UNIVERSITY OF NOVA GORICA
GRADUATE SCHOOL

# LIPIDS IN FRESHWATER ECOSYSTEMS: CASE STUDIES FROM LAKE BOHINJ (SLOVENIA) AND LAURENTIAN GREAT LAKES (NORTH AMERICA)

DISSERTATION

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## Summary

The aim of this research has been to reveal the seasonal dynamics of lipids in freshwater ecosystem of Lake Bohinj (Slovenia). Different pools of lipids, ranging from natural seston, zooplankton and fish, were used to reveal the main lipid groups there and to reveal the trophic interactions and energy transfer across the food web within a growing season. Along with the improved techniques used to analyze the lipid classes, we tentatively indicated several compounds as potential taxonomic biomarkers.

The results of the research on Lake Bohinj show that analyzed aquatic organisms are subjected to seasonal effects and they have distinct fatty acids' profiles by which can be detected down to the species level.

In the second part of the thessis, the focus was on the recent discovery of polymethylene interrupted fatty acids in freshwater ecosystems, characteristic for invasive and native mollusks of the Laurentian Great Lakes (Canada). For the first time, the existence of these fatty acids was proven in freshwater molluscs and their transfer into higher trophic levels confirmed.

**Keywords:** benthic invertebrates, biomarkers, fatty acids, freshwater ecosystem, fish, invasive species, lipids, mussels, poly methylene interrupted fatty acids, zooplankton

## Povzetek

Namen doktorske disertacije z naslovom: "Lipidi v sladkovodnih ekosistemih na primeru Bohinjskega jezera (Slovenija) in Velikih jezer (Severna Amerika)" je bil razkriti dinamiko lipidov različnih organizmov. V raziskavo smo vključili organizme rastlinskega in živalskega planktona in ribe, na katerih smo izvedli analize vsebnosti lipidov. Predstavili smo uporabo novo razvite metode in nadalje iz rezultatov analiz sklepali na energijski pretok preko prehranjevalne verige preko vegetacijske sezone. Podali smo predloge za uporabo nekaterih markerjev, za katere se je izkazalo, da so značilni za posamezne taksonomske skupine.

Drugi del raziskave je bil usmerjen v odkritje maščobnih kislin (MK), t.i. »Polymethylene Interrupted Fatty Acids – PMI-FA«, v invazivnih in nativnih vrstah školjk, prisotne v Velikih jezerih v Kanadi. Razkrili smo obstoj teh MK v invazivnih vrstah in nadalje ugotovili prehajanje teh MK preko prehranjevalne verige.

**Ključne besede:** bentoški nevretenčarji, biomarkerji, maščobne kisline, sladkovodni ekosistem, ribe, invazivne vrste, lipidi, školjke, zooplankton

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## ABBREVIATIONS

All abbreviations are used as presented for singular and plural.

| | |
|---|---|
| ALA | C18:3n3c (α-linolenic acid) or C18:3ω3 |
| ALC | fatty alcohol |
| AMPL | acetone mobile polar lipids |
| ARA | C20:4n6c (arachidonic acid) or C20:4ω6 |
| AU | absorption unit |
| DHA | C22:6n3c (docosahexaenoic acid) or C22:6ω3 |
| EFA | essential fatty acid |
| EPA | C20:5n3c (eicosapentaenoic acid) or C20:5ω3 |
| FA | fatty acid |
| FAME | fatty acid methyl esters |
| FFA | free fatty acids |
| GC /MS | gas chromatography / mass spectroscopy |
| HC | hydrocarbons |
| HUFA | highly unsaturated fatty acid |
| KET | ketones |
| LIN | C18:2n6c (linoleic acid) or C18:2ω6 |
| LOD | limit of detection |
| LOQ | limit of quantitation |
| MUFA | mono unsaturated fatty acid |
| m/z | mass-to-charge ratio |
| PL | phospholipids |
| P/N | Part number |
| PMI-FA | poly methylene interrupted fatty acid |
| PUFA | poly unsaturated fatty acid |
| RF | retention factor |
| SAFA | saturated fatty acid |
| SD | standard deviation |
| SE | standard error |
| ST | sterol |
| TAG | triacylglycerols |
| WE | wax esters |
| UI | unsaturation index |
| C15:0 | pentadecanoic acid |
| C16:0 | hexadecanoic acid |
| C16:1n7c | 9-hexadecenoic acid |
| C17:0 | heptadecanoic acid |
| C18:0 | octadecanoic acid |
| C18:1n9c | cis-9-octadenoic acid |

µg • $mg^{-1}$ dw *or* µg • mg dw means microgram of particular lipids per milligram of dry weight

# 1 INTRODUCTION

## 1.1 Problem description, purpose of the research and relevance

The focus of this work is to reveal the seasonal dynamics of lipids in seston, zooplankton and fish of Lake Bohinj, Slovenia, and to determine their lipid profiles within definite taxonomic/trophic level. Another goal is to discuss the recently described new group of FA (i.e polymethylene interrupted fatty acids (PMI-FA)) which could be used as biomarkers in freshwater ecosystems.

The environment in which we are living in is subject to change within time. Living organisms tend to preserve their inner environment as stable as possible, contrary to the unstable and constantly changing outer environment. Whether factors influencing environmental conditions are derived from human or natural sources, living organisms must cope with them in order to survive. All living organisms, as a response to their changed environmental conditions, adopt a survival strategy (including their life cycle). Wilson (1975) wrote that species are prisoners of past evolution (phylogenetic revolt) without adaptations for survival in all circumstances. Therefore, if the conditions in the environment are changing too fast to be adopted, organisms are being pushed into a marginal niche, and eventually die out. Changed temperatures due to global warming (Walther et al., 2002), changed nutrients levels (eutrophication) (Butzler and Chase, 2009), changed salinity levels (Arts et al., 1993), changed metal concentrations (Arts and Sprules, 1987), elevated levels of UVB radiation (De Lange and Van Donk, 1997), changes in biodiversity due to invasive species (i.e. Nalepa et al., 2006) or contamination with organic pollutants (Arts et al., 2009) are only few out of many factors which can have influence on species distribution, abundance, survival strategy, and life history traits in natural ecosystems, including freshwater.

Freshwater ecosystems, due to their smaller size compared to marine water, are more sensitive natural ecosystems (Gleick, 1996). This is true of alpine lakes in particular, with Slovenia being no exception. Organisms which are particularly prone to alterations are those at the bottom of the food web, i.e. phytoplankton or one step higher, – herbivorous zooplankton. Zooplankton thus holds a key position in the

pelagic food web by providing a carbon source available to higher trophic levels (mainly through lipids). Their abundance and relatively fast reproduction rate is resulting in a fast response to changes in the environment within a growing season (i.e. heat wave (Maazouzi et al., 2008), earthquakes and climate change (Brancelj et al., 2000a).

Ecosystems largely depend on energy derived from the Sun (Lang, 2001). In temperate climates the duration of sun insolation is directly linked to the rate of primary production in summer (Lang, 2001). As biochemical processes of aquatic animals are in majority linked to water temperature, lakes within temperate climates and lakes with ice cover during winter should have different dynamics compared to others which are not seasonal. On a micro-scale, local topographic effects can have significant influences on seasonal dynamics too (Livingstone et al., 1999).

As food resources change within season (growing) in temperate climates, this should reflect on the dynamics of the lake itself, on primary producers, on the food web, and especially on the higher trophic levels. Until recently, even humans were heavily dependant and subject to seasonal change – preparing food reserves for less favorable winter months.

The same can be concluded for our study sites, Lake Bohinj (Slovenia) with the surrounding Alpine space (Brancelj, 2002 a, b), as well as the Laurentian Great lakes in Canada.

Numerous methods can be implied to measure and track seasonal dynamics and food chain interactions between species due to varying environmental conditions. One possible method can also be by measuring lipids in a qualitative and quantitative manner.

The carbon transfer across trophic levels in food webs, starting from primary producers and bacteria, has a central position in all ecosystems. Herbivorous and detritivorous (i.e filter-feeding) animals play a critical role in carbon transfer along the food web. However, there are many unknown factors regulating this flow (Shurin et al., 2002).

Therefore, there is a need to know how growth intensity and carbon flow efficiency are regulated within the aquatic food web. Patterns of carbon flow in certain ecosystems can offer a closer look on how global warming or/and the introduction of invasive species effects natural ecosystems (Persson, 2007). Also, by revealing

certain species-specific (bio) markers or tracers, scientists might better understand inter- and intra-species interactions, how ecosystems function and they might be able to better estimate the effects of invasive species on the ecosystems invaded.

Rates of primary production, and thus circulation of matter in the water column, could be to some extent, tracked by analysis of sediment. Paleolimnological surveys can help reconstructing past and recent changes in lake ecosystems (Brancelj et al., 2002b) as well as revealing alochtonous inputs (Muri et al., 2006). However, sediments offer limited insights into ecosystem dynamics, or in the qualitative and quantitative values of organic molecules originating from the bottom of the food web. Aquatic animals do bio-accumulate lipids for their energy reserves, thus making these organic molecules especially appropriate to be used as tracers, despite being utilized by different organisms and transformed on a species and/or taxa specific basis.

## 1.2 Introduction to Lipids

Energy reserves in aquatic organisms are mostly lipids (*senso lato)*. They are the key energy reserve of aquatic organisms (Arts and Wainman, 1999). Lipids have higher energy storage capacity of 39.4 J $mg^{-1}$ then proteins (23.6 J $mg^{-1}$) and carbohydrates (17.2 J $mg^{-1}$) (Hagen and Auel, 2001). Lipids also represent the major carbon reservoir in aquatic organisms, especially by preserving or metabolizing wax esters (80 % of carbon), triacylglycerols (77 %), phospholipids (65 %) or other lipid based substances (Oller Ventura, 2005). However, functions of lipids are not limited to energy reserve functions (for details see Arts and Wainman, 1999; Arts et al., 2009; Vance and Vance, 1996).

Lipids are important in thermal insulation and influence the specific density (buoyancy) of aquatic organisms. They play a role at the molecular level forming structural elements of cell membranes and are involved in many biochemical processes, such as signal transduction and as carriers of chemical signals (Vance and Vance, 1996, 2002).

Chemically, they range from simple hydrocarbons (HC) to steroids and complex amphiphilic (polar) lipids (i.e. phospholipids (PL)). Functionally, their range is from

fat storage to hormones and vitamins. Different functions of lipids prevent a straight-forward definition of this group although "lipid scientists" have a firm definition what is meant by this term (Christie, 2003). A most widely accepted definition defines them as a group of naturally occurring compounds, soluble in organic solvents such as chloroform, benzene, ethers and alcohols (Christie, 2003; Budge et al., 2006). Due to their complex nature, the previous definition does not apply in all circumstances. A more concise definition is based on their chemical structure, which divides lipids onto neutral, amphiphilic and redox lipids (Johnson and Davenport, 1971). For the purposes of this study lipid terminology on the basis of their chemical structure is employed, also referred to as different moieties of lipids; neutral (non-polar) lipids (hydrocarbons (HC), wax esters (WE), ketones (KET), triacylglycerols (TAG), free fatty acids (FFA), fatty alcohols (ALC), sterols (ST) and polar lipids (amphiphilic) (acetone-mobile polar lipids (AMPL), phospholipids (PL)).

Nevertheless, the most abundant lipids are those that contain fatty acids (FA) as part of their structure. FA usually esterify to a glycerol backbone or other compounds forming moieties of lipids of different polarity. Any lipid containing an esterified FA is called an acyl lipid. Common FA of animal and plant origin usually have even-numbered straight carbon chains, with a methyl terminus ($-CH_3$) at one end, and an acid (carboxyl) terminus at the other (-COOH) (fig. 1). Carbon chains in FA are typically from 12 to 24 carbon atoms and containing 0 to 6 double bonds (predominantly in the *cis* configuration). Non esterified FA, known as free fatty acids (FFA) are rather low in organisms. It was demonstrated that certain FA are essential for growth, health and development and also their hold on ecological roles at higher trophic levels (Kainz et al., 2004; Arts et al., 2009). Essential fatty acids (EFA) are usually referred to arachidonic acid (ARA; C20:4ω6), eicosapentaenoic acid (EPA; C20:5ω3) and docosahexaenoic acid (DHA; C22:6ω3). As α-linolenic acid (ALA; C18:3ω3) can be converted into EPA (Von Elert, 2002) and linoleic acid (LIN; 18:2ω6) to ARA (Stanley-Samuelson, 1994), those two can be designated as EFA, too (fig. 1c).

FA with single double bond can be referred as MUFA, with two or more double bonds PUFA and those with three or more HUFA. SAFA are FA without double bonds.

Despite the numerous possible configurations relating to degree of unsaturation,

position of double bonds, carbon chain length, and constituents attached to the FA molecule, FA found in nature most commonly contain a single methylene group between double bonds. These FA are referred to as methylene interrupted FA (MI-FA) (fig. 1a). Recently discovered in freshwater ecosystems, PMI-FA (poly methylene interrupted fatty acids) contain more than one methylene group between double bonds within the FA molecule (fig. 1b).

FA are among the most important bio-molecules transferred across the plant-animal interface, including in the aquatic food web. Due to the fact that animals cannot alter the position of a double bond on aliphatic chain closest to the terminal methyl group (closer than n-9), as well as to *de novo* synthesize certain FA, it is possible to trace certain FA up the food web, revealing the prey-predator relationships. It was demonstrated that the vast majority of animal lipids (lipid classes and FA) are taken directly from their diet, and only limited by *de novo* synthesis occurances. One of the few exceptions is the *de novo* synthesis of substantial amounts of energy reserve lipids (WE) in marine Arctic copepods of the genus *Calanus* (Arts & Wainman, 1999; Graeve et al., 2005). This makes certain lipids – (lipid class) useful as biomarkers/trophic markers in marine ecosystems.
However, as the amount of lipids used as an energy reserve in aquatic animals varies by season, lipids might be particularly suitable to study their dynamics from primary producers up the food web (Dygert, 1990; Arts et al., 1992, 1993, 1997).

Fig. 1a – MI-FA
20:4Δ5,8,11,14 = 20:4ω-6
Arachidonic acid

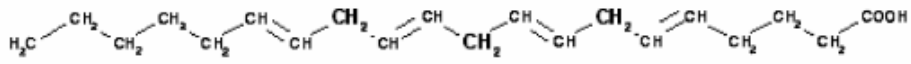
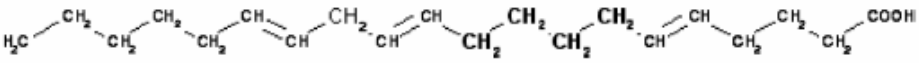


Fig. 1b – PMI-FA
20:4Δ5,11,14 = 20:3ω-6
Sciadonic acid



**1c**

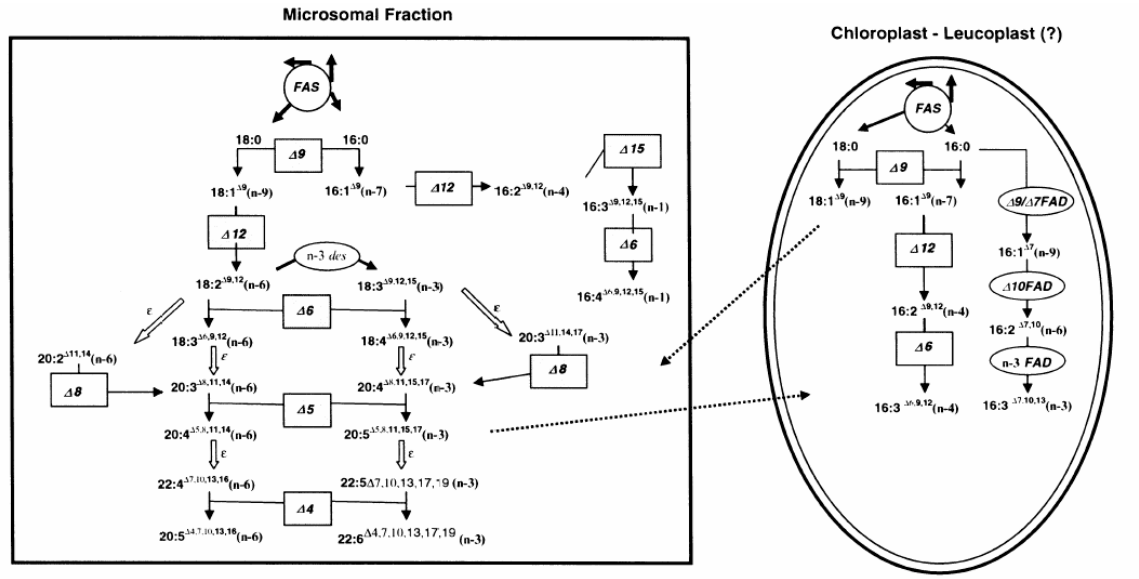



**Figure 1**: *Structure and metabolism of fatty acids (FA);* ***a)*** *Methylene interrupted FA with single methylene group between double bonds on the aliphatic chain (indicated in bold),* ***b)*** *Poly-methylene interrupted FA with more than one methylene groups between at least 2 of the double bonds on the aliphatic chain (indicated in bold) (from Mezek et al., 2009a),* ***c)*** *Diagrammatic representation of the proposed pathways of PUFA synthesis in several groups of protists (from Arts et al., 2009).*

## 1.3 Lipids as biomarkers

Fatty acids are rapidly gaining importance because these compounds have been shown to be valuable trophic markers (Desvilettes et al., 1997; Napolitano, 1997) and/or indices of health and nutritional status (Gulati and Demott, 1997; Arts et al., 1999; Arts et al., 2001; Stubling and Hagen, 2003; Stubling et al., 2003). They have been recently shown to track long term changes in the structure of pelagic ecosystems of the lakes (Hebert et al., 2006, 2008) and correlate both directly and indirectly with the health/nutrition of cornerstone aquatic invertebrates - e.g. *Mysis relicta* Loven, 1862 (Schlechtriem et al., 2008; Johannsson et al., 2008).

The perfect trophic marker is a compound whose origin can be uniquely and easily identified, is inert and non-harmful, is not selectively processed during food uptake and incorporation, and it is metabolically stable and hence transferred from one trophic level to the next in both a qualitative and quantitative manner. Such a marker would provide essential insight into the dynamics of ecosystems by presenting unique information on pathways of energy flows, i.e. crucial information on which all ecosystem models are eventually built. However, such markers are rare or even nonexistent. Instead we have to be content with less ideal components, a category to which FA belong (Dalsgaard et al., 2003). Fatty acids are usually only partially successful as trophic markers because they exhibit species-specific bioconversion and utilization rates. At the same time many FA are common and ubiquitous; they can be found from phytoplankton up to the top predatory fish. One of the first publications from this regard published in 1997 by Napolitano and co-workers, demonstrated a connection between diatom blooms; actually their FA profile and the FA profile of primary consumers (calanoid copepod). However, there are still concerns regarding the usage and interpretation of the data, as a majority of FA originate from primary producers.

An exception to this might be a group of uncommon FA termed PMI-FA (Mezek et al., 2009a). PMI-FA are uncommon FA, found in various types of seed oils (Smith et al., 1969; Plattner et al., 1975; Madrigal et al., 1975), plants or plant oils (Fore et al., 1966; Jamieson et al., 1972), human milk (Murawski et al., 1971), beef and mutton tallow (Hoffmann et al., 1969) and various marine invertebrates (Jefferts et al., 1973; Ackman et al., 1973; Paradis et al., 1974; Pearce et al., 1976; Garrido et al.,

2002; Saito, 2007; Budge et al., 2007). In marine systems, PMI-FA are believed to be synthesized *de novo* only in bivalves and carnivorous gastropods (Zhukova, 1986; Saito, 2007; Budge et al., 2007 cit op. Joseph, 1982). It was demonstrated that proportions of PMI-FA among certain marine species vary, suggesting that these compounds might be suitable biomarkers in food web studies (Budge et al., 2007), especially because they are *de novo* synthesized at a trophic level higher than phytoplankton, what is unique.

## 1.4 Lipid Analysis

### 1.4.1 Lipid classes separation

Thin-layer chromatography (TLC) was developed in the 1960's for the separation and semi-quantitative determination of the individual components of more or less complicated mixtures (Fried and Sherma, 1996; Sherma and Fried, 2003). Latest techniques and improved instrumentation coupled with TLC analyses (TLC-UV-VIS, TLC-MS, TLC-FID, densitometry …) enhances the reproducibility of TLC methods, which enables the use of TLC methods also for quantitative purposes. Various stationary and mobile phases which can be used makes TLC analyses extremely flexible.

The general principle of TLC analyses is that a "sample" is applied on a pre-prepared TLC or High Performance Thin Layer Chromatoghraphy (HPTLC) plate at the starting position. This plate is further immersed into a mobile phase. According to the relative polarity of the stationary and mobile phase, the sample is separated into different groups of compounds with similar characteristics (within progress of mobile phase along the plate). The plate is removed from the mobile phase when the mobile phase reaches a pre-determined top line. In parallel, the retention factor for each group of compounds (solutes) is calculated. The position of a particular solute spot during TLC analysis is characterized by the retention factor (Rf). It is calculated as a distance between the center of spot (zs) and the starting line for a selected compound divided by the distance between the front of mobile phase and its start line (zf). The value of the retention factor is between 0 (solute remains at the start) and 1 (solutes move with front of the mobile phase).

The main factors influencing retention factor are physical and chemical processes – adsorption, partition and ion exchange, which determines retention capacity and selectivity. However, separation mechanisms can be divided in two groups according to the relative polarity of the stationary and mobile phases.

Stationary phases are generally well-defined inorganic materials (although sometimes replaced with organic ones) with well defined porous structures and with relatively high specific surface areas bounded to a supporting medium (usually glass or aluminum – also termed sorbents).

Connection is achieved by means of various organic (polyvinyl alcohols with various molecular mass) or inorganic (gypsum) binders. In theory, a smaller particle size of the stationary phase and its size distribution enhances separation efficiency and improves resolution, decreases time of analysis, and increases detection sensitivity, characteristics also of HPTLC plates.

Mobile phases in TLC analyses are usually organic solvents (or a mixture of organic solvents) with well defined solvent strength (elution capacity). Elution capacity of solvents is defined by their ability to transport a given solute (or a given series of solutes) from a starting point to the compound-specific distance along a plate. The elution strength is higher when the mobility of a solute is higher. Elution strength depends both on the sorbent and on the chemical character of solutes used for its determination. For example, elution strength for non-polar compounds is higher in dichloromethane than in methanol, which is again higher than water. Solvents with low boiling point, low viscosity, and low toxicity (as a human health risk factor) are preferable for TLC analysis. In TLC analyses compounds remain separated on the surface of the sorbent. In addition, some compounds absorb visual or UV-light and can be visualized by measuring absorption, but some of them need to be visualized by using derivatization reagents.

Chromathograms are, in a next step, quantitatively evaluated by means of a densitometer or camera. Densitometers measure remission, i.e. the intensity of diffused reflected light, where the intensity of the signal is dependent on the concentration of molecules which absorb light in the layer of sorbent and from light scattering on the sorbent particles. By means of a camera, a photograph of the developed TLC plate is taken to obtain a high resolution picture, which is evaluated by computer software (Fried and Sherma, 1996).

Quantitation in TLC analysis is done on the calibration curve principle. Standards are applied to a plate next to the samples, usually lower and higher in concentration than expected in the sample. Computer software evaluates the results, according to the chosen statistical technique.

### 1.4.2 Reasons for HPTLC method development

There has been a great interest in the development of rapid and simple methods for separating complex lipids into fractions corresponding to their polarity. Different chromatographic techniques exist (aminopropyl-bonded silica gel column chromatography (Alvarez et al., 1992), thin layer chromatography (TLC) (Touchstone, 1995), Chromarod-Latroscan (Parrish, 1987)), for separating complex lipids into fractions of similar polarity. However, some, e.g. solid phase extraction (SPE) (Pernet et al., 2006) and recently also TLC (Christie, 2003) offer only limited quantitative measures of the separated compounds.

Modern enhanced TLC analytical techniques offer semi-quantitative and quantitative analyses that can be routinely used in pharmaceutical industry (i.e. Vovk et al., 2005) and include high performance thin layer chromatography (HPTLC) coupled with densitometry, which applications in terms of lipid separation and analysis are numerous (Olsen and Henderson, 1989; Sek et al., 2001; Vovk et al., 2005). Despite the quantity of published papers and books (Johnson and Davenport, 1971; Fried and Sherma, 1996; Christie, 2003) about lipid analysis by means of such techniques, the vast majority of cases do not separate lipid classes on a single TLC or HPTLC plate. Therefore, more chemicals, peripherals and human effort are needed to perform the analyses. Published papers mainly focus on:

- separation of particular lipid classes: PL (Ramadan and Morsel, 2003), TAG (Cavaletto et al., 1996; Fontecha et al., 2000; Sek et al., 2001),
- certain lipid fractions: acidic and neutral lipids (Alvarez and Touchstone, 1992), neutral-, glycol- and phospholipids (Ramadan and Morsel, 2003), neutral and polar lipids (Varljen et al., 2003),
- whole fraction of lipids on a qualitative basis (Giacometti et al., 2002; Pinturier-Geiss et al., 2002; Meireles et al., 2003; Jeffs et al., 2004; Yoshida et al., 2004; Jayasinghe et al., 2006),
- whole fraction of lipids on a wet weight basis (Murphy et al., 2002),
- whole fraction of lipids on a dry weight basis (Arts et al., 1992, 1993; Copeman and Parrish, 2004).

A limited number of publications focus on separation of lipid classes by using HPTLC except for separating lipids by fractions (i.e. Olsen and Henderson 1989; Sek et al., 2001). So far, according to our knowledge, there is no method that focuses on separation of neutral and polar lipid classes on a single HPTLC, plate by which quantitative analyses are possible.

The aim of the present work was to develop a HPTLC method for separating complex lipid mixtures that could be effectively used for investigation of lipid classes in different organisms. We wanted to separate lipid classes which are, according to the literature, important in freshwater organisms. Thus we needed to develop a new method, which should replace the method developed by Parrish (1987) (that used the nowadays less available Chromarod-Latroscan).

#### 1.4.2.1 Lipid classes of developed HPTLC method

Below are briefly presented the lipid classes (Johnson and Davenport, 1971; Parrish, 1978; Christie, 2003; Vance and Vance, 2002; Arts et al., 2009), according to increasing polarity and which can be separated and quantified with the newly developed method (for details see Materials and Methods).

**Hydrocarbons – HC**

HC are the simplest form of lipids although the initial carbon chain may be linear (normal), branched, saturated or unsaturated. They contain only carbon and hydrogen. Those organic compounds include alkanes and alkenes as well as carotenoids (not evaluated in our study). Living organisms, eukaryotic or prokaryotic, contain frequent hydrocarbons which are directly derived from FA. HC are also formed as products of fatty acid cleavage during peroxidation processes. Alkanes as well as alkenes appear during hydroperoxide decomposition. However, an important member of hydrocarbons is squalene ($C_{30}H_{50}$) which is a metabolic precursor of sterols and steroids and was used as a laboratory standard (CAS: 111-02-4) (fig. 2).

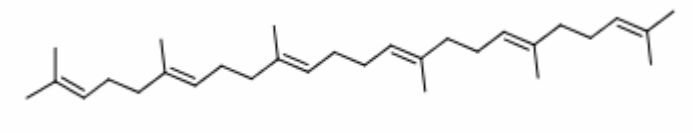


**Figure 2:** *Skeletal formula of squalene.*

**Wax esters – WE**

WE are a group of compounds relevant to diet studies. WE consist of a FA esterified to a fatty alcohol. In theory, both the FA and the fatty alcohol can have any structure, but WE almost always contain saturated or monounsaturated fatty alcohols, unlike the FA portion which may have a variety of polyunsaturated structures. Functions of WE are uncertain but, like TAG, they are thought to be involved in energy storage in certain species of crustaceans, fish and marine mammals. It was demonstrated by several authors that marine copepods store almost all their energy reserves as WE (Lee et al., 1971, 2006; Budge et al., 2006). We used stearyl palmitate (CAS: 2598-99-4) as a laboratory standard (fig. 3).

**Figure 3:** *Skeletal formula of stearly palmitate.*

**Ketones – KET**

KET are produced by reduction of carboxyl group during pyrolysis of FFA or TAG. They can be referred to a group of compounds by which we can measure the rate of metabolic activity. Also, when lipids are utilized (i.e TAG) certain fragments are not completely oxidized and fat fragments as KET arise. KET can also be as a waste product of fat when being metabolized. They can be found in both animal tissues and higher plant leaf waxes. We used 3-hexadecanone as a laboratory standard (CAS: 187878-64-9) (fig. 4).

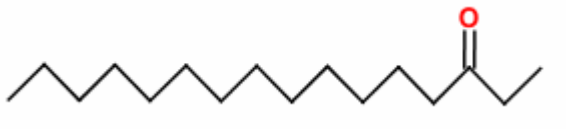


**Figure 4:** *Skeletal formula of 3-hexadecanone.*

### Triacylglycerols – TAG

TAG represents the common form of storage lipids and make up the majority of lipids found in aquatic organisms (Arts et al., 2009). TAG consists of three FA molecules esterified to a glycerol backbone. Animals mobilize stored TAG if FA requirements are not met through the diet or, conversely, deposit TAG when dietary FA and energy intake exceed demands. Thus, the composition of TAG FA in major lipid storage depots is relatively dynamic and readily influenced by diet; therefore we can assign them as short-term energy reserves in organisms. Tripalmitin (CAS: 555-44-2) was used as a laboratory standard (fig. 5).

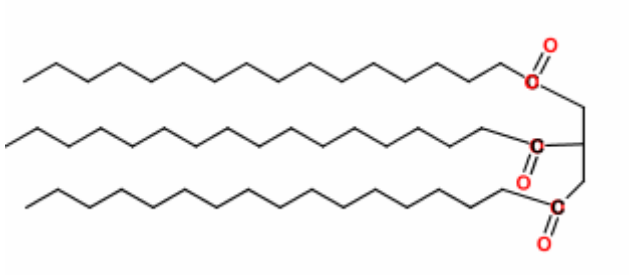


**Figure 5:** *Skeletal formula of tripalmitin.*

### Free Fatty Acids – FFA

FFA (also un-esterified FA) are ubiquitous but not abundant components of living tissues. In animals, much of the dietary lipid is hydrolyzed to free acids before it is absorbed and utilized for lipid synthesis. Intact lipids in tissues can be hydrolyzed to free acids by a variety of enzymes (*e.g.* lipoprotein lipase, hormone-sensitive lipase, phospholipase A), before being metabolized in various ways including oxidation, desaturation, elongation or re-esterification. FFA are transported in plasma as a complex with albumin to other organs, where they can diffuse or be actively

transported across cell membranes for incorporation into lipids and be used in intracellular processes. However, if analyses reveal elevated levels of FFA, this can be an index of improper sample integrity in terms of stability or treatment (Arts, M.T. pers. comm.). We used palmitic acid (CAS: 57-10-3) as a laboratory standard (fig. 6).

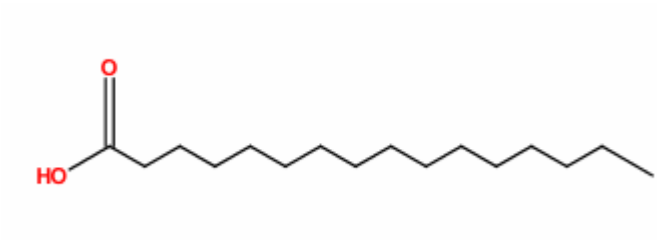



**Figure 6:** *Skeletal formula of palmitic acid.*

**Fatty alcohols - ALC**

ALC are aliphatic alcohols, counterparts of FA and fatty aldehydes. Aliphatic alcohols occur naturally in a free (component of the cuticular lipids in plants) but usually in esterified form to form WE or etherified form (glyceryl ethers). Several ALC can be used effectively as specific biomarkers, especially in paleolimnological surveys (i.e. Meyers and Ishiwatari, 1993). We used cetyl alcohol (CAS: 36653-82-4) as a laboratory standard (fig. 7).

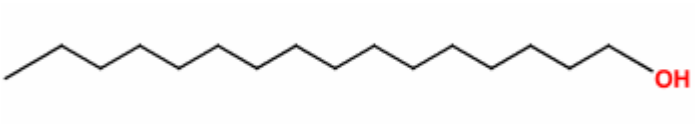



**Figure 7:** *Skeletal formula of cetyl alcohol.*

**Sterols -ST**

ST forms an important group among the steroids. ST are lipids resistant to saponification and are found in an appreciable quantity in all eukaryotes (Arts et al., 2009). The most abundant sterol in the animal kingdom is Cholesterol. While it became clear very early that cholesterol plays an important role in controlling cell membrane permeability by reducing average fluidity, it appears now that it has a key

role in the lateral organization of membranes and free volume distribution too. We used Cholesterol (CAS: 57-88-5) as a laboratory standard (fig. 8).

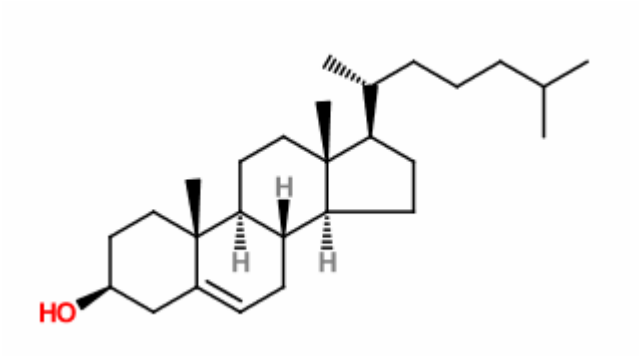



**Figure 8:** *Skeletal formula of cholesterol.*

### Acetone Mobile Polar Lipids -AMPL

AMPL or monoacylglycerols (monoglycerides) are fatty acid monoesters of glycerol. They are found in low amounts in cell extracts but are intermediates in the degradation of TAG or diacylglycerols. We used α-monopalmitin (CAS: 542-44-9) as a laboratory standard (fig. 9).

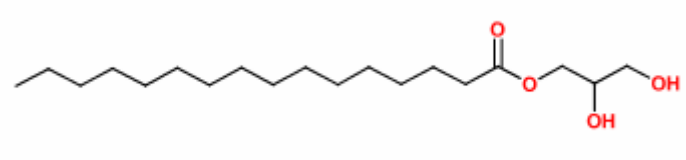



**Figure 9:** *Skeletal formula of α-monopalmitin.*

### Phospholipids - PL

PL are the structural components of all cell membranes and normally consist of two FA esterified to a glycerol molecule. Because of the specialized functions of these lipids and membranes, organisms tend to conserve PL (FA therein), making this lipid class relatively robust to dietary changes and thus not informative as an indicator of diet. We used L-α-lecithin (CAS: 8002-43-5) as a laboratory standard (fig. 10).

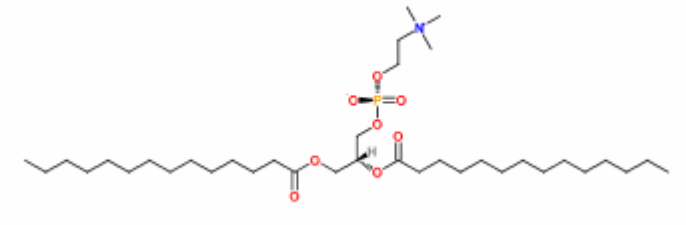


**Figure 10:** *Skeletal formula of phosphatidylcholine.*

### 1.4.3 Additional separation techniques before chromatographic analyses

In order to reduce the complexity of the total lipids or/and if only certain fraction of lipids (i.e. lipid class) is of interest or needs to be analyzed, a sample can undergo various preparative techniques.

FA can be separated and purified before analysis (i.e. on GC) by first subjecting the derivatized FA, i.e. fatty acid methyl esters (FAME) to various preparative procedures, such as thin layer chromatography (TLC) and/or silver ion - thin layer chromatography ($AgNO_3$-TLC) (Tanaka et al. 1997, 1999, 2007). Despite the possibility of using high performance liquid chromatography (HPLC), in order to reduce the complexity of the lipid sample, HPLC is not yet routinely used by analysts. The reasons are partly due to the higher cost of HPLC compared to TLC, and the fact that HPLC columns improved just recently (i.e. higher capacity and reduced amounts of sample residues within the column). Following earlier successes with the $AgNO_3$-TLC method the technique has been adapted to HPLC ($AgNO_3$-HPLC) analyses. However, this new method has not yet been successfully applied to questions of interest to freshwater or marine researcher.

TLC method is used for preparative purposes (removal of unsaponifiable material; i.e. glycerol moieties) which can interfere with GC and/or coupled with $AgNO_3$-TLC analyses to separate lipid extract (usually their FAME derivatives) by their degree of unsaturation. The common goal of such preparative procedures is to remove sample-related matrix material which may confound analyses and to enhance separation and resolution; although the increase in analytical steps can result in an increase of analytical variability.

$AgNO_3$-HPLC can, compared to $AgNO_3$-TLC, offer better and more consistent separation, cleaner fractions and, on modern columns, higher sample applications at minimal/no loss of resolution, but at much higher equipment cost (Christie, 2003).
Lately, an emerging technique for separating complex lipid mixtures or/and to remove sample matrix prior to GC analysis is solid phase extraction (SPE). In the example by using SPE, total lipids (non derivatized) can be separated into several lipid classes – fractions by using Supelco P/N 504483 or P/N 57014, separation of PL by using Sep-Pak P/N WAT043400, separation of CIS/TRANS isomers of FAME by using Supelco, P/N 54225-U. The SPE cartridges are small, cost-effective, and provide better qualitative outcomes (small void and residual volumes) when used with the appropriate controls compared to TLC methods. However, it is difficult to do exact quantitative measure of the analytes by using SPE.

### 1.4.4 Derivatives of FA

Organisms contain complex mixtures of different lipid moieties; the most abundant are those containing FA as part of their molecular structure. FA can be analytically isolated from lipid classes (moieties from which FA are released from, i.e. glycerol), followed by derivatization, by which they are converted to volatile, non-polar derivatives such as fatty acid methyl esters (FAME), picolynyl esters or dimethyloxazoline (DMOX) derivatives (Christie, 1989, 2003), before analysis on a GC.
Preparation of FAME derivatives is simpler and so is their analysis. However, by using FAME derivatives only limited insight into the exact position of the double bonds on the aliphatic FA chain is obtained; DMOX and picolynyl esters are much more informative, especially regarding the double bond position. However, they are commonly used for qualitative analyses only; picolynyl esters sample preparation is relatively simple, but their analysis requires higher oven temperatures on GC (approx. 50 °C higher compared to FAME) and thus use of non-polar capillary columns and longer run times. DMOX derivatives require more complicated analytical procedures and may degrade during storage (Christie, 2003).

Therefore FAME derivatives are the most common method for analysis of FA and when positioning and identification of the FA is no longer the question. Also by using FAME derivatives one can use available standard solutions (e.g. Supelco's 37-component FAME mix; P/N 47015-U), thus enabling quantization.

### 1.4.5 Analyses of FA by GC/MS: theory and praxis

Derivatives of fatty acid can be directly analyzed by a GC equipped with different types of detectors. Most common are flame ionization detectors (FID), or a mass spectrometer (MS). Each procedure is highly specific to the type of analyses. Most of the routine analyses are done using FID as it is cheaper to purchase and to maintain, but it is not possible to reveal any new FA different from those pre-determined previously by an appropriate standard. Thus, the results are derived entirely from the elution time (i.e. retention time), which can sometimes be misleading when analyzing complex samples. For example, PMI-FA can not be analyzed by using FID unless the exact retention time is known.

Gas chromatography coupled with mass spectrometry (GC/MS) is an optimized analytical technique, which combines two techniques into a single analytical method. Gas chromatography separates the components of a mixture (separation is column specific) and mass spectroscopy characterizes each of the components individually. Combining the two techniques, both qualitative and quantitative data can be obtained from a solution containing a number of different chemicals. The modes of use of GC/MS are numerous; from trace level analyses of polycyclic aromatic hydrocarbons (PAH), pesticides, to analyses of fatty acid methyl esters (FAME) or other derivatives.

The sample is injected into the injection port, where it is vaporized and transferred on to a column, where chromatographic separation of the components is obtained, depending on experimental condition, column selection and other factors. When effluent from GC column enters the MS, molecules are ionized. Various ionization techniques exists (electron ionization (EI), chemical ionization (positive or negative) or inductively coupled plasma). Most commonly used ionization method is EI. In EI, molecules are bombarded with fast electrons (70 eV), resulting in fragmentation of

molecules, which provides specific structural information about that molecule. Positively charged ions move further to the mass analyzer, all other species (none charged, negatively charged) are removed by a vacuum pump. There are many types of mass analyzers (mass filters) (i.e. Magnetic sector, Quadrupole, Ion trap, Time of flight). Amongst them the Quadrupole is the most common. Its function is to separate previously formed positively charged molecular fragments based upon mass/charge ratio. The theoretical background is complex. Molecular species leaving the mass analyzer proceed into an electron multiplier, followed by data collection. Data collection can be in SIM (Selected Ion Monitoring mode reports only specified m/z results over a scanned range) mode or in SCAN (all spectral information is recorded as TIC (Total Ion Chromatogram) mode). Elapsed time between injection and elution is called retention time, which can help to differentiate between some compounds, especially if retention time locking (RTL) is used. The size of the peaks is proportional to the quantity of the corresponding substances in the specimen analyzed. The peak is measured from the baseline to the tip of the peak. Quantification is done by comparing peak size of analyte with corresponding internal standard or calibration curve.

### 1.4.6 Retention Time Locking (RTL)

Retention time locking is the ability to precisely match chromatographic retention times in any Agilent 6890 or 6850 GC system to at in another Agilent 6890 or 6850 GC system with same nominal capillary column, regardless of detector type. It is also the ability to lock analyte retention time to GC or GC/MS method, regardless of column maintenance or/and replacement. Retention time locking is done by adjusting the column head pressure via electronic pressure control (EPC) using an Agilent Chemstation module. It is a new approach for qualitative and quantitative analyses, not yet widely used but fully accepted by analytical laboratories.

## 1.5 Principles for Hypotheses development

Zooplankton is a community of invertebrates inhabiting fresh and marine waters throughout the world. Their importance in ecosystems and thus in food webs/chains is well known, but not fully understood. Many studies tried to interconnect and measure energy flow within food webs/chains, to measure zooplankton nutritional values, their bioaccumulation capacity of essential poly-unsaturated fatty acids (PUFA) and trophic transfer of these compounds. The content of PUFA could be used as a basis for environmental studies, such as linking changes in environment (including seasonal), to shifts in zooplankton community structure and occurrence. Apart from the rate of primary production (i.e. analyses of seston or/and chlorophyll *a*) which is directly linked to zooplankton dynamics, also the water column vertebrates (fish) should follow seasonal dynamics to reflect their amount of lipids. The results of qualitative and quantitative studies of lipid content of fish could be also applied to fish-farming (e.g. for fish oil production). The information could be also useful for anglers and the broader community as a guide when and which fish is the best to be "put on the saucer" in terms of their EFA content.

Freshwater zooplankton taxa exhibit diverse life history traits. A major force determining their life strategies in the temperate zone is the seasonal variability in the food supply, more pronounced in regions with extreme fluctuations in primary production. Normally there is a change in primary production along the seasons. Until recently it was thought that zooplankton feed without preference i.e. they perform mechanical filtering and ingestion of particles. Recent studies have shown that copepods as well as cladocerans can detect by chemo– and mechanoreceptors, handle, select and ingest particles (Poulet and Marsot, 1978; DeMott, 1982; Dussart and Defaye, 1995; Dumont and Silva-Briano, 1997).

Seasonal adaptations are closely related to the trophic level of zooplankton, with strongest pressures occurring on herbivorous species. The dominant grazers, daphniids and calanoid copepods (along with a minor role of other zooplankton groups) have developed efficient solutions for successful over-wintering. In order to escape periods of harsh environmental conditions zooplankton can; **a**) exhibit storage and utilization of energy reserves to reduce the effect of oscillatory seasonal primary

production (Hagen and Auel, 2001) or **b**) over-winter in a stage of dormancy or diapause (Gyllstrom and Hansson, 2004), between which it is difficult to distinguish, because they can be induced by biological clock or by periodic variations in environmental conditions (i.e. temperature, light) (Alekseev et al., 2007).

Fluctuations in the life history traits of zooplankton are governed by environmental variables such as temperature and photoperiod (directly linked to rate of primary production as seasonally dependent factor) as well as interspecific competition and predation. From that reason, seasonality of lipids, their dynamic and differences in their amounts are expected. Moreover, pronounced seasonality in terms of lipids should be detected also on the next trophic level, planktivorous fish in particular.
However, if certain FA can be used as markers (incl. trophic markers) in food webs/chains, their transfer and dynamics along the food chain can be (partly) revealed.
As a model, there is the example of alien/introduced species. If FA would originate from a particular (alien) species it should be possible to trace these compounds along the food web (actually trophic levels) and consequently the effects would be monitored in more predictable manner. An example for this could be invasive zebra and quagga mussels (*Dreissena spp.*) native to the Caspian, Black and Azov seas of Eastern Europe (Nalepa and Schloesser, 1993). Zebra mussel (*Dreisenna polymorpha*) was first discovered in North America in Lake St. Clair of the Laurentian Great Lakes in June 1988 (Nalepa and Schloesser, 1993) after being introduced, presumably in 1986, through ballast water discharge from ocean-going ships. Their adaptable lifestyle and prolific breeding has ensured their invasive status in the Great Lakes and has contributed to their predominance over native mussels in some parts of the Great Lakes and elsewhere (Zanatta et al., 2002; Schloesser et al., 2006). The damage and ecosystem alterations that zebra and quagga mussels cause are extensive and, as yet, not fully understood (Wu and Culver 1991; Holland, 1993; Klerks et al., 1996; Stoeckmann et al., 1997; Connelly et al., 2007; Karatayev et al., 2007). Thus, new methods are needed, so that a more comprehensive assessment can be made of the full extent of their impact on native species and on the ecosystem they invaded.

The following presumptions have been put forward:

- Each zooplankton and fish species, due to its food preferences, metabolism, and different environmental factors in the water column, has a specific temporal composition of lipid classes as well as FA profile.
- Lipid dynamics of fish should follow the dynamics of their predominant food source.
- PMI-FA are *de novo* synthesized in freshwater benthic mollusks. Trophic transfer of these compounds is expected, so they can be tracked along a food web.

## 1.6 Outline

In order to reveal inter and intra-specific differences between four dominant zooplankton species in Lake Bohinj, their lipids were analyzed for major lipid classes and FA content within the growing season. A novel method was developed, by which only single HPTLC plate is used to separate all major lipid classes. Along with the primary consumers (zooplankter's) samples of natural seston and three fish species from Lake Bohinj were analyzed for their FA and lipid content. Seasonality of the particular lipid or its group was also of interest, both in qualitative and quantitative manner.

The research done on the Great Lakes was focused on the discovery of unusual fatty acids (PMI-FA) in invasive benthic mollusks. By systematic analysis of water column and benthic invertebrates and vertebrates, we tried to reveal the origin of these lipids – at which trophic level appears for the first time. Within the three case studies in the Laurentian Great Lakes we detect the trophic transfer of PMI-FA and infer on prey-predator relationships. At the same time this is the first record of the existence of PMI-FA in the Laurentian Great Lakes and the first report of PMI-FA in freshwater mussels.

# 2 MATERIALS AND METHODS

## 2.1 Sampling sites

### 2.1.1 Lake Bohinj, Slovenia

Slovenia is located in southern Central Europe. Its geographical position is resulting in temperate climate with four seasons and three main and distinct climate regions; **a**) continental climate in the eastern and central part of the country, **b**) a Mediterranean zone in western and coastal part, and **c**) an Alpine climate region, located in the north-west, where the south-eastern part of the Alps determines climate. Lake Bohinj is located in the central part of the Alpine climate region (fig. 11).

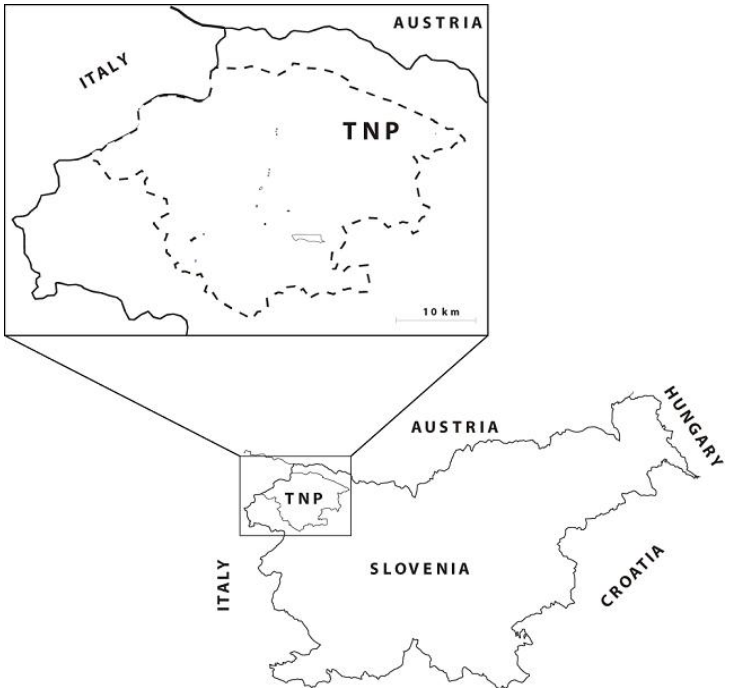



**Figure 11:** *Position of the Triglav National Park (TNP) and Lake Bohinj within Slovenia.*

Lake Bohinj is the biggest permanent lake of tectonic-glacial origin in north-western Slovenia. It is part of the Triglav national park (fig. 11). The lake is 4350 m long, 1250 m wide, with maximum depth 44.6 m and average depth 29.5 m. The length of the lake's coastline is 10.900 m and the total area of the lake is 3.18 $km^2$. Volume of the lake is estimated to 92.5 mio $m^3$ (ARSO, 2006).

The main inflow to the lake is Savica river (fig. 12), at the eastern-most part of the lake. A catchment area of the Savica river covers partly the Triglav Lakes Valley and partly the region of Pršivec. Along the rest of the northern part of the Bohinj lake there are several underwater springs – e.g. the karst spring Govic. Outflow is on the east – as the river Jezernica. Calculations and measurements of the lake water parameters show that turn-over time is two to three times per year (i.e retention time is 4 – 6 months). During winter the lake is ice-covered, usually from January to late March, followed by the highest water levels in late spring (May) when a significant water inflow is caused by snow melt. In 2007, the lake did not get ice covered. The lowest water level is normally during late winter. Lake Bohinj is thermally stratified from approx. April to October with mean surface temperature of 17 °C (maximum up to 21 °C). The lake is ranked as oligotrophic (OECD criteria), despite increased levels of total phosphorus (annual average) which were averaging at 3.6 μg $l^{-1}$ and 0.7 μg $l^{-1}$ of Chl-*a* in 2006. Temporal and spatial distribution of chlorophyll *a* are suggesting that May to September are the most productive months, with its maximum between 6 – 15 m of depth peaking at 4.1 μg $l^{-1}$, and annual average of 1.0 μg $l^{-1}$ of phosphorus (data for year 2005) (ARSO, 2006).

Phytoplankton in Lake Bohinj is represented by six main taxonomic groups with overall 53 recognised algal taxa. Dynophyceae representing the majority of overall algal biomass throughout the season, except in April and May, when Bacillariophyceae (=Diatoms) predominates. In addition, there are 18 species of macrophytes, over 60 taxa of benthic invertebrates and 16 fish species, approximately half of them introduced (ARSO, 2006, 2007).

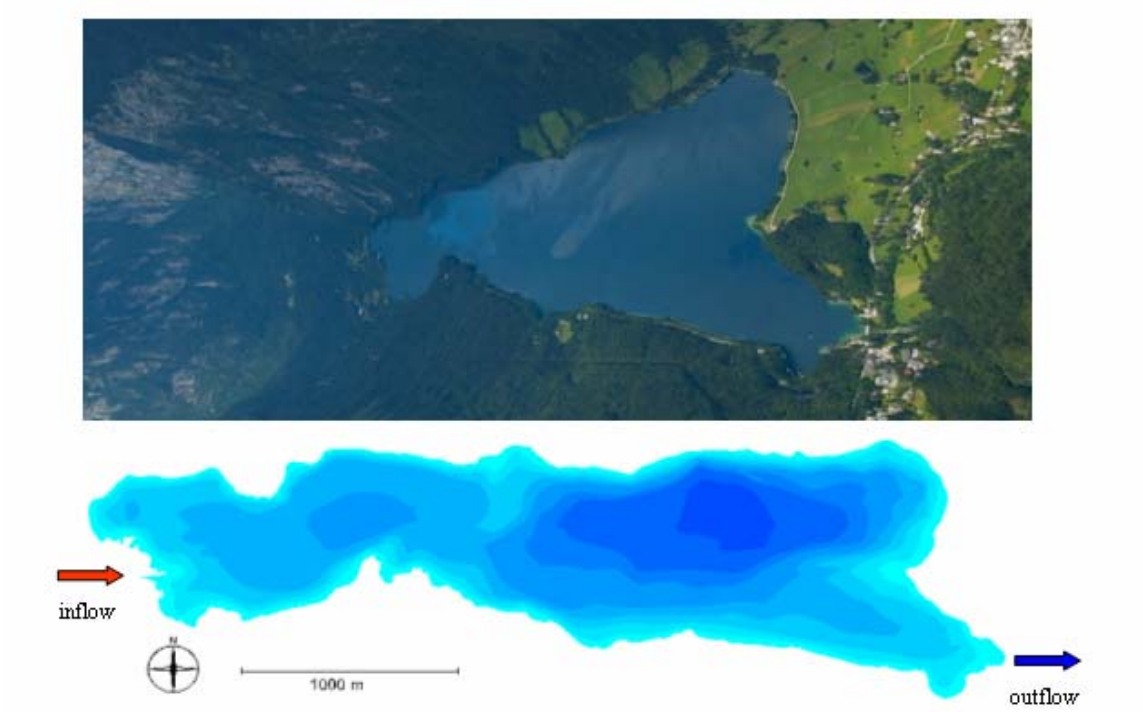



**Figure 12:** *Bathymetric map of Lake Bohinj with indicated main inflow and outflow (bottom), panoramic aerial view of Lake Bohinj – July 2007 (top) (source: photo documentation of NIB).*

### 2.1.2 The Laurentian Great Lakes, North America

The Laurentian Great Lakes (fig. 13) are a chain of freshwater lakes located in the eastern part of North America – partly representing the border between Canada and the United States of America (USA). In the present form they were formed at the end of the last ice age about 10.000 year ago (Pleistocene) (Rousmaniere, 1979). They consist of five big lakes located from north-west to south-east: the Lakes Superior, Michigan, Huron, Erie, and Ontario and numerous minor lakes and rivers in between. The combined surface area is approximately 244.100 $km^2$ (77.000 times of the size of Lake Bohinj). The length of the coastline combined measures approximately 16.900 km. Due to their size the lakes have substantial effect on climate and weather (Burnett et al., 2003). Hot summers and cold, snow-full winters are generally determining the weather of the region.

The lakes form the largest group of freshwater lakes on Earth and contain approximately one-fifth of the world's fresh surface water. However, they represent only a small portion of North America's drinking water supply. The lakes are

extensively used for water transport mainly for bulk loads and are also an important pathway for ocean-going vessels. The economy is based on water transport, recreational boating, tourism and fishing. Despite the diversity richness ecosystems is being altered by several invasive species, also by an invasive dreissenids (Mollusca: Bivalvia).


**Figure 13:** *The Laurentian Great Lakes (Grady, 2007).*

## 2.2 Field work, sample treatment and preparation for lipid extraction

### 2.2.1 Lake Bohinj, Slovenia

a) Field Sampling

Sampling of zooplankton was carried out from April to October 2006 (HPTLC and FAME analysis) and from February to December 2007 (total lipids), at approximately monthly intervals. Seston was sampled from February to December in 2007 only. Samples were collected above the deepest part of the lake (GPS coordinates: 46° 17' N; 13° 52' E). Samples for Chl-*a* analyses were collected by hauling a plankton net (diameter 20 cm; mesh size 30 μm) from a depth of 15 m to the surface along with measurements of temperature at 1 m vertical resolution.

Zooplankton and seston samples were collected with a plankton net (zooplankton: diameter 20 cm; mesh size 100 μm; seston: diameter 20 cm; mesh size 30 μm). Samples of both types were collected from 20 m to the surface. For each type of samples several tows were made. Zooplankton and seston samples were transferred separately in lipid-free glass vial (zooplankton: volume of 0.8 l, seston: volume of 0.2 l), and stored in cool box during transport to the laboratory.

Sampling of fish was performed in collaboration with the angling club of Bohinj from May to September 2007 and 2008. Three fish species (chub (*Leuciscus cephalus* Linnaeus, 1758), perch (*Perca fluviatilis* Linnaeus, 1758), and arctic char (*Salvelinus alpinus* Linnaeus, 1758)) were used for analyses. After the fish have been caught, their gender was determined and the length of each individual was measured to the nearest 0.1 cm. Subsequently, their wet mass was measured to the nearest gram (g) followed by removal of the stomachs. Fish were wrapped individually and stored in the cool box until being brought into the laboratory.

**Figure 14:** *Transfer of zooplankton sample from the net to the lipid - free glass bottle on Lake Bohinj (Slovenia).*

b) Laboratory preparation for lipid extraction

Immediatelly after the transfer of samples into the laboratory they were stored:

a) zooplankton in refrigerator at 8 °C until being separated to species

b) seston stored at -85 °C as being prepared in the field.

Just before further processing zooplankton samples were concentrated to a smaller volume with a sieve (mesh size 100 μm), transferred to a Petri dish and anaesthetized with $CO_2$. Four main species of zooplankton (*Arctodiaptomus laticeps* G.O. Sars, 1863, *Cylops abyssorum prealpinus* Kiefer, 1939, *Daphnia hyalina s.l.* (*Daphnia hyalina* Leydig, 1860, *Daphnia cucullata* G.O. Sars, 1862, *Daphnia galeata* G.O. Sars, 1864) and *Bosmina longirostris* O.F. Müller, 1776) were used for further analyses. Results obtained from selected four species of zooplankton were afterwards (in statistical analyses) amalgamated in two broader taxonomic groups: Cladocera (including *B. longirostris* and *D. hyalina s.l.*) and Copepoda (*A. laticeps* and *C. abyssorum prealpinus*). We are defining seston as the fraction of material in the water column which includes phytoplankton, micro-zooplankton (incl. naupli), organic and inorganic material smaller than 30 μm.

Separation of species was done within 24 h after sample collection. Animals were separated to species level under a dissecting microscope and transferred into lipid free, pre-weighted vials containing 0.5 ml of deionized water. Adult Copepoda, collected in 2007, were in addition separated also by gender (ovigerous females *vs.* males). Only live animals were picked for subsequent lipid analysis. The number of individuals per sample was seasonally - and per-species related and varied from 100 to 600. When a final number of specimens of each zooplankton species had been achieved, a sample was immediately frozen at -85ºC before further preparation (see 2.3).

Relative abundance of examined zooplankton species in 2007 was evaluated from samples brought into the laboratory for lipid analysis. An aliquot of the zooplankton sub-sample were separated, and speciments theirein counted.

In fish, skin just below the dorsal fin was cut and about 1 g of dorsal muscle was removed, stored in a lipid-free vial and stored at -85 ºC until further processing.

### 2.2.2 The Laurentian Great Lakes, North America

a) Field sampling

Seston samples were obtained from a water column of 1 m depth collected in Lake Erie (near Port Colbourne) and from Hamilton Harbor during the first week of June, 2008 by submerging the water containter with a volume of 20 l.

Zooplankton samples were collected from the epilimnetic waters of Lake Ontario in July 2001 and separated into several subsamples, corresponding to main groups of zooplankton. Approximately 100 specimens represented a sample: daphnids (3 replicates), calanoid copepods (3 replicates), cyclopoid copepods (3 replicates). Two macro-invertebrates (*Mysis relicta* Loven, 1862 and *Diporeia* spp.) were collected from the deepest basin of Lake Ontario at the same time.[1]

Zebra mussels (*Dreissena polymorpha* Pallas, 1771) were collected from Hamilton Harbor in April 2007. Quagga mussels (*Dreissena bugensis* Andrusov, 1897) were collected from Lakes Ontario and Huron in June-July 2007.

Native mussels, including the fat mucket (*Lampsilis siliquoidea* Barnes, 1823) and plain pocketbook (*Lampsilis cardium* Rafinesque, 1820) were collected from Lake Saint Clair in 2003 – 04 as part of a larger study to determine the effectiveness of natural refuge areas for native mussels (McGoldrick et al., 2008).[2]

Five species of fish were collected from various locations in Hamilton Harbour in July and September 2006. The fish examined were: alewife (*Alosa pseudoharengus* Wilson, 1811; 3 specimens), largemouth bass (*Micropterus salmoides* Lacepede, 1802; 10 specimens), emerald shiner (*Notropis atherinoides* Rafinesque, 1818; 8 specimens), pumpkinseed (*Lepomis gibbosus* Linnaeus, 1758; 9 specimens) and brown bullhead (*Ameiurus nebulosus* Lesueur, 1819; 10 specimens). Lake whitefish (*Coregonus clupeaformis* Mitchill, 1818) were collected from two locations in the Bay of Quinte – Big Bay (18 specimens) and Lake Ontario off Point Pelee (9 specimens) in November 2006. Additional lake whitefish samples were obtained from three locations in Lake Michigan from October to November 2005 (the Naubinway – 20 specimens, the Big Bay de Noc – 19 specimens, and the Bailey's

[1] These samples were obtained from the lipid bank of Environment Canada (EC) - Canadian Centre for Inland Waters (CCIW) either as lipid extracts ready to be injected into GC/MS or as animals being only freeze dried and stored cryogenically. The same sample preparation methods were used as being described here.

[2] See footnote number 1.

Harbor – 19 specimens). The same storage procedure was used as for zooplankton[3].

b) Laboratory monocultures of algae

Monocultures of algae raised in defined media, originally isolated from Lake Ontario were obtained from Environment Canada (Susan Watson) in May – June 2008 for the subsequent analysis of PMI-FA. The selected taxa were cyanobacteria (*Aphanizomenon flos-aquae* sp. and *Synechococystis* sp.), diatoms (*Asterionella Formosa* Hassal, 1850 and *Diatoma elongatum* (Lyngbye) C. Agardh, 1824 and chrysophyte (*Dinobryon cylindricum* Sandgren, 1983).

c) Laboratory preparation for lipid extraction

Water containing seston was first screened through a 64 μm mesh to remove zooplankton and then filtered onto a pre-combusted lipid-free GF/F filter followed by lipid extraction. Similarly, laboratory raised algal cultures were collected on GF/F filters before undergoing lipid extraction (see 2.3).
Several representatives of each mussel species were dissected and body parts of each representatives pooled (i.e foot, mantle,) followed by freeze-drying.
After the fish have been brought into the laboratory, their overlying skin was removed and dorsal muscle on either side of the dorsal fin collected for lipid analysis.

[3] See footnote number 1.

## 2.3 Lipid extraction

a) Pre-extraction procedures

Before lipid extraction samples undergo freeze-drying (i.e. lyophilization). The duration of the process varied from 24 to 48 hours - depending on type of sample. After freeze drying samples were prepared for homogenization. They were homogenized by grinding, using mortar-pestle and liquid nitrogen or using Fluka Ultra Turax T8.

After homogenization the amount of sample (homogenate – aliquot) used for lipid analyses were weighed on a microbalance Sartorius BP210S to the nearest 0.1 mg (in 2006) and on Sartorius ME-5 microbalance to the nearest 1 μg (in 2007) and all of the samples from 2.2.2.

b) Extraction process

Lipid extraction was performed in the fume hood. Homogenised samples were kept cold by keeping them on ice throughout the whole extraction and re-extraction procedures. Only clean, i.e. lipid free, glassware was used along with the GC grade chemicals. Laboratory glasses, gloves and a coat were worn at all times. Extractions were performed with procedural blanks.

Lipids were extracted according to modified Folch procedure (Folch et al., 1956) in chloroform/methanol (2 : 1, v/v) or dichloromethane/methanol (2 : 1, v/v) as extraction solvent. Synthetic lipid (cholestane) or nonadecanoic acid C19 (CAS: 1731-94-8) was added to all samples (1 mg $mL^{-1}$) as a recovery standard (spiking reagent - surrogate) to provide an indication of extraction efficiency prior to extraction (Sigurgisladottir et al., 1992).

The extraction mixture was homogenized to enhance the extraction by means of Teflon grinding pestle or by means of Fluka Ultra Turax T8 (45 s). Supernatant was transferred and pooled into a new vial after the vial has been centrifuged at 3300 rpm for 8 min at 4 °C (repeated three times). A 0.9 % NaCl solution was added to the pooled supernatant (containing lipids) to get the final volume ratio chloroform/methanol/NaCl solution (2 : 1 : 0.2, v/v/v). The procedure was repeated

three times for each sample.
Sample was then centrifuged 10 min at 3300 rpm at 4 °C and upper salt-water-methanol layer discarded. The lipid solution (total lipid fraction) was evaporated in a nitrogen evaporator, recapped and stored at -85 °C until further analysis (= total lipid extract (TLE)).
For the determination of zooplankton lipid class analyses, lipids were extracted as presented except without adding the recovery standard into the sample prior extraction.

c) Gravimetric determination of total lipids.

TLE was re-dissolved in the exact quantity of solvent solution of chloroform/methanol (2 : 1, v/v) using calibrated dispenser (2000 µl) and two aliquots, each consisted of 100 µl, was transferred into pre-weighted vessels (Elemental Microanalysis Limited P/N 4057), re-weighted after solvent has been evaporated and discarded. The remaining organic solvent (1800 µl) inside the vessels containing TLE from where the aliquots were taken was evaporated by using nitrogen evaporator followed by interesterification.

d) Interesterification/Methylation/Derivatization of lipids.

The remaining TLE was re-suspended in a solution of methanol/toluene (2 : 1, v/v) in 1 % sulfuric acid and (methylation reagent) and incubated 24 h at 50 °C according to Christie (1989) or by using 3 M methanolic HCl (incubating 15 min at 60 °C) according to Von Elert and Stampfl (2000). Interesterification resulted in the production of FAME, which were re-extracted three times from methylation reagent with 2 % $KHCO_3$ (w/v) in hexane/diethyl ether (1 : 1, v/v) containing butylated hydroxytoluene (0.01% w/v) or by hexane (2 : 1, v/v). Upper organic layer containing FAME were transferred and pooled into new collecting vial. Pooled organic layer containing FAME were then evaporated by using nitrogen evaporator and redissolved in 2 ml of hexane.

## 2.4 HPTLC analyses

### 2.4.1 Chemicals, standard solutions, preparation of standard stock solution and application onto HPTLC plates.

All chemicals, including solvents, were GC grade purchased from Sigma (Steinheim, Germany) and Merck (Darmstadt, Germany). A standard stock solution which included all standards from tab. 1 was prepared. A standard stock solution was stored in a glass vial with Teflon lined caps at -85 $^{o}$C until applied on a HPTLC plate.

Prior to sample application onto the HPTLC plate, the plate was pre-washed with a solution of chloroform : methanol (1 : 1, v/v) in order to remove the possible contaminants.

An automated sample applicator (Linomat IV, Camag, Muttenz, Switzerland) was used to apply standard stock solution and samples (TLE) onto the HPTLC plates. Standard stock solution and TLE were applied onto the HPTLC plate (17 tracks) under steady flow of nitrogen blanket (delivery rate of 0.39 µl $s^{-1}$ and syringe size of 25 µl) 15 mm from the side and 5 mm from the lower edge of the plate as 4 mm long bands separated by a 10 mm space. TLE of the samples were applied two times onto the HPTLC plate to the corresponding tracks in between the tracks of standard stock solution in varying application volume. The application volume for a standard stock solution was from 1-40 µl in 5 µl increments representing 9 point (level) calibration curve. The lowest of all 9 calibration levels (level 1) represented limits of quantitation (LOQ). The application volume for samples was varying from 8 - 16 µl respectively.

**Table 1:** *Compounds representing the lipid classes which can be resolved by a four-step separation.*

| Lipid Class | Abbreviation | Compound used | CAS NUMBER | Retention factor [mm] | Detection limits [µg] | Standard stock solution [µg/µl] |
|---|---|---|---|---|---|---|
| Hydrocarbons | HC | Squalene | 111-02-4 | 82.48 | 0.0625 | 0.0500 |
| Wax Esters | WE | Stearyl Palmitate | 2598-99-4 | 75.51 | 0.0153 | 0.0900 |
| Ketones | KET | 3-Hexadecanone | 187878-64-9 | 64.57 | 0.0406 | 0.1650 |
| Triacylglycerols | TAG | Tripalmitin | 555-44-2 | 49.27 | 0.1578 | 1.4000 |
| Free Fatty Acids | FFA | Palmitic acid | 57-10-3 | 36.18 | 0.0810 | 0.1850 |
| Fatty alcohols | ALC | Cetyl Alcohol | 36653-82-4 | 25.79 | 0.0096 | 0.0900 |
| Sterols | ST | Cholesterol | 57-88-5 | 22.62 | 0.0180 | 0.0750 |
| Acetone-mobile polar lipids | AMPL | α-monopalmitin | 542-44-9 | 14.33 | 0.0795 | 0.2350 |
| Phospholipids | PL | Phosphatidylcholine | 8002-43-5 | 9.08 | 0.0360 | 0.0550 |

### 2.4.2 HPTLC method accuracy, precision and detection limits

Intra assay accuracy was determined by replicate analysis (N= 6) of stock solution between all 9 calibration levels as presented in tab. 2.

Inter assay accuracy (i.e. repeatability = system precision) was tested by four applications of standard stock solution at LOQ level (level 1). Calculated inter assay accuracy was expressed in per centage of standard error (SE) - was calculated as a ratio between average values of peak areas to relative standard error of the mean.

**Table 2:** *Intra (n=6) and inter (n=4) assay accuracy [%], values over 10% are in bold text.*

| Lipid classes | Level 1 | Level 2 | Level 3 | Level 4 | Level 5 | Level 6 | Level 7 | Level 8 | Level 9 | Average | ST DEV | Inter |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| PL | **10.3** | 4.4 | 6.8 | 8.4 | 3.6 | 3.6 | 4.0 | 4.3 | 4.0 | 5.5 | 2.4 | 8.3 |
| AMPL | 4.6 | 4.9 | 3.9 | 5.9 | 4.2 | 3.2 | 3.7 | 3.0 | 3.7 | 4.1 | 0.9 | 5.6 |
| ST | 3.2 | 2.0 | 1.3 | 7.3 | 3.0 | 2.1 | 3.1 | 2.5 | 2.9 | 3.0 | 1.7 | 6.7 |
| ALC | 7.9 | 6.4 | 3.1 | 7.9 | 5.3 | 3.8 | 4.1 | 4.0 | 4.9 | 5.3 | 1.8 | 7.1 |
| FFA | 6.4 | 3.3 | 0.9 | 8.3 | 5.2 | 4.6 | 3.8 | 3.5 | 3.4 | 4.4 | 2.1 | 8.3 |
| TAG | **14.6** | **11.6** | 0.3 | **18.6** | **10.8** | 8.7 | 9.1 | 7.6 | 6.3 | 9.7 | 5.2 | 5.86 |
| KET | 14.2 | 5.2 | 4.6 | 9.8 | 5.4 | 4.4 | 4.4 | 3.6 | 3.1 | 6.1 | 3.6 | 6.22 |
| WE | **13.9** | **19.2** | 1.2 | **14.0** | **18.0** | **15.1** | **16.8** | **14.1** | **13.6** | 14.0 | 5.2 | 4.58 |
| HC | **15.5** | **18.3** | **10.3** | **15.3** | 8.1 | 7.4 | 5.5 | 7.6 | 5.8 | 10.4 | 4.7 | 2.87 |

### 2.4.3 Multiple development method

Three different mobile phases of decreasing polarity in a sequence of five steps were used to separate the varying concentrations of standard stock solutions and/or complex lipid mixtures (migration distances (RF) for compounds are presented in tab. 1). Saturated twin trough chamber (Camag) was used as the development chamber. The first two developments to a distance of 10 mm were performed in a mobile phase consisted of chloroform/methanol/water (65 : 25 : 4, v/v/v), while the next two developments to a distance of 50 mm were performed by hexane/diethyl ether/acetic acid (80 : 20 : 1.5, v/v/v). The last final development to a distance of 90 mm was performed using hexane/diethyl-ether/acetic acid (90 : 11 : 1, v/v/v). Between each development step, the plate was dried with a flow of nitrogen. The total development time for plate was approximately 65 min.

### 2.4.4 Post-chromatographic derivatisation

The derivatisation solution for the detection of separated lipid classes was comprised of molybdatophosphoric acid/ethanol (10 : 90, w/v). Immediately after development, HPTLC plates were dipped into the derivatisation solution for 2 s using manual immersion device. The plates were then drained of the excess derivatisation solution and charred by heating them at 150 °C for 35 min on a temperature regulated heating plate.

Separated lipid classes from natural samples appeared almost immediately after the plates have been charred. However, separated lipid classes from standard stock solution needed more time to appear. Lower detection limits precluded us to use $H_2SO_4$/EtOH (1 : 1, v/v) as derivatization agent (especially of AMPL and FFA) although a plate can be fully developed within charring it at 150 °C for 10 min on a temperature regulated heating plate (fig. 15).

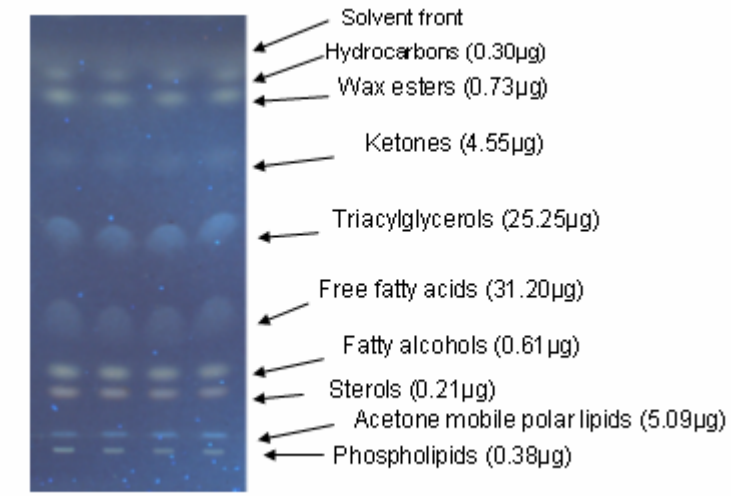



**Figure 15:** *HPTLC plate derivatized with $H_2SO_4$.*

### 2.4.5 Densitometry

Densitometric measurements were performed on each plate using the CAMAG TLC Scanner 3 immediately after charring. For each plate all applied tracks were scanned, starting 5 mm from the bottom of the plate to the solvent front of 90 mm. The measurement mode was Absorption/Remission; with a 2.0 x 0.2 mm slit dimensions at scanning speed of 20 mm $s^{-1}$ and a data resolution of 100 μm $step^{-1}$. Tracks were scanned under W lamp at 500 nm wavelength.

### 2.4.6 Quantification

The Camag TLC evaluation software (CATS V1.4.2) was used to record the peak height and area of each densitometric chromatogram. Calibration curves for each of 9 target lipid classes (and 9 levels) were prepared by using the data of the peak area for each of the corresponding lipid class. The relationship between the peak area and the amount of lipid class applied per track was best described by a polynomial regression and were thus used for quantification (average calibration curve correlation R = 0.99 ± 0.01 and average RSD = 6.19 ± 2.93 %). Quantification range was from 0.05 – 56 μg depending on the particular lipid class. Unknowns were determined by the comparison of peak areas (mean of all replicates) to standard calibration curves run simultaneously on the same plate for each of the 9 target lipid class analyzed.

## 2.5 Gas chromatograpy / mass spectrometry (GC/MS) analyses

### 2.5.1 Equipment and experimental conditions

FAME were analyzed by gas chromatography coupled to mass spectrometer (GC/MS) (Agilent Technologies 6890N GC) equipped with a polar capillary column (Agilent Technologies; 60-m x 0,25 mm id x 0.15 μm DB-23 (P/N 122-2361), an Agilent 7683B injector, and a mass selective quadrupole detector (Agilent 5973N). Helium was used as the carrier gas at constant pressure (~ 180 kPa at 33 cm $s^{-1}$ at 50 °C). Injection was done at an oven temperature of 50 °C. After 1 min the oven temperature was raised to 175 °C at a rate of 25 °C $min^{-1}$, then to 235 °C at a rate of 4 °C $min^{-1}$ and held for 5 min. Transfer line temperature was 180 °C. Retention time (RT) locking was used in order to obtain elutions of FA with very little retention time shifting of the peaks (methyl stearate was retention time locked to 14.0 min). Samples were injected in split and/or splitless mode depending on the requirements of individual samples. Samples were analyzed in both SIM and SCAN mode.

### 2.5.2 Quantification and accuracy

Samples were quantified using a calibration curve, by linear regression through zero or by measuring peak ratio method (C19). By using Supelco 37 Component FAME mix (Supelco, P/N 47885-U), DPA (Supelco, P/N 47563-U) and Cholestane (Sigma, P/N C8003) we prepared standard solutions in four different concentration ranges (4 point calibration curve) as presented in tab. 3.
On every fifth sample injected, the equipment accuracy was controlled by injecting a standard solution used for the calibration curve (Standard level 1 or Standard level 2) in SIM or/and SCAN mode.

Final concentrations of FAME [µg FAME • mg $dw^{-1}$] were calculated according to the formula:

$$[\mu g\ FAME \bullet mg\ dw^{-1}] = (X_{\mu g/ml} \cdot V / Mef \bullet Cr / Xr) / Ms$$

$X_{\mu g/ml}$ – concentration provided by GC

V – volume of derivatized fatty acid extract

$M_{ef}$ – methylation efficiency

$C_r$ – correction factor

$X_r$ – percent recovery of cholestane

$M_s$ – mass of sample

**Table 3 :** *Concentrations of standard solutions for the calibration curve.*

| **FAME [%]** | **37 Component Level's [µg/ml]** | | | |
|---|---|---|---|---|
| | I | II | III | IV |
| 2 | 2.5 | 5.0 | 10.0 | 20.0 |
| 4 | 5.0 | 10.0 | 20.0 | 40.0 |
| 6 | 7.5 | 15.0 | 30.0 | 60.0 |
| | **DPA Level's [µg $ml^{-1}$]** | | | |
| | 2.5 | 10.0 | 20.0 | 60.0 |
| | **5 - α cholestane [µg $ml^{-1}$]** | | | |
| | 5.0 | 10.0 | 20.0 | 40.0 |

Repeatability, i.e. inter-assay accuracy was determined on a daily basis by comparing the FA responses of the standard solutions which were injected for controlling the equipment accuracy. Detection limits were ranging from 10 ng to 0.2 ng, respectively.

## 2.6 Poly-methylene interrupted fatty acid (PMI-FA) analyses

PMI-FA were identified by comparing their corresponding mass spectra, by retention time matching and comparing their degree of unsaturation (Ag-ION solid phase extraction (SPE)) with our designated laboratory standard *Mytilus edulis* Linnaeus, 1758 for which was reported to contain PMI-FA (Budge et al., 2007).

As no laboratory standard for PMI-FA exists and because the mass spectra of FA with the same degree of unsaturation are very similar and so is their response, we were quantifying PMI-FA in samples to the nearest neighbor principle according to tab. 4. Overall, we were quantifying 6 PMI-FA and 1 novel tentatively identified PMI-FA found only in quagga mussels and native mussels (20:2PMID*).

**Table 4:** *PMI-FA corresponding to the FA on which they were quantified.*

| PMI-FA | Quantified on | Assumed double bond position |
|---|---|---|
| 20:2PMID* | 20:2 n-6 | not known |
| 20:2 n-9 | 20:2 n-6 | 20:2Δ5,11 |
| 20:2 n-7 | 20:2 n-6 | 20:2Δ5,13 |
| 20:3 n-6 | 20:3 n-6 | 20:3Δ5,11,14 |
| 22:2 n-9 | 22:2 n-6 | 22:2Δ7,13 |
| 22:2 n-7 | 22:2 n-6 | 22:2Δ7,15 |
| 22:3 n-6 | 22:2 n-6 | 22:3Δ7,13,16 |

## 2.7 Chlorophyll-a measurements

Samples containing Chl-*a* were extracted and analyzed according to Standard methods (APHA, 1991). Briefly Whatman GF/C glass fibre filters containing a known amount of pre-filtrated water were extracted in acetone followed by spectrophotometric measurement of absorbance in the extract at a wavelength of 663 nm.

## 2.8 Data analysis

### 2.8.1 Fulton's K

Relative robustness or fish condition was expressed by a "coefficient of condition" – Fulton's K (Williams, 2000) and was calculated according to the equation;

$$K = \frac{100.000 \cdot W}{L^3}$$

where: W = the weight of the fish in grams

L = the standard length of the fish in millimeters

### 2.8.2 Unsaturation index, grouping of data and FA

The unsaturation index (UI) was calculated as the sum of the percentage contributions of individual FA (to total quantified FAME) multiplied by the number of double bonds in that FA.

FA were grouped and summarized according to their degree of unsaturation to SAFA, MUFA, PUFA, ω3, ω6 (termed FA classes), if not otherwise stated. Relative proportions expressed in percent of FA classes were calculated as a percentage of the sums of the FA in each group to total quantified FAME. Examined FA with corresponding names and expected retention times are presented in tab. 5.

In order to present seasonal dynamics of total lipids in certain species we grouped certain data into groups by months; by quartiles (i.e [January - April], [May - August], [September - December]) or by splitting the months into the spring, fall and main growing season in between ([April - May], [June - August], [September - October]).

**Table 5:** *Examined FA with their corresponding names and expected retention times.*

| Molecular formula | Scientific Name | Common Name | Expected RT |
|---|---|---|---|
| C4:0 | butanoic acid | butyric acid | 4.191 |
| C6:0 | hexanoic acid | caproic acid | 5.481 |
| C8:0 | octanoic acid | caprylic acid | 6.825 |
| C10:0 | decanoic acid | capric acid | 8.010 |
| C11:0 | undecanoic acid | undecanoic acid | 8.595 |
| C12:0 | dodecanoic acid | lauric acid | 9.156 |
| C13:0 | tridecanoic acid | tridecanoic acid | 9.744 |
| C14:0 | tetradecanoic acid | myristic acid | 10.390 |
| C14:1n5c | 9-tetradecenoic acid | myristoleic acid | 10.703 |
| C15:0 | pentadecanoic acid | pentadecanoic acid | 11.095 |
| C15:1c | cis-10-pentadecanoic acid | cis-10-pentadecanoic acid | 11.460 |
| C16:0 | hexadecanoic acid | palmitic acid | 11.898 |
| C16:1n7c | 9-hexadecenoic acid | palmitoleic acid | 12.201 |
| C17:0 | heptadecanoic acid | heptadecanoic acid | 12.784 |
| C17:1c | cis-10-heptadecenoic acid | cis-10-heptadecenoic acid | 13.144 |
| C18:0 | octadecanoic acid | stearic acid | 13.780 |
| C18:1n9t | trans-9-octadenoic acid | elaidic acid | 13.970 |
| C18:1n9c | cis-9-octadenoic acid | oleic acid | 14.102 |
| C18:2n6t | trans-9,12-octadecadienoic acid | linolelaidic acid | 14.356 |
| C18:2n6c | cis-9,12-octadecadienoic acid | linoleic acid | 14.695 |
| C18:3n6c | 6,9,12-octadecatrienoic acid | γ-linolenic acid | 15.082 |
| C18:3n3c | 9,12,15-octadecatrienoic acid | α-linolenic acid (ALA) | 15.463 |
| C20:0 | eicosanoic acid | arachidic acid | 16.036 |
| C20:1n9c | cis-11-eicosenoic acid | eicosenoic acid | 16.406 |
| 20:2PMID | PMI-FA | PMI-FA | 16.614 |
| 20:2n9c | PMI-FA | PMI-FA | 16.700 |
| 20:2n7c | PMI-FA | PMI-FA | 16.771 |
| C20:2n6c | cis-11,14-eicosadienoic acid | cis-11,14-eicosadienoic acid | 17.105 |
| C21:0 | heneicosanoic acid | heneicosanoic acid | 17.253 |
| 20:3n6c | PMI-FA | PMI-FA | 17.378 |
| C20:3n6c | 8,11,14-eicosatrienoic acid | homo-γ-linolenic acid | 17.534 |
| C20:4n6c | 5,8,11,14-eicosatetraenoic acid | arachidonic acid (ARA) | 17.814 |
| C20:3n3c | 11,14,17-eicosatrienoic acid | eicosatrienoic acid (ETA) | 17.963 |
| C22:0 | docosanoic acid | behenic acid | 18.529 |
| C20:5n3c | 5,8,11,14,17-eicosapentaenoic acid | eicosapentaenoic acid (EPA) | 18.704 |
| C22:1n9c | 13-docosenoic acid | erucic acid | 18.953 |
| 22:2n9c | PMI-FA | PMI-FA | 19.339 |
| 22:2n7c | PMI-FA | PMI-FA | 19.429 |
| C22:2n6c | cis-13,16-docosadienoic acid | cis-13,16-docosadienoic acid | 19.700 |
| C23:0 | tricosanoic acid | tricosanoic acid | 19.811 |
| 22:3n6c | PMI-FA | PMI-FA | 20.113 |
| C24:0 | tetracosanoic acid | lignoceric acid | 21.100 |
| C22:5n3 | *cis*-7,10,13,16,19-Docosapentaenoic methyl ester | docosapentaenoic acid (DPA) | 21.400 |
| C24:1n9c | 15-tetracosenoic acid | nervonic acid | 21.570 |
| C22:6n3 | 4,7,10,13,16,19-docosahexaenoic acid | docosahexaenoic acid (DHA) | 21.750 |
| | α Cholestane | | 22.829 |
| | | | **Σω3** |
| | | | **Σω6** |
| | | | **ΣSAFA** |
| | | | **ΣMUFA** |
| | | | **ΣPUFA** |
| | | | **ΣPMI** |
| | | | **Total** |

### 2.8.3 Statistical analyses

Statistical analyses was performed by Sigmastat for Windows version (v3.5, Dundas Software, Ltd. and TE Subsystems Inc. Germany) or/and by SAS JMP version 7.0 (SAS Institute Inc., Cary, NC, USA). The later was also used to chart, plot the results along with the Sigmaplot for Windows version (v10.0, Dundas Software, Ltd. and TE Subsystems Inc. Germany).

Pairwise comparisons were perfomed using student's t-test. One way analysis of variance (ANOVA) was used do determine inter- and intraspecific taxonomic differences. When ($P < 0.05$) the data was further analyzed using the post-hoc Tukey-Kramer highly significantly different (HSD) test.

The data is presented as a mean ± SD (standard deviation) or by using the mean ± SE (standard error) when presenting error of replicate analyses. Results of the post-hoc Tukey-Kramer HSD are denoted with capital letters above the boxplot (box and whisker diagram) on figures which undergo ANOVA – a different letter denotes significant differences between groups (i.e. [A], [AB], [B] can be interpreted that there was significant difference between means of [A] and [B], however means of [AB] were not significantly different from neither [A] or [B]. At several occasions (when arguments are not supported by figure) we are providing the results of the post-hoc Tukey-Kramer HSD test within the text as demonstrated below. The same principle was used when t-test was performed.

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EXAMPLE:

"*However on the basis of relative proportions of AMPL we revealed that* Bosmina longirostris ***[A]*** *has highest relative average proportion of AMPL yearround (5.7 ± 1.7%) compared to* Arctodiaptomus laticeps ***[AB]*** *(4.3 ± 1.7 %),* Daphnia hyalina s.l. ***[AB]*** *(4.3 ± 3.2 %) and* Cyclops abyssorum prealpinus ***[B]*** *(3.1 ± 1.9 %) (ANOVA and Tukey-Kramer HSD,* $R^2 = 0.15$, $DF = 51$, $F = 2.8$, $P = 0.04$)"

---

The box plots summarize the distribution of points at each factor level. The ends of the box are the 25th (lower quartile – Q1) and 75th (upper quartile – Q3) quartiles defining the interquartile range. The line across the middle of the box indentifies the median (Q3) sample values. The whiskers extend from the ends of the box to the outermost data point that falls within the distances computed (the smallest observation (sample minimum) and the largest observation (sample maximum).

# 3 RESULTS

## 3.1 Thin-layer chromatographic method for the determination of lipid classes

The photographed HPTLC plate before the densitometry was performed is presented in fig. 16. The numbers at the bottom of the plate are denoting the number of applications on the plate (blank not denoted at the end). Detection limits, retention factors and the results of inter and intra calibration results are presented in tab. 1 and tab. 2, respectively. Densitograms for standard stock solutions and two zooplankton species are presented in figs. 17-19.

Each of the plates involved in the intra assay accuracy testing was developed on a different day. Lowest average intra assay accuracy was detected by quantification of ST 3.0 ± 1.7 % followed by quantification of AMPL where it was averaging at 4.1 ± 0.9 %. An increase of average intra assay accuracy was detected from RF= 0 up to the solvent front ($R^2 = 0.57$, $y = 0.9987\ x + 1.9564$) (tab. 2).

System precision was ranging from 2.8 % for HC up to 8.3 % for PL and was not significantly correlated to the elution RF (tab. 2).

Detection limits (LOD) were obtained from applying 32-times the diluted standard stock solution onto the HPTLC plate from 1 – 26 µl (1 – 10 µl on 1µl interval, 10 – 26 µl on interval of 2 µl). LOD for each of the compounds being separated are presented in tab. 1.

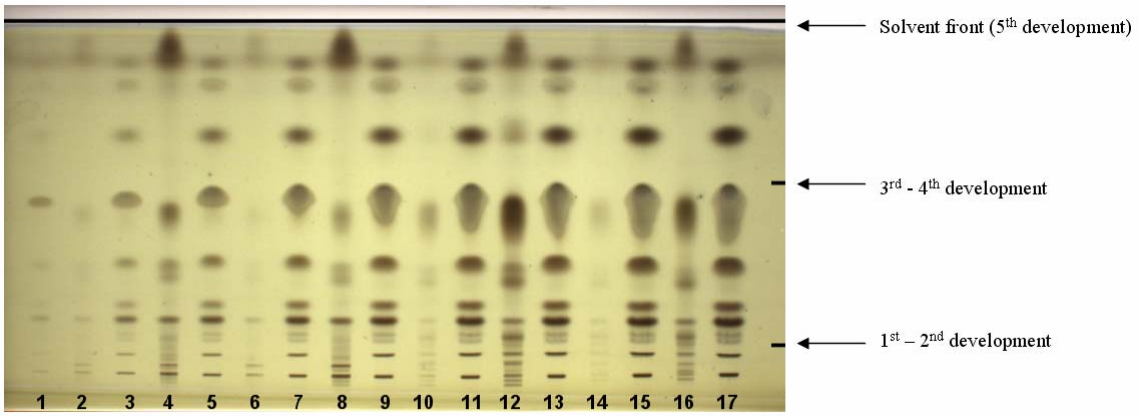


**Figure 16:** *Developed and derivatized HPTLC plate; Odd numbers are denoting the tracks of separated lipid classes of the standard stock solution (level 1 - 9). Even numbers are presenting the separated lipid classes of examined zooplankton species; Tracks 2 and 4* = B. longirostris, *6 and 8* = D. hyalina s.l., *10 and 12* = A. laticeps, *14 and 16* = C. abyssorum prealpinus.

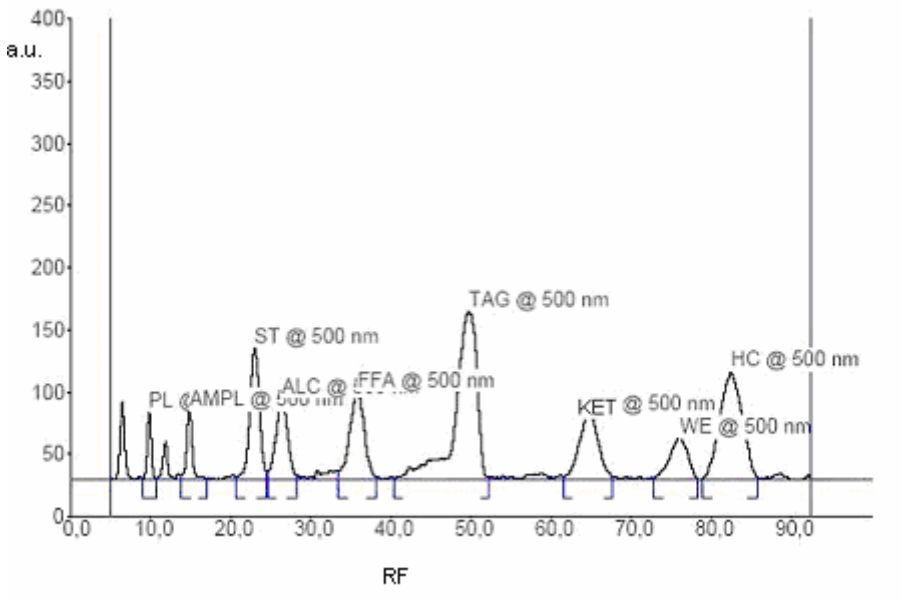


**Figure 17:** *Scanned track* (*densitogram) of Level 1 standard stock solution – track 1 from fig. 16. X axis represent retention factor (RF), Y axis represent absorption units (AU).*

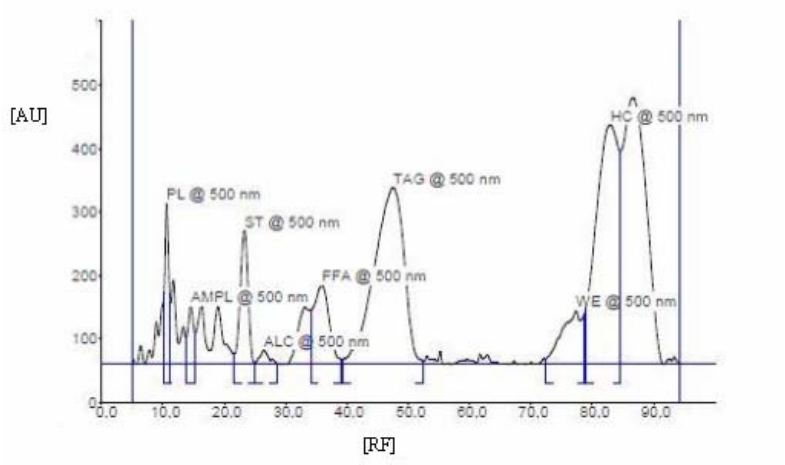


**Figure 18***: Typical scanned track* (*densitogram) of* Bosmina longirostris *(Cladocera) – track 2 from fig. 16. X axis represent retention factor (RF), Y axis represent absorption units (AU).*

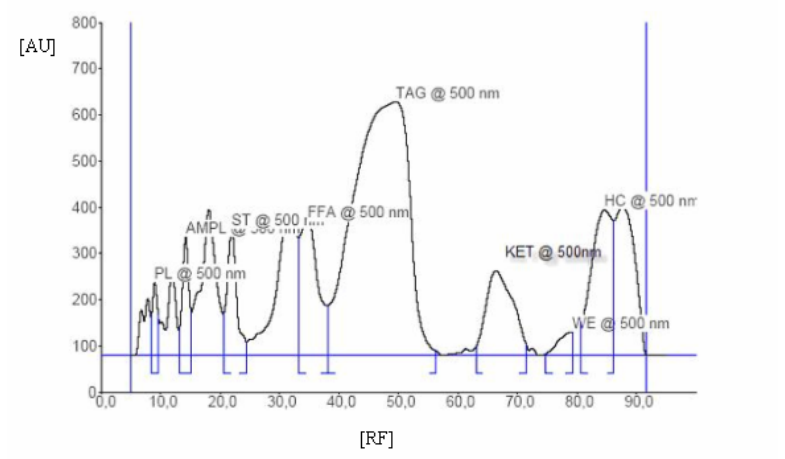


**Figure 19:** *Typical scanned track (densitogram) of* Cyclops abyssorum prealpinus *(Copepoda) – track 10 from fig. 16. X axis represent retention factor (RF), Y axis represent absorption units (AU).*

## 3.2 Seasonal variations of temperature and chlorophyll a and distribution of zooplankton species

In 2007 an average concentration of Chl-*a* in Lake Bohinj measured at the 15 m depth was 1.6 µg • $L^{-1}$. Hypolimnetic water temperature was ranging from 4 – 10 °C (fig. 20 left).

The abundance of Cladocera was higher in summer months compared to Copepoda while this trend was reversed during winter when Copepoda predominate (fig. 20 right).

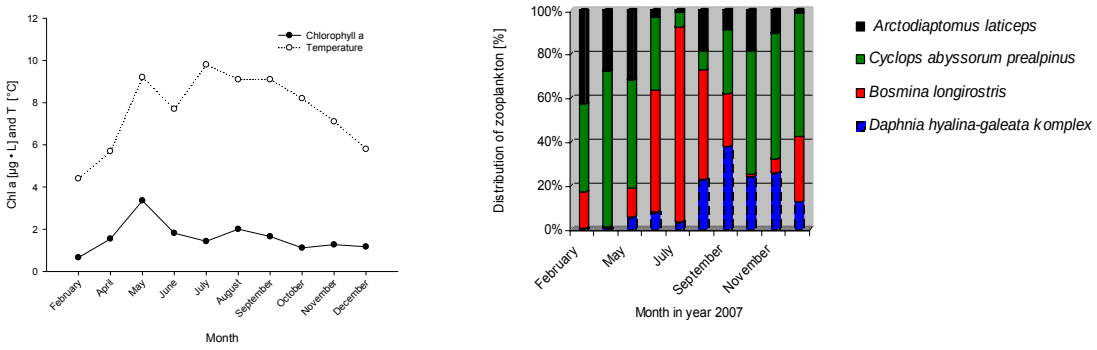


**Figure 20:** *Seasonal values of hypolimnetic water temperature and Chlorophyll-a concentrations (left side of the graph) and distribution of zooplankton species from Lake Bohinj (Slovenia) (right side of the graph).*

## 3.3 Total lipids in seston and zooplankton from Lake Bohinj

The total lipids per unit of dry weight in seston ranged from 7 up to 15 % with an annual average of 11.6 ± 2.68 (mean ± SE) %. Lowest values were measured in May and June (fig. 21 a) followed by a sharp increase up to the season peak (July), followed by a gradual decline up to October. Relative proportions of lipids were gaining again up to December.

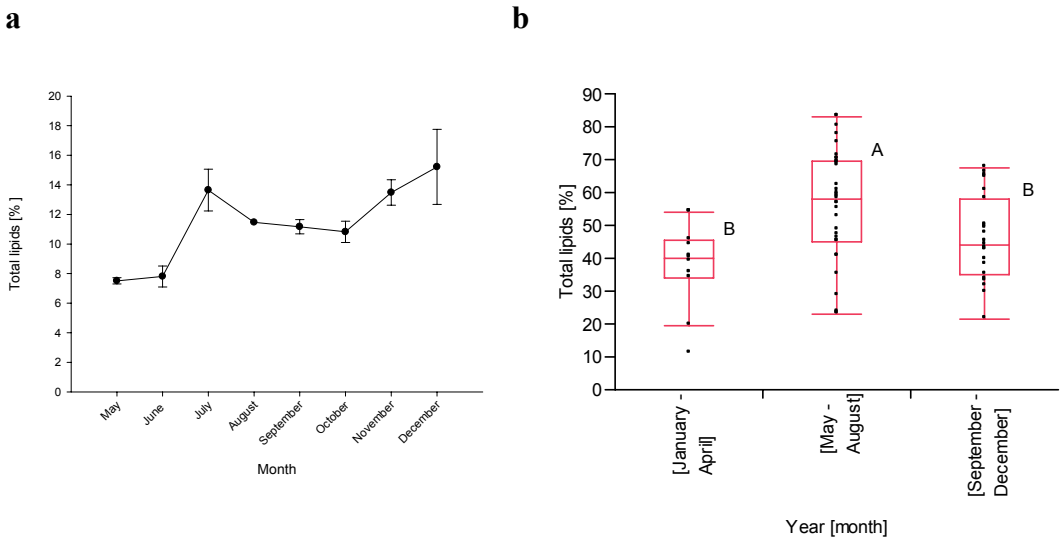


**Figure 21:** *Seasonal dynamics of total lipids in seston of Lake Bohinj (Slovenia);* **a)** *relative abundance of total lipids in seston (vertical error bars representing mean ± SE);* **b)** *relative abundance of total lipids (gravimetric measurements) in zooplankton species (Copepoda and Cladocera pooled) (ANOVA and Tukey-Kramer HSD, $R^2$= 0.18, DF= 67, F= 7.0, P= 0.0017). Different letters above data groups mean that groups are significantly different (p< 0.05).*

The seasonal increase of total lipids during the growing season was measured in zooplankton species as well. These values were highest in May-August and significantly lower before (January-April) and after (September-December) (fig. 21 b).

### 3.3.1 Lipid classes of four species of zooplankton from Lake Bohinj (Slovenia)

Triacylglycerols (TAG) were the principle lipid class of all four zooplankton species with higher relative proportions (of detected and quantified lipid classes) and amounts in Copepoda (*C. abyssorum prealpinus:* 85.6 ± 5.4 %, 551.8 ± 36.9 µg TAG · $mg^{-1}$ dw; *A. laticeps:* 85.2 ± 2.1 %, 546.4 ± 36.9 µg TAG · $mg^{-1}$ dw) compared to Cladocera (*B. longirostris:* 63.9 ± 10.1 %, 192.1 ± 39.9 µg TAG · $mg^{-1}$ dw; *D. hyalina s.l.* 72.72 ± 9.7 %, 199.5 ± 39.9 µg TAG · $mg^{-1}$ dw) (fig. 22 a, b, c, d), between the later two (Cladocera) significant difference was detected (t-test, DF= 23, P= 0.025).

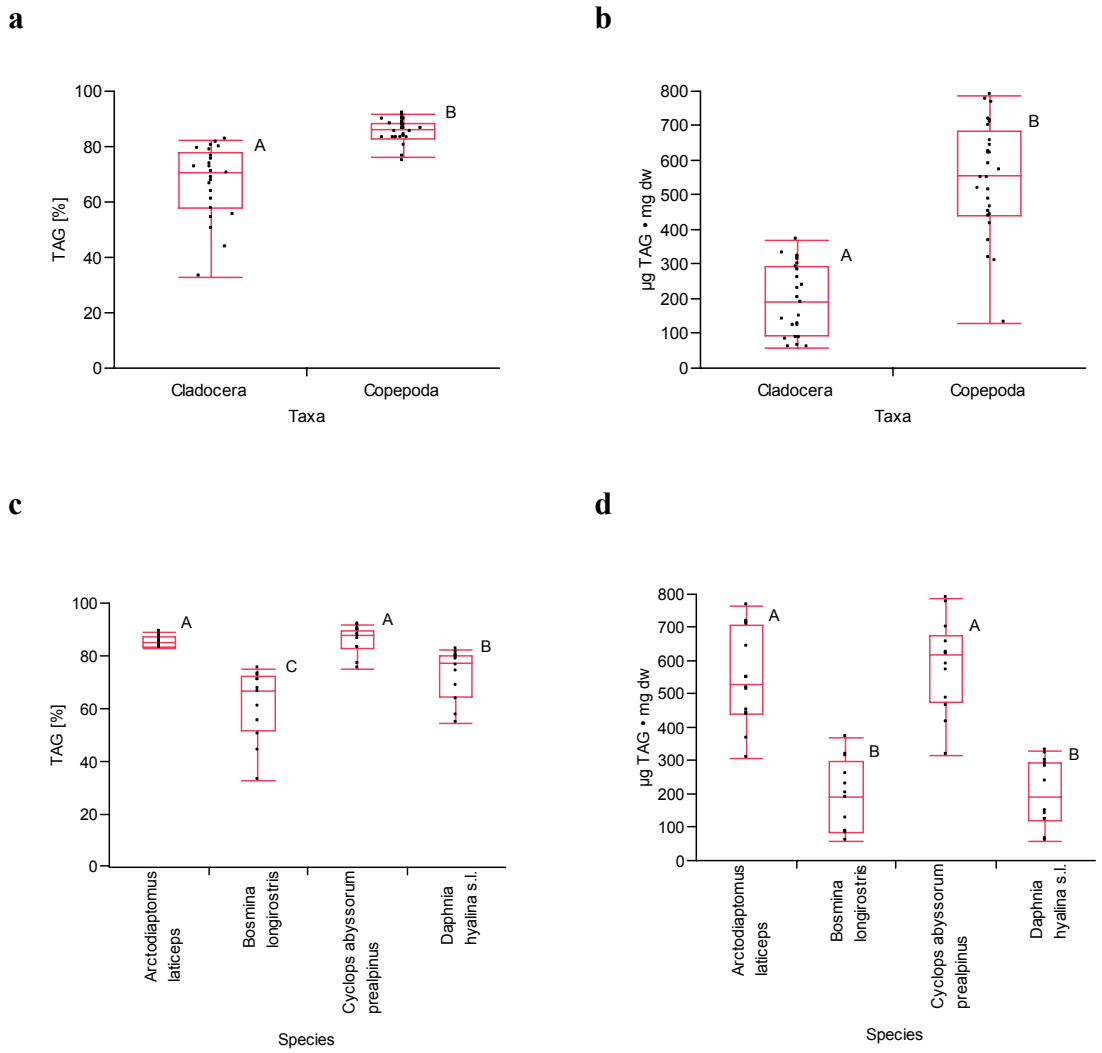


**Figure 22:** *Seasonal mean values of triacylglycerols (TAG) in zooplankton species from Lake Bohinj (Slovenia);* ***a)*** *relative proportions of TAG [%] of four zooplankton species merged by taxa (t-test, t-ratio= 7.19, DF= 51, P= 0.0001),* ***b)*** *amounts of TAG of four zooplankton species merged by taxa (t-test, t-ratio= 9.36, DF= 51, P< 0.0001),* ***c)*** *relative proportions of TAG [%] of four zooplankton species (ANOVA and Tukey-Kramer HSD, $R^2$= 0.59, DF= 51, F= 23.95, P< 0.0001),* ***d)*** *amounts of triacylglycerols (TAG) in four zooplankton species (ANOVA and Tukey-Kramer HSD, $R^2$= 0.70, DF= 51, F= 37.48, P< 0.0001). Different letters above data groups mean that groups are significantly different (p< 0.05).*

Total lipids (total content of quantified lipid classes) were higher in Copepoda (640.8 ± 28.9 µg total lipids · $mg^{-1}$ dw) compared to Cladocera (279.6 ± 31.3 µg total lipids · $mg^{-1}$ dw (fig. 23). Across the seasons, the amounts of TAG in all four species were lower in spring, gaining up to August, followed by a sharp decrease in September and increase in October (fig. 24).

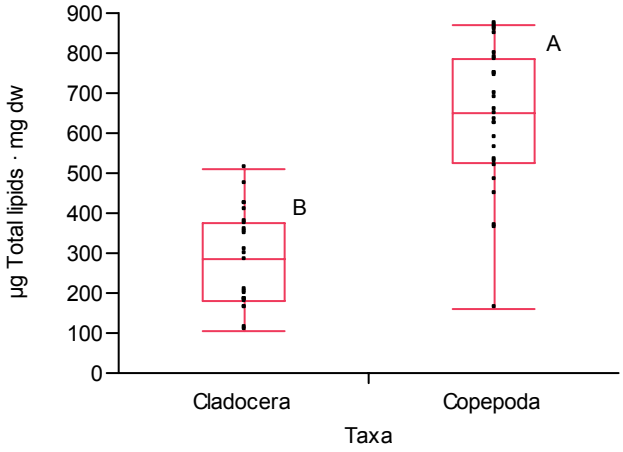


**Figure 23:** *Differences between Cladocera and Copepoda from Lake Bohinj (Slovenia) on the amounts of total lipids (sum of quantified lipid classes) (t-test, t-ratio= 8.4, DF= 51, P< 0.0001). Different letters above data groups means that groups are significantly different (p< 0.05).*

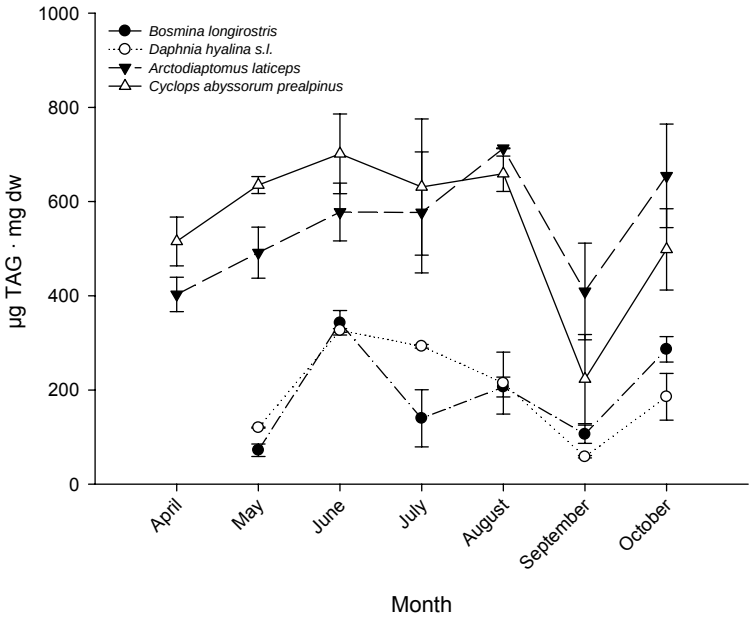


**Figure 24:** *Seasonal dynamics of triacylglycerols in four zooplankton species from Lake Bohinj (Slovenia). Vertical error bars representing ± SE.*

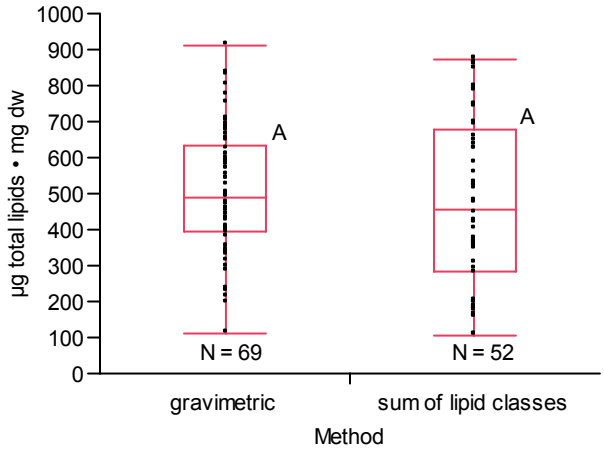


**Figure 25:** *Comparing effectiveness of two methods for estimating the total lipid content in zooplankton (t-test, DF= 120, F= 0.5, P> 0.05). Different letters above data groups mean that groups are significantly different (p< 0.05).*

Both methods, gravimetric and summarizing all quantified lipid classes, indicate no significant difference in the amounts of total lipids as they gave identical results (fig. 25).

Hydrocarbons (HC) were the second the most abundant lipid class with higher amounts detected in *B. longirostris* **[A]** 53.9 ± 5.2 µg HC · $mg^{-1}$ dw, compared to *D. hyalina s.l.* **[B]** 21.2 ± 5.2 µg HC · $mg^{-1}$ dw, *C. abyssorum prealpinus* **[B]** 30.9 ± 4.87 µg HC · $mg^{-1}$ dw and *A. laticeps* **[B]** 22.6 ± 4.8 µg HC · $mg^{-1}$ dw (ANOVA and Tukey-Kramer HSD, $R^2$= 0.34, DF= 51, F= 8.43, P= 0.0001). Thus the relative proportions [%] of HC were on average higher in *B. longirostris* **[A]** 19.6 ± 9.4 % compared to *D. Hyalina s.l.* **[B]** 9.2 ± 4.8 % (Cladocera), *C. abyssorum prealpinus* **[BC]** 4.8 ± 2.9 % followed by *A. laticeps* **[B]** 3.5 ± 1.3 %) (ANOVA and Tukey-Kramer HSD, $R^2$= 0.34, DF= 51, F= 8.43, P= 0.0001).
Cladocera exhibit higher amounts and relative proportions of HC than Copepoda (fig. 26 a, b). On seasonal basis an overall decrease of HC (Cladocera and Copepoda pooled) was detected throughout the season. The highest amounts were detected in the spring period [April-May] **[A]** (40.6 ± 6.0 µg HC · $mg^{-1}$ dw) and lowest in [September-October] **[B]** (19.1 ± 5.0 µg HC · $mg^{-1}$ dw), while a period [June-August] **[AB]** (34.5 ± 4.1 µg HC · $mg^{-1}$ dw) being in between (ANOVA and Tukey-Kramer HSD, $R^2$= 0.16, DF= 51, F= 4.49, P= 0.0162), with a seasonal maximum detected in June in *B. longirostris* (102.5 ± 9.3 µg HC · $mg^{-1}$ dw) with a gradual decline towards the end of the growing season (fig. 27). Generally, smaller variations were detected in Copepoda compared to Cladocera.

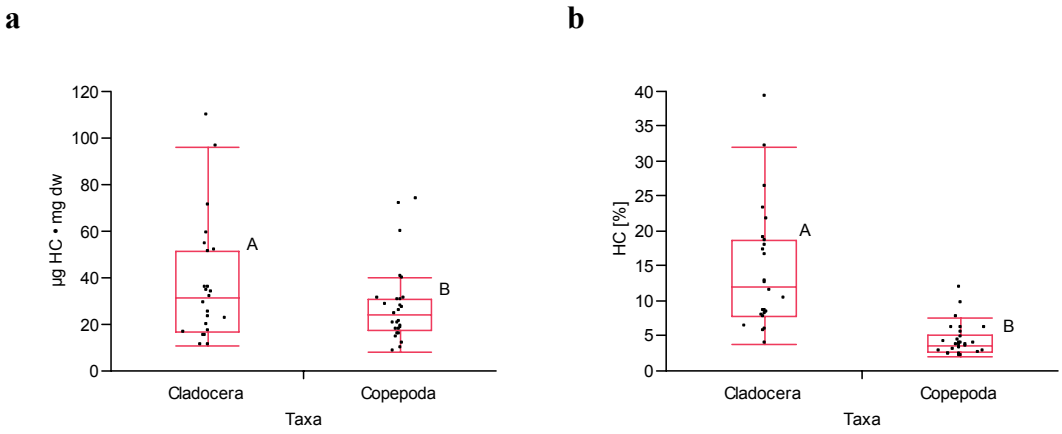


**Figure 26:** *Differences between Cladocera and Copepoda from Lake Bohinj (Slovenia) on the amounts and relative proportions of hydrocarbons (HC);* ***a)*** *amounts of HC (t-test, t-ratio= 4.65, DF= 51, P= 0.03),* ***b)*** *relative proportions of HC [%] (t-test, t-ratio= -5.82, DF= 51, P< 0.0001). Different letters above data groups mean that groups are significantly different (p< 0.05).*

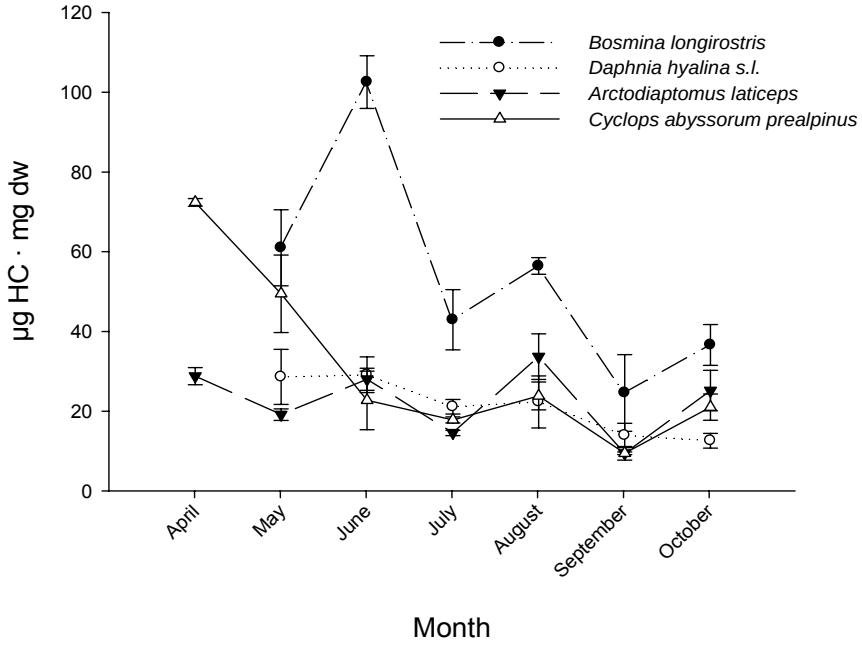


**Figure 27:** *Seasonal dynamics of hydrocarbons in four zooplankton species from Lake Bohinj (Slovenia). Vertical error bars are representing ± SE.*

Ketones (KET) were below detection limits (LOD) in Cladocera. Seasonal average of 7.7 ± 2.0 µg KET · $mg^{-1}$ dw and relative proportions of 1.1 ± 0.3 % was detected in Copepoda, with a seasonal maxima in August (*A. laticeps;* 53.4 ± 1.9 µg KET · $mg^{-1}$ dw, 6.2 ± 0.2 %, *C. abyssorum prealpinus;* 20.5 ± 3.6 µg KET · $mg^{-1}$ dw, 2.7 ± 0.2 %) (fig. 28). No significant differences were detected between both Copepoda on their seasonal average of KET (amounts and relative proportions) (t-test, DF= 27, P> 0.05).

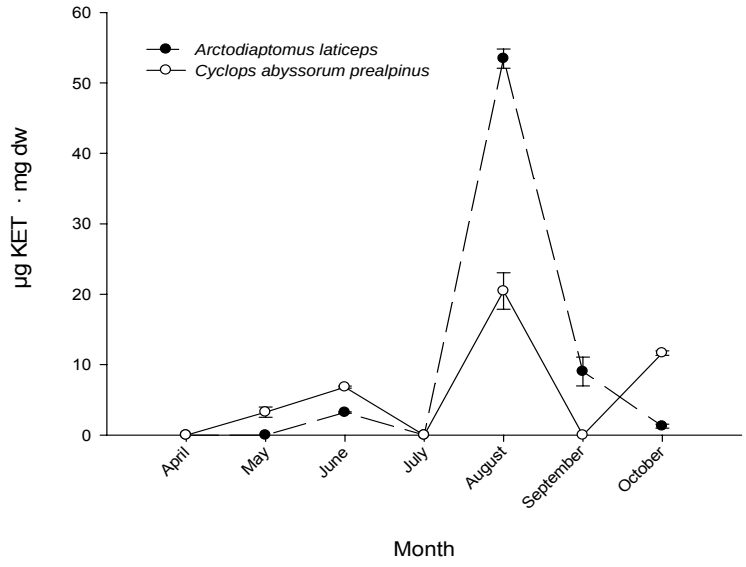


**Figure 28:** *Seasonal dynamics of ketones in zooplankton species from Lake Bohinj (Slovenia). Vertical error bars representing ± SE.*

By comparing temporal dynamics of wax esters (WE) between four zooplankton species from Lake Bohinj (Slovenia) we revealed that the amounts and proportions of WE in *A. laticeps* are constant throughout the growing season, with a peak in June (ANOVA and Tukey-Kramer HSD, amounts: $R^2$= 0.94, DF= 13, F= 17.3, P= 0.0007, proportions: $R^2$= 0.90, DF= 13, F= 11.7, P= 0.0024). Similarly, *C. abyssorum prealpinus* is gaining in amounts and proportions of WE up to June, followed by a decline (ANOVA and Tukey-Kramer HSD, amounts: $R^2$= 0.98, DF= 13, F= 74.0, P< 0.0001, proportions $R^2$= 0.86, DF= 13, F= 7.23, P= 0.009). However, both Cladocera (*B. longirostris* and *D. Hyalina s.l.*) contained significantly higher amounts of WE in October (end of the growing season) compared to May (start of growing season) and both two periods from the other months (fig. 29) (*B. longirostris* (ANOVA and

Tukey-Kramer HSD, $R^2$= 0.91, DF= 11, F= 12.5, P= 0.0040; *D. Hyalina s.l.* (ANOVA and Tukey-Kramer HSD, $R^2$= 0.96, DF= 11, F= 16.2, P= 0.0003).

By comparing the relative proportions of WE between both taxa, we detected them to be higher in Cladocera (2.0 ± 0.3 %) than Copepoda (0.7 ± 0.2 %) (t-test, DF= 51, t-ratio= 11.3, P= 0.002) (fig. 30) while there was no significant difference between the amounts of WE (Copepoda: 5.0 ± 0.9 µg WE · $mg^{-1}$ dw; Cladocera: 4.9 ± 0.9 µg WE · $mg^{-1}$ dw) (t-test, DF= 51, t-ratio = 0.12, P> 0.05).

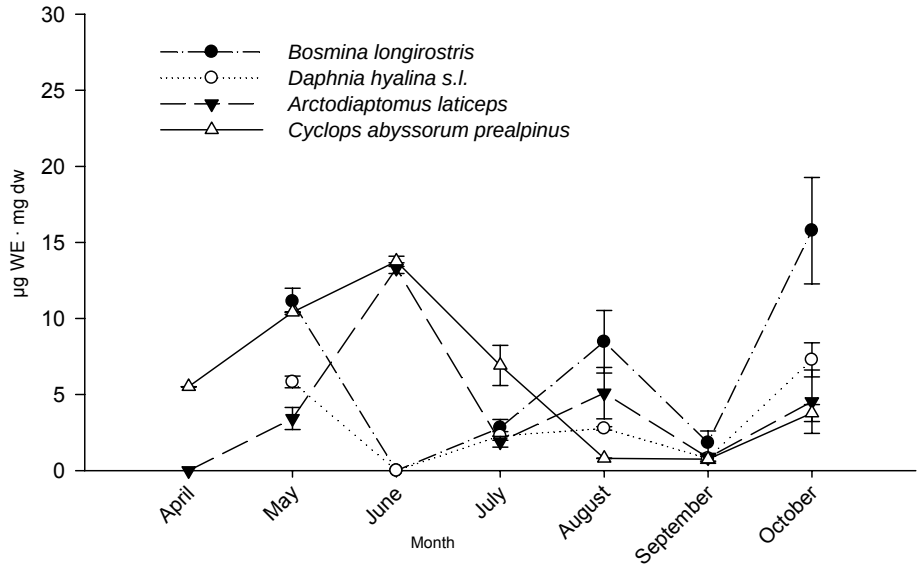


**Figure 29:** *Seasonal dynamics of wax esters in zooplankton species from Lake Bohinj. Vertical error bars representing ± SE.*

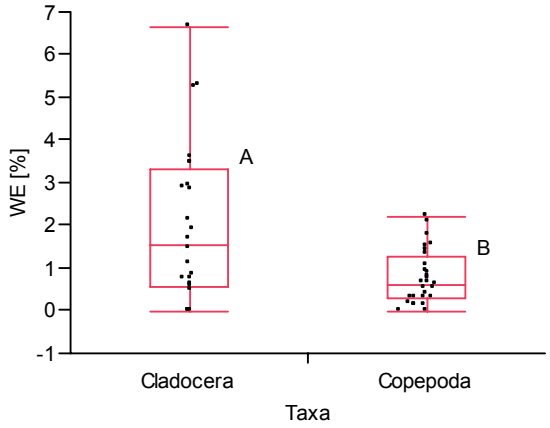


**Figure 30:** *Relative proportions of wax esters [%] between Cladocera and Copepoda (t-test, t-ratio= -3.36, DF= 51, P= 0.0015). Different letters above data groups mean that groups are significantly different (p< 0.05).*

On a temporal basis the values of free fatty acids (FFA) were closely following the dynamics of TAG (fig. 31). The amounts of FFA were on average highest in *A. laticeps* **[A]** 13.9 ± 8.0 µg FFA · $mg^{-1}$ dw followed by *B. longirostris* **[A]** 13.3 ± 4.5 µg FFA · $mg^{-1}$ dw, *C. abyssorum prealpinus* **[AB]** 10.2 ± 4.7 µg HC · $mg^{-1}$ dw and *D. Hyalina s.l.* **[B]** 6.2 ± 2.2 µg HC · $mg^{-1}$ dw (ANOVA and Tukey-Kramer HSD, $R^2$= 0.25, DF= 51, F= 5.3, P= 0.003).

On the basis of relative proportions of FFA [%] the differences were significant between *B. longirostris* **[A]** 5.0 ± 2.0 % and the other three species **[B],** all three averaging at 2.2 ± 1.5 % (ANOVA and Tukey-Kramer HSD, $R^2$= 0.53, DF= 51, F= 18.1, P< 0.0001), thus the relative proportions between both taxa were significantly different (fig. 32).

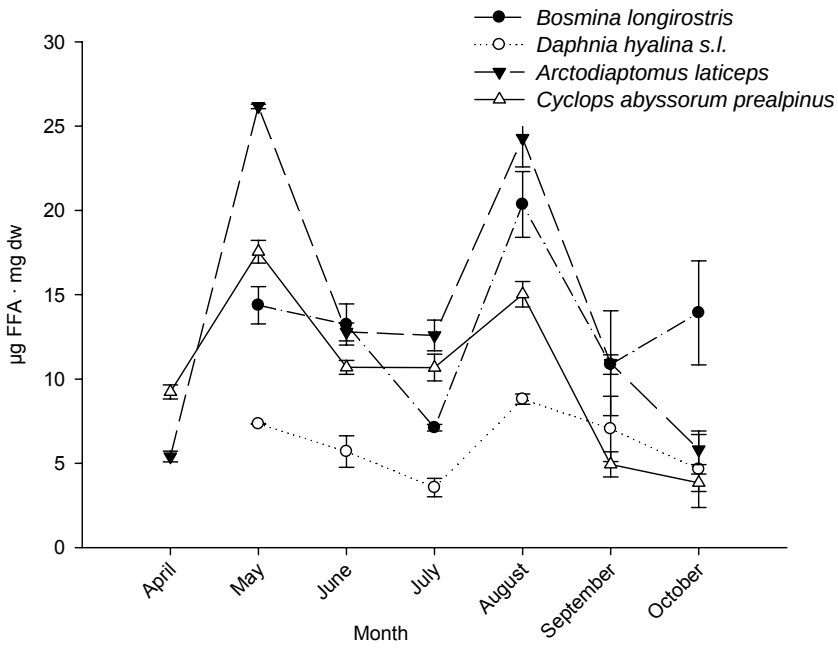


**Figure 31:** *Seasonal dynamics of free fatty acids in zooplankton species from Lake Bohinj (Slovenia). Vertical error bars representing ± SE.*

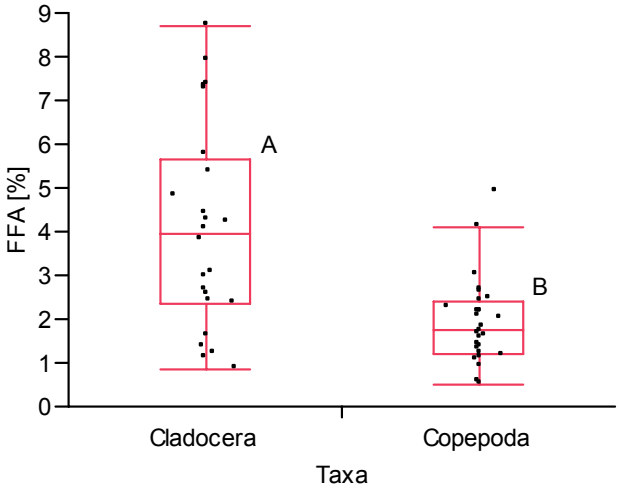


**Figure 32:** *Relative proportions of free fatty acids between Cladocera and Copepoda (t-test, t-ratio= -4.38, DF= 51, P< 0.0001). Different letters above data groups mean that groups are significantly different (p< 0.05).*

The temporal dynamics of sterols (ST) (fig. 33) were linked to the dynamics of PL throughout the season (fig. 34); from a sharp decline of amounts in both Copepoda from April to May, the amounts of ST were ranging within the value of 5.0 ± 1.6 µg ST • $mg^{-1}$ dw (mean of all four species of zooplankton) throughout the growing season. There were no significant differences between all four zooplankton species on the basis of the amounts of ST (ANOVA, DF= 51, P> 0.05), but differences were detected in relative proportions of ST [%]. Relative proportions of ST were higher in both Cladocera; *D. Hyalina s.l.* (2.45 ± 1.0 %) **[A]** and *B. longirostris* (2.01 ± 0.8 %) **[A]** compared to Copepoda; *C. abyssorum prealpinus* (0.88 ± 0.5 %) **[B]** and *A. laticeps* (0.75 ± 0.2 %) **[B]** (ANOVA and Tukey-Kramer HSD, $R^2$= 0.39, DF= 51, F= 10.6, P< 0.0001), thus significant differences between both taxa were detected on the basis of the amounts and proportions of ST (fig. 35 a, b). The latter were almost three-fold higher in Cladocera (2.2 ± 0.9 %) then in Copepoda (0.8 ± 0.38 %), whereas the amounts of ST averaged at 5.4 ± 0.26 µg ST • $mg^{-1}$ dw in Cladocera and 4.5 ± 0.24 µg ST • $mg^{-1}$ dw in Copepoda, respectively.

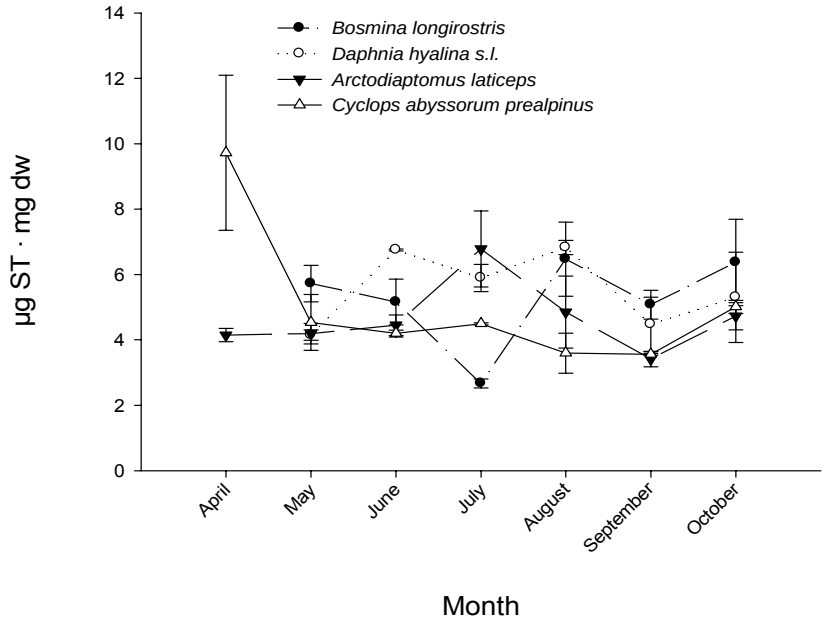


**Figure 33:** *Seasonal dynamics of sterols in four zooplankton species from Lake Bohinj (Slovenia). Vertical error bars representing ± SE.*

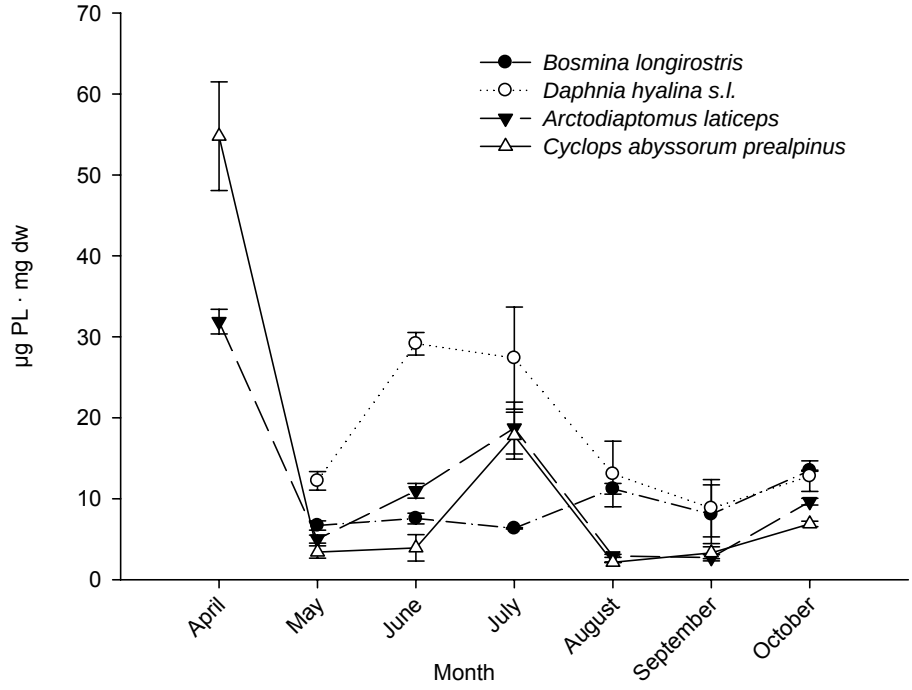


**Figure 34:** *Seasonal dynamics of phospholipids in four zooplankton species from Lake Bohinj (Slovenia). Vertical error bars representing ± SE.*

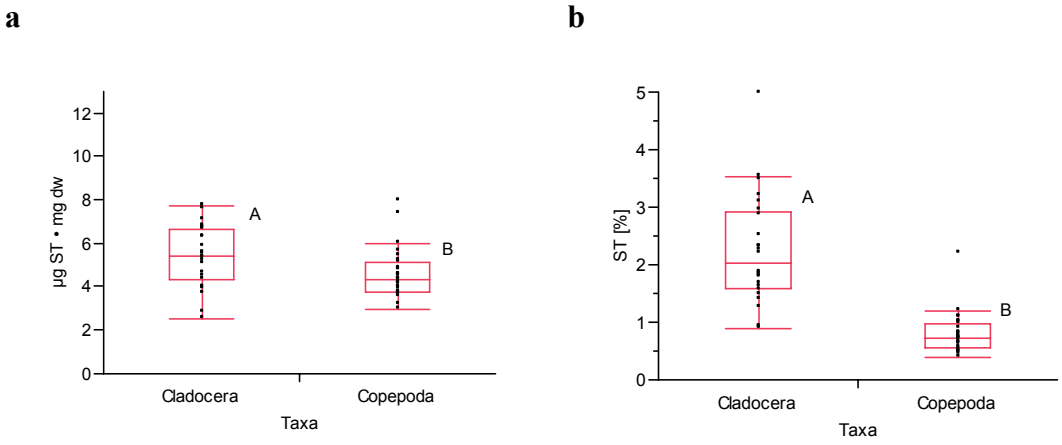


**Figure 35:** *Differences between Cladocera and Copepoda on:* ***a)*** *amounts of sterols (t-test, t-ratio= -2.19, DF= 51, P= 0.033),* ***b)*** *relative proportions of sterols (t-test, t-ratio= -7.16, DF= 51, P< 0.0001). Different letters above data groups mean that groups are significantly different (p< 0.05).*

We were unable to link phospholipid (PL) temporal dynamics to species or/and taxonomic specificity. However, the relative proportions of PL between Cladocera (4.9 ± 2.3 %) and Copepoda (2.0 ± 2.3 %) were different (fig. 36), on account of the higher relative proportions of PL in *D. hyalina s.l.* (Cladocera), compared to the other three species between which there was no significant difference (ANOVA and Tukey-Kramer HSD, $R^2$= 0.45, DF= 51, F= 13.13, P< 0.0001).

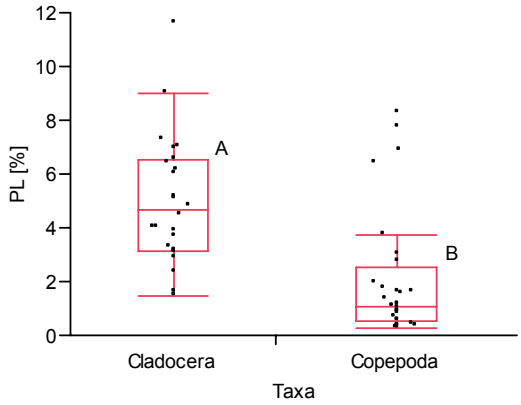


**Figure 36:** *Differences between Cladocera and Copepoda based on their relative proportions of phospholipids [%] (t-test, t-ratio= -4.42, DF= 51, P< 0.0001). Different letters above data groups mean that groups are significantly different (p< 0.05).*

Mean seasonal amounts of acetone mobile polar lipids (AMPL) in zooplankton species from lake Bohinj were highest in *A. laticeps* (27.3 ± 2.3 µg AMPL · $mg^{-1}$ dw) **[A]** compared to other three species (*C. abyssorum prealpinus* (17.9 ± 2.3 µg AMPL · $mg^{-1}$ dw, *B. longirostris* 16.6 ± 2.5 µg AMPL · $mg^{-1}$ dw and *D. hyalina s.l.* 9.4 ± 2.5 µg AMPL · $mg^{-1}$ dw) **[B]** (ANOVA and Tukey-Kramer HSD, $R^2$= 0.36, DF= 51, F= 9.07, P< 0.0001) (fig. 37). Similarly, the relative proportions of AMPL in zooplankton species were the highest in *B. longirostris* (5.7 ± 1.7 %) **[A]** as compared to *A. laticeps* (4.3 ± 1.7 %) **[AB]**, *D. hyalina s.l.* (4.3 ± 3.2 %) **[AB]** and *C. abyssorum prealpinus* (3.1 ± 1.9 %) **[B]** (ANOVA and Tukey-Kramer HSD, $R^2$= 0.15, DF= 51, F= 2.8, P= 0.04).

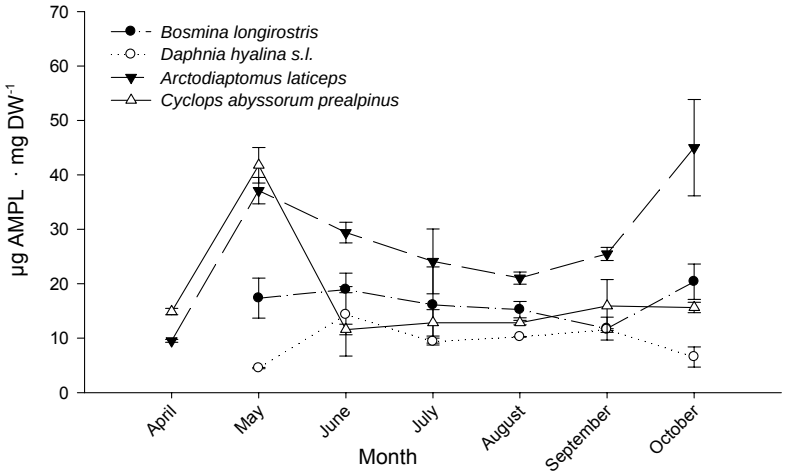


**Figure 37:** *Seasonal dynamics of acetone mobile polar lipids in four zooplankton species from Lake Bohinj (Slovenia). Vertical error bars representing ± SE.*

The amounts of AMPL in both Copepoda were lower at the beginning of the growing season (April) (2.0 ± 0.2 %) followed by a sharp increase of amounts and relative proportions of AMPL in May, peaking at 5.8 ± 2.7 %. As the season progressed the amounts of AMPL remained constantly higher in *A. laticeps* compared to the other three species. This reflected overall on higher amounts of AMPL in Copepoda (22.6 ± 1.8 µg AMPL · $mg^{-1}$ dw) than in Cladocera (13.1 ± 1.9 µg AMPL · $mg^{-1}$ dw). The reverse trend was recorded when comparing relative proportions of AMPL of both taxa (Cladocera (5.2 ± 0.4 %), Copepoda (3.7 ± 0.4 %) (fig. 38 a, b).

**a** **b**

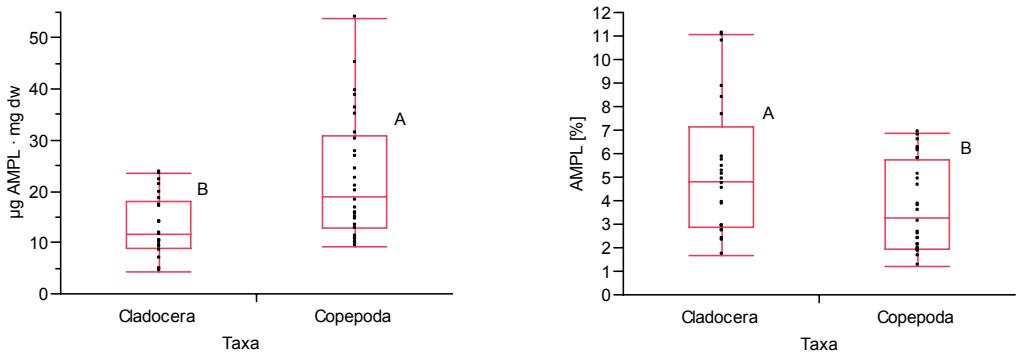


**Figure 38:** *Differences between Cladocera and Copepoda on:* ***a)*** *amounts of acetone mobile phospholipids (AMPL) (t-test, t-ratio= 3.56, DF= 51, P= 0.0008);* ***b)*** *relative proportions of AMPL [%] (t-test, t-ratio= -2.3, DF= 51, P= 0.02). Different letters below data groups mean that groups are significantly different (p< 0.05).*

Fatty alcohols (ALC) were below the detection limit in all of the species analyzed in any time interval.

### 3.3.2 Total lipids in ovigerous Copepoda

Ovigerous females of *A.laticeps* exhibited significantly higher amounts of total lipids (based on gravimetric measurements of total lipid content) and thus the proportions of total lipids (65.8 ± 15.2 % which equals to 658.2 ± 152 µg of total lipids • $mg^{-1}$ dw, respectively) compared to males of (44.5 ± 12.0 %). No significant differences were detected between males and females in *C. abyssorum prealpinus* (fig. 39).

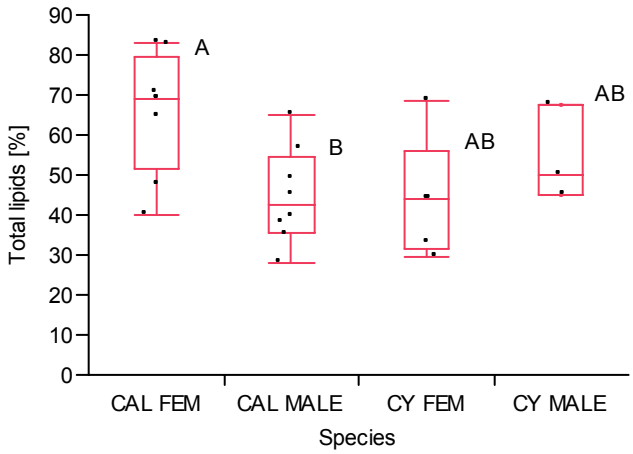


**Figure 39:** *Total lipids in ovigerous Copepoda with and without egg sacs from Lake Bohinj (Slovenia). Legend: CAL FEM = ovigerous female of* A. laticeps, *CAL MALE = male of* A. laticeps, *CY FEM = ovigerous female of* C. abyssorum prealpinus, *CY MALE = male of* C. abyssorum prealpinus. *(ANOVA and Tukey-Kramer HSD, $R^2$= 0.37, DF= 24, F= 4.0, P= 0.02). Different letters above data groups mean that groups are significantly different (p< 0.05).*

### 3.3.3 Fatty acid methyl esters (FAME) analyses of zooplankton from Lake Bohinj

#### 3.3.3.1 Intra and interspecific differences between species

Both copepod species exhibited significantly higher amounts of FAME compared to Cladocera (Copepoda: 405.3 ± 29.9 µg FAME • $mg^{-1}$ dw, Cladocera: 262.4 ± 29.9 µg FAME • $mg^{-1}$ dw, respectively (fig. 40 and tab. 7).

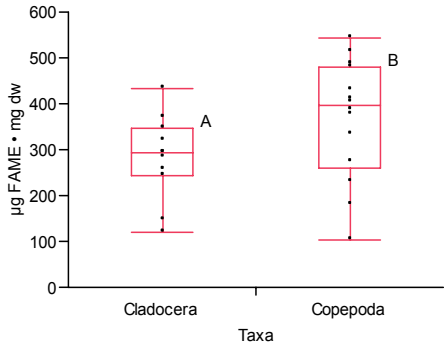


**Figure 40:** *Amounts of fatty acid methyl esters in Cladocera and Copepoda from Lake Bohinj (Slovenia) (t-test, t-ratio= 2.18, DF= 25, P= 0.038). Different letter above data groups mean that groups are significantly different (p< 0.05).*

Similarly, the overall amounts of poly-unsaturated fatty acids (PUFA) between Copepoda and Cladocera, ω3, ω6 and thus the unsaturation index (UI) (tab. 6) were overall detected to be higher in Copepoda over to Cladocera (fig. 41 a-d). These differences at the species level are presented in tab. 7.

**Table 6:** *Differences between Copepoda and Cladocera from Lake Bohinj (Slovenia) in groups of FA.*

| [mean ± SD] | Copepoda | Cladocera |
|---|---|---|
| | [µg · $mg^{-1}$ dw] | [µg · $mg^{-1}$ dw] |
| PUFA | 201.2 ± 13.0 | 73.2 ± 13.0 |
| ω3 | 160.4 ± 11.7 | 53.4 ± 11.7 |
| ω6 | 40.7 ± 3.5 | 19.7 ± 3.5 |
| UI | 242.8 ± 8.3 | 132.1 ± 8.3 |

**Table 7:** *Differences between total fatty acid methyl esters (FAME) and groups of fatty acids (FA) between zooplankton species from Lake Bohinj (Slovenia). Calculated st. error uses a pooled estimate of error variance.*

| [mean ± SE] | *A. laticeps* | *C. abyssorum prealpinus* | *B. longirostris* | *D. hyalina s.l.* | ANOVA |
|---|---|---|---|---|---|
| FAME [µg • mg$^{-1}$ dw] | 455.1 ± 40.5 | 355.1 ± 40.5 | 288.7 ± 40.5 | 236.0 ± 40.5 | $R^2$= 0.44, DF= 25, F= 5.4, P= 0.0067 |
| Tukey-HSD$_{FAME}$ | **[A]** | **[A]** | **[B]** | **[B]** | |
| PUFA [µg • mg$^{-1}$ dw] | 238.0 ± 15.3 | 164.4 ± 15.3 | 75.5 ± 15.3 | 70.8 ± 15.3 | $R^2$= 0.44, DF= 25, F= 5.4, P= 0.0067 |
| Tukey-HSD$_{PUFA}$ | **[A]** | **[B]** | **[C]** | **[C]** | |
| w3 [µg • mg$^{-1}$ dw] | 199.7 ± 12.7 | 121.1 ± 12.7 | 56.0 ± 12.7 | 50.8 ± 12.7 | $R^2$= 0.85, DF= 25, F= 38.4, P< 0.0001 |
| Tukey-HSDw$_3$ | **[A]** | **[B]** | **[C]** | **[C]** | |
| w6 [µg • mg$^{-1}$ dw] | 38.2 ± 5.2 | 43.2 ± 5.2 | 19.5 ± 5.2 | 20.0 ± 5.2 | $R^2$= 0.44, DF= 25, F= 5.42, P= 0.0068 |
| Tukey-HSDw$_6$ | **[AB]** | **[A]** | **[A]** | **[A]** | |
| UI | 261. 1 ± 10.9 | 224.5 ± 10.9 | 132. 1 ± 10.9 | 132. 1 ± 10.9 | $R^2$=0.84, DF=23, F=36.26, P< 0.0001 |
| Tukey-HSD$_{UI}$ | **[A]** | **[A]** | **[B]** | **[B]** | |

Relative proportions of PUFA were also significantly higher in Copepoda (49.5 ± 1.7 %) as compared to Cladocera (26.1 ± 1.7 %) (t-test, t-ratio= 10.6, DF= 25, P< 0.0001) on the account of relative proportions of ω3 (Copepoda: 39.2 ± 1.6 %, Cladocera: 18.9 ± 1.6 %) (t-test, t-ratio= 9.1, DF= 25, P< 0.0001) and relative proportions of docosahexaenoic acid (DHA) (Copepoda: 21.5 ± 0.8 %, Cladocera: 0.8 ± 0.8 %) (t-test, t-ratio= 17.5, DF= 25, P< 0.0001). Contrary, relative proportions of SAFA were significantly higher in Cladocera (48.38 ± 1.9 %) than in Copepoda (36.95 ± 1.9 %) on account of C15:0 (Cladocera: 2.0 ± 0.1 %, Copepoda: 1.0 ± 0.1 %) (t-test, t-ratio= -5.7, DF= 25, P< 0.0001), C16:0 (Cladocera: 23.8 ± 0.7 %, Copepoda: 20.7 ± 0.7 %) (t-test, t-ratio=-2.1, DF= 25, P= 0.04), C17:0 (Cladocera: 2.4 ± 0.2 %, Copepoda: 1.5 ± 0.2 %) (t-test, t-ratio= -2.8, DF= 25, P= 0.009) and C18:0 (Cladocera: 6.1 ± 4.2 %, Copepoda: 4.2 ± 0.3 %) (t-test, t-ratio= -3.3, DF= 25, P= 0.002).

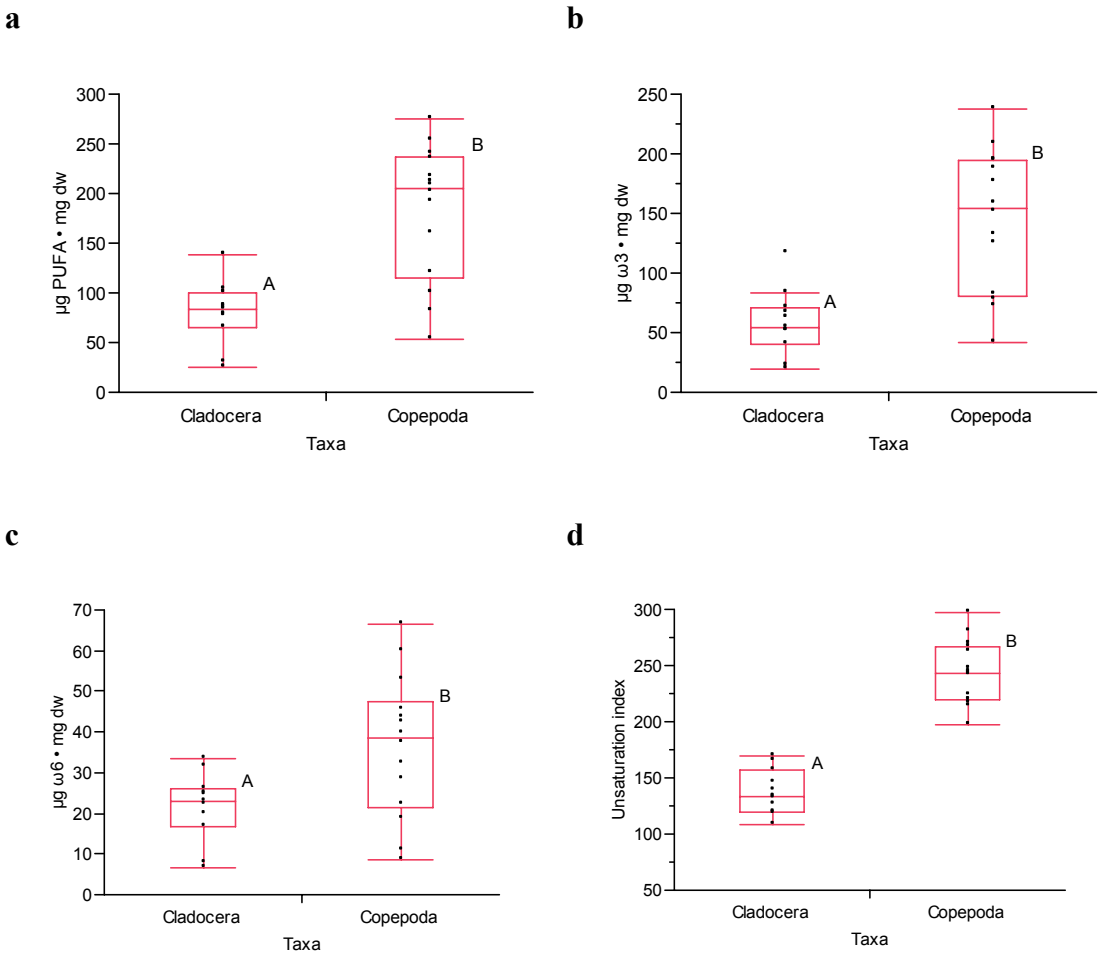


**Figure 41:** *Differences between Cladocera and Copepoda from Lake Bohinj (Slovenia) in:* ***a)*** *amounts of poly-unsaturated fatty acids (t-test, t-ratio= 4.90, DF= 25, P< 0.0001),* ***b)*** *amounts of ω3 (t-test, t-ratio= 4.88, DF= 25, P< 0.0001),* ***c)*** *amounts of ω6 (t-test, t-ratio= 2.89, DF= 25, P= 0.008),* ***d)*** *unsaturation index (t-test, t-ratio= 10.19, DF= 25, P< 0.0001). Different letter above data groups mean that groups are significantly different (p< 0.05).*

The amounts of mono-unsaturated fatty acids (MUFA) were higher in Cladocera (68.3 ± 7.4 µg MUFA · $mg^{-1}$ dw) as compared to Copepoda (56.0 ± 7.4 µg MUFA · $mg^{-1}$ dw) (fig. 42 a) on account of C18:1n9c (Cladocera: 33.3 ± 2.8 µg C18:1n9c · $mg^{-1}$ dw, Copepoda: 24.4 ± 2.7 µg C18:1n9c · $mg^{-1}$ dw ) (fig. 42 b).

In contrast, the relative proportions of MUFA were higher in Cladocera (25.4 ± 0.7 %) than in Copepoda (13.5 ± 0.7 %) (t-test, t-ratio= -12.2 DF= 25, P< 0.0001) on account of higher relative proportions of C16:1n7c (Cladocera: 12.0 ± 0.9 %, Copepoda: 5.7 ± 0.9 %) (t-test, t-ratio= -5.5, DF= 25, P< 0.001) and C18:1n9c (Cladocera: 11.5 ± 0.6 %, Copepoda: 6.0 ± 0.6 %) (t-test, t-ratio= -7.5, DF= 25, P< 0.001).

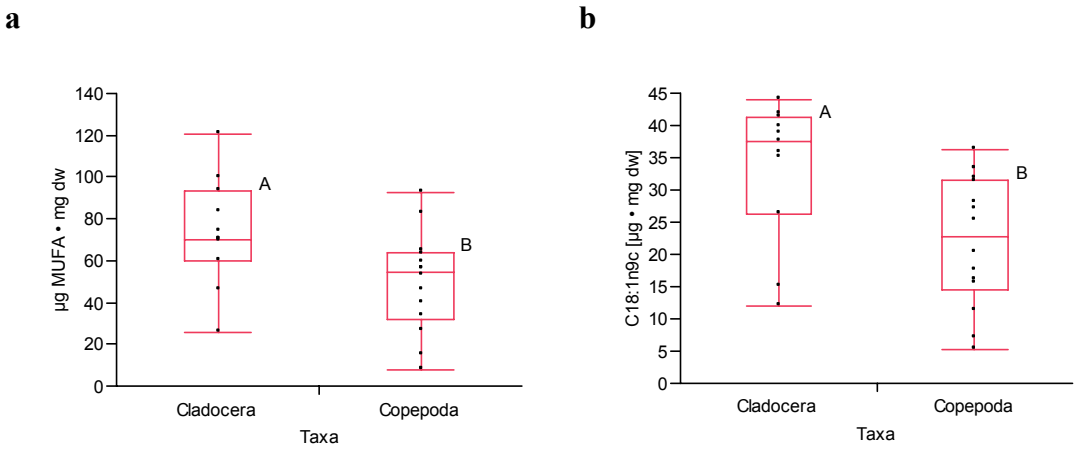


**Figure 42:** *Differences between Cladocera and Copepoda from Lake Bohinj (Slovenia) in the amount of;* ***a)*** *mono-unsaturated fatty acids (t-test, t-ratio= -2.43, DF= 25, P= 0.02),* ***b)*** *C18:1n9c (t-test, t-ratio= -2.74, DF= 25, P= 0.01). Different letter above data groups mean that groups are significantly different (p< 0.05).*

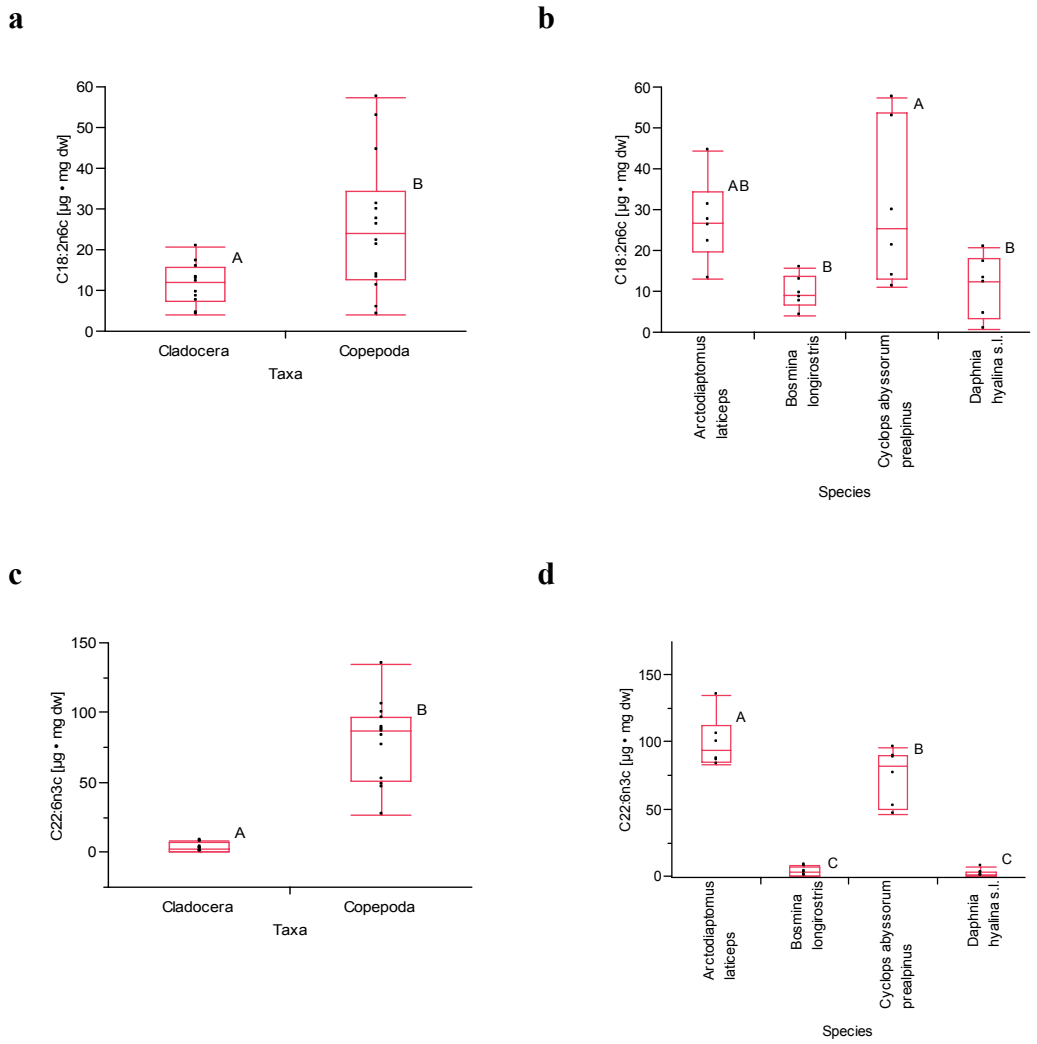


**Figure 43:** *Differences between four zooplankton species from Lake Bohinj (Slovenia) in:* ***a)*** *amount of linoleic acid (LIN) between Cladocera and Copepoda (t-test, t-ratio= 2.75, DF= 25, P= 0.01),* ***b)*** *amount of LIN between all four species (ANOVA and Tukey-Kramer HSD, $R^2$= 0.42, DF= 23, F= 4.9, P= 0.009),* ***c)*** *amount of docosahexaenoic acid (DHA) between Cladocera and Copepoda (t-test, t-ratio= 9.40, DF= 25, P< 0.0001),* ***d)*** *amount of DHA between all four zooplankton species (ANOVA and Tukey-Kramer HSD, $R^2$= 0.91, DF= 25, F= 72.14, P< 0.0001). Different letter above data groups mean that groups are significantly different (p< 0.05).*

The amounts of LIN and DHA were lower in Cladocera over to Copepoda (fig. 43 a-d and tab. 9). In contrast, the amounts of ARA were higher in Cladocera compared to Copepoda (fig. 44 a and tab. 9), while the amounts of EPA were not significantly different between both taxa (fig. 44 b and tab. 9), being the highest in *A. laticeps* 65.8 ± 8.9 µg EPA · $mg^{-1}$ dw compared to the other three species of zooplankton (*D. hyalina s.l.* 24.8 ± 15.1 µg EPA· $mg^{-1}$ dw, *C. abyssorum prealpinus* 26.5 ± 7.7 µg EPA· $mg^{-1}$ dw, *B. longirostris* 38.5 ± 16.6 µg EPA· $mg^{-1}$ dw) (fig. 44 c).

Interspecific differences between four zooplankton species from the lake were also revealed for amounts of α-LA. The highest amounts were detected in *A. laticeps* 34.9 ± 14.4 µg α-LA · $mg^{-1}$ dw followed by *C. abyssorum prealpinus* 14.8 ± 9.5 µg α-LA · $mg^{-1}$ dw, *B. longirostris* 14.0 ± 5.1 µg α-LA · $mg^{-1}$ dw and *D. hyalina s.l.* 12.1 ± 9.8 µg α-LA · $mg^{-1}$ dw (fig. 44 d).

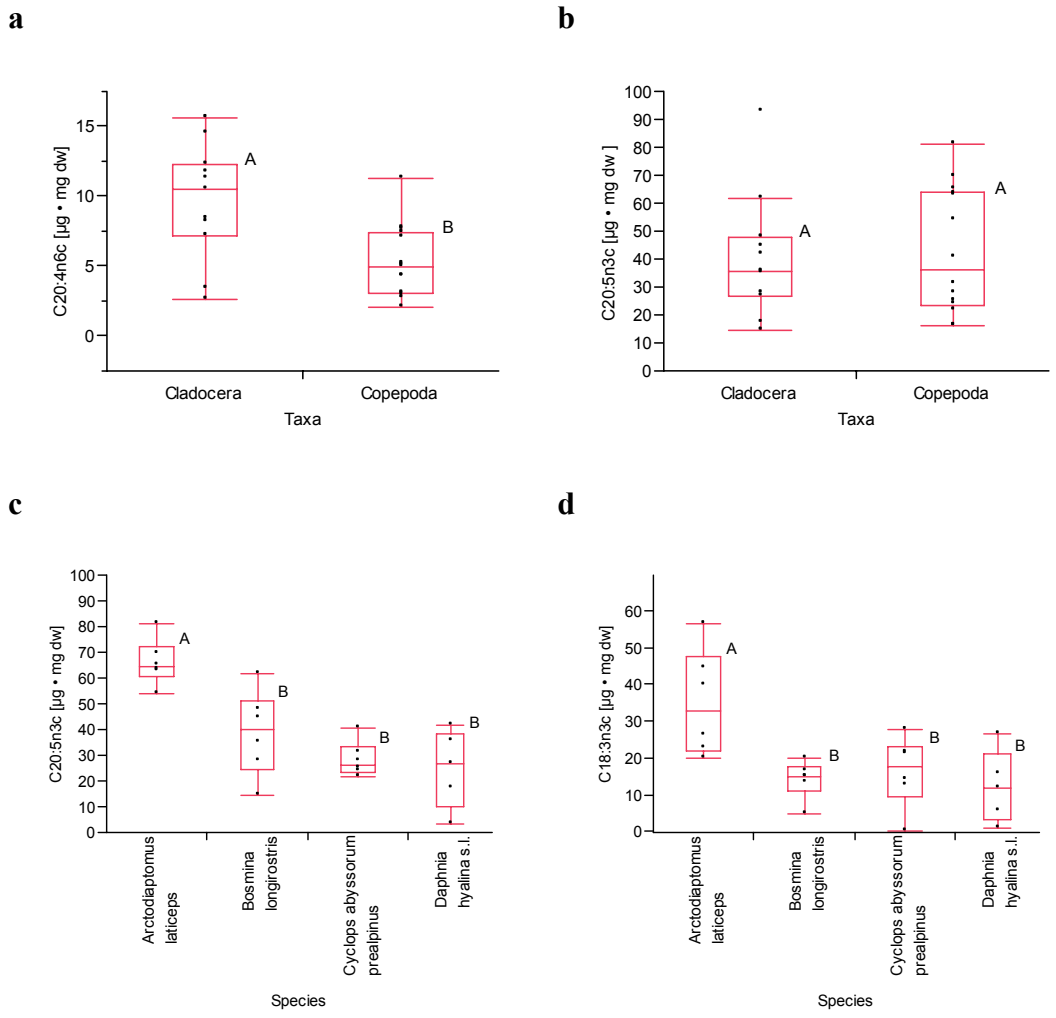


**Figure 44:** *Differences between four zooplankton species from Lake Bohinj (Slovenia) in:* ***a)*** *amount of ARA between Cladocera and Copepoda (t-test, t-ratio= -3.12, DF= 25, P= 0.004),* ***b)*** *amount of EPA between Cladocera and Copepoda (t-test, t-ratio= 0.25, DF= 24, P> 0.05),* ***c)*** *amount of EPA between all four species (ANOVA and Tukey-Kramer HSD, $R^2$= 0.69, DF= 25, F= 14.5, P< 0.0001),* ***d)*** *amount of α-LA between all four species (ANOVA and Tukey-Kramer HSD, $R^2$= 0.48, DF= 25, F= 6.32, P= 0.0034). Different letter above data groups mean that groups are significantly different (p< 0.05).*

### 3.3.4 Lipids in fish from Lake Bohinj

#### 3.3.4.1 Weight – length relationship as an indicator of fish lipid status

Good correlation was obtained between length and body mass for the cumulative data of the three fish species (fig. 45 a). However, Fulton's K was not correlating with total lipids [%] in *S. alpinus* ($y = 6.3270763 + 4.0046953 \cdot x$, DF= 36, R= 0.03, P> 0.05) and *L. cephalus* ($y = 10.53186 - 0.4034687 \cdot x$, DF= 26, R= 0.03, P> 0.05). A negative correlation was obtained for specimens of *P. fluviatilis* ($y = 14.245631 - 3.2937485 \cdot x$, DF= 34, R= - 0.50). Body mass and length of fish analyzed (*L. cephalus* (mean body mass: 182 ± 71 g; mean body length: 27 ± 2 cm), *P. fluviatilis* (64 ± 22 g; 17 ± 2 cm), *S. alpinus* (111 ± 41 g; 25 ± 3 cm) (fig. 45 b) resulted in significant differences between species, evaluated by their Fulton's K (fig. 46). The highest Fulton K was detected in *P. fluviatilis* (1.1 ± 0.04) followed by *L. cephalus* (0.9 ± 0.04) and *S. alpinus* (0.7 ± 0.03), respectively.

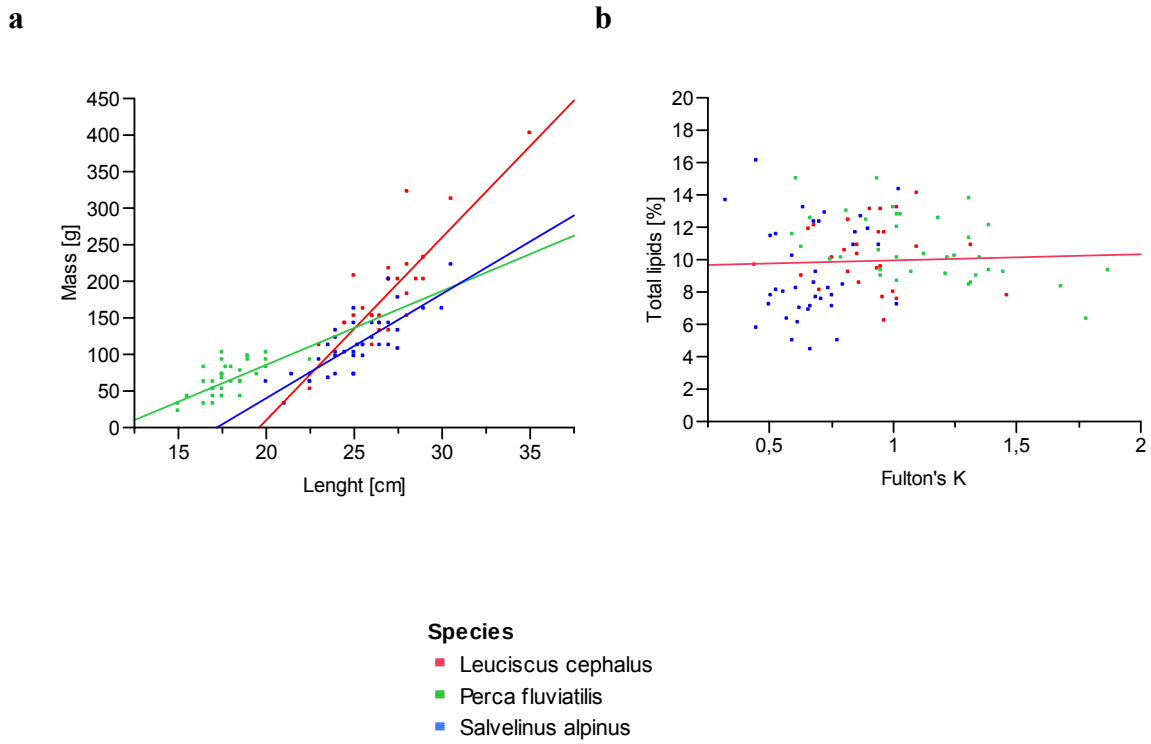


**Figure 45:** *Relationships of three fish species from Lake Bohinj (Slovenia) in:* ***a)*** *length* vs. *body mass relationships (*L. cephalus *(y = -492.3149 + 25.079097 · x, $R^2$=0.71 )*, P. fluviatilis *(y = -115.8278 + 10.122008 · x [cm], $R^2$= 0.45)*, S. alpinus *(y = -210.536 + 12.827507 · x, $R^2$= 0.62),* ***b)*** *Fulton's K* vs. *total lipids [%] comparison ( y = 9.608699 + 0.341753 · x, $R^2$=0.00181).*

### 3.3.4.2 Interspecific differences between fish from Lake Bohinj

Mean total lipids [%] were significantly higher and different in *L. cephalus* (10.1 ± 2.0 %) as compared to *S. alpinus* (mean 9.2 ± 2.9 %) (fig. 47 a). The mean value of the unsaturation index (UI) of *S. alpinus* (150.0 ± 17.4) was significantly higher, as compared to UI in *L. cephalus* (140.5 ± 14.1). Mean values of total lipids [%] in *P. fluviatilis* were 10.5 ± 1.9 % and UI 144.2 ± 14.6 what is just in between the values of other two fish species (fig. 47 a, b). The highest amounts of FAME were detected in *L. cephalus* and *P. fluviatilis* followed by *S. alpinus* (fig. 48 and tab. 8).

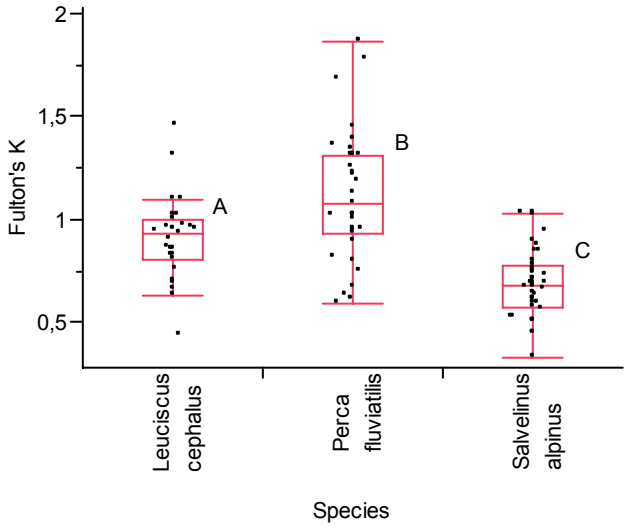


**Figure 46:** Comparing *Fulton's K of three fish species from Lake Bohinj (Slovenia); (ANOVA and Tukey-Kramer HSD, $R^2$= 0.38, DF= 100, F= 30.67, P< 0.0001). Different letters above data groups mean that groups are significantly different (p< 0.05).*

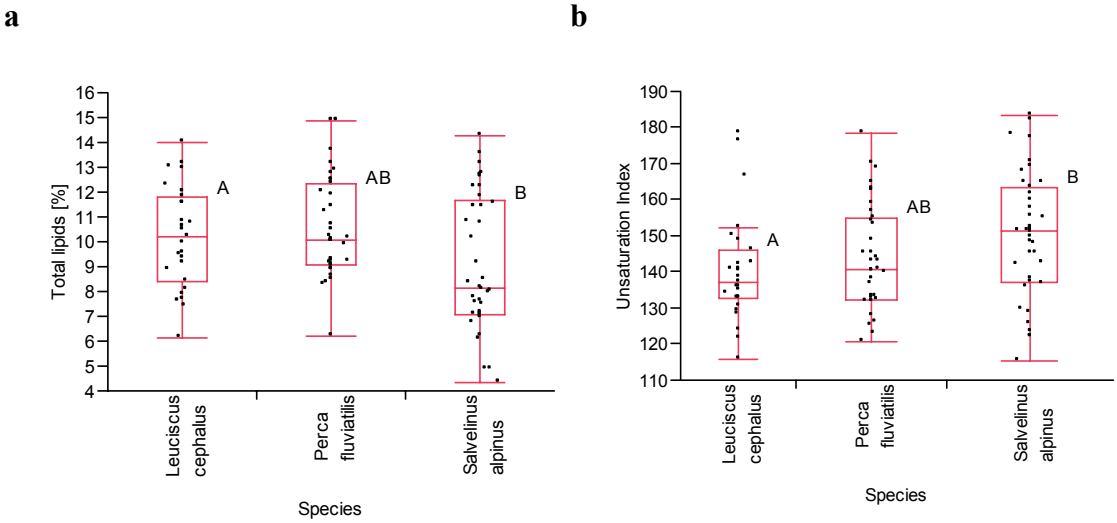


**Figure 47:** *Interspecific differences between three fish species from Lake Bohinj (Slovenia) on:* ***a)*** *amount of total lipids (ANOVA and Tukey-Kramer HSD, $R^2$= 0.07, DF= 100, F= 3.71, P= 0.02),* ***b)*** *unsaturation index (ANOVA and Tukey-Kramer HSD, $R^2$= 0.059, DF= 100, F= 3.07, P= 0.05). Different letters above data groups mean that groups are significantly different (p< 0.05).*

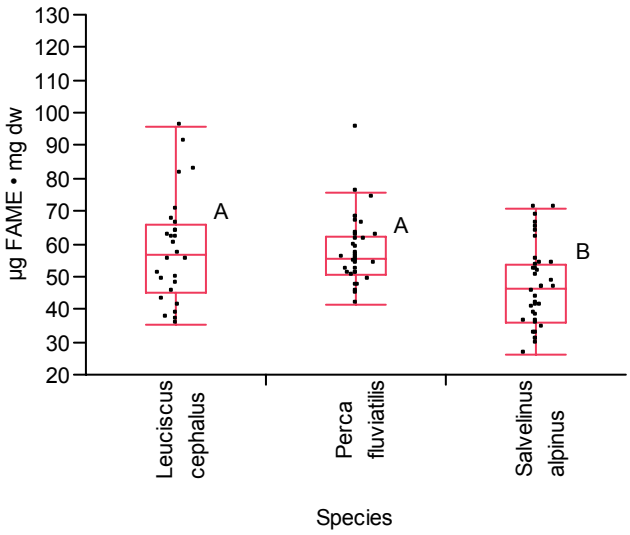


**Figure 48:** *Amount of fatty acid methyl esters in three fish species from Lake Bohinj (Slovenia); (ANOVA and Tukey-Kramer HSD, $R^2$= 0.15, DF= 100, F= 8.55, P= 0.0004). Different letters above data groups mean that groups are significantly different (p< 0.05).*

The amounts of unsaturated ω3 FA were higher in *S. alpinus* and *P. fluviatilis* compared to *L. cephalus* (fig. 49 a, tab. 8). The amounts of ω6 FA were highest in *L. cephalus* compared to *P. fluviatilis* and *S. alpinus* (fig. 49 b, tab. 8), which is in reverse order with the results of ω3. The highest levels of ω6 FA detected in *L. cephalus* are due to the high amounts and relative proportions of LIN (ANOVA and Tukey-Kramer HSD, $R^2$= 0.30, DF= 100, F= 21.7, P< 0.0001). A strong positive correlation was detected between the amounts of ω3 and ω6 for *S. alpinus* (y = 1.7527 + 0.2922 · x, $R^2$= 0.41). Relative proportions of ω3 FA were highest in *S. alpinus* followed by *P. fluviatilis* and lowest in *L. cephalus* (tab. 8). The highest proportions of ω6 FA were detected in *L. cephalus*; the lowest in *S. alpinus* and *P. fluviatilis* (fig. 49 c, d, tab. 8).

Relative proportions of PUFA were thus highest in *S. alpinus* and *L. cephalus* and lowest in *P. fluviatilis* (tab. 8). Absolute amounts of PUFA were highest in *L. cephalus* followed by *P. fluviatilis* and *S. alpinus* (fig. 50 a, b, tab. 8).

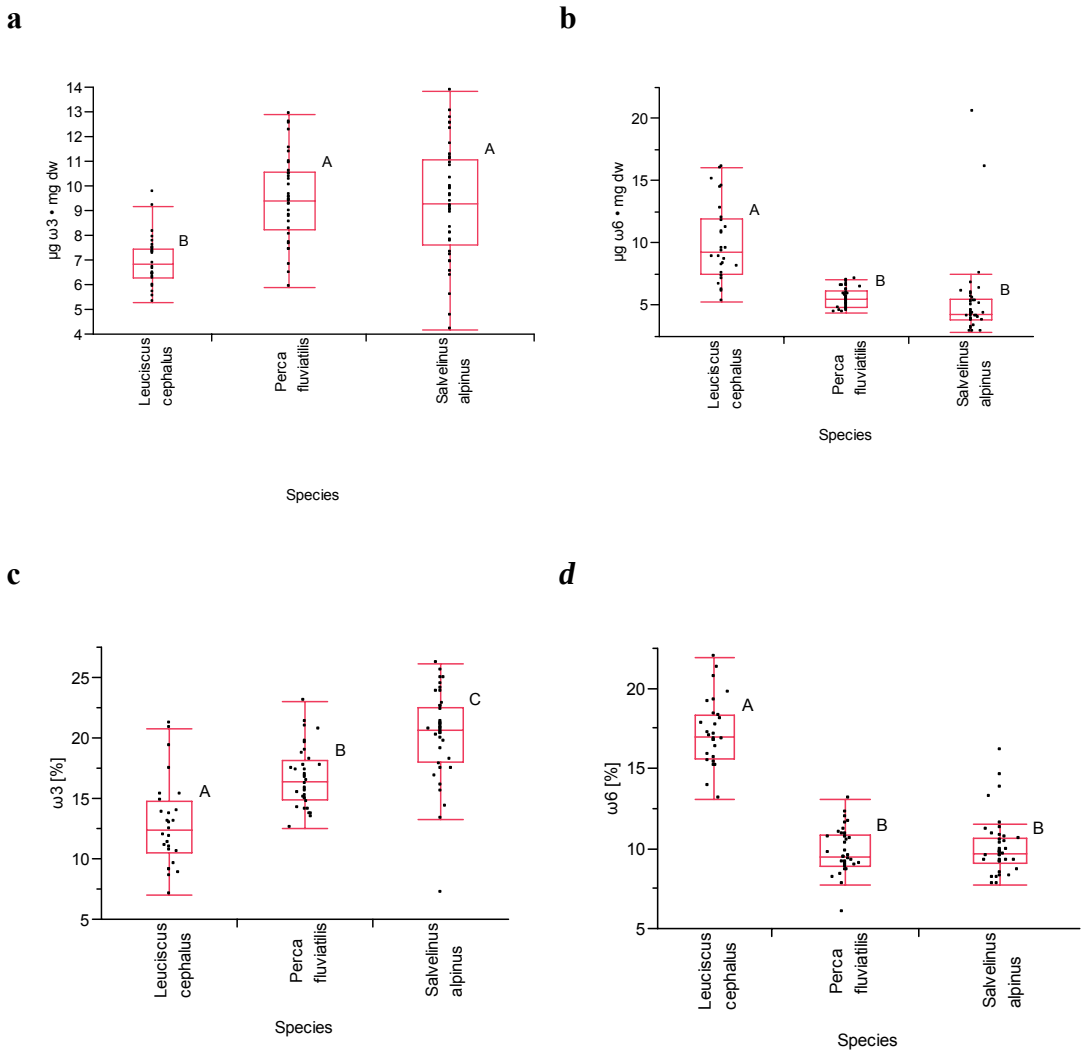


**Figure 49:** *Concentrations of fatty acids (FA) in three fish species from Lake Bohinj (Slovenia) in***: *a) amounts of ω3 FA (ANOVA and Tukey-Kramer HSD, $R^2$= 0.25, DF= 100, F= 16.43, P< 0.0001), b) amounts of ω6 FA (ANOVA and Tukey-Kramer HSD, $R^2$= 0.40, DF= 100, F= 30.00, P< 0.0001), c) relative proportions of ω3 FA (ANOVA and Tukey-Kramer HSD, $R^2$= 0.39, DF= 100, F= 31.41, P< 0.0001), d) relative proportions of ω6 FA (ANOVA and Tukey-Kramer HSD, $R^2$= 0.75, DF= 100, F= 147.30, P< 0.0001). Different letters above data groups mean that groups are significantly different (p< 0.05).*

No differences between species were detected in the amount of SAFA (tab. 8) (ANOVA, $R^2$= 0.031, DF= 100, F= 1.85, P> 0.05), but were significantly different by comparing their relative proportions: *S. alpinus* **[A]**, followed by *P. fluviatilis* **[B]** and *L. cephalus* **[B]** (tab. 8) (ANOVA and Tukey-Kramer HSD, $R^2$= 0.033, DF= 100, F= 24.28, P< 0.0001). Differences were detected also in the amount of MUFA: *S. alpinus* **[A]** exhibiting significantly lower amounts compared to *P. fluviatilis* **[B]** and *L. cephalus* **[B]** (tab. 8) (ANOVA and Tukey-Kramer HSD, $R^2$= 0.117, DF=

100, F= 6.52, P= 0.0022), which comply with the relative proportions of MUFA being the lowest in *S. alpinus* **[A]** followed by *L. cephalus* **[B]** and *P. fluviatilis* **[B]** (tab. 8) (ANOVA and Tukey-Kramer HSD, $R^2$= 0.42, DF= 100, F= 34.7, P< 0.0001).

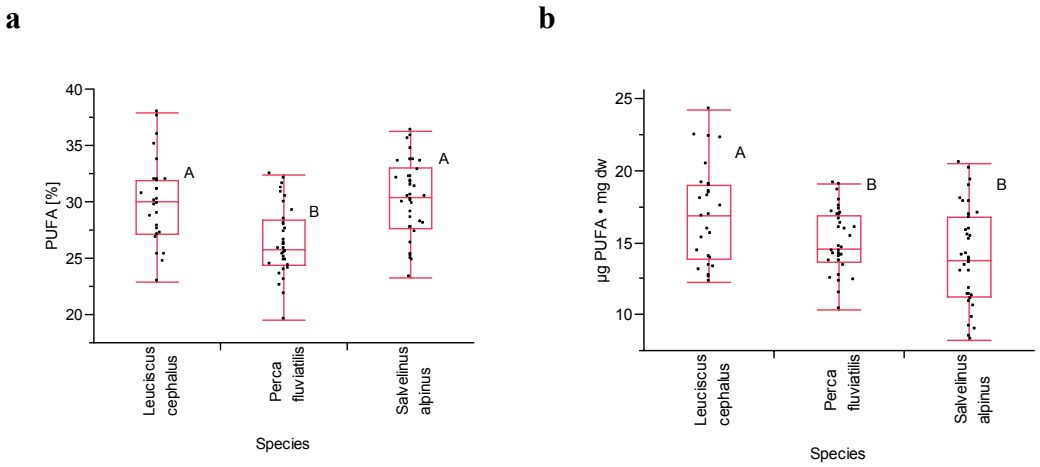


**Figure 50:** *Differences between three fish species from Lake Bohinj (Slovenia) in;* ***a)*** *relative proportions of PUFA (ANOVA and Tukey-Kramer HSD, $R^2$= 0.13, DF= 100, F= 7.50, P= 0.0009,* ***b)*** *amounts of PUFA (ANOVA and Tukey-Kramer HSD, $R^2$= 0.23, DF= 100, F= 15.10, P< 0.0001). Different letters above data groups mean that groups are significantly different (p< 0.05).*

**Table 8:** *Differences between three fish species from Lake Bohinj (Slovenia) in FA groups.*

| [mean ± SD] | *S. alpinus* | | *L. cephalus* | | *P. fluviatilis* | |
|---|---|---|---|---|---|---|
| | [µg · mg$^{-1}$ dw] | [%] | [µg · mg$^{-1}$ dw] | [%] | [µg · mg$^{-1}$ dw] | [%] |
| ω3 | 9.4 ± 2.5 | 20.5 ± 3.1 | 6.9 ± 1.0 | 12.8 ± 3.6 | 9.4 ± 1.7 | 16.6 ± 2.5 |
| ω6 | 4.5 ± 1.1 | 9.8 ± 1.4 | 10.0 ± 3.1 | 17.2 ± 2.1 | 5.5 ± 0.7 | 9.8 ± 1.5 |
| SAFA | 21.9 ± 5.4 | 46.5 ± 5.4 | 20.1 ± 3.7 | 35.7 ± 4.1 | 22.3 ± 5.4 | 39.4 ± 8.6 |
| MUFA | 10.5 ± 1.3 | 21.9 ± 1.1 | 20.7 ± 1.5 | 34.1 ± 1.3 | 20.0 ± 1.3 | 34.1 ± 1.1 |
| PUFA | 14.0 ± 3.3 | 30.4 ± 3.1 | 16.7 ± 3.4 | 30.0 ± 3.8 | 15.0 ± 2.1 | 26.4 ± 3.0 |
| Total FAME | 46.5 ± 12.4 | / | 57.9 ± 16.2 | / | 57.4 ± 10.3 | / |

3.3.4.3 Intraspecific seasonal differences

**Chub (*Leuciscus cephalus*)**

The amounts of total lipids [%] were relatively constant in *L. cephalus* throughout the season, averaging at 10.16 ± 2.04 % (fig. 51). Consequently, the amounts of FAME were not varying either (ANOVA, $R^2$= 0.23 DF= 26, F= 1.64, P= 0.19).

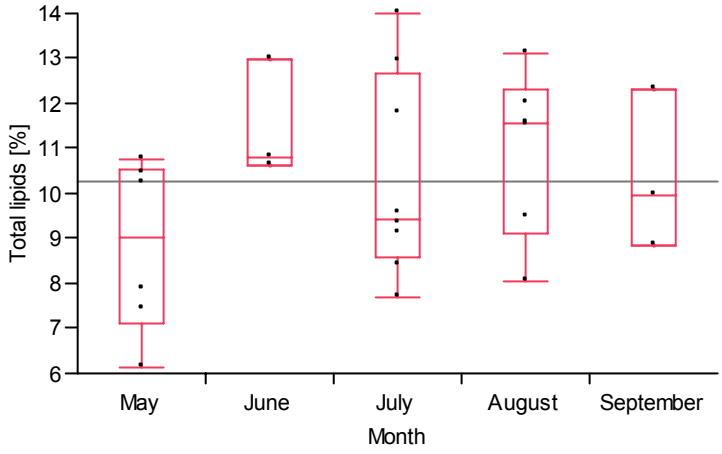


**Figure 51:** *Seasonal dynamics of total lipids [%] in* L. cephalus *from Lake Bohinj (Slovenia); (ANOVA and Tukey-Kramer HSD, $R^2$= 0.187, DF= 26, F= 1.20, P> 0.05). Horizontal line is presenting the grand mean.*

A drop in UI (fig. 52 a) was detected from June onwards and it correlated with the increase of amounts of SAFA (fig. 52 b) and with a decrease of relative proportions of ω3 FA and PUFA (fig. 52 c, d). No significant differences (P> 0.05) within a season were observed in other FA classes regarding their degree of unsaturation.

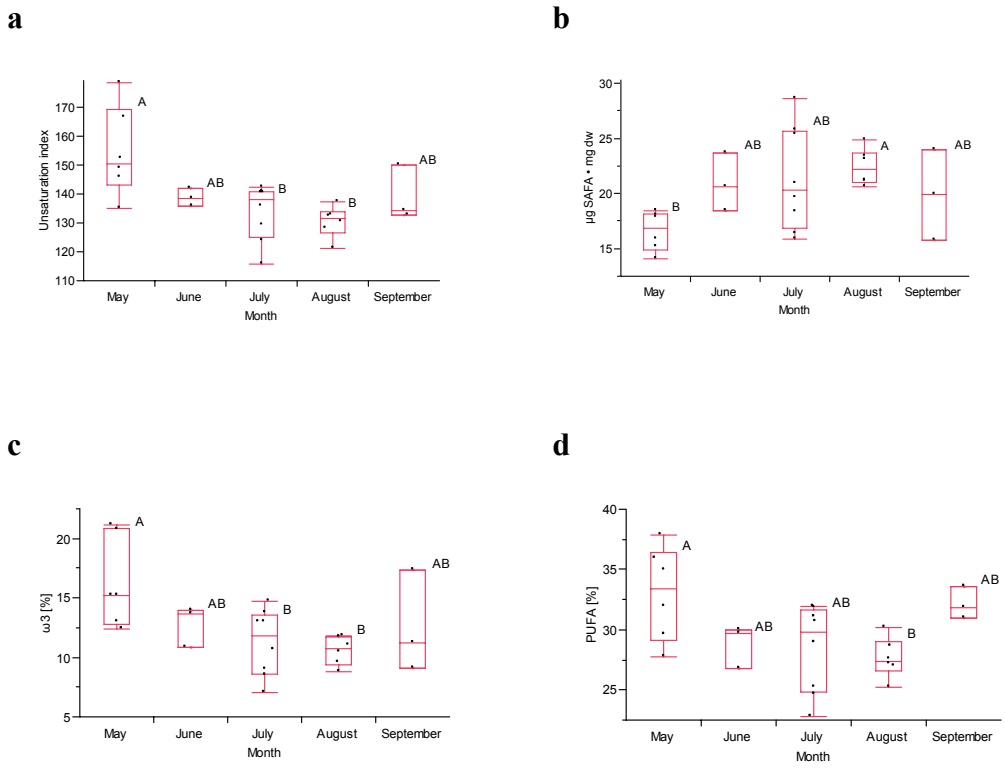


**Figure 52:** *Seasonal dynamics of fatty acids in* L. cephalus *from Lake Bohinj (Slovenia) in;* ***a)*** *unsaturation index (ANOVA and Tukey-Kramer HSD, $R^2$= 0.48, DF= 26, F= 4.9, P= 0.0055),* ***b)*** *amounts of saturated fatty acids (ANOVA and Tukey-Kramer HSD, $R^2$= 0.341, DF= 26, F= 2.72, P= 0.05),* ***c)*** *relative proportions of ω3 [%] (ANOVA and Tukey-Kramer HSD, $R^2$= 0.40, DF= 26, F= 3.62, P= 0.02),* ***d)*** *relative proportions of poly-unsaturated fatty acids [%] (ANOVA and Tukey-Kramer HSD, $R^2$= 0.39, DF= 26, F= 3.4, P= 0.02). Different letters above data groups mean that groups are significantly different (p< 0.05).*

**Perch (*Perca fluviatilis*)**

There was no significant intra-seasonal changes of UI in *P. fluviatilis* (ANOVA, $R^2$= 0.068, DF= 35, F= 0.73, P> 0.05), amounts of ω6 (ANOVA, $R^2$= 0.139, DF= 35, F= 1.61, P> 0.05), amounts of MUFA (ANOVA, $R^2$= 0.113, DF= 35, F= 1.28, P> 0.05), and relative proportions of MUFA (ANOVA, $R^2$= 0.03, DF= 35, F= 0.35, P> 0.05), relative proportions of SAFA (ANOVA, $R^2$= 0.04, DF= 35, F= 0.49, P> 0.05) and relative proportions of PUFA (ANOVA, $R^2$= 0.04, DF= 35, F= 0.52, P> 0.05).

However, a significant increase in total lipids [%] was detected across the season. In June the relative mean value of total lipids in *P. fluviatilis* was 9.7 ± 1.7 % with a peak in August (12.4 ± 1.6 %) followed by a decrease in September (10.4 ± 2.4 %) (fig. 53 a). The overall values of total lipids [%] corresponded to the values on the overall amounts of FAME detected in the fish (fig. 53 b). This corresponded to the gains in amounts of ω3 FA (fig. 54 a), gains of SAFA (fig. 54 b) and PUFA (fig. 54 c) and in a parallel a drop of ω6 FA (fig. 54 d).

A significant difference was detected in Fulton's K in August and September. Data on length and mass of fish caught revealed that representatives in that period were significantly lighter (ANOVA and Tukey-Kramer HSD, $R^2$= 0.325, DF= 35, F= 4.83, P= 0.007), at the same length (ANOVA, $R^2$= 0.147, DF= 35, F= 1.73, P> 0.05) compared to representatives caught in June and July (fig. 55).

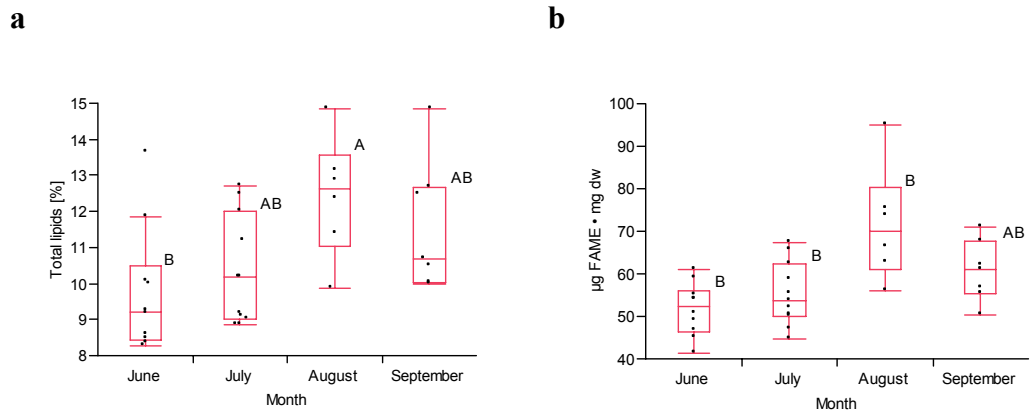


**Figure 53:** *Seasonal dynamics of some lipids in* P. fluviatilis *from Lake Bohinj (Slovenia):* ***a)*** *total lipids [%] (ANOVA and Tukey-Kramer HSD, $R^2$= 0.255, DF= 35, F= 3.54, P= 0.02),* ***b)*** *amounts of fatty acid methyl esters (ANOVA and Tukey-Kramer HSD, $R^2$= 0.43, DF= 35, F= 7.61, P= 0.0006). Different letters above data groups mean that groups are significantly different (p< 0.05).*

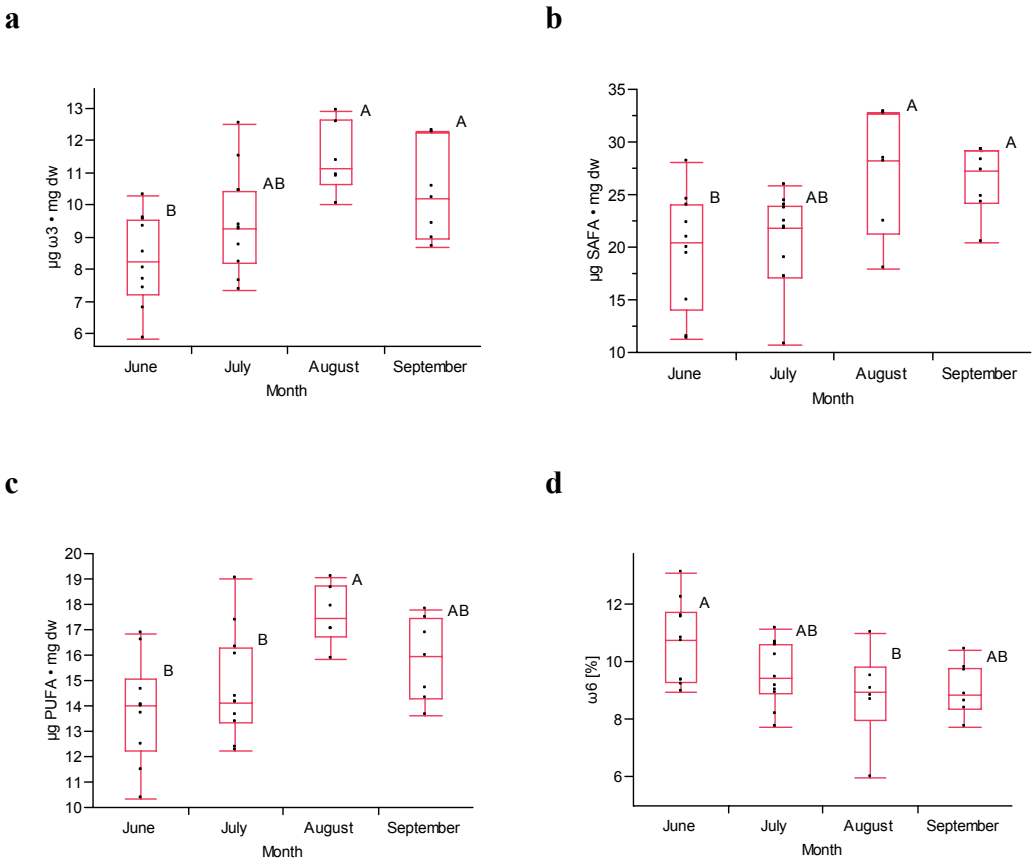


**Figure 54:** *Seasonal dynamics of some lipid classes in* P. fluviatilis *from Lake Bohinj (Slovenia):* ***a)*** *amounts of ω3 (ANOVA and Tukey-Kramer HSD, $R^2$= 0.397, DF= 35, F= 6.60, P= 0.001),* ***b)*** *amounts of saturated fatty acid (ANOVA and Tukey-Kramer HSD, $R^2$= 0.320, DF= 35, F= 4.72, P= 0.008),* ***c)*** *amounts of poly-unsaturated fatty acid (ANOVA and Tukey-Kramer HSD, $R^2$= 0.397, DF= 35, F= 6.60, P= 0.001),* ***d)*** *relative proportions of ω6 (ANOVA and Tukey-Kramer HSD, $R^2$= 0.256, DF= 35, F= 3.44, P= 0.02). Different letters above data groups mean that groups are significantly different (p< 0.05).*

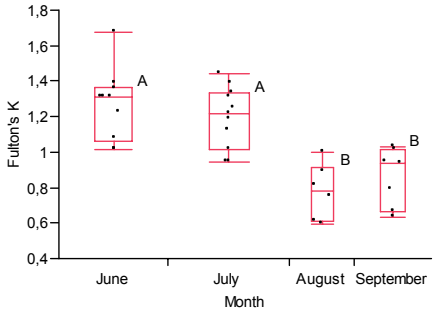


**Figure 55:** *Seasonal dynamics of Fulton's K of in* P. fluviatilis *from Lake Bohinj (Slovenia). (ANOVA and Tukey-Kramer HSD, $R^2$= 0.59, DF= 35, F= 14.88, P< 0.0001). Different letters above data groups mean that groups are significantly different (p< 0.05).*

## Arctic char (*Salvelinus alpinus*)

Total lipids in *S. alpinus* increased from spring till autumn. The lowest values were detected in May (mean 7.2 ± 1.2 %) and June (mean 6.7 ± 2.1 %) and significantly higher ones were detected in the following months with a peak in September (mean 11.5 ± 2.0 %) (fig. 56 a). Along with the total lipids [%], a significant increase of some lipid classes were detected as season progressed; like SAFA (fig. 56 b), PUFA (fig. 56 c), FAME (fig. 56 d), ω3 (fig. 57 a) and ω6 (fig. 57 b).

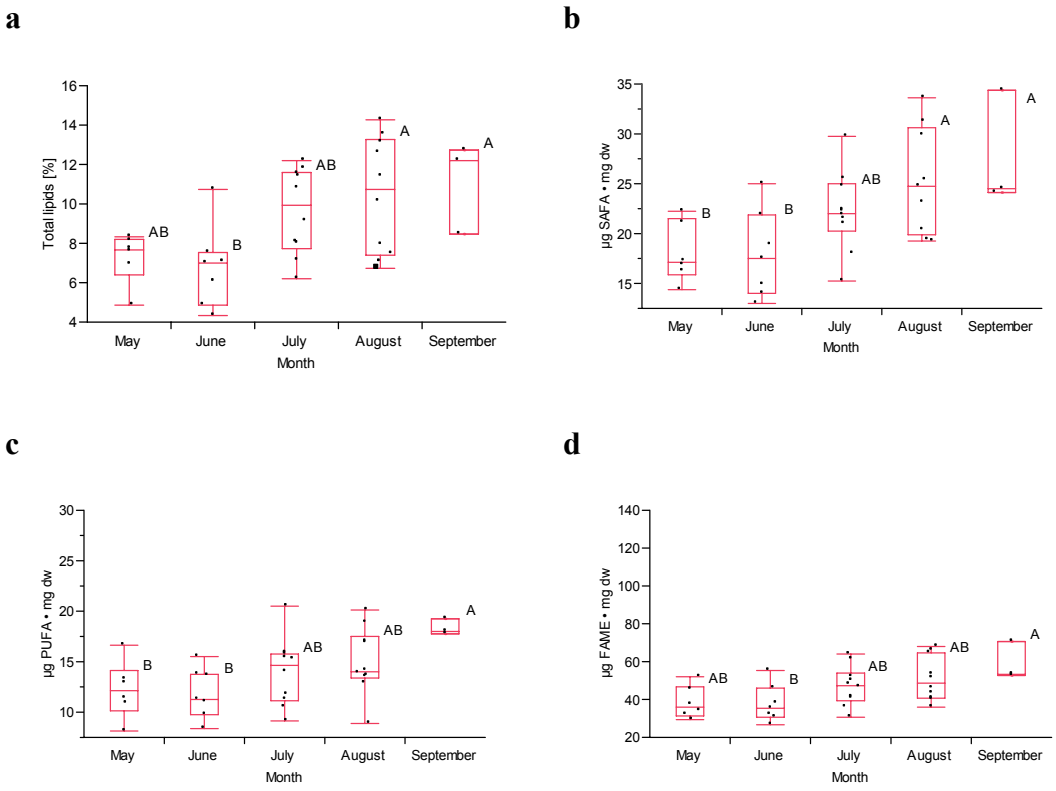


**Figure 56:** *Seasonal dynamics of lipids of* S. alpinus *in Lake Bohinj (Slovenia) in:* ***a)*** *total lipids [%] (ANOVA and Tukey-Kramer HSD, $R^2$= 0.535, DF= 37, F= 8.06, P= 0.0002),* ***b)*** *amounts of saturated fatty acid (ANOVA and Tukey-Kramer HSD, $R^2$= 0.351, DF= 37, F= 4.20, P= 0.007),* ***c)*** *amounts of poly-unsaturated fatty acid (ANOVA and Tukey-Kramer HSD, $R^2$= 0.357, DF= 37, F= 4.17, P= 0.008),* ***d)*** *amounts of fatty acid methyl esters (ANOVA and Tukey-Kramer HSD, $R^2$= 0.354, DF= 37, F= 4.10, P= 0.008). Different letters above data groups mean that groups are significantly different (p< 0.05).*

***a*** ***b***

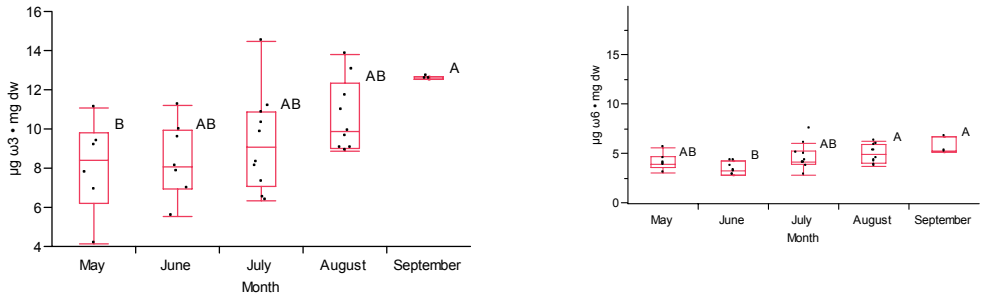


**Figure 57:** *Seasonal dynamics of lipids of* S. alpinus *in Lake Bohinj (Slovenia) in:* ***a)*** *amounts of ω3 (ANOVA and Tukey-Kramer HSD, $R^2$= 0.327, DF= 37, F= 3.77, P= 0.012),* ***b)*** *amounts of ω6 (ANOVA and Tukey-Kramer HSD, $R^2$= 0.32, DF= 37, F= 3.6, P= 0.01). Different letters above data groups mean that groups are significantly different (p< 0.05).*

No differences were detected in Fulton's K (ANOVA, $R^2$= 0.04, DF= 37, F= 0.38, P> 0.05), UI (ANOVA, $R^2$= 0.14, DF= 37, F= 1.32, P> 0.05), amounts of MUFA (ANOVA, $R^2$= 0.070, DF= 37, F= 0.58, P> 0.05), relative proportions of MUFA [%] (ANOVA, $R^2$= 0.081, DF= 37, F= 0.64, P> 0.05), relative proportions of ω3 (ANOVA, $R^2$= 0.125, DF= 37, F= 1.03, P> 0.05), relative proportions of PUFA (ANOVA, $R^2$= 0.119, DF= 37, F= 0.98, P> 0.05) and relative proportions of SAFA (ANOVA, $R^2$= 0.005, DF= 37, F= 0.03, P> 0.05) in *S. alpinus* along the season.

### 3.3.5 Comparing lipids along a food web

The average total lipids [%] were significantly higher in zooplankton (all zooplankton pooled) (50.2 ± 17.1 %) **[A],** as compared to fish (all fish species pooled) (9.9 ± 2.4 %) **[B]** or seston 11.6 ± 2.6 % **[B]** (ANOVA and Tukey-Kramer HSD, $R^2$= 0.77, DF= 202, F= 303, P< 0.0001) and fig. 58.

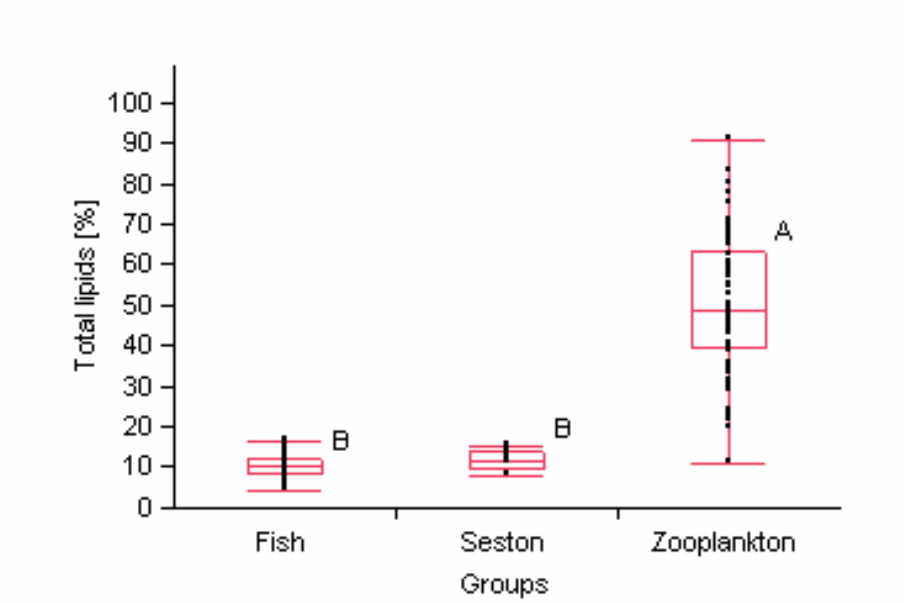


**Figure 58:** *Comparing total lipids of seston, zooplankton and fish species from Lake Bohinj (Slovenia); (ANOVA and Tukey-Kramer HSD, $R^2$= 0.76, DF= 177, F= 289.0, P< 0.0001). Different letters above data groups mean that groups are significantly different (p< 0.05).*

Similarly, the amounts of corresponding FA classes are significantly higher in zooplankton compared to seston (ω3 zooplankton: 29.9 ± 11.7 %, seston: 14.4 ± 3.8 %, PUFA zooplankton: 38.6 ± 5.4 %, seston: 24.4 ± 5.4 %) and fish (figs. 59 a, b, 60) with an exceptions. The relative proportions of SAFA (seston: 53.0 ± 5.2 %, zooplankton: 42.7 ± 8.5 %) and relative proportions of ω6 (seston: 10.1 ± 2.6 %, zooplankton: 8.6 ± 3.7 %), were higher in seston than in zooplankton. No differences were detected in the relative proportions of MUFA between seston and zooplankton (t-ratio= 2.41, DF= 25, P> 0.05).

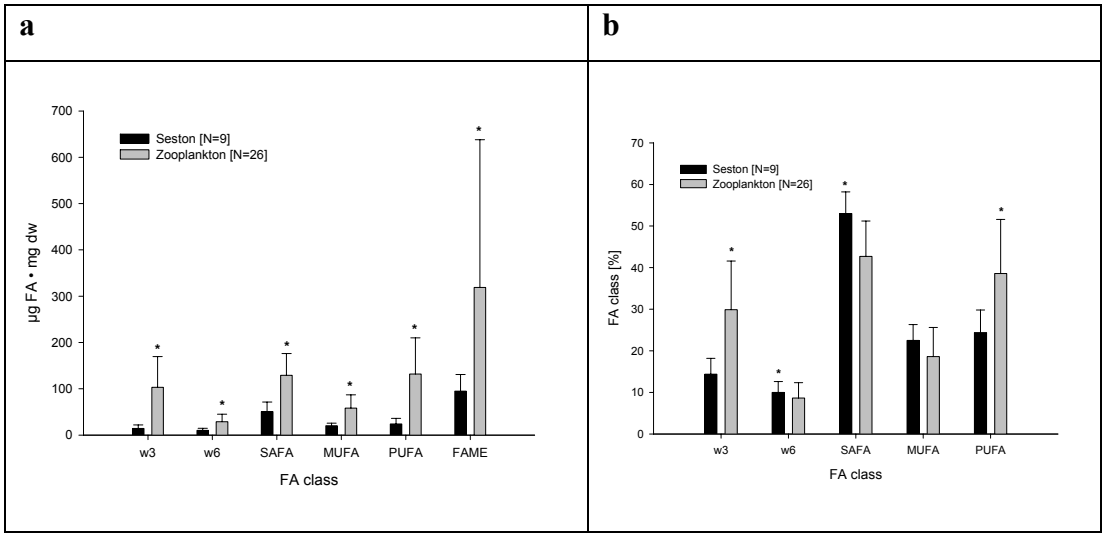


**Figure 59:** *Comparing fatty acids classes of seston and zooplankton (Cladocera and Copepoda pooled) from Lake Bohinj (Slovenia) in:* ***a)*** *amounts of fatty acids classes,* ***b)*** *relative proportions [%] of fatty acids class. Significant differences (t-test, P< 0.05) between FA classes are denoted with a star above the vertical bar which means are higher. Vertical error bars represent ± SD.*

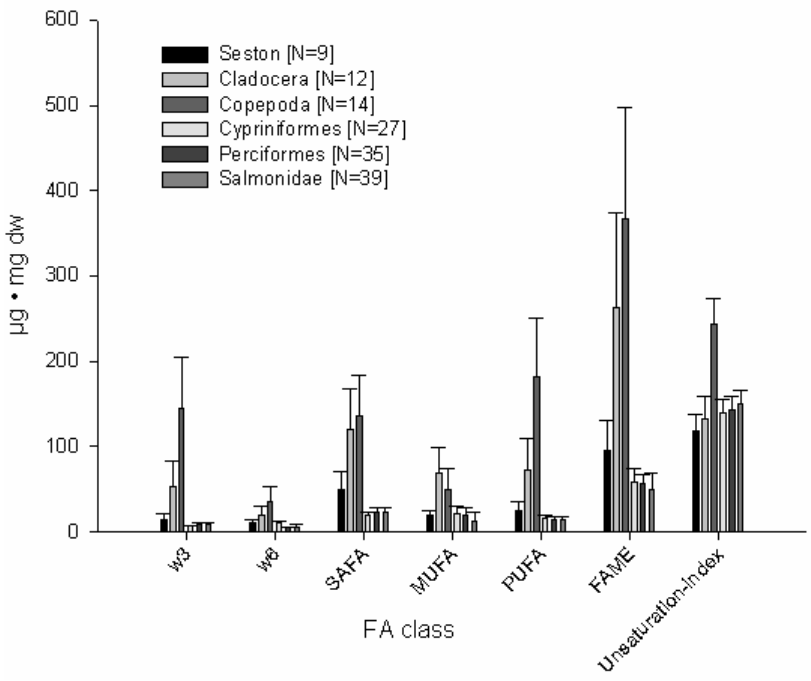


**Figure 60:** *Comparing fatty acids classes of seston, zooplankton and fish from Lake Bohinj (Slovenia). Vertical error bars represent ± SD.*

**Table 9:** *Amounts* [µg • mg dw] *and relative proportions [%] of essential fatty acids (EFA) in examined seston and taxa from Lake Bohinj (Slovenia).*

| | [µg • mg dw] | [%] | [µg • mg dw] | [%] | [µg • mg dw] | [%] | [µg • mg dw] | [%] | [µg • mg dw] | [%] | [µg • mg dw] | [%] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Seston | | Cladocera | | Copepoda | | Cypriniformes | | Perciformes | | Salmonidae | |
| LIN | 3.4 ± 1.7 | 3.4 ± 1.7 | 10.4 ± 5.8 | 3.8 ± 1.7 | 27.3 ± 15.9 | 6.6 ± 3.6 | 5.9 ± 2.9 | 1.2 ± 0.9 | 1.9 ± 0.7 | 1.1 ± 0.4 | 2.4 ± 0.4 | 1.1 ± 0.4 |
| α - LA | 3.3 ± 1.7 | 3.4 ± 0.6 | 13.1 ± 7.2 | 4.9 ± 1.7 | 24.0 ± 15.5 | 5.3 ± 3.2 | 0.9 ± 0.3 | 1.8 ± 0.5 | 1.5 ± 0.6 | 1.8 ± 0.5 | 1.8 ± 0.9 | 2.1 ± 0.9 |
| ARA | 2.3 ± 1.8 | 2.4 ± 1.2 | 9.5 ± 4.1 | 3.1 ± 1.6 | 5.3 ± 2.5 | 1.5 ± 0.6 | 0.6 ± 0.0 | 1.0 ± 0.3 | 0.6 ± 0.1 | 1.3 ± 0.9 | 0.5 ± 0.1 | 1.2 ± 0.4 |
| EPA | 2.4 ± 1.8 | 2.5 ± 1.3 | 40.6 ± 22.1 | 13.2 ± 3.7 | 42.6 ± 22.3 | 11.4 ± 3.6 | 0.5 ± 0.0 | 0.9 ± 0.3 | 0.5 ± 0.2 | 0.9 ± 0.2 | 0.5 ± 0.1 | 1.0 ± 0.4 |
| DHA | 4.6 ± 2.0 | 4.8 ± 1.2 | 2.6 ± 3.0 | 0.8 ± 0.8 | 83.6 ± 24.5 | 22.1 ± 3.9 | 4.2 ± 1.3 | 7.5 ± 2.7 | 5.8 ± 1.5 | 10.1 ± 2.5 | 5.4 ± 1.6 | 11.7 ± 3.6 |

## 3.4 Polymethylene interrupted fatty acids (PMI-FA)

### 3.4.1 Origin of PMI-FA from the lake

Polymethylene interrupted fatty acids (PMI-FA) were not detected in samples of seston from Lake Ontario (n= 3) and Hamilton Harbor (n= 3) or from other sources, including various laboratory cultures of phytoplankton spp. (n= 15) and zooplankton species, including Cladocera and Copepoda. PMI-FA were detected only in macroinvertebrates and detected amounts and proportions of PMI-FA were higher in *Diporeia* spp. compared to *Mysis* spp. and were averaging at 0.06 ± 0.01 µg PMI-FA • $mg^{-1}$ dw; 0.04 ± 0.01 µg PMI-FA • $mg^{-1}$ dw and 0.1 ± 0.01 % PMI-FA • $mg^{-1}$ dw; 0.06 ± 0.02 % PMI-FA, respectively (fig. 61 a, b).

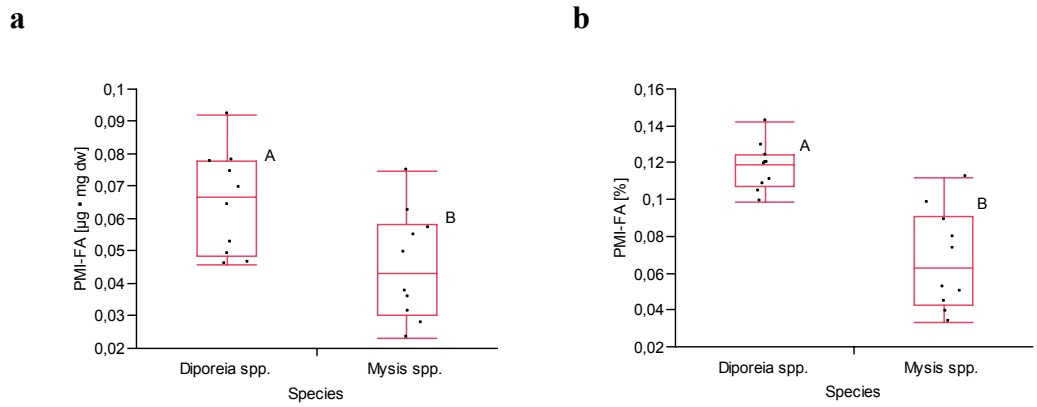


**Figure 61:** *Amounts of polymethylene interrupted fatty acids (PMI-FA) in macroinvertebrates from Lake Huron (Canada):* ***a)*** *total amounts of PMI-FA (t-test, t-ratio= - 2.66, DF= 18, P= 0.0158),* ***b)*** *relative proportions of PMI-FA (t-test, t-ratio= -5.33, DF= 18, P< 0.0001). Different letters above data groups mean that groups are significantly different (p< 0.05).*

PMI-FA were detected in the invasive mussels as well as in native mussels from the Laurentian Great Lakes. Highest amounts of PMI-FA were detected in *D. polymorpha* from Hamilton Harbon (Lake Ontario) and were averaging at 2.0 ± 0.04 µg PMI-FA • $mg^{-1}$ dw which represented on average of 6.5 ± 0.4 % of all FAME (fig. 62 a, b). There were no significant differences between the same species invading different lakes (fig. 62 a, b) and neither between both co-occurring native

mussels. However, significant differences were detected in the amounts and proportions of PMI-FA between different body parts of the mussels examined; significantly higher amounts of PMI-FA were detected in the foot (fig. 63 a, b) of both native molluscs (*L. siliquoidea* (0.53 ± 0.03 µg PMI-FA • $mg^{-1}$ dw ) and *L. cardium* (0.90 ± 0.04 µg PMI-FA • $mg^{-1}$ dw) compared to the amounts of PMI-FA in mantle (*L. siliquoidea* 0.85 ± 0.03 µg PMI-FA • $mg^{-1}$ dw, *L. cardium* 0.64 ± 0.04 µg PMI-FA • $mg^{-1}$ dw). Similarly the amounts of PMI-FA were higher in the mantle and foot (pooled) *vs.* whole body of invasive species *D. rostriformis bugensis* (mantle and foot 1.29 ± 0.02 µg PMI-FA • $mg^{-1}$ dw *vs.* whole 1.17 ± 0.02 µg PMI-FA • $mg^{-1}$ dw ) (fig. 63 c).

The only significant difference in terms of the relative proportions of PMI-FA was detected by comparing foot and mantle of native *L. siliquoidea* (mantle 6.92 ± 0.37 %, foot 5.35 ± 0.37 %) (fig. 63 d).

Freshwater mussels exhibited lower amounts and relative proportions of PMI-FA than the marine mussel (*M. edulis*), which served as the laboratory standard (see materials and methods). Relative proportions of PMI-FA in the marine mussel were averaging at 7.98 ± 0.11 % of total FAME which represented on average 2.75 ± 0.02 µg PMI-FA • $mg^{-1}$ dw.

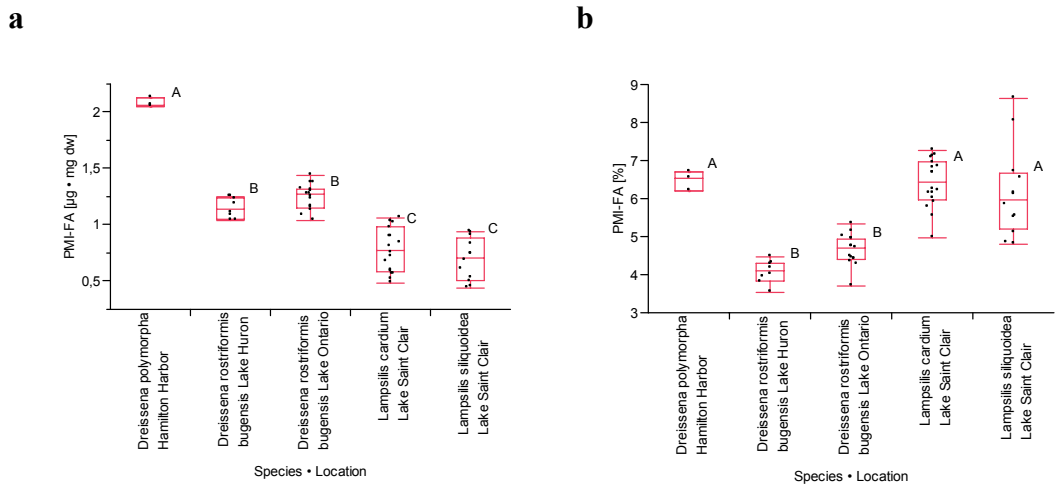


**Figure 62:** *Polymethylene interrupted fatty acids (PMI-FA) in freshwater mussels from the Great Lakes (Canada):* ***a)*** *amounts of PMI-FA (ANOVA, $R^2$= 0.83, DF= 55, F= 64.9, P< 0.0001),* ***b)*** *relative proportions [%] of PMI-FA (ANOVA, $R^2$= 0.66, DF= 55, F= 24.8, P< 0.0001). Different letters above data groups mean that groups are significantly different (p< 0.05).*

Several differences in FA profiles of PMI-FA of freshwater mussel species examined were detected. The most prominent PMI-FA in *D. polymorpha* was C22:2:n-9c (fig. 64). The ratio between the later and C22:3n-6c was different in *D. rostriformis bugensis* (≈ 2.7 µg PMI-FA • $mg^{-1}$ dw) and both native *L. siliquoidea* (≈ 2.2 µg PMI-FA • $mg^{-1}$ dw) and *L. cardium* (≈ 2.9 µg PMI-FA • $mg^{-1}$ dw). We tentatively identified novel PMI-FA 20:2 PMID, which was not detected in *D. polymorpha* and neither in *M. edulis* (laboratory standard), thus being unique for *D. rostriformis bugensis* and both native *L. siliquoidea* and *L. cardium*.

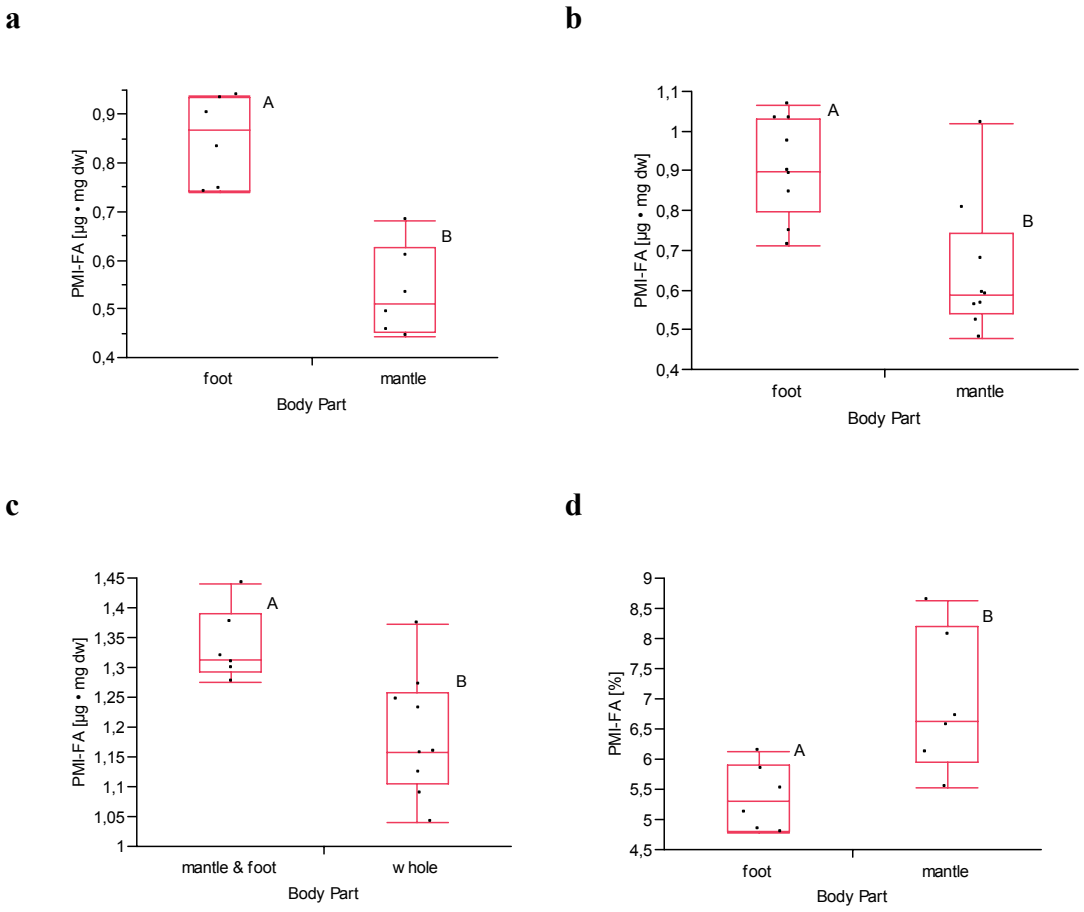


**Figure 63:** *Polymethylene interrupted fatty acids (PMI-FA) in different body parts of the mussels from the Great Lakes (Canada):* ***a)*** *amounts of PMI-FA in* L. siliquoidea *foot vs. mantle (t-test, t-ratio= -5,91, DF= 11, P= 0.0001),* ***b)*** *amounts of PMI-FA in* L. cardium *PMI-FA (t-test, t-ratio= -3.76, DF= 17, P= 0.0017),* ***c)*** *amount of PMI-FA in* D. rostriformis bugensis *(Lake Ontario) (t-test, t-ratio= -3.18, DF= 14, P = 0.0072),* ***d)*** *relative proportions [%] of PMI-FA in* L. siliquoidea *Lake Saint Clair different body parts (t-test, t-ratio= 2.93, DF= 11, P= 0.01).* ***Different letters above data groups*** *mean that groups are significantly different (p< 0.05).*

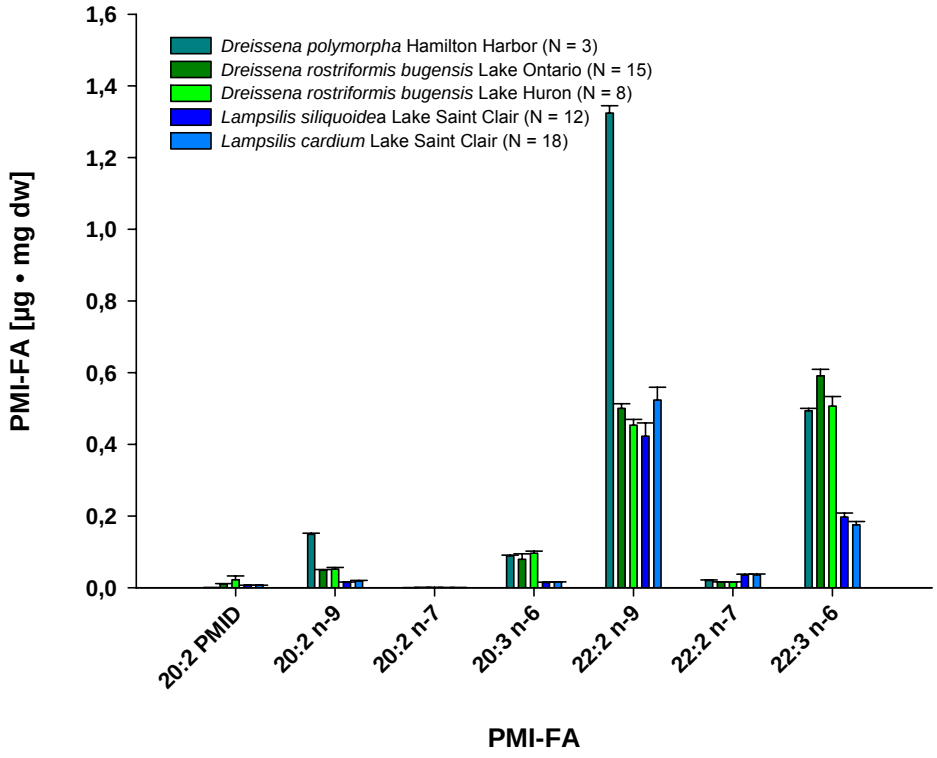


**Figure 64:** Fatty acid (*FA) profiles of polymethylene interrupted fatty acids (PMI-FA) in freshwater mussel species from the Great Lakes (Canada) (vertical lines represents ± SE).*

### 3.4.2 Trophic transfer of PMI-FA

#### 3.4.2.1 Presence of PMI-FA in fish from Hamilton Harbor (Lake Ontario)

The amounts of PMI-FA in all fish species analyzed were approximately twenty five-fold lower than in mussels (tab. 9). However, there were differences between fish species. Planktivorous *A. pseudoharengus* has the lowest amounts and proportions of PMI-FA compared to omnivorous *A. nebulosus* and other, predominantly predatory species, including *L. gibbosus, M. salmoides* and *N. atherinoides* (figs. 65, 66 a, b) in Hamilton Harbor (Lake Ontario). Fish also differentiate on the amounts of 22:3n-6 PMI-FA, being the highest in *N. atherinoides* and lowest in *L. gibbosus* (ANOVA and Tukey-Kramer HSD, $R^2$= 0.25, DF= 42, F= 2.95, P= 0.033).

**Table 10:** *Average amounts of polymethylene interrupted fatty acids (PMI-FA) detected in freshwater mussel and in fish species from Hamilton Harbor (Lake Ontario) and Lake Michigan.*

| | [µg PMI-FA • $mg^{-1}$ dw] Mean ± SD | [ PMI-FA - % ] Mean ± SD |
|---|---|---|
| Freshwater mussels examined (pooled) | 1.00 ± 0.3 | 5.54 ± 1.2 |
| Fish from Hamilton Harbor (pooled) | 0.04 ± 0.02 | 0.19 ± 0.11 |
| Fish from Lake Ontario (pooled) | 0.06 ± 0.04 | 0.20 ± 0.12 |
| Fish from Lake Michigan (pooled) | 0.03 ± 0.02 | 0.11 ± 0.08 |

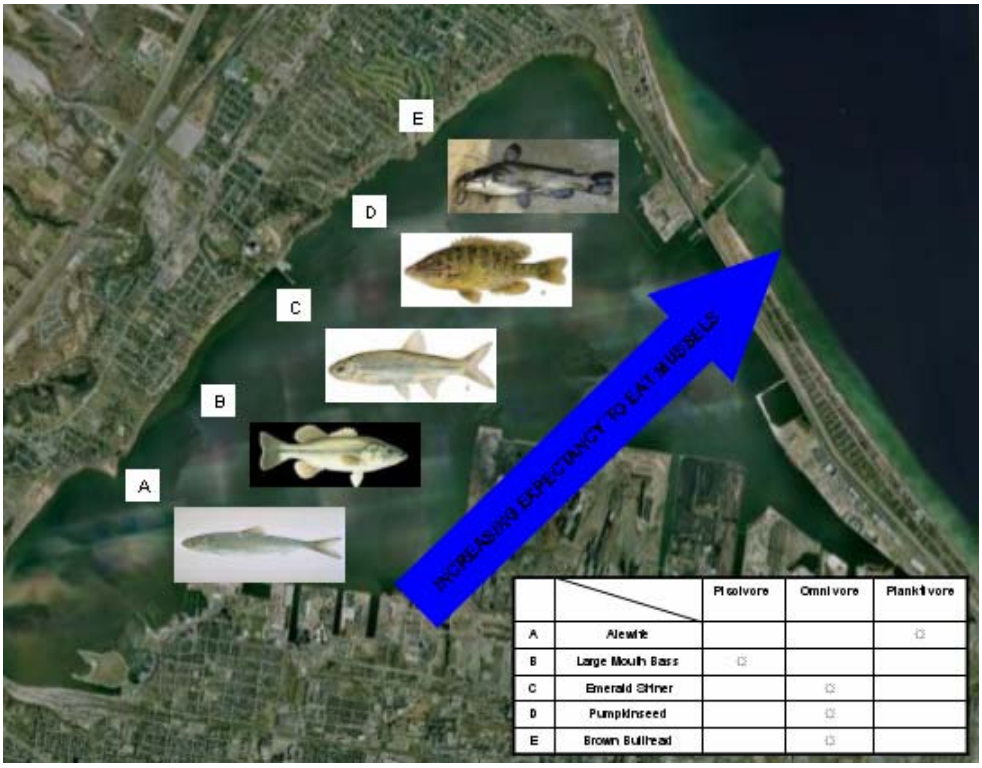


**Figure 65:** *Examined fish species from Hamilton Harbor (Lake Ontario).*

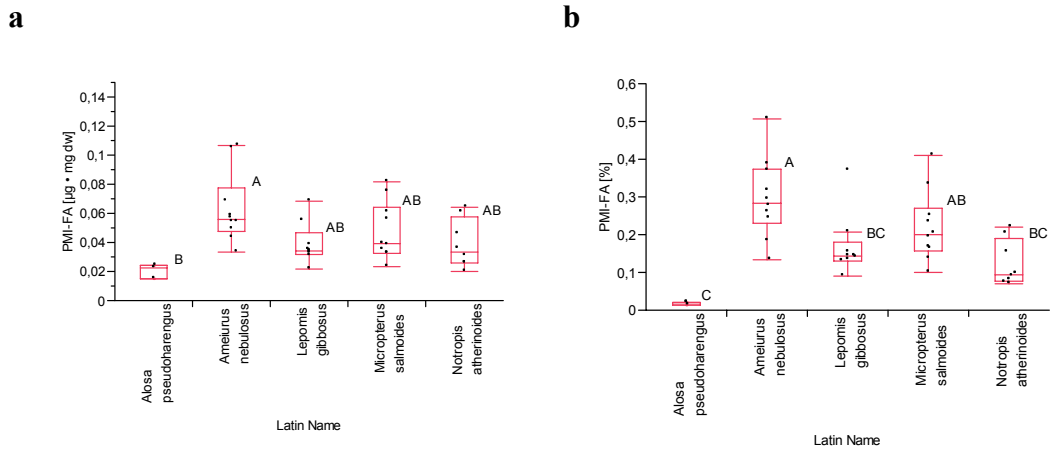


**Figure 66:** *Polymethylene interrupted fatty acids (PMI-FA) in different fish species from Hamilton Harbor (Lake Ontario);* ***a)*** *amounts of PMI-FA (ANOVA and Tukey-Kramer HSD, $R^2$= 0.31, DF= 42, F= 3.98, P= 0.0091),* ***b)*** *relative proportions of PMI-FA (ANOVA and Tukey-Kramer HSD, $R^2$= 0.49, DF= 42, F= 8.51, P< 0.0001). Different letters above data groups mean that groups are significantly different (p< 0.05).*

PMI-FA in *C. clupeaformis* (lake whitefish) from Lake Ontario's Point Pelee were 2-fold higher compared to *C. clupeaformis* inhabiting the area of the Big Bay in both, amounts (fig. 67 a) and relative proportions of PMI-FA (t-test, t-ratio= 2.32, DF= 23,

P= 0.029). Fish inhabiting the Point Pelee area exhibited also significantly higher amounts of total lipids [%] (fig. 67 b).

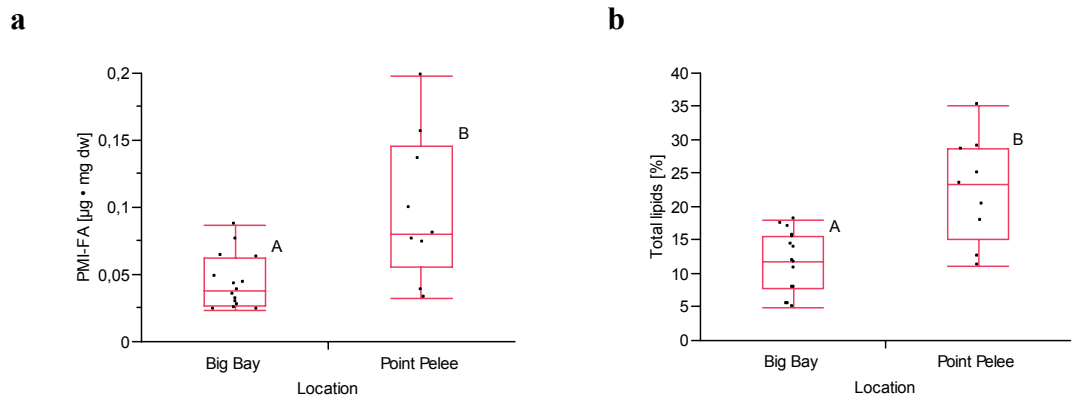


**Figure 67:** *Polymethylene interrupted fatty acids (PMI-FA) in different locations within Lake Ontario:* ***a)*** *amounts of PMI-FA (t-test, t-ratio= 3.55, DF=23, P= 0.0018),* ***b)*** *total lipids in fish between different sampling locations (t-test, t-ratio= 3.55, DF= 22, P= 0.0018). Different letters above data groups mean that groups are significantly different (p< 0.05).*

### 3.4.2.2 PMI-FA content of *C. clupeaformis* in Lake Michigan

Analysis of PMI-FA on *C. clupeaformis* originating from different locations in Lake Michigan revealed that *C. clupeaformis* from Bailey's Harbor and Big Bay de Noc exhibit significantly lower amounts of PMI-FA compared to specimens within the Naubinway region, both in quantitative and qualitative manner (fig. 68 a, b). Moreover, the PMI-FA content in all of the specimens of Lake Michigan's *C. clupeaformis* were not related to gender. On the basis of the amount of 20:2n-9 PMI-FA there were significant differences also between individuals inhabiting the Naubinway region compared with those from the Big bay de Noc (ANOVA and Tukey-Kramer HSD, $R^2$= 0.33, DF= 57, F= 14.49, P< 0.0001).

**a** **b**

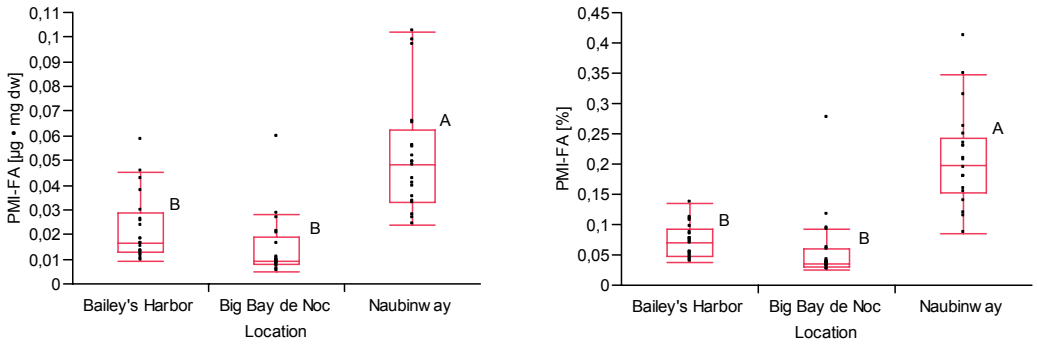


**Figure 68:** *Polymethylene interrupted fatty acids (PMI-FA) in fish from Lake Michigan:* ***a)*** *amounts of PMI-FA in* C. clupeaformis *from Lake Michigan (ANOVA and Tukey-Kramer HSD, $R^2$= 0.47, DF= 59, F= 26.1, P< 0.0001),* ***b)*** *relative proportions of PMI-FA (ANOVA and Tukey-Kramer HSD, $R^2$= 0.58, DF= 59, F= 39.7, P <0.0001). Different letters above data groups mean that groups are significantly different (p< 0.05).*

# 4 DISCUSSION

## 4.1 Sample preparation procedures

Numerous papers on lipid extraction from organisms and tissues exist, including those specialized on aquatic invertebrates where a special micro method was developed (Folch, 1956; Bligh and Dyer, 1959; Parrish, 1985; Booij and Van den Berg, 1994; Honeycutt, 1995; Iverson, 2001). The most frequently employed methods for lipid extraction are after Bligh and Dyer (1959) or/and Folch method (Folch, 1956) with many modifications. Papers suggest that samples containing more than two per cent of lipids should use the Folch method for lipid extraction, which result in significantly higher lipid content compared to using soxhlet extraction or Bligh and Dyer extraction method (Booij and Van den Berg, 1994; Iverson, 2001). As aquatic organisms are rich in lipids (Arts et al., 2009) we used the Folch method for the extraction of lipids.

Some extraction procedures include ultrasound treatment in order to speed up the extraction process by enhancing the hydrolysis of lipids. However, it was demonstrated that this enhances the lipid oxidation (Chemat, 2004). For that reason, ultrasound was not used during sample preparation.

Reduction of non-polar lipids was observed by using freeze drying, with no significant changes for polar lipids (Dunstan et al., 1993). However, by not using freeze drying we would not be able to report the results in quantifiable amounts [µg lipids • $mg^{-1}$ dw] but only on a relative proportional basis [as a per centage]. That would speed up the sample preparation procedures by three to five fold, but with a significant loss of information.

It was demonstrated that the presence of ALC might alter the FAME analysis on GC (Budge et al., 2006). Thus, their removal from the sample before GC analyses is needed in order to obtain clean and verifiable data. In the samples from Lake Bohinj ALC were under detection limits, thus we excluded additional pre-cleaning steps in

sample preparation procedures and did not encounter any problems from this regard.

## 4.2 HPTLC method characteristics

A five step development program with decreasing polarity of mobile phases was required to separate complex lipids of animal or plant origin in this thesis. For separation of lipids from a prepared stock solution, a three step development process would be sufficient: one development in each of the three of the mobile phases being used. However, our analysis revealed that complex lipid mixtures (natural samples) need a more sophisticated development program in order to obtain appropriate resolution of separated compounds.

For the purpose of the thesis the best method for separation of polar lipids was by using a solution of chloroform/methanol/water (65 : 25 : 4, v/v/v). Good separations of our target compounds, PL and AMPL, were obtained by a single development procedure up to a distance of 10 mm (fig. 16). However, polar lipid classes were not resolved (i.e. well separated) in samples of animal origin by using a single development procedure. A second development procedure to the same migration distance enabled the separation of lipid classes of "real" samples without measurable effects on a standard stock solution which was well enough separated within the first development procedure. The advantage of the second development procedure was, that up to now no identified lipid classes within the polar fraction were also well separated (fig. 16). As an alternative possibility, or if a polar lipid fraction is the only interest, is that the only single development procedure is used up to a distance of 30 mm. In that distance all of the polar lipids will be well separated.

The third and fourth development step uses a less polar mobile phase separating the neutral lipids (neutral lipid fraction), which in the vast majority of cases are the most abundant fraction (Arts and Wainman, 1999; Christie, 2003; Arts et al., 2009) which includes ST, ALC, FFA and TAG. The fourth (second development with the second mobile phase) development procedure is needed in order to separate ST and ALC which migration distances are relatively close (see tab. 1 for migration distances).

We are ascribing these additional developments to the complex nature of lipids which are extracted from organisms, compared to the standard stock solution which is comprised of only nine individual compounds.

As with the separated polar fraction of the lipids, several unidentified peaks were detected within the neutral fraction of zooplankton lipids.

The third mobile phase (fifth development) separated the least polar compounds of lipids including KET, WE and HC. The later being just below the solvent front.

Exact volume ratios of mobile phases are mandatory in order to separate lipid classes properly. For example, by using the third mobile phase in the ratio of hexane/diethyl-ether/acetic acid (90 : 10 : 1 v/v/v) instead of suggested (90 : 11 : 1, v/v/v) we were not able to obtain a separation between ALC and ST (by separation of standard stock solution).

Thus, caution and much accuracy is needed when preparing the mobile phases.

Several publications suggest using petrol-ether/diethyl-ether/acetic acid (i.e. 80 : 20 w/o acetic acid v/v) (Varljen et al., 2003), 80 : 20 : 1 (v/v/v) (Johnson and Davenport, 1971, p. 182) as a mobile phase for separating neutral lipids. However, we found no combination that separated all of the neutral lipids including non-polar HC and enabled densitometry – and moreover use a single HPTLC plate. We tested several v/v/v ratios of this mobile phase with no clear results. With a ratio of 80 : 10 : 10 (v/v/v) only ALC were separated, while other compounds were moving with the solvent front. By using a mobile phase with a ratio of 80 : 20 : 1.5 (v/v/v) the WE and HC were not well separated. Blurred separation of lipids were obtained also by using a ratio of 85 : 15 : 1 (v/v/v) (no separation from TAG onwards the solvent front), 90 : 10 : 1(v/v/v) by which pairs of ST/ALC and WE/HC were not separated. By increasing the proportions of diethyl-ether in the mobile phase solution from 90 : 10 : 1 (v/v/v) to 90 : 11 : 1(v/v/v), 90: 12 : 1(v/v/v) and 90 : 13 : 1(v/v/v) we were unable to separate ALC and ST, while above this ratio (i.e. 90 : 14 : 1 (v/v/v), 90 : 15 : 1 (v/v/v)) other lipid classes (i.e. HC) were not separated or/and did start to move with the solvent front.

The described method can be effectively used to separate lipid classes from animal and plant origin but caution and accuracy are needed within the whole process.

The assay could be used as an alternative to developed method by Parrish (1987) for separating aquatic lipid classes as the procedure is simpler and hardware needed for analyses is accessible and widespread. The presented assay can be used effectively also for the separation of lipids from i.e. egg yolk, plants and macro invertebrates.

Even though all lipid classes from stock standard solution are well separated, there are a number of differences between separated stock solution and separated lipid classes from biota.

Tripalmitin, a lipid standard, used for quantifying TAG (tab. 1), behaves slightly differently on developed HPTLC plates compared to TAG in the samples taken from biota (comp. figs. 17, 18, 19). The changes occur in RF as well as in the response to the HPTLC plate and thus on the densitogram. An explanation is in the complex nature of TAG originating from biota. TAG, collected directly from live samples (biota) comprise of numerous different fatty acids bound to a glycerol backbone. This is different to the standard used – tripalmitin uses only one type of FA bound to glycerol. Without taking this drawback into account, quantification of TAG would not be possible in real time as there is currently no complex standard solution of TAG on the market. But, as it turned out, the results obtained in lipid class analysis of zooplankton, TAG in particular, agree with published information (Arts, 1992, 1993). Also, we compared two different techniques (i.e. gravimetric measurements of total lipids *vs*. HPTLC separation and quantification of lipid classes and summing them) and did not find significant differences between both methods when seasonal average quantities of total lipids from zooplankton species were analyzed (fig. 25). Thus, the results of both do comply with other studies on this topic (Arts, 1992, 1993; Arts et al., 2009).

Additional support for the correctness of our method is the fact that our data from intra assay accuracy and precision (tab. 2), averaging 6.9 ± 3.6 % for all of the analyzed lipid classes, fit well with Sek (2001). He reported reproducibility being below 12 % for all of compounds separated by his method. We conclude that our methods provide reliable estimates of the compounds analyzed, with detection limits in a ng [$10^{-9}$ g] range (tab. 1) as reported by Vovk and Simonovska (2005) for chloramphenicol (3 ng).

HPTLC assays are by now well accepted and are accurate enough to be used for quantitative purposes as shown by the validation experiment. Various possible applications of the method on different biota revealed that our method separates also other up for now unidentified lipid classes. Therefore, there are possibilities for improvements of the current method or/and re-application of current one for specific needs.

## 4.3 Lipids in the ecosystem of Lake Bohinj (Slovenia)

Lake Bohinj (Slovenia) has distinctive seasonal patterns in rates of primary production reflecting the qualitative and quantitative abundance and dynamics of lipids from zooplankton to fish.

In four zooplankton species examined, TAG were the most abundant lipid class throughout the growing season both in quantitative [μg TAG • $mg^{-1}$ dw] and qualitative manner [%]. Overall values were higher in Copepoda than in Cladocera (fig. 22 a, b), in agreement with Arts (1993) who reported that relative proportions of TAG in Copepoda species (*Leptodiaptomus* sp.) were higher (≈ 60% of total lipids) than in Cladocera (*Daphnia pulex*) (≈ 40%). Thus, the majority of lipids are represented by TAG, which consequently mirrored the seasonal patterns in total lipids and overall, was higher in Copepoda than in Cladocera (fig. 23).

Other studies which were studying the lipid classes of various taxa have revealed that TAG are the principle and most abundant lipid class: from seeds (Yoshida et al., 2004), marine fish (Varljen et al., 2003) or seafood (Copeman and Parrish, 2004) to other benthic invertebrates (Arts et al., 2009).

As TAG are short term energy reserves (i.e. Arts et al., 1992, 1993; Hagen and Auel, 2001; Arts et al., 2009) it is expected that their amounts will be the most variable group. This is indeed to what we find, as we recorded variations of over 100 %, despite the fact that the amounts of total lipids, and thus TAG as a dominating lipid class in zooplankton, are a direct link to dietary input. There is little *de novo* synthesis of FA in biota, excluding primary producers (Goulden and Place, 1990; Arts and Wainman, 1999; Arts et al., 2009). By now it is well accepted that certain FA, their groups or differences between them, can be used as trophic markers and moreover have species specificity or/and are typical of a certain trophic level (i.e. Persson and Vrede, 2006; Smyntek et al., 2008). Sekino (1997) also reported that inter- and intraspecific differences in fatty acid composition exist between Crustacean zooplankton.

We were able to ascribe the amount or/and the relative proportions [%] of TAG of zooplankton to species level (figs. 22 a-d) thus these molecular species might be used as trophic or/and taxonomic markers.

According to Persson and Vrede (2006), phylogenetic origin and/or trophic position explains more of the variation in zooplankton FA content than does the FA content of their food. Despite efforts to explain the variation within each zooplankton group using the variation in seston (or/and phytoplankton) FA content, such relationships are hard to be found due to the species-specific utilization and/or transformation of certain FA (but see Napolitano et al., 1997).

In agreement with the seasonal oscillations of total lipids in zooplankton species analyzed and generally in the water column (also in other benthic communities, see Cavaletto et al., 1996) are our finding regarding total lipids (and thus TAG) in zooplankton. Overall we found that total lipids (and thus TAG) are significantly higher within the growing season than in periods before and after (fig. 21 b). The amounts of total lipids in seston averaged at 11.6 ± 2.7 %, in agreement with Kainz (2004) who reported 12 ± 3.6 % of total lipids for the same size fraction.

However, it was demonstrated that there is only a weak correlation between net primary production and secondary production. For any given level of primary production there is a great variation in the secondary production along trophic levels (McNaughton et al., 1989). So if primary production increases, this is not necessarily correlated to an increase in consumer production (Persson, 2007). Thus much information is lost during the trophic transfer due to species specific metabolic requirements.

Exceptions to the dominative position of TAG compared to other lipid classes are representative of the north temperate marine zooplankton within polar marine ecosystems (Lee, 1971, 2006). High-latitude (marine) zooplankton contains significant amounts of WE (Hagen and Schnack-Schiel, 1996), which can be greater than TAG. According to Lee (2006) they can be as high as high 56 ± 32 % of total lipids and TAG as low as 13 ± 19 % of total lipids. Hagen and Kattner (1998) reported their values to be even higher (66.6%). WE probably originate from feeding on short and intense phytoplankton blooms and they have probably a physiological function too. Lee et al. (2006) has demonstrated that WE due to thermal expansion and compressibility allows copepods to maintain neutral buoyancy while diapausing. Hagen and Kattner (1998) demonstrated on Antarctic euphasiids that WE are rich in C18:1n9c. In contrast, this FA was detected in our samples of zooplankton in significantly higher amounts in Cladocera than in Copepoda (fig. 42 b). This also

complies with overall higher relative proportions of WE in Cladocera as compared to Copepoda (figs. 29, 30), complementing our FAME analysis with HPTLC lipid class analysis. Also, our analyses did not reveal a pronounced seasonality in the amounts of WE in all four zooplankton species. However, several papers reported significant abundance of these lipids (WE) in freshwater species (i.e. Cavaletto et al., 1989) too. It remains unclear why our analysis revealed higher amounts of WE in Cladocera over Copepoda, contrary to what was found for marine Copepoda.

Apart from other, already mentioned functions of WE, they can be hydrolyzed into PUFA and then incorporated into cell membranes maintaining membrane fluidity during winter, during periods of food scarcity or/and dormancy or diapause. Thus in the future, there should be more attention to the physiological effects of WE. A key question should be whether the amount of oxygen and insolation are really the ultimate factors which interrupt dormancy (for details see Hansson et al., 2004). It was demonstrated that WE are highly preserved during starvation and thus are used only in exceptional conditions (Lee et al., 2006). This is in agreement with Olsen (2004) which found significant amounts of ω3 FA within the WE fraction of the lipids, which are highly preserved and exploited conservatively, also during starvation (i.e. Arts et al., 2009) or during laboratory-induced fasting experiments (Mezek et al., 2009b). More detailed analyses of WE would be needed in order to reveal the exact amounts of ω3 within this fractions of the lipids in our analyzed zooplankton species. The amounts of TAG and WE are both storage compounds that allow animals to save energy resources for either for reproduction or over-wintering (Hagen and Schnack-Schiel, 1996). Lee at al. (1971) suggests that relative abundances of WE *vs.* TAG in marine zooplankton are in reversed correlation to each other. However, in zooplankton from Lake Bohinj (Slovenia) there was no correlation between TAG and WE content with any of the four species in any of the given periods. While a majority of Cladocera do not overwinter as adults, the possible explanation for their higher relative proportion of WE is that they invest these (assumable) PUFA rich compounds in ephippia. Also, it is possible that these compounds prolong the periods for Cladocera being an active inhabitant of the water column (before producing ephippia). In addition, we find that ALC, common in marine copepods, were below detection limits (in ng range) in all of our zooplankton samples examined. ALC, if esterified to FA, are structural elements of WE (Hagen

and Auel, 2001). At this moment we do not know whether they are present in non-detectable amounts or are they re-transformed to WE. In the marine environment metabolic pathways exist for such transformations immediately after uptake (Budge et al., 2006).

Cladocera convert certain long chain PUFA, and DHA in particular, into EPA (see fig. 1) (von Elert, 2002), which supports the results of this study. While it was not possible to reveal differences between both taxa (Cladocera and Copepoda) on the basis of the amounts of EPA (fig. 44 b), DHA levels were significantly higher in Copepoda (fig. 43 c, d, tab. 7). A possible reason for the fact that planktonic Cladocera do not overwinter as adults in the temperate zone is a lack of HUFA (actually DHA), which maintain the physiological fluidity of cell membranes at low temperatures. Accumulation of DHA in Copepoda is thus an adaptation for over-wintering in an active life stage (Smyntek et al., 2008). However, these statements are not without constrains as Scott (2002) and Persoon and Vrede (2006) have reported that the high content of DHA in Copepods might be associated with the more sophisticated nerve system of Copepoda compared to other zooplankton (i.e. Cladocera). DHA is particularly associated with neural tissue (Persson and Vrede, 2006), eye structures in particular (Sargent et al., 1993). Copepods have highly developed prey-attack and predator-avoidance strategies which allow them to respond to visual stimuli within milliseconds (Lenz et al., 2000). Laboratory experiments have shown that increasing values of DHA in Cladocera diets did not increase the amounts in the bodies (von Elert, 2002). A hypothesis on species specific metabolism, which only partly reflects the diet holds also for the amounts of the EFA in salmonids (*S. alpinus*). Despite the fact that they feed mainly on Cladocera (in Lake Bohinj) which is poor in DHA, their levels were within the limits found in two other fish species analyzed (tab. 9). This is suggesting that there must be metabolic pathway(s) along with the developed retaining mechanisms for synthesis of EFA (i.e. Bell et al., 1986; Sargent et al., 1989, 1993, Von Elert, 2002; Arts et al., 2009).

In contrast to Copepoda, the amounts of ARA and thus their relative proportions were significantly higher in Cladocera which might give them an advantage over Copepods in higher reproductive potential (Persson and Vrede, 2006; Smyntek et al., 2008).

Pelagic Cladocera which start new populations mainly by hatching from ephippia had significantly higher amounts and proportions of HC compared to Copepoda (figs. 26 a, b, 27). It was demonstrated that squalene (lipid standard on which HC were quantifyed) can function also as a neutral buoyancy regulator (in sharks) (Deprez, 1990). The same role they can play in Cladocera, also, to support their position in the water column.

Concentrations of HC measured in this study are higher than literature data for zooplankton (Arts et al., 1992, 1993) and *Diporeia spp*. (Quingley, 1989). However, one of the conclusions from the environmental study from the Great Lakes (Casey et al., 1976) was that zooplankton exhibited definite HC contamination, probably related to floating micro-tarballs. Thus elevated levels of HC are due to environmental pollution (Napolitano et al., 1995) and these molecular species enter the body of zooplankton (and food web) through infiltration, followed by bioconcentration. In agreement with the fact that elevated levels of HC can be due to pollution is also the fact that pronounced seasonality of the HC were detected. The highest values were detected in spring with gradual decline up to fall. The concentration range of detected HC in zooplankton was similar to the amounts detected in sediments of nearby lakes (Muri et al., 2002).

Differences between both zooplankton groups were revealed in the amounts and relative proportions of KET, detected only in representatives of Copepoda with a significant increase in August (fig. 28). Parallel analysis of FAME revealed that in late summer both copepod species contain highest values of FAME, SAFA, MUFA but lowest UI, PUFA and ALA. As the amounts of KET correlate well with the amount of lipids it is obvious that the Copepods were within the most active stage during the growing season. They have enough resources to build up their lipids and invest those into reproduction. As the amounts of ALA, which can be converted into EPA (Von Elert, 2002), were the lowest in that period it is possible that changes in terms of their FA composition (and KET) were due to metabolic hyper activity interconnected with maternal investments, preparing for the reproduction, typically

occurring after summer. Seasonal dynamics of reproductive parameters (i.e. number of eggs per ovigerous female) of Copepoda are usually associated with two peaks within the season (Ceballos and Alvarez-Marques, 2006). Similar information was reported for ovigerous Cladocera of the nearby Lake Bled (≈ 10 km air distance), where the spring peak was higher (April) compared to the one in late summer (August) (Simčič and Brancelj, 2001). However, our datasets revealed only one maximum for each of the co-occurring zooplankton species within Lake Bohinj (fig. 20 right), possibly due to a more pronounced Alpine climate in Lake Bohinj as compared to Lake Bled.

Changes in lipid classes might also be induced by specific taxonomic composition of phytoplankton. In September, there was normally a bloom of Dynophyta (*Ceratium hirudinella*) with the bio-volume of $0.13 mm^3 L^{-1}$ (ARSO, 2007). That group was the most probable driver for a significant reduction of TAG, and thus total lipids, in all four species of zooplankton in September (fig. 24) as dinoflagellates can cause crashes of zooplankton population during their blooms (Iwakuma and Fujii, 1998). A drop in the amount of lipid classes was reflected also in a drop of zooplankton FAME.

After a drop of TAG and thus total lipids in September an overall increase was detected in October just at the start of homothermy (fig. 21 a). It is a period, when zooplankton taxa are preparing for over-wintering (Copepoda) or produce resting eggs (Cladocera). A similar increase of total lipids in seston at the period of homothermy was reported also by Hagen and Schnack-Schiel (1996) (fig. 21 a).

The amounts of FFA (fig. 31) correlated with two seasonal peaks, the first one from May to June and the second one from July to August. This is in agreement with an overall higher rate of metabolism in the most productive months. The dynamics of FFA follow the pathways of TAG and other lipid classes. The spring peak of elevated amounts of FFA is connected with the amounts of AMPL (fig. 37), which were, on average, in absolute values, higher in Copepoda (fig. 38 a) but they were in relative proportions higher in Cladocera (fig. 38 b).

PL (fig. 34) are group of lipids which should not be prone to external disturbances, including seasonal changes, due to their prime role of being an integral part and

constituents of cell membranes (Vance and Vance, 1996; Arts et al., 2009). This is in agreement with the results as well as complies with the dynamics of ST (fig. 33). Elevated levels of ST were detected in productive months, from May to August, and resulted resulted in bigger lipid droplets in a body of zooplankton specimens (especially Cladocera). This is in agreement with Arts et al. (1992, 1993) where the most productive months were associated with an increase of ST and other lipids involved into metabolic processes or/and pathways. However, higher amounts of PL, and thus ST, were detected in early spring (April) in *C. abyssorum prealpinus.* We are assuming that the reason for this might be a smaller overall lipid content in this period (and thus TAG) as compared to periods after – thus organisms comprised of relatively higher amounts of PL compared to TAG as PL are a lipid class being not utilized – but if usually lastly.

On the other hand, elevated levels of PL compared to TAG could indicate food scarcity or/and exposure to contaminants (i.e. petroleum hydrocarbons or other organic polutants) (Capuzzo et al., 1984; Arts and Wainman, 1999), that could be linked to elevated levels of HC in that period too. On an annual basis in Lake Bohinj the amounts and relative proportions of ST are greater in Cladocera than in Copepoda (fig. 35 a, b). Arts (1993) also reported higher amounts of PL in Cladocera (*Daphnia pulex*) over Copepoda (*Leptodiaptomus sicilis*). The proportions of ST detected in Lake Bohinj were within the range expected in invertebrates (Cavaletetto et al., 1996; Arts et al., 2009) and were generally following the dynamics of PL.

While temporal patterns of lipid classes are informative in terms of revealing the intra and inter specific variations in zooplankton and possibly to infer on species or taxa specificities (i.e. differences between zooplankton taxa), information on FA can be even more informative.

Zooplankton, including from Lake Bohinj, play a distinct role in aquatic food webs, especially due to their lipid content. In some cases, the amount of the lipids could be as high as 80 % of their dry mass (figs. 21 b, 22 d) and with average values of 51 ± 17 % (Cladocera and Copepoda pooled). Thus zooplankton can be ascribed also as bio-concentrators of lipids. However, some differences can be ascribed to species or/and taxonomic specificity. Especially females allocate significant amounts of lipids to their eggs to provide their offspring with material and energy for growth and development and to offset the potential of starvation during the early life stages (Arts

and Wainman, 1999). Allocation of lipids to eggs is especially intensive in daphniids (Wacker and Creuzburg, 2007), but we could not confirm this during our study in Lake Bohinj. However, we could confirm that egg-carying (ovigerous) females of *A. laticeps* had comparatively higher amounts of total lipids compared to males, but could not confirm the same for *C. abyssorum prealpinus*. Thus we can conclude, that egg-carying cyclopoids invest less total lipids into eggs compared to calanoids (mean total lipids 43.7 ± 15.1 % *vs*. 65 ± 15.3 %) what could be to some extent linked to differences in life-history between both Copepods (Gilbert, 1983).

In fish from Lake Bohinj, body length *vs*. body mass correlated well (fig. 45 a). However, we found no correlation comparing condition factor (Fulton's K) with the relative amount of total lipids (fig. 45 b). An exception was *P. fluviatilis* where a slight negative correlation was recorded. No correlation indicates that the relative amount of total lipids (usually referred to fish fitness) can not be estimated by Fulton's K. This is in agreement with the survey done on the *C. clupeaformis* in the Laurentian Great Lakes (Kinnunen et al., 2007). Fulton's K has been often assumed to indicate lipid content/energy reserves in salmonids (Neavdal et al., 1981; Herbinger and Friars, 1991). Dutil (1986) also demonstrated strong correlations between Fulton's K and lipid content in adult arctic char (*S. alpinus*), which is opposite to the results of our study. It was also demonstrated that in less complex fish community structures where arctic char (*S. alpinus*) is the only salmonid fish in the community, their predominant food source is mainly zoobenthos (Curtis, 1985). However, it needs to be considered that Lake Bohinj is an oligotrophic lake located in the Alpine region, where the growing season is short.

While we found that Fulton's K is a poor predictor of total lipids in the fish analyzed (fig. 45 b), it turned out to be a useful tool for discriminating between these three species (fig. 46).

Species-specific ecology of fish (i.e. predominant food) and their physiological specificities reflect on the qualitative and quantitative amounts and relative proportions of lipids in their tissues, similar as for zooplankton (Sekino et al., 1997; Kainz et al., 2004; Smyntek et al., 2008).

Stomach content analyses of fish specimens, which undergo lipid analyses (presented here) revealed that *L. cephalus* was feeding predominantly on water macrophytes (≥ 33 %) followed by insects larvae (≥ 27 %). Insects larvae were the predominant food

of *P. fluviatilis* (> 90 %) while *S. alpinus* was planktivorous, preying mostly on Cladocera (> 90 %) throughout the season (Magjar, 2009). Findings of Magjar (2009) comply well with the lipid analysis presented here. Total lipids [%] were found to be the lowest in *S. alpinus* where energy investment for food-search is higher compared to *L. cephalus* whose predominant food source (macrophytes and insects larvae) within the main growing season did not change the position (fig. 47 a). This is in agreement with the amounts of FAME (fig. 48), which were detected to be the lowest in *S. alpinus*.

While quantity of total lipids is highest in *L. cephalus* (fig. 47 a) its quality in terms of essential ω3 (fig. 49 c) is the lowest. It is the highest in planktivorous *S. alpinus* (fig. 49 a) and predatory *P. fluviatilis*. The highest UI of *S. alpinus* (fig. 47 b) agree also with the highest amounts of ω3 FA (fig. 49 a) and also with the highest relative proportion of ω3 FA (fig. 49 c) averaging at 20.5 ± 3 %. This is in agreement with several studies (i.e. Arts et al., 2009) revealing that the amounts of total lipids (and also PUFA) of zooplankton are significantly higher then on the trophic level below (primary producers) or/and trophic level of planktivorous fish (i.e. Arts and Wainman, 1999) (fig. 59 a, b, 60). Conversely, the amounts and relative proportions of ω6 FA (fig. 49 b, d) were lowest in *S. alpinus*, while highest in *L. cephalus*, what reflects its predominant food source and its feeding mode (Magjar, 2009) - ω6 FA are the markers for food source of terrestrial and/or plant origin, C18:2n6c FA in particular (Arts and Wainman, 1999; Arts et al., 2009). This confirms the origin of ω6 FA in *L. cephalus* as of plant origin (majority). Despite the fact that both *L. cephalus* and *S. alpinus*, contained similar relative proportions of PUFA (fig. 50 a), the overall amounts of PUFA were higher in *L. cephalus* compared with *S. Alpinus* (fig. 50 b), what can be ascribed to overall higher amounts of ω6 FA detected in *L. cephalus* (fig. 49 b, d).

While no differences were detected in fish in the amounts of SAFA, they were detected on their relative proportions. Specimens of *S. alpinus* have the highest relative proportion of SAFA compared to the other two species. In opposition, the lowest relative proportions of MUFA were detected in *S. alpinus* compared to other two species. Both differences can be explained in the context of the food web of *S. alpinus* - its trophic position of and/or prevailing food source – zooplankton (i.e. Cladocera). Zooplankton filter the water column, thus their lipid FA profile should

be to some extent, linked to the lipid FA profile of seston (fig. 59 a, b) where the dominative FA class is SAFA (fig. 59 b). Relative proportion of SAFA in seston is averaging at 53 ± 5.2 % and at 42.7 ± 8.5 % in zooplankton (fig. 59 a, b). This is in agreement with the highest SAFA content (amounts and relative proportions) in *S. alpinus* compared to other two fish species anaylzed. Our findings fit well with the stomach content analysis which revealed *S. alpinus* being chiefly planktivorous fish, which is rich in SAFA. Major SAFA, and thus major FA, apart from certain PUFA, is C16:0 which gives 22.5 ± 3.1 % of total FA in zooplankton examined (pooled) in Lake Bohinj. Fatty acid C16:0 is known as major SAFA of aquatic origin (Arts and Wainman, 1999) and can be used effectively in paleo-limnological surveys as a marker (the ratio between C16:0 and C24:0) for revealing the rate of alochtonous inputs into aquatic ecosystems too (Meyers et al., 1984).

Similarly, however, the situation reverses regarding the relative proportion of MUFA. MUFA are one of the least abundant FA in seston. Diet of *S. alpinus* is thus poorer in the amounts of MUFA while the retaining mechanism is not so strongly developed as for PUFA. It was demonstrated that certain MUFA from phytoplankton (seston) can originate from specific taxa (Napolitano et al., 1997).

Despite the fact, that *S. alpinus* is feeding mainly on Cladocera which are poor in DHA, their amounts and proportions of this FA are on the same level as with the two other fish species analyzed. A possible source of high values of DHA is that besides highly retaining DHA, *de novo* biosynthesis occurs through converting ALA and α-LA into DHA (Bell et al., 1986; Sargent et al., 1989, 1993) (tab. 9).

*S. alpinus* is an alochthonous species introduced into Lake Bohinj during the Second World War (Svetina and Verce, 1969). Soon after introduction they out-competed autochthonous salmonid species for food resources. It turned out that introduced salmonid is a significantly healthier food, in terms of human health, than autochthonous one. Salmonid *S. alpinus* from Lake Bohinj is a low trophic level fish (short food web chain) with high amounts of ω3 FA thus being more appropriate for food, however being unaware of the amount of bio-accumulated (biomagnified) toxic substances (like heavy metals, PCBs) (Rasmussen, 1990; Arts and Wainman, 1999; Van der Oost., 2003; Arts et al., 2009).

### 4.3.1 Seasonal dynamics of lipids in *Leuciscus cephalus*

The omnivorous feeding character of *L. cephalus* (Magjar, 2009) is reflected in a low seasonal variability in total lipids (fig. 51) and amounts of FAME, which both did not vary within the season. *L. cephalus* incorporates more macrophytes into its diet when they are available – in summer months 43 %, in spring 5 % and in fall 14 % (Magjar, 2009).

Although total lipids remained constant throughout the growing season (fig. 51) a drop of the unsaturation index (fig. 52 a) was detected due to an increase of SAFA, a drop of relative proportions of PUFA and amounts of ω3 FA (fig. 52 b, c, d).

The drop of the unsaturation index, as a result of a drop of some FA classes is connected with the prevailing food source, i.e.macrophytes. As the seasons progress, more and more macrophytes are available in the habitat of *L. cephalus*. Rather than investing energy into searching for insect larvae and other benthic invertebrates consumed by other fish, especially *P. fluviatilis*, they incorporate more and more macrophytes into their diet. When the warm weather season is over and young shoots of macrophytes are no longer available, they start to consume insect larvae and other benthic invertebrates, incl. molluscs (Povž and Sket, 1990).

### 4.3.2 Seasonal dynamics of lipids in *Perca fluviatilis*

*P. fluviatilis* is a typical predatory fish, which feeds more or less constantly on one particular type of food throughout the season (Willock, 1969; Povž and Sket, 1990). In agreement with this is a rather constant unsaturation index throughout the growing season. This suggests that the food source in terms of EFA did not change in comparison with *L. cephalus*. During the peak of the growing season, the diet of *P. fluviatilis* in Lake Bohinj almost entirely consists of insect larvae (Magjar, 2009). Preying on insect larvae is maximal in spring (98 % of diet) and minimal in fall where diet consists of 47 % of insect larvae and other benthic invertebrates (34 %), which densities are seasonal dependant (Khrennikov, 1987; Baryshev, 2007).

The amounts of total lipids [%] (fig. 53 a) varied along the season; Amounts of total lipids were the lowest in June, highest in August and intermediate in July and September; but lower than in August. This is in agreement with the amounts of FAME (fig 53 b). This is also in agreement with slight increase of the amounts of PUFA (fig. 54 c) as a result of elevated ω3 FA (fig. 54 a). At the same time a drop of relative proportions of ω6 FA (fig. 54 d) was observed. In parallel, amounts of SAFA (fig. 54 b) were also increased towards the end of the season, as a result of preying on aquatic animals.

In August and September a significant decrease in Fulton's K (fig. 53 b) occured, as a result of caught individuals which were lighter but not longer. Fish were caught in different parts of the lake, thus we excluded the possibility that fish at both sampling dates were caught from specific location where lighter (younger?) animals would predominate. A possible reason for this might be that these differences are due to late spawning of females, usually occurring from March up to June (Hofer, 1991). However we were unable to reveal differences between female and male representatives in Fulton's K and in total lipids content (t-test; P= 0.78, P = 0.33).

Another possible explanation for this might be competition between *P. fluviatilis* and *L. cephalus* (and possibly other fish species) for food resources. We are supporting this statement with the fact that we detected a shift in feeding mode of other fish species from Lake Bohinj (Slovenia) *L. cephalus* which changed their diet from insect larvae to macrophytes during the warm season. Similarly *P. fluviatilis* feed with supplemental food to their predominance in spring and fall, at the same time when their overall weight was significantly lower. That might be a clear sign of food shortages they are possibly facing with. However, opportunism of fish could not be excluded too.

A more dedicated study is needed in order to reveal whether these shifts occur due to inter specific species interactions within Lake Bohinj (Slovenia) or there are other unknown reasons for such behaviour.

### 4.3.3 Seasonal dynamics of lipids in *Salvelinus alpinus*

*S. alpinus* was, similarly to *P. fluviatilis,* gaining total lipids throughout the period April - September thus their value were highest at the end of the growing season in September (11.1 ± 2.3 %) and lowest in May (7.2 ± 1.2 %) (fig. 56 a). The amount of total lipids followed the density of zooplankton. It was the highest in summer (especially predominant food of *S. alpinus*: Cladocera), and lower during late fall and winter, when the probability of fish to find zooplankton was decreased. During the height of the season, *S. alpinus* is gaining total lipids to overcome the unfavorable condition which follows – winter and moreover to later invest them into reproduction (spawning). Spawning usually occurs from October up to January (Svetina and Verce, 1969; Hofer et al., 1997).

No differences were detected in the unsaturation index throughout the season what is in agreement with Magjar (2009) who has reported very stable food intake of *S. alpinus* mainly consisted of pelagic Cladocera (year average ≈ 96 %).

The increase of total lipids [%] corresponded to gained levels of FAME, the amounts of PUFA (as a result of increased level of ω3 FA), and amounts of SAFA on the account of the previously described planktivorous feeding mode, which are rich in SAFA derived from primary producers (figs. 56 a-c, 57 a, b).

## 4.4 Polymethylene interrupted fatty acids (PMI-FA) in biota from Laurentian Great Lakes

Polymethylene interrupted fatty acids (PMI-FA) in seston, various cultures of phytoplankton and zooplankton, including Cladocera and Copepoda, were under LOD. However, we confirmed the existence of PMI-FA in the marine mussel *M. edulis*, as reported by Budge et al. (2007) and several other authors (Ackman, 1973; Berge and Barnathan, 2005) which served us as a laboratory standard. In addition, we detected these compounds in freshwater mussel species, the first report of such kind. PMI-FA were detected in invasive mussels (*Dreissena polymorpha* and *D. rostriformis*) and in native mussels (*Lampsilis cardium* and *L. siliquoidea*) of the Laurentian Great Lake as well.

This important information reveals that these molecular compounds are not transferred up the food web through a plant-animal interface (through primary producers - algae) but enter the food web at a higher trophic level. It has been previously reported that PMI-FA are synthesized by marine mollusks and gastropods (Zhukova, 1986; Zhukova and Svetashev, 1986; Zhukova, 1991), and their existence was also reported in freshwater snails (Dembitsky et al., 1992; Fried and Sherma, 1993). However, new data confirms their common distribution in mollusks of marine and freshwater origin. PMI-FA is thus a taxonomic specificity of molluscs, with *de novo* synthesis of these compounds (Zhukova, 1991).

According to Budge et al. (2007) the values of PMI-FA in bearded seals (*Erignathus barbatus* Erxleben, 1777) and Pacific walruses (*Odobenus rosmarus* Linnaeus, 1758), feeding intensively on marine mollusks, are close to 1 % of total FA expressed as weight percentages. Much higher values of PMI-FA (6.6 %) were reported for the shallow water clam (*Mactra chinensis* Philippi, 1846) and the deep-sea clam (*Calyptogena phaseoliformis* Metivier, Okutani and Ohta, 1986) - up to 30 % of total FA expressed as weight percent of total FA (Saito, 2007). Results regarding the shallow water clam are in agreement with what we have found for marine *M. edulis*, our laboratory standard.

There is no published data available on the content of PMI-FA in freshwater mollusks on a $\mu g \bullet mg^{-1}$ dw. There is only limited information on these compounds in freshwater ecosystems.

Fried et al. (1993) reported that the weight percentages of PMI-FA in aquatic gastropods were ranging ≈ 3 %. However, he revealed only one PMI-FA (20:2 PMI-FA), which he is referring as 20:2 NMID and moreover also as total content of PMI-FA. He also observed that laboratory-raised snails contain significantly less PMI-FA (≈ 1 %) compared to animals taken from nature. However, Dembitsky (1992) reported that weight percentages of PMI-FA in *Vivaparus viviparus* Linnaeus, 1758 and *Radix auricularis* Linnaeus, 1758 (Gastropoda) to be 4.7 % and 6.9 %, what is close to what we have detected in examined freshwater mussels.

Comparing data for marine and freshwater mollusks indicates that relative proportions of PMI-FA are greater in marine species (up to ≈ 8% of total quantified FAME in *M. edulis*) than in freshwater species (≈ 6.4 ± 0.4 % in *D. polymorpha,* 4.3 ± 0.2 % in *D. rostriformis bugensis* and 6.3 ± 0.1 % in *L. cardium* and *L. siliquoidea*) (fig. 62 b). Total amounts of PMI-FA were highest in invasive *D. polymorpha* 2.0 ± 0.09 µg PMI-FA $mg^{-1}$ dw and *D. rostriformis bugensis* 1.1 ± 0.05 µg PMI-FA $mg^{-1}$ dw and both native *L. cardium* 0.7 ± 0.03 µg PMI-FA $mg^{-1}$ dw and *L. siliquoidea* 0.6 ± 0.04 µg PMI-FA $mg^{-1}$ dw. Thus clear differences exist between marine, both native and introduced, mussels. At the same time no significant difference were observed by comparing *D. rostriformis bugensis* from two different lakes, thus we concluded that the amount of PMI-FA in tissue is species-specific, determined by species metabolism rather than location, similarly to what has been found for lipids of zooplankton species (i.e. Arts et al., 2009). Similar conclusions were reached by Sekino et al. (1997) and Smyntek et al. (2008) for the FA taxonomic biomarkers. An additional support is that we were unable to reveal a difference in the amounts of PMI-FA in mussels sampled from different locations within the same lake (up to ≈ 10 km offshore).

In the marine mussel (*M. edulis*), C20 carbon PMI-FA (20:2n-9) is the most prominent PMI-FA, while in freshwater mussels major PMI-FA was of C22 carbon chain length (22:2n-9c in *D. polymorpha* and 22:3n-6c in *D. rostriformis bugensis*) (fig. 64). In contrast, Dembitsky (1992) reported that in the freshwater snails from his assay the most prominent PMI-FA was of C20 chain length. At the moment it is not known whether these differences are suggesting new taxonomic markers (snails *vs*. mussels) or that there are other possible reasons. We also tentatively identified novel PMI-FA detected only in invasive *D. rostriformis bugensis* and both native (*L.*

*cardium* and *L. siliquoidea*) mussels. It is eluting just before 20:2n-9 PMI-FA, however the exact position of double bond is still up to be determined thus we termed it 20:2PMID*.

In very few references on PMI-FA (Dembitsky, 1992; Fried, 1993) authors used only qualitative approaches and they did not report which body parts were used for the analysis of PMI-FA. Detailed analyses of the mussels from the Great Lakes have demonstrated that different body parts contain different amounts and relative proportions of total PMI-FA. According to data (fig. 63 a-d), the foot contains significantly more PMI-FA compared to the mantle, presumably due to different metabolic activity of both tissues. Berge and Barnathan (2005) reported that PMI-FA could be found in the organs which are more exposed to the immediate environment, i.e. the gills, mantle and foot, at least in the marine environment.

The presence of PMI-FA, first discovered in freshwater mussels, was also confirmed now in fish. The mussels become prey of the fish due to their limited food resources which were affected by the invasion of Dreissenids (Dermott et al., 2003). Three small scale case studies from this thesis revealed that trophic transfer of PMI-FA exists also in freshwater ecosystems (figs. 66 - 68). Such transfer was earlier confirmed for the marine environment only (Budge et al., 2007).

### 4.4.1 PMI-FA in food webs of the Laurentian Great Lakes

In fish from Hamilton Harbor (Lake Ontario) the highest amounts of PMI-FA were detected in samples of an opportunistic bottom feeder *A. nebulosus* (brown bullhead) whose diet can include mollusks (Massengill, 1973), mussels and snails in particular. The lowest amounts of PMI-FA were detected in planktivorous *A. pseudoharengus* (alewife) (fig. 66 a, b). While it is unlikely that planktivorous *A. pseudoharengus* would prey on mussels, however, it is possible that PMI-FA enters the alewife food web through preying on macro-invertebrates containing PMI-FA (i.e. *Mysis* spp., *Diporeia* spp.) (Mills et al., 1992). Certain fish, i.e. piscivorous *M. salmoides* (large mouth bass), prey on other fish, thus PMI-FA enters the fish through a fish-fish interface.

Significant differences were found in the PMI-FA content in *C. clupeaformis* (lake whitefish) from the two contrasting sampling spots: the Big Bay, where the biomass of food resources for *C. clupeaformis* consists of a large population of chironimids, oligochaetes and dreissenids whose biomass is lower in that region since 2005 (Dermott et al., 2003, Dermott, pers. comm.). In contrast, the food source for *C. clupeaformis* inhabiting the area of the Point Pelee consists of invasive Dreissenidea and Oligochaeta only. Dreissenidae and Oligochaeta contain less total lipids; 6 - 18 % and 8 - 20 % compared to chironomids which contain 8 - 35 % total lipids (Arts and Wainman, 1999). Also significantly higher concentrations of total lipids were detected in *C. clupeaformis* inhabiting Point Pelee compared to Big Bay (22.48 ± 2.0 % and 11.75 ± 1.5 %) (t-test, t-ratio= 4.23, DF= 26, P= 0.0003), in agreement with Pathovel et al. (2006). We suggest that the higher total lipids in *C. clupeaformis* inhabiting Point Pelee are due to their lower investment of energy into food search.

An ecological survey on Lake Michigan, focused on fish refuges during their life cycle, revealed that *C. clupeaformis,* inhabiting the Naubinway region (where dreissenids predominate) remain there throughout the whole life cycle while *C. clupeaformis* from Bailey's Harbor and Big Bay de Noc move all along the western coast of the lake searching for food, where there still are some *Diporeia spp*. (fig. 69 a, b). This major food source for *C. clupeaformis* (*Diporeia* spp.) was drasticaly reduced by invasive mollusks, spreading from Naubinway region towards the south (Dermott et al., 2005; Nalepa et al., 2006). Our results are in agreement with the distributional (ecological) patterns of *C. clupeaformis* during their annual life cycle. Results suggest that *C. clupeaformis*, inhabiting Naubinway region exhibit 2-fold higher amounts of PMI-FA compared to the Big Bay de Noc and the Bailey's harbor in both a qualitative and quantitative manner (fig. 68 a, b)

**a** **b**

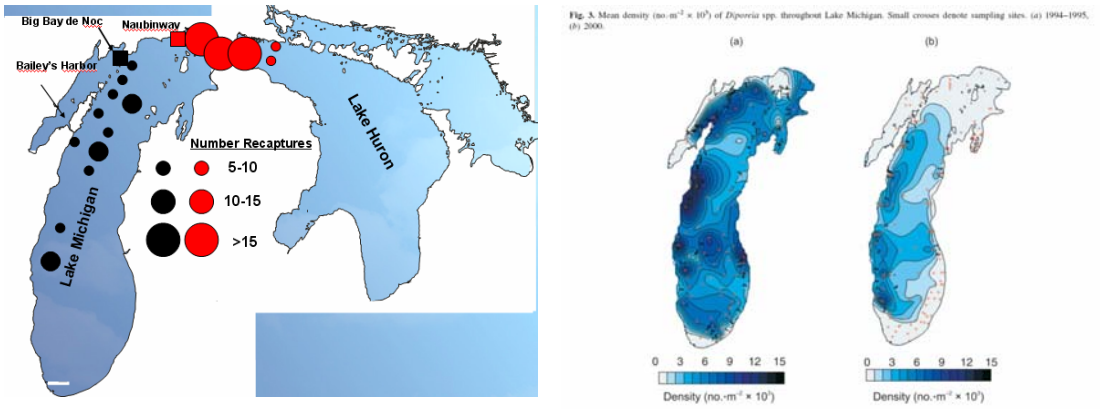


**Figure 69:** *Sutton-Koops study revealed the fish refuge during their life cycle (from Sutton-Koops)* ***(a)****,* ***b)*** *spatial and temporal distribution of Diporeia spp (from Nalepa et al., 2006).*

The PMI-FA content in fish can thus be effectively correlated to the distribution and occurrence of invasive mussels, as confirmed with three independent case studies. However, we know little about the species (or higher taxa) specific conversion rates and utilization of these compounds.

Utilization of PMI-FA certainly occurs in the examined fish as significantly lower amounts and proportions of PMI-FA were detected in fish than in mussels (tab. 10). The data are in agreement with Tanaka (2007) who reported the existence of metabolic pathways from PMI-FA to EFA. So far it is not known what exact amount of mussel carbon (and corresponding amount of lipids) is present in examined fish. This prevents one to determine which fish species prey on which particular type of mussels and in what quantity.

The fact that certain amounts of PMI-FA were detected in two macro-invertebrates is a consequence of their feeding mode. Both *Mysis* spp. and *Diporeia* spp. are scavengers, temporary scavenging on sediment. At the same time it was demonstrated that *Mysis* spp. is a cannibal (Nordin et al., 2008). By scavenging on lake sediment, where exotic invaders are present, a certain amount of mussel remaining particles are ingested and enter the next trophic level.

### 4.4.2 PMI-FA TERMINOLOGY

Although several authors (Tanaka et al. 1997, 1999; Budge et al., 2007) designate PMI-FA as NMI-FA (non-methylene interrupted fatty acids), we propose the usage of the term PMI-FA (Mezek et al., 2009a) to be more consistent. The term PMI-FA more specifically reflects the possibility of multiple (2 or more) methylene groups between contiguous double bonds. We also believe that by using NMI-FA terminology, incorrect interpretation is more likely to occur (because of the absence of a methylene group between contiguous double bonds.

### 4.4.3 GENERAL CONCLUSIONS ON PMI-FA

Although PMI-FA represents a small portion of total fatty acids they have a potential to be effectively used as a biomarker in food web studies and trophic ecology, especially where the food web includes mollusks. A major difference of PMI-FA with other MI-FA is that these FA are exclusively *de novo* synthesized in mollusks and not originating from the lowest trophic levels where the majority of FA are synthesized and move up the food web conservatively. In certain aspects PMI-FA are better markers for revealing trophic interactions and prey-predator relationships when mollusks are part of them. Moreover, when utilization rates of fish will be determined (or other species preying on mussels or other mollusks) we will be able to quantify the effects they are causing to the food webs and possibly be able to identify the quantity of mollusk carbon (PMI-FA) in predators and correlate it to corresponding amounts in mollusks. Also, there is no data available on fish preferring to prey on a particular mussel type, or whether there are preferences in consuming mussels between male and female fish. It is also unkonown, whether veligers, which can be a prey of planktivorous fish, contain PMI-FA or not.

# 5 CONCLUSIONS

Lipids can be used effectively to track seasonal dynamics of organisms at different trophic levels, to reveal a species niche, trophic interactions, prey-predator dynamics and foraging behaviour. They represent an integration of dietary intake for certain period of time to provide fuel for short or long-term energy demands.

With the newly developed HPTLC method it has become possible to separate the lipids of four dominant zooplankton species of Lake Bohinj and track their seasonal dynamics. Moreover, the method was also useful for lipid class separation of other biota.

While triacylglycerols (TAG) were the principle energy reserve of all four zooplankton species examined, they were also the most variable. Several intra- and inter- taxonomic differences were detected in zooplankton on the basis of their lipid classes and/or fatty acids (FA) analyses. They are potential taxonomic discriminators and could also be used as taxonomic markers. Previously, some differences, regarding the FA analyses of seston and zooplankton were already observed, and this study confirmed that the pattern holds also for species inhabiting Lake Bohinj. This is a good indication that a phenomenon is wide spread and common in freshwater ecosystems.
As a general pattern emerges, a large difference in composition of lipid classes as well as FA correlates with phylogenetic origin or/and life history traits of zooplankton and other aquatic species. This results in revealed interspecific differences between examined species.

Analyses of lipid profiles of egg-carrying females indicate that the maternal investment is significantly higher in Calanoida than in Cyclopoida.

Lipids of three fish species from Lake Bohinj were less variable than those of zooplankton. However, the variation of lipids was higher in planktivorous than in omnivorous fish. There was a good correlation between stomach content analysis and

the analysis of lipids in terms of fish food selection. Both approaches are complementary as they both reveal different data. The quantity of lipids is not necessarily correlated with their quality in terms of EFA (i.e. ω3 FA). Planktivorous fish show higher amounts of ω3 compared to omnivorous species, thus being more appropriate for human health and nutrition.

The results indicate that zooplankton is predominately a bio-concentrator rather than a producer of lipids. Concentration of lipids in zooplankton, expressed as a per centage of body mass is much higher, compared to concentrations in seston or fish.

This study revealed for the first time the existence, origin, trophic transfer and utilization of PMI-FA in species inhabiting the Laurentian Great Lakes. There are important differences between poly-methylene interrupted fatty acids (PMI-FA) content and PMI-FA profiles of the species analyzed. These, so far less well known lipid compounds of FA, are one of few *de novo* bio-synthesized lipids in freshwater organisms, specifically mollusks. While PMI-FA are *de novo* bio-synthesized in a trophic level above primary producers, these might become useful for studies on food-web interactions.

There are indications that PMI-FA are utilized or/and biotransformed during transport through the food web (mussel-fish relation). At the moment there is no information how intensive this flow is, and it is not possible to quantify the amount of mollusk carbon (PMI-FA) ingested by fish. The problem can be solved by the feeding experiment under controlled conditions. This recruitment study on the Laurentian Great Lakes has revealed many important data about the PMI-FA, yet there is still much to be determined, especially the harmfull effects of invasive molluscs (g. *Dreissena*) on the environment.

We concluded that lipids, FA in particular, can serve as an affective tool to estimate the food-web (seasonal) dynamics or to reveal trophic position within well defined food web, culminating in vertebrates (fish). Coupled with other information (i.e. stable isotope analysis) even more valuable insights about the species ecology or/and ecosystem structure can be revealed.

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