always less than 0.14 ‰ for δ^{13}C, 0.24 ‰ for δ^{15}N and 2.41 ‰ for δ^{2}H.

Statistical analyses

We used Generalized Linear Mixed Effect Models (GLMM) in the statistical software R (version 4.1.2; 2022-09-06; R Core Team, 2022) to test for the effects of ethanol preservation (unpreserved samples at time point 0 and preserved samples at time point 6; hereafter *Preservation*) and preservation time (preservation for 1, 2, 4, and 6 months; hereafter *Time*) on the δ^{13}C, δ^{15}N and δ^{2}H values and C:N ratios of FPM foot and gonad tissues (hereafter *Tissue*). For the GLMMs, we used *lme* function with default settings in the *nlme* package (Pinheiro et al., 2021) and set the δ^{13}C, δ^{15}N and δ^{2}H values and C:N ratios as the response variables and ethanol preservation (*Preservation*), preservation time (*Time*), tissue type (*Tissue*) and the *Preservation* x *Tissue* and *Time* x *Tissue* two-way interactions as the explanatory variables and FPM individual as the random factor (random = ~ 1|Individual). In the case of significant differences ($P < 0.05$), Tukey-tests were used for pairwise post hoc comparisons to compare the δ^{13}C, δ^{15}N and δ^{2}H values and C:N ratios between different time points, using the emmeans package in R (Lenth et al., 2022).

Table 1 Results of Generalized Linear Mixed Effect Models (GLMM) predicting δ^{13}C, δ^{15}N and δ^{2}H values and C:N ratios of foot and gonad tissues of freshwater pearl mussel, with *Preservation* [unpreserved (t = 0) and ethanol-preserved samples (t = 6)], *Tissue* (foot and gonad) and the two-way *Preservation* x *Tissue* interaction as the explanatory variable

	NumDF	denDF	*F* value	*P* value
δ^{13}C				
Intercept	1	67	9164.78	< 0.001
Preservation	1	67	33.64	**< 0.001**
Tissue	1	67	230.35	**< 0.001**
Preservation × Tissue	1	67	0.19	0.666
δ^{15}N				
Intercept	1	67	285.41	< 0.001
Preservation	1	67	1.093	0.300
Tissue	1	67	97.204	**< 0.001**
Preservation × Tissue	1	67	2.253	0.138
δ^{2}H				
Intercept	1	67	13,723.47	< 0.001
Preservation	1	67	111.69	**< 0.001**
Tissue	1	67	46.65	**< 0.001**
Preservation × Tissue	1	67	13.87	**0.001**
C:N ratio				
Intercept	1	55	3770.063	< 0.001
Preservation	1	55	16.27	**< 0.001**
Tissue	1	55	197.05	**< 0.001**
Preservation × Tissue	1	55	7.27	**0.009**

Significant differences ($P < 0.05$) are highlighted in bold

Results

As indicated by the significant two-way interactions (Table 1), the positive effect of ethanol preservation on δ^{2}H values and the negative effect on C:N ratios depended on the tissue type, with the effects being larger in the lipid-rich gonad than in the foot tissue (Fig. 1). However, no significant *Preservation x Tissue* interaction effects were detected for δ^{13}C or δ^{15}N, as gonads were consistently depleted in both ^{13}C and ^{15}N compared to foot tissue, and the significant positive main effect of *Preservation* on δ^{13}C was comparable in both tissue types (i.e., 0.3–0.4 ‰; Tables 1, 2, Fig. 1).

Ethanol preservation increased δ^{13}C values in both tissues but with a slightly higher magnitude in foot (0.4 ‰) than in gonad (0.3 ‰; Table 2). In contrast, preservation had minor and inconsistent effects on δ^{15}N, with the shift in δ^{15}N ranging from −0.1 ‰ to 0.2 ‰ in gonad and foot, respectively (Table 2). Both tissues showed elevated δ^{2}H values in preserved samples as compared to the unpreserved samples by a mean difference of 7.1 ‰ and 14.5 ‰ in foot and gonad (Table 2), respectively. C:N ratios were overall higher in gonad than foot and decreased 0.1 in foot and 0.5 in gonad after 6 months' preservation.

The GLMM results indicated no significant *Time x Tissue* interaction effects for $\Delta\delta^{13}$C, $\Delta\delta^{15}$N, $\Delta\delta^{2}$H or ΔC:N ratios (Table 3). In contrast, the results show a significant main effect of *Time* on $\Delta\delta^{15}$N, but not for $\Delta\delta^{13}$C, $\Delta\delta^{2}$H and ΔC:N ratios (Table 3). However, as indicated by the pairwise comparisons,

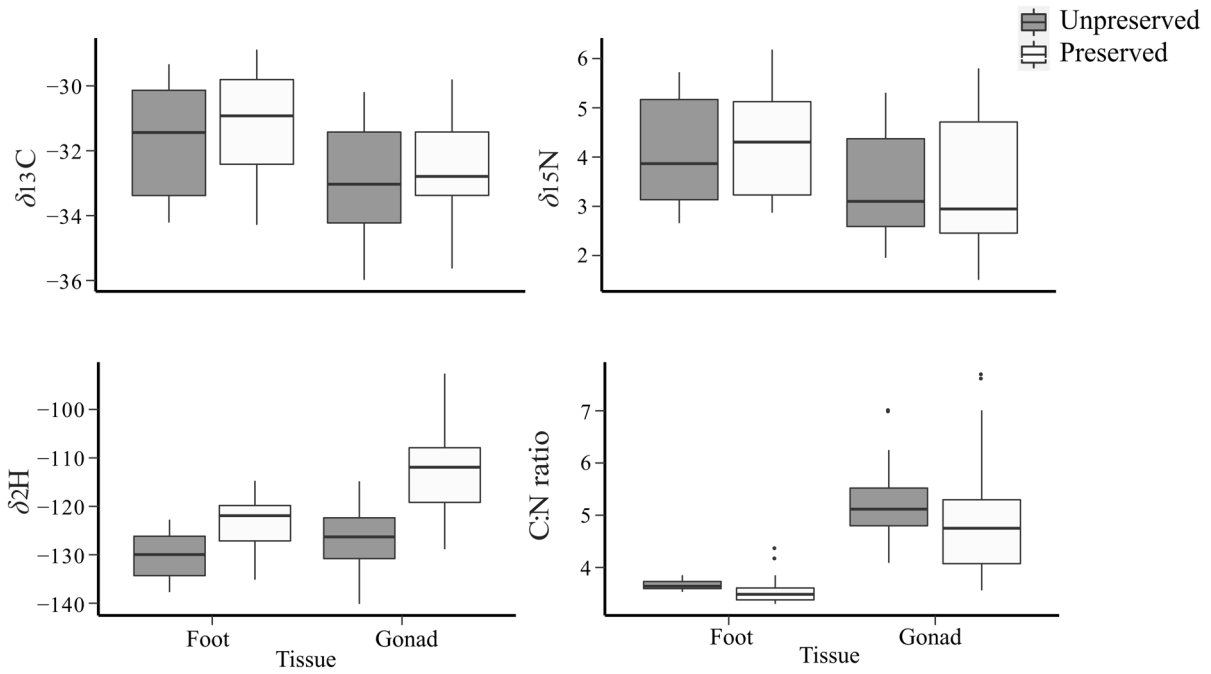

Fig. 1 Boxplots showing $\delta^{13}C$, $\delta^{15}N$, $\delta^{2}H$ values (‰) and C:N ratios of unpreserved (time = 0) and preserved foot and gonad (time = 6) in ethanol of freshwater pearl mussel. The central box spans the interquartile range with the middle line denoting the median and whiskers defining minimum and maximum range

Table 2 Mean ± standard deviation (SD) and the observed minimum (min) and maximum (max) values of $\delta^{13}C$, $\delta^{15}N$, $\delta^{2}H$ and C:N ratios of unpreserved (time 0) and preserved (time 6) foot and gonad tissues of freshwater pearl mussel

	Unpreserved				Preserved				Differences (Δ)			
	Mean	SD	Min	Max	Mean	SD	Min	Max	mean	SD	min	max
Foot												
$\delta^{13}C$	−31.6	1.7	−34.2	−29.3	−31.2	1.6	−34.3	−28.7	0.4	0.1	−0.7	1.3
$\delta^{15}N$	4.1	1.1	2.7	5.7	4.3	1.1	2.7	6.2	0.2	0.1	−0.3	0.9
$\delta^{2}H$	−130.1	4.8	−137.7	−122.8	−123.0	5.2	−135.1	−101.3	7.1	0.5	−0.8	16.0
C:N ratio	3.7	0.1	3.5	3.9	3.6	0.3	3.3	4.9	−0.1	0.2	−0.4	1.4
Gonad												
$\delta^{13}C$	−32.9	1.8	−36	−30.2	−32.6	1.6	−35.6	−29.2	0.3	0.2	−1.1	2.1
$\delta^{15}N$	3.5	1.1	2.0	5.3	3.4	1.3	1.5	6.0	−0.1	0.2	−0.9	1.0
$\delta^{2}H$	−126.9	6.2	−140.2	114.8	−112.4	9.6	−133.6	−79.8	14.5	3.4	−13.3	27.8
C:N ratio	5.0	0.6	4.1	6.2	4.6	0.6	3.6	6.7	−0.5	0.1	−1.7	1.7

The Δ values represent the differences between unpreserved and preserved samples

the effect of *Time* on $\Delta\delta^{15}N$ was not significant over the preservation period and resulted from the lower $\Delta\delta^{15}N$ values at months 2 (−0.1 ‰) and 6 (−0.2 ‰) (Fig. 2). Finally, gonads showed higher $\Delta\delta^{2}H$ values (13.3 ‰) but lower ΔC:N ratios (0.5) than the FPM foot tissue at month 6 (Fig. 2, Table 3).

Table 3 Results of Generalized Linear Mixed Effect Model (GLMM) repeated measures for $\delta^{13}C$, $\delta^{15}N$, $\delta^{2}H$ and C:N ratios of foot and gonad tissues of freshwater pearl mussel preserved in ethanol for different time periods (month 1–6)

	numDF	denDF	*F* value	*P* value
$\delta^{13}C$				
Intercept	1	122	65.88	< 0.001
Time	3	122	0.60	0.614
Tissue	1	122	0.38	0.537
Time × Tissue	3	122	0.35	0.789
$\delta^{15}N$				
Intercept	1	117	36.5	< 0.001
Time	3	117	6.80	**< 0.001**
Tissue	1	117	0.04	0.851
Time × Tissue	3	117	0.54	0.658
$\delta^{2}H$				
Intercept	1	120	182.01	< 0.001
Time	3	120	1.87	0.138
Tissue	1	120	22.67	**< 0.001**
Time × Tissue	3	120	2.30	0.080
C:N ratio				
Intercept	1	118	2770.58	< 0.001
Time	3	118	1.28	0.283
Tissue	1	118	137.57	**< 0.001**
Time × Tissue	3	118	0.32	0.813

Significant differences ($P < 0.05$) between time periods and tissues are highlighted in bold

Discussion

Our results demonstrate that ethanol preservation can affect the $\delta^{13}C$ and $\delta^{2}H$ values and the C:N ratios of FPM, with the effects being particularly evident in the lipid-rich gonad tissues. The shifts in isotope values and C:N ratios were generally minor, with the largest shifts being observed in the $\delta^{2}H$ values (ca. 7–14 ‰ increase).

The reasons for preservation-induced changes in isotope values are not fully understood, but they can be associated with lipid extraction (Syväranta et al., 2008), exchange of heavy and light isotopes between samples and preservatives (Hobson et al., 1997; Edwards et al., 2002), or protein hydrolysis during preservation (Arrington & Winemiller, 2002; Sarakinos et al., 2002; Lecea et al., 2011). Although this study was not designed to explain the causative mechanisms altering the stable isotope values in FPM tissues, most of the shifts in isotope values could be caused either by loss or uptake of materials during preservation. As compared to proteins and carbohydrates, lipids are depleted in ^{13}C and ^{2}H (Park & Epstein, 1961; DeNiro & Epstein, 1978; Hobson et al., 1999). Several studies have shown that the extraction of isotopically lighter lipids from the samples may increase the $\delta^{13}C$ and $\delta^{2}H$ values of an organism and decrease the C:N ratios (DeNiro & Epstein, 1978; McConnaughey & McRoy, 1979; Gloutney & Hobson, 1998; Kiljunen et al., 2006). The effect of ethanol preservation can arise through either hydrolysis of lipids (Hobson et al., 1997; Gloutney & Hobson, 1998; Bosley & Wainright, 1999) or uptake of ethanol into the tissues (Sarakinos et al., 2002). The increase in $\delta^{13}C$ and $\delta^{2}H$ values of preserved foot and gonad tissues may be explained by ethanol-induced lipid extraction as indicated by the decreased C:N ratios (Kaehler & Pakhomov, 2001; Arrington & Winemiller, 2002; Sweeting et al., 2004). C:N ratios in animals, including aquatic species, provide a strong predictor of lipid content within tissues. C:N ratios of > 4 typically indicate lipid-rich whereas C:N ratios of < 4 indicate lipid-poor tissues (Post et al., 2007). In our study, lipid-rich gonads showed lower $\delta^{13}C$ values and higher C:N ratios in both unpreserved and preserved samples as compared to the foot. Surprisingly, $\delta^{2}H$ values showed an opposite pattern with higher $\delta^{2}H$ in lipid-rich gonads, but both tissues were clearly more enriched after 6 months' preservation. ^{2}H-enriched was larger in the gonads, suggesting that lipid-rich tissues are more likely to lose lipids which are depleted in ^{13}C and ^{2}H (Kaehler & Pakhomov, 2001; Sweeting et al., 2004; Carabel & Verísimo, 2009). Our results correspond with previous studies (Stallings et al., 2015; Hogsden & McHugh, 2017; Umbricht et al., 2018; Le Bourg et al., 2020), but contrast with the previously observed ethanol-induced decrease in $\delta^{13}C$ values of a soft-shell clam species (Umbricht et al., 2018). While ethanol is known to alter isotopic values, recent reviews suggest that preservative effects on different tissues, species, and organisms can be variable and inconsistent across studies, taxa and environments (Hogsden & McHugh, 2017).

The differences in $\delta^{13}C$ values between unpreserved and preserved FPM foot and gonad tissues were + 0.4 ‰ and + 0.3 ‰, respectively. This preservation effect was smaller in magnitude than previously reported for preserved fish muscle tissue (i.e.,

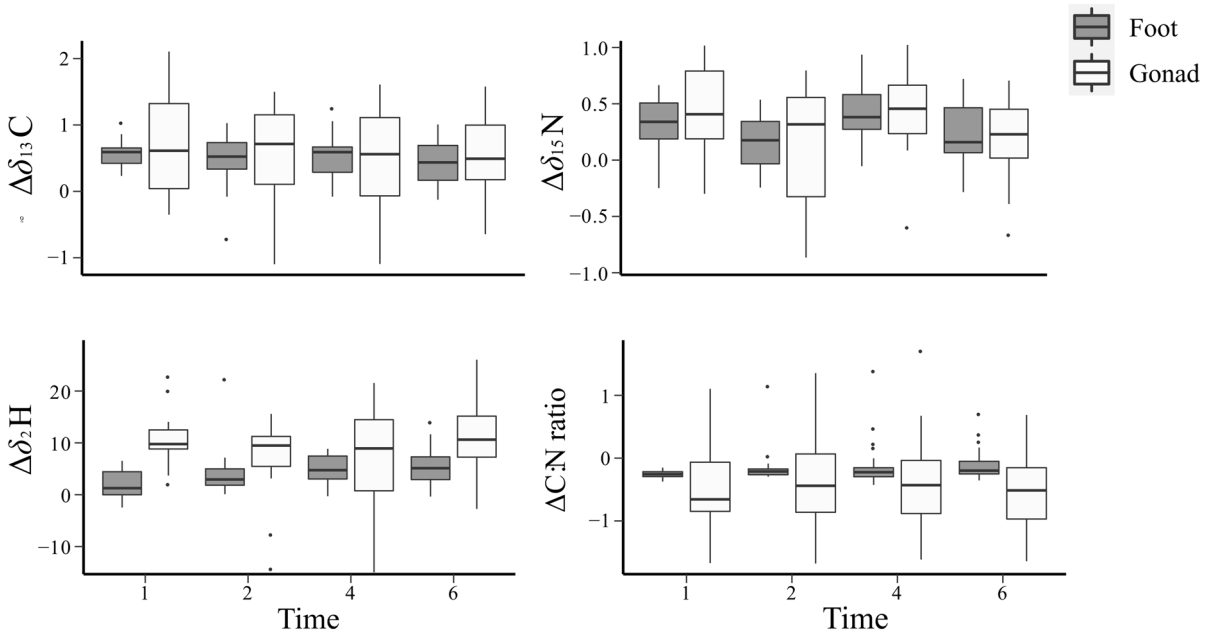

Fig. 2 Boxplots showing the relative differences (Δ) in δ^{13}C, δ^{15}N, δ^{2}H values and C:N ratio of preserved samples (time points 1–6 months) to the values of unpreserved samples (time 0) of freshwater pearl mussel. The central box spans the interquartile range with the middle line representing the median and whiskers defining minimum and maximum range

1.3–1.4 ‰ increase; Vizza et al., 2013), probably due to differences in tissue composition. Similar enrichment was also found in an Asiatic clam [*Corbicula fluminea* (O. F. Müller, 1774)] (Sarakinos et al., 2002) with a higher magnitude of 2.2 ‰, while an increase of 0.8 ‰ was observed in dorsal muscle tissue of Arctic charr [*Salvelinus alpinus* (Linnaeus, 1758)] (Kelly et al., 2006). Thus, the degree of ethanol preservation effect on δ^{13}C values varies among tissue types, species and preservation times. δ^{13}C values in animal tissues usually provide information about the consumed food sources (McConnaughey & McRoy, 1979) and the trophic fractionation between the diet and consumer tissues is assumed to be ca. 0–1 ‰ per trophic level (Post, 2002). If preservation causes a shift that exceeds the trophic discrimination factor (TDF), this may cause mis-interpretations in food web studies (DeNiro & Epstein, 1978; Bosley & Wainright, 1999; Bugoni et al., 2008). Thus, the changes in δ^{13}C observed here (i.e., 0.3–0.4 ‰ enrichment) are comparable to TDFs generally applied in ecological stable isotope studies (Post, 2002).

Ethanol preservation altered the δ^{15}N values of FPM foot and gonad tissues in different directions. These shifts in δ^{15}N values of foot (+0.2 ‰) and gonad (– 0.1 ‰) were relatively small when considering the analytical precision here (0.24 ‰ for δ^{15}N) as well as the commonly used TDF values (i.e., 3–4 ‰ increase with each trophic level) (Deniro & Epstein, 1981; Minagawa & Wada, 1984). This suggests that the δ^{15}N values of preserved FPM tissues can be used in food web studies if unpreserved samples are not available. Findings from our and previous studies (Hobson et al., 1997; Ponsard & Amlou, 1999; Sarakinos et al., 2002; Lau et al., 2012; Olin et al., 2014) demonstrate a minimal effect of ethanol on δ^{15}N values. However, some other studies have found relatively high increase in δ^{15}N values, such as 0.7 ‰ for squid muscle tissue (Ruiz-Cooley et al., 2011) and 0.9–1.0 ‰ for Asiatic clams (Sarakinos et al., 2002; Syväranta et al., 2011).

The largest effects of ethanol preservation were observed in δ^{2}H values, with a significantly higher ^{2}H-enrichment in the lipid-rich gonad (+14.5 ‰) than in the foot tissue (+7.1 ‰). The significant increase of δ^{2}H in both tissues is likely derived from the loss of ^{2}H-depleted lipids (Hobson et al., 1999). It is possible that structural changes, such as shrinkage or hardening of the tissues, could have caused lipid-release and isotopic enrichment (Singhal et al., 2016;

Panzacchi et al., 2019). The TDFs of δ^2H are yet poorly studied, but there are substantial differences in δ^2H values between terrestrial and aquatic primary producers, with an average difference of ~ 100 ‰ (Doucett et al., 2007). Although the ethanol-induced shifts in δ^2H values of FPM foot and gonad tissues were relatively large as compared to those observed for δ^{13}C and δ^{15}N values, the marked differences between the terrestrial and aquatic isotopic endmembers may allow the use of δ^2H values of ethanol-preserved FPM tissues in isotopic mixing models estimating the reliance of FPM on various food sources (Doucett et al., 2007; Brauns et al., 2021).

Some previous studies demonstrate that preservation time can significantly change stable isotope values (Sweeting et al., 2004; Ruiz-Cooley et al., 2011; McConnaughey & McRoy, 1979), whereas other studies have found no significant effect on δ^{13}C and δ^{15}N values (Gloutney & Hobson, 1998; Barrow et al., 2008). Our results showed significant effects of preservation time only on $\Delta\delta^{15}$N. Our results indicated that the most preservation time-induced changes in $\Delta\delta^{13}$C happened after 1 month of storage for both tissues, although the effect on $\Delta\delta^{13}$C was not significant, as was the case also in some previous studies (Sweeting et al., 2004; Syväranta et al., 2008). Few studies have investigated temporal changes in δ^{13}C and δ^{15}N values of ethanol-preserved samples, but it appears that changes can occur almost instantly (e.g. an increase of δ^{13}C within a week for freshwater clams; (Syväranta et al., 2011). The most striking and temporally stable shifts occurred in the $\Delta\delta^2$H values and ΔC:N ratios of the FPM gonad tissue after 6 months' ethanol preservation. The $\Delta\delta^{13}$C and $\Delta\delta^{15}$N values showed inconsistent and unexplained fluctuations during the preservation experiment but the fluctuations were within the range of analytical error (i.e., 0.14 ‰ for δ^{13}C and 0.24 ‰ for δ^{15}N) and thus may simply represent instrument noise.

Our results indicate that ethanol preservation affects δ^2H values more than δ^{13}C and δ^{15}N values of FPM foot and gonad tissues. Although significant and potentially ecologically relevant shifts were observed for all elements, the effects on δ^{15}N seemed small enough to facilitate the use of ethanol-preserved FPM samples to infer e.g. the trophic position of consumers in food web studies. Considering our findings, we suggest that ethanol should be used with caution and potential isotopic shifts should be accounted for when using preserved bivalves as tracers of environmental changes (Glibert et al., 2018). While our findings are encouraging for samples preserved for short periods, more research is needed to determine whether archived samples of endangered and long-lived FPM can be used to evaluate long-term changes in freshwater ecosystems (Schöne, 2013; Fritts et al., 2017).

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Author contributions MH, JT, and MK all conceived of and designed the experiments, performed the experiments, and collected the data. MH, APE and MK analyzed the data. MH, JT, APE, and MK contributed to writing the manuscript.

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Declarations

Conflict of interest The authors declare that there is no conflict of interest.

Data availability All the data and materials will be provided, if requested.

Code availability Not applicable.

Ethical approval Applicable.

Consent to participate All authors have given their consent to participate.

Consent for publication All authors have given their consent for publication.

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III

ENVIRONMENTAL FACTORS IMPACT TERRESTRIAL-AQUATIC ECOSYSTEM CONNECTIVITY AND AFFECT THE CONDITION OF CRITICALLY ENDANGERED FRESHWATER PEARL MUSSELS IN THE FIELD

by

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