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**Historical changes in species composition and interspecific hybridization of the *Daphnia longispina* species complex (Crustacea: Cladocera) in Lago Maggiore**

Historické změny v druhovém složení a mezidruhová hybridizace perlooček druhového komplexu *Daphnia longispina* (Crustacea: Cladocera) v Lago Maggiore

**Master thesis**

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Prague, 2018

**Declaration:**

I declare that this thesis is a result of my own work, except for the contribution of people stated in the acknowledgments. I properly cite all information sources used. The work contained in the thesis has not been used to obtain any other academic degree.

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## Abstrakt

Hybridizující druhy komplexu *Daphnia longispina* jsou klíčovými taxony v planktonních společenstvech mnoha evropských jezer. V několika těchto jezerech bylo zdokumentováno, že taxonomická struktura komplexu se během 20. století podstatně změnila následkem člověkem vyvolaných změn životního prostředí, zejména eutrofizací a re-oligotrofizací a změnami rybích populací. Tyto změny pozorujeme v jižním Alpském jezeře Lago Maggiore (Itálie/Švýcarsko), které také prošlo změnami životního prostředí zprostředkovanými člověkem.

Lago Maggiore je jedním z nejvíce a nejdéle studovaných evropských jezer, tudíž je k dispozici velké množství historických vzorků perlooček r. *Daphnia* z pravidelného sledování. Vzhledem k tomu, že lokální populace r. *Daphnia* nevytvářejí dormantní vaječné banky vhodné pro genetickou analýzu (díky schopnosti přezimovat ve vodním sloupci), používáme kombinaci morfologie a geometrické morfometrie (eliptická Fourierova analýza) k vyhodnocení taxonomických a fenotypových změn v komplexu *Daphnia longispina* v Lago Maggiore od poloviny 20. století do současnosti (roky 1948-2012) a dále k pokusu o charakterizaci vlivu změn životního prostředí v daném období na tvar a velikost těla *Daphnia*. Zkoumání fenotypu *Daphnia* naznačuje skutečně převládající přítomnost *D. longispina* ve čtyřicátých letech minulého století, dominanci morfotypů *D. galeata* po roce 1980, pouze výjimečnou přítomnost *D. cucullata* v letech 1986 a 1992, a také přechodné fenotypy představující zřejmě mezidruhové hybridy od čtyřicátých let minulého století. Mnohazměrná analýza výsledků geometrické morfometrie naznačuje, že většina variability tvarů těla místních populací r. *Daphnia* může být shrnuta jednou hlavní osou PCA. Bylo možno charakterizovat sezónní a meziroční změny ve tvaru těla, a dále pak vliv taxonomické příslušnosti. Jednoznačný vztah tvaru perlooček ke specifickým charakteristikám prostředí se nepodařilo nalézt.

**Klíčová slova:** *Daphnia longispina* komplex, Lago Maggiore, mezidruhová hybridizace

## Abstract

Hybridizing species of the *Daphnia longispina* complex are key taxa in plankton communities of many European lakes. In several of these lakes, it has been documented that the taxonomic structure of the complex during the 20<sup>th</sup> century has substantially changed following human-mediated environmental changes, particularly eutrophication and re-oligotrophication and fish stock changes. We characterize these changes in Lago Maggiore (Italy/Switzerland), a southern pre-alpine lake, which also passed through the human-mediated environmental changes.

Lago Maggiore is one of best and longest studied European lakes so a large number of historical *Daphnia* samples from regular monitoring are available. Because local *Daphnia* do not form dormant egg banks suitable for genetic analysis (being able to overwinter in the water column), we used a combination of morphology and geometric morphometrics (elliptic Fourier analysis) to evaluate the taxonomic and phenotypic changes in the *Daphnia longispina* complex in Lago Maggiore since the mid-20<sup>th</sup> century (1948-2012), and attempted to characterize the impact of environmental changes over the respective period on *Daphnia* body shape and size. Examination of *Daphnia* phenotype indicates indeed the prevailing presence of *D. longispina* (*hyalina* morph) in the 1940s, dominance of helmeted *D. galeata* morphs after 1980s, only occasional presence of *D. cucullata* in 1986 and 1992, as well as common intermediate phenotypes that likely represent interspecific hybrids since the 1940s. Geometric morphometric analyses indicate that most of the variation in *Daphnia* body shapes can be summarized along a single multivariate axis. Inter-annual and seasonal variation in *Daphnia* body shapes have been characterized, as well as the relationship between taxonomic assignment and body shape. However, we could not document a clear impact of specific environmental characteristics in the lake on the *Daphnia* shape.

**Key words:** *Daphnia longispina* complex, Lago Maggiore, interspecific hybridization

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# 1 Introduction

The *D. longispina* species complex is one of the dominant components of the zooplankton of permanent freshwaters in the Holarctic region and represents the most common hybridizing group from the *Daphnia* genus. In several European lakes, it has been documented that taxonomic structure of the complex has substantially changed following human-mediated environmental changes, particularly eutrophication and re-oligotrophication (Keller et al., 2008). Lago Maggiore, one of best and longest studied European lakes, also underwent environmental changes in the 20<sup>th</sup> century. Local *Daphnia* population overwinters in the water column, thus dormant egg banks accessible for paleolimnological and paleogenetic studies are not available. Consequently, historical samples from regular monitoring are the only means for studying past changes in zooplankton composition. To characterize past *Daphnia* population changes I combined morphology and morphometrics for specification of species and hybrids. And because of available long-term environmental it was possible to interpret few observed shifts of phenotypes.

## 1.1 *Daphnia* as a model organism

*Daphnia* (Cladocera) is a prominent model organism among freshwater invertebrates and is frequently used for studying interspecific hybridization in inland waters (e.g., Yin et al., 2010). *Daphnia*, a genus of small (0.2-6 mm) crustaceans, is widespread almost all over the world playing the role of a keystone species in many continental water bodies (Seda & Petrusek, 2011). They are central in pelagic food webs as harvesters of phytoplankton and prey for planktivorous fish. Some characteristics making *Daphnia* species a model organism are their cyclical parthenogenetic life cycle, easy culturing and handling and short generation time with the production of a large number of offspring. Thank to these, *Daphnia* is a suitable organism in the field of ecology, evolutionary biology, ecotoxicology and many other areas of interest (Seda & Petrusek, 2011).

Ecological and evolutionary studies on *Daphnia* focused, among others, on their trophic ecology and predator-prey interactions (e.g., Guilizzoni et al., 2012; Juračka et al., 2011; Manca et al., 2008), hybridization (e.g., Alric et al., 2016a; Griebel et al., 2016; Yin et al., 2014), host-parasite interactions (e.g., Corradi et al., 2009; Decaestecker et al., 2007; Wolinska et al., 2007a), or evolutionary relationships (e.g., Gießler & Englbrecht, 2009; Taylor et al., 1996).

Most of *Daphnia* species can reproduce via cyclical parthenogenesis, which offers a great advantage in adapting to continually changing environmental conditions (Hebert, 1987; Seda & Petrusek, 2011). This type of reproduction represents an alternation of asexual and sexual cycle during the season (Fig. 1). During periods of population growth under appropriate environmental conditions, diploid female offspring are

asexually produced in the parthenogenetic phase. When environmental conditions deteriorate, *Daphnia* females start to produce diploid eggs developing into males. It usually happens under certain conditions, e.g., shortening of photoperiod, decrease of temperature or rapidly dropping phytoplankton density (Macháček et al., 2012). At that point, produced males mate with ephippial females, fertilize haploid sexual eggs and the sexual cycle begins. Resulting diploid embryos are enclosed inside a chitinous capsule called an ephippium, which is cast off at the female's next moult and serves as a protective sheath for dormant stages – diapausing embryos. When conditions improve, females hatch from the ephippia and the cycle restarts.

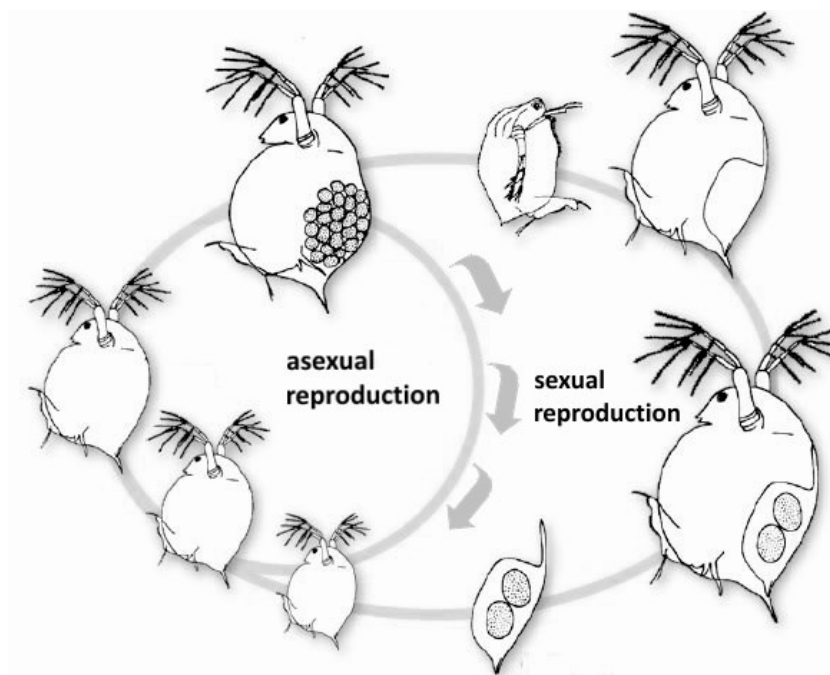


Fig. 1 Cyclical parthenogenesis of *Daphnia* – alternation of asexual and sexual reproduction (taken from Petrusek 2010, drawings by Katleen Van der Gucht)

A scenario of alternation of asexual and sexual reproduction is common for most *Daphnia* species (as well as other cladocerans), but asexual production of ephippia was noticed in various taxa of the *Daphnia pulex* group with obligate parthenogenesis (Černý & Hebert, 1993; Tucker et al., 2013). Sometimes, *Daphnia* species reproduce asexually during long part of the year. This fact was observed for example in population of *D. galeata* in Tatra mountain lakes, which is able to survive under ice cover as adult females (Hamrová et al., 2011).

The production of ephippia provides a great advantage in a study of *Daphnia* ecology, evolution and reaction on changes in the environment because of the preservation of dormant eggs in the sediment (Brendonck & De Meester, 2003; Cáceres, 1998; Jankowski & Straile, 2003). Ephippia preserved in the sediment represent



an egg bank which can be hatched by suitable stimuli. Eggs can remain viable for several years, decades or even in some cases for several hundred years (Frisch et al., 2014; Mergey et al., 2007). Thanks to this, the dormant stages represent a reservoir of genetic variability of *Daphnia* populations and can preserve a genetic footprint of past events even after they passed (De Stasio, 1989; Keller et al., 2008).

Ecological and paleolimnological studies that benefited from analyses of dormant egg banks include, among others, research on *Daphnia* microevolution (Jankowski & Straile, 2003; Weider et al., 1997), *Daphnia* responses to changes in trophic levels or other habitat characteristics (Brede et al., 2009; Jeppesen et al., 2002; Schilder et al., 2015) and studies showing the impact of long-term human activities on *Daphnia* populations (Guilizzoni et al., 2012; Pollard et al., 2003).

## **1.2 *Daphnia longispina* complex**

The *Daphnia longispina* complex is widespread in the Holarctic region and inhabits a wide range of freshwater habitats. Its members are ordinarily found in lakes and ponds with various extents of fish predation, but also in small fishless pools and ephemeral rock-pools, and they occur even in high-altitude alpine ponds. The Nearctic taxa from *Daphnia longispina* complex comprise *D. mendotae*, *D. dentifera* and *D. thorata* (which might be conspecific with *D. dentifera*; Petrusek et al., 2008). The remaining species of this complex, including, e.g. *D. longispina*, *D. cucullata*, *D. galeata*, *D. lacustris* and “*D. umbra*”, are known especially in the Western Palearctic (Adamowicz et al., 2009; Petrusek et al., 2008a). The lineage diversity of *Daphnia longispina* complex in Europe seems much higher than expected in the last century. More lineages were distinguished (Petrusek et al., 2012) and as was later discovered by a closer study of its mitochondrial and nuclear genome, hybridization in this complex is frequent even across large phylogenetic distances (Thielsch et al., 2017).

The species which I focused on in this thesis are *D. longispina* (O. F. Müller), *D. galeata* Sars and *D. cucullata* Sars. They are all widespread in Europe, ecologically very important and they commonly coexist together. All three show a considerable phenotypic plasticity in response to changing environmental conditions. *D. longispina* was taxonomically revised by Petrusek et al. (2008), who synonymized with it three other previously recognized taxa: *D. hyalina*, *D. rosea* and *D. zschokkei*.

These three European species of the *Daphnia longispina* complex have different demands on environmental conditions and they also differ in their preferred type of habitat. *Daphnia longispina* (in many previous studies labelled also as “*D. hyalina*”) occurs mostly in oligo- to mesotrophic lakes with lower predation pressure and lower food supply (Spaak et al., 2012). For this reason, the species is frequently found in European alpine lakes (Hamrová et al., 2012). In terms of life-histories, *D. longispina* may show depth-specific preferences and diel vertical migrations in the pelagic zone

of vertically stratified environments (Sed'a et al., 2007; Stich & Lampert, 1981). Vertical migration may respond to predatory pressure but due to this strategy, *D. longispina* is getting to the colder water with a poor food supply each day and it consequently suffers a huge disadvantage in its reproduction success (Stich & Lampert, 1981). As one of the phenotypically variable *Daphnia* species, a number of *D. longispina* morphotypes were distinguished and specified (Petrušek et al., 2008a).

*D. galeata* is the most widespread species from the *D. longispina* complex (across Europe as well as globally), it is extremely ecologically plastic and can be found in almost all types of permanent water bodies (Sed'a & Petrušek, 2011; Spaak et al., 2012; Yin et al., 2014). *D. galeata* is typically found in temperate, relatively warm and eutrophic reservoirs and lakes with a higher predation pressure. This species generally requires ample food to persist, but interestingly it was documented also in low-trophy fishless alpine lakes (Petrušek et al., 2007). *Daphnia galeata* is closely a related species to *D. longispina*, but unlike the latter, *D. galeata* typically inhabits warmer epilimnion near the surface and usually does not migrate into crosswise water layers. However, in some studies of Czech and Swiss lakes and reservoirs, *D. galeata* was found in both the epilimnion and in the deeper water layers (Sed'a et al., 2007; Winder et al., 2004). In terms of phenotypic plasticity, helmet formation is one of the most noticeable morphological traits of *D. galeata* (Oda et al., 2011).

The third species of the complex I am dealing with, *D. cucullata*, is no less prominent in its phenotypic responses than *D. galeata*. It occurs in the epilimnion of moderately meso- to eutrophic lakes across Europe with intermediate temperatures and food availability (Flößner & Kraus, 1986). One of *D. cucullata* adaptations against fish predation is its small, transparent body and small clutch size (reviewed by Laforsch & Tollrian, 2004). The induced development of helmets is the most prominent ability for this species. In fact, *D. cucullata* is a textbook example of cyclomorphosis. The helmet formation can be initiated by increased microturbulence of water (Laforsch & Tollrian, 2004a) or as a response to the presence of infochemicals of invertebrate predators (Tollrian & Laforsch, 2006) or fish (Brooks, 1965).

Individual species from the *Daphnia longispina* complex can differ from each other in responses to changes of the environment (Keller & Spaak, 2004; Macháček et al., 2012; Wolf, 1987). Especially in the presence of fish predators they have different reactions to predation pressure (Rautio et al., 2003). Predation pressure can induce reduction of *Daphnia* adult body size or reduce *Daphnia* fecundity. Especially zooplanktivorous fish play a dominant role as zooplankton predators (de Bernardi et al., 1987; Manca et al., 2000a).

### 1.3 Interspecific hybridization in the *Daphnia longispina* complex

As mentioned in the previous chapters, species from the *Daphnia longispina* complex may differ in their habitat characteristics. A single species can dominate in small reservoirs or even in larger lakes. However, multi-species lakes are frequent and commonly studied (e.g., Alric et al., 2016b; Vaníčková et al., 2010; Yin et al., 2010; Yin et al., 2012). Such water bodies allow us to study not only taxonomical changes of *Daphnia* populations but also interspecific hybridization, which is an important phenomenon in ecology and evolutionary history of the complex.

Hybridization is a crossing of individuals from two populations or groups of populations that are recognizable by one or more hereditary characters. Interspecific hybridization is common in natural populations, especially in plants. About 25 % of plant species are involved in hybridization and consequently in potential introgression with other species (Mallet, 2005). In animal species, this proportion has been estimated to be approximately 10 % (Mallet, 2005; Schwenk et al., 2008). Hybridization can be followed by the coexistence of parental species and their hybrids. It can lead to the reinforcement of reproductive barriers through selection against hybrids, but the merging of hybridizing gene pools is more frequently observed (Arnold, 1992).

Animal hybridization is frequently studied also in groups that reproduce by cyclical parthenogenesis (Delmotte et al., 2003; Hebert, 1985). Therefore, it is a long-studied phenomenon in the *Daphnia longispina* complex (Yin et al., 2010). But several conditions have to be realized for interspecific hybridization to take place (Hobæk & Larsson, 1990; Schwenk et al., 2000). One of them is a physical contact of species. Their habitats must be overlapping spatially and sexually reproducing individuals must get into contact for at least some time (Schwenk & Spaak, 1995; Wolf, 1987).

As was detected on lakes in Germany, F1 hybrids may have lower diversity than parental taxa due to some reproductive barriers between parental genomes (Yin et al., 2010). The reproductive barriers have been studied a few times in the *D. longispina* complex (Yin et al., 2012). It was also found, that the potential hybrid sterility can be bypassed by asexual reproduction (Keller et al., 2007; Yin et al., 2010). And what's more, the degree of asexual reproduction in hybrids may be in some conditions, much higher than in parental species (Yin et al., 2010). Hybrids also tend to have intermediate phenotypes, combine parental traits and their environmental requirements are similar to the parental ones (Fig. 2) (Schwenk & Spaak, 1995; Wolf & Mort, 1986). As hybrids mostly reproduce parthenogenetically and often combine life-history traits of their parents in a beneficial way (e.g., Schwenk & Spaak, 1995) this may under some conditions lead to hybrid dominance (e.g., Schwenk & Spaak, 1995; Keller & Spaak, 2004). The hybrid populations are also sometimes involved in backcrossing and introgression (Hebert, 1985; Keller et al., 2007; Schwenk & Spaak, 1995; Spaak, 1995).

Several theories about the coexistence of hybrids and parental taxa were described especially for terrestrial habitats, which exhibit gradients in ecological parameters like temperature, humidity and vegetative structure. Aquatic habitats seem to be more homogenous, but they show some gradients too. The structure of *Daphnia* populations in long and narrow reservoirs was recently studied (Petrusek et al., 2008b). Such types of reservoirs are characterized by a tributary-to-dam longitudinal gradient of both abiotic and biotic factors. Environmental gradients affect not only the species themselves and interspecific hybrids (Petrusek et al., 2008b; Seda et al., 2007), but also individual *Daphnia* clones within one species (Petrusek et al., 2013; Yin et al., 2012). In this type of water bodies, hybrid dominated zones, usually located at points when the parental species habitats are overlapping, may emerge (Petrusek et al., 2008b).

For example, *D. galeata* sometime inhabits waters with high predation pressure together with *D. cucullata*, so they usually coexist and form hybrids (Spaak & Hoekstra, 1997). The habitat of *D. longispina* may be overlapping with the *D. galeata* habitat as well. Their hybrids are often found in European lakes and reservoirs in contrast with *D. longispina* x *D. cucullata* hybrids, which are relatively scarce (Hebert et al., 1989; Petrusek et al., 2008b; Spaak & Hoekstra, 1997), although locally they may be abundant (Gießler, 1997a). The scarcity or absence in some sites may be explained by preferences of the parental species for contrasting predation and trophic levels (Spaak, 1997; Wolf & Mort, 1986).

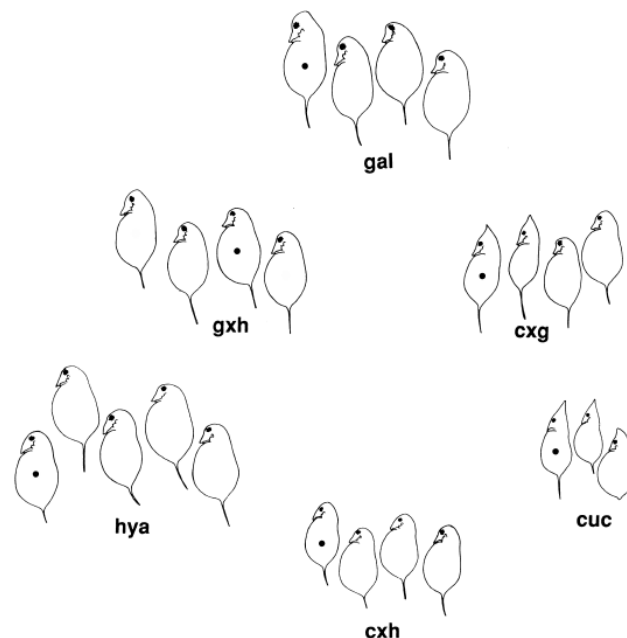


Fig. 2 Morphological phenotypes in *Daphnia longispina* complex involving pelagic morphotype of *D. longispina* (here marked as *hyalina* morph), *D. galeata*, *D. cucullata* and their hybrids. Full circles indicate typical morphs of each cluster (Gießler, 1997b).

Each species from the *D. longispina* complex was several times distinguished from the others using molecular methods, most frequently based on allozyme electrophoresis, microsatellite genotyping, or RFLP analysis, but SPN-based markers or other molecular markers have been used as well (Dlouhá et al., 2010; Keller et al., 2007; Rusek et al., 2015; Yin et al., 2010). However, morphological approaches may be useful for species identification too, even despite difficulties, especially with recognition of hybrids (Dlouhá et al., 2010). Individuals may be distinguished by morphological characters (e.g., body shape, body length, helmets, spine and seta lengths, cuticular pigmentation, eye position, naupliar eye, hemoglobin levels and shape of rostrum). Some of them are environmentally induced (e.g., body shape and length, helmets, neckteeth), but most of them are hereditary (Jacobs, 1961).

As environmental conditions and habitat states change, the structure of *Daphnia* populations may be strongly affected. Especially, the human-mediated disturbances can be a generator of environmental changes, which may possibly be followed by changes in taxa composition of aquatic populations (Keller et al., 2008). A lot of European lakes passed by these changes during the past half century and the taxonomical changeover occurred (Brede et al., 2009; Rellstab et al., 2011; Spaak et al., 2012).

#### **1.4 Changes of pre-alpine lakes in the 20<sup>th</sup> century**

Many European lakes underwent a eutrophication phase during the past century and some of them have returned to their original trophic state (Correll, 1998). In many pre-alpine lakes in Central Europe species shifts occurred owing to anthropogenetic disturbances which took place in the middle of the last century (Brede et al., 2009; Rellstab et al., 2011). Even the scenario of eutrophication is very similar to the Lago Maggiore one (Marchetto et al., 2004; Salmaso et al., 2007).

A snapshot study from year 2003-4 (Keller et al., 2008) comparing the taxonomic structure and patterns of *Daphnia* hybridization of lakes both north and south of the Alps highlighted differences between these two regions, with *D. longispina* being much more frequent in the north, and *D. galeata* being dominant in the south. This difference likely stems from differences in the species' ecology, and is facilitated by climatic factors. Nevertheless, this biogeographic separation regarding the Alps as geographical barriers must not be taken too seriously because of human activity during the last century and increased mobility though the Alps serving as a transferring factor (Keller et al., 2008).

Deep lakes north of the Alps, such as Lake Constance (Austria, Germany and Switzerland) or Greifensee (Switzerland) show similar taxonomical changes within the *Daphnia longispina* complex (Brede et al., 2009). Plankton ecology of these two mentioned pre-alpine lakes has been particularly well studied (Keller & Spaak, 2004; Keller et al., 2008; Sommer et al., 1993). Both lakes underwent an increase in water temperature and phosphorus level in the middle of the 20<sup>th</sup> century

(Correll, 1998; Straile et al., 2003). The anthropogenetic nutrient loading causing the eutrophication started in many alpine and pre-alpine lakes about one hundred years ago (Löffler, 1983). This event led in the establishment of populations of *D. galeata* into the originally oligotrophic lakes north of the Alps and was noticed in years between the 1940s and 1950s (Brede et al., 2009). The trophic state of the lakes and the magnitude of eutrophication defined the success of colonization or rediscovery of the *D. galeata*, which prefers lakes with higher trophy (Rellstab et al., 2011). Thus, the population structure of both lakes changed and hybrids started to occur (Brede et al., 2009; Löffler et al., 2004).

Despite the fact, that both lakes have different magnitudes of phosphorus concentrations (lake Greifensee used to have a considerably higher P concentration in the period of the 20<sup>th</sup> century than Lake Constance), egg banks of *Daphnia* species were usually formed in these lakes facilitating studies of past taxonomical changes in the *Daphnia* population (Brede et al., 2009; Keller et al., 2002; Weider et al., 1997). The evidence of *D. galeata* appearance documented in resting eggs noted by Brede et al. (2009) suggests that it was the variation in trophic status of the lakes, which allowed the establishment of the species and subsequent interspecific hybridization with *D. longispina* causing long-lasting changes in the genotypic architecture of the *Daphnia* population (Fig. 3).

Lake Constance, a mesotrophic prealpine lake, underwent eutrophication from the 1950s to 1970s together with trophic change from oligotrophic to meso-/eutrophic conditions (Rellstab & Spaak, 2009; Straile & Geller, 1998; Thomas & Eckmann, 2007). The originally copepod-dominated lake changed to cladoceran-dominated at the end of the 1980s (Anneville et al., 2005; Straile & Geller, 1998). But the shift in zooplankton composition in the lake started in the mid-1950s, when *D. galeata* invaded. It was an invasion from lower Lake Constance, a more eutrophic and shallow basin, to an upper Lake Constance. And because both *D. galeata* and *D. longispina* have apparently low levels of prezygotic or postzygotic isolation and *D. galeata* has a high ability to hybridize with local species, hybridization may occur (Brede et al., 2009; Schwenk et al., 2001).

The impacts of *D. galeata* appearance in the waters of Greifensee in the mid-1940s, a highly eutrophic prealpine lake of medium size, were similar to the ones that occurred in Lake Constance, though with some differences (Keller et al., 2002; Rellstab & Spaak, 2009; Wolinska et al., 2007b). Interspecific hybridization with *D. longispina* probably started immediately and newly formed hybrids co-occurred with both parental taxa (Brede et al., 2009; Keller et al., 2002; Wolinska et al., 2004). So the process of invasion of *D. galeata* is more unclear than for Lake Constance because no detailed historic observations exist to prove the presence of *D. galeata* before the 1940s. However, there is some evidence for the presence of some interspecific hybrids in Greifensee in old sediments layers from the beginning of the 20<sup>th</sup> century.

Because of this finding, it can be assumed that *D. galeata* occurred in the lake before the 1940s, but the abundance of available ephippia was not high enough (Brede et al., 2009; Keller et al., 2002).

For the example of Lake Constance and Greifensee it is possible to figure the environmental changes through the 20<sup>th</sup> century in other prealpine lakes in the north of the Alps. Salmaso et al. (2007) reported a comparative analysis of few Italian lakes on the basis of the environmental variables alternation during the second half of the 20<sup>th</sup> century, including Lago Maggiore as well.

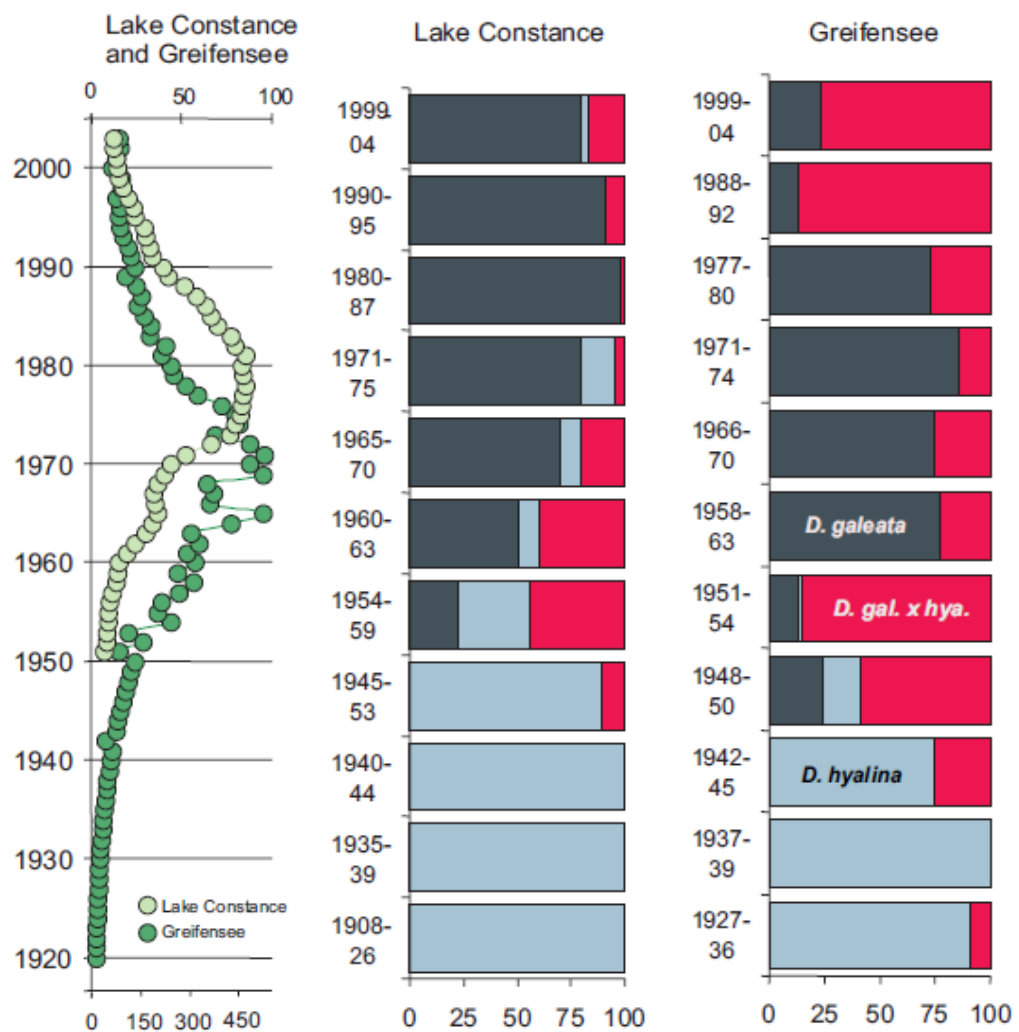


Fig. 3 Phosphorus concentration ( $\mu\text{g/l}$ ) and temporal variation in relative abundances of *Daphnia* taxa [%] in Lake Constance and Greifensee over time (Brede et al., 2009).

## **1.5 Lago Maggiore**

### **1.5.1 General knowledge**

One of the many prealpine Italian lakes, particularly known for its beauty, is Lago Maggiore. This lake is located on the south of the Alps on the border of Italy with Switzerland. With an altitude of 194m a.s.l. and plenty of surrounding alpine peaks the lake offers beautiful scenery. The climate is mild temperate (Cfa, according to the Köppen climate classification) so the 65km long water surface usually does not freeze up in the winter period. The average depth of the lake is 177 meters and it reaches 370 meters in some parts (the deepest point called Ghiffa, Fig. 5). The lake is holo-oligomictic (Ambrosetti & Barbanti, 1999). From the north to the south of Lago Maggiore the main tributaries comprise these rivers: Ticino, Maggia, Tresa and Toce and the watershed contains several natural lakes. The only outlet is Ticino, the main tributary of the river Po.

Lago Maggiore is one of the best and longest studied European lakes, thanks to long-term research based especially at the Istituto per lo Studio degli Ecosistemi (formerly named as Istituto Italiano di Idrobiologia), located on the waterfront of Verbania Pallanza and founded in 1938. The institute manages a collection of plankton samples from the lake (Museo di Plancton) which affords an opportunity for research of the plankton community in the lake over a long timescale from the 20<sup>th</sup> to the 21<sup>th</sup> century.

### **1.5.2 Changes in Lago Maggiore throughout the 20<sup>th</sup> century**

As other deep southern prealpine lakes, Lago Maggiore underwent several changes in trophic state and biota composition during the 20<sup>th</sup> century (de Bernardi et al., 1990; Marchetto et al., 2004; Salmaso et al., 2007). The Post-World War II economic expansion in the mid-20<sup>th</sup> century and Green Revolution caused dramatic changes in the environment. The spread of this rapid industrialisation and economic development affected biodiversity, nutrient cycling and climate warming.

#### **1.5.2.1 Eutrophication and pollution**

Large scale postwar application of mineral fertilizers all around the world represented the crucial impulse, together with climate warming, for eutrophication of inland waters globally, with Lago Maggiore being no exception (Visconti et al., 2008). Eutrophication is defined as an enrichment of a water body by nutrients – with phosphate playing the prime role. The main sources of phosphorus released to the lake in the middle of the 20<sup>th</sup> century were sewage discharge, detergents and industrial pollution (Manca et al., 2007a).

Lago Maggiore is naturally oligotrophic (Guilizzoni et al., 1983). Early indications of raising level of phosphorus measured as TP (total phosphorus) or RP (reactive



phosphorus) came at the end of the 1960s and the lake started to be mesoeutrophic (Manca et al., 1992). In this period the total water body heat content started to escalate as well (Guilizzoni et al., 2012). Due to water temperature increasing, faster growth rates of algae populations, displayed as a chlorophyll-*a*, were detected (Ruggiu et al., 1998).

As mentioned above, the eutrophication of Lago Maggiore started in the early 1960s and this stage persisted until the late 1970s, when there was a peak of trophic conditions (TP 35µg/l measured in 1977-8; Manca et al. 1992) (de Bernardi et al., 1990; Manca et al., 2007a). Afterwards in the 1990s, when industrial waste waters and phosphorus in detergents became efficiently regulated, the lake initiated its re-oligotrophication stage. A significant role in TP reduction was also played by the construction of treatment plants for domestic sewage around the lake and in the watershed (Mosello & Lami, 2012; Ruggiu et al., 1998). However, oligotrophication phase has been slightly delayed by further industrial pollution and of course by increased water warming, which may affect phosphorus recycling from sediments and related oxygen conditions in the hypolimnion (Ambrosetti & Barbanti, 1999; Genkai-Kato & Carpenter, 2005).

Industrial pollution of Lago Maggiore was caused foremost by persistent organic pollutants (POPs), which have been used in the middle of the 20<sup>th</sup> century as agricultural pesticides or industrial chemicals. The point sources of pollution were usually manufacturing plants around the lake (Mosello & Lami, 2012). But as Lei & Wania (2004) explored and Bettinetti et al. (2008) confirmed on the nearby Italian lakes, Como and Iseo, snow and consequently glaciers and high-mountain glacial lakes are also considered to be secondary pollution sources for POPs (Bettinetti et al., 2008; Lei & Wania, 2004).

One of the POPs, which have been polluting Lago Maggiore, is DDT (dichlorodiphenyltrichloroethane). The peak of DDT production in Italy was in the 1960s as Galassi et al. (1995) pointed out. The concentration of DDT, its homologues and other organochlorine compounds decreased in the environment after legal restrictions took effect in the 1970s. Nevertheless, depending on conditions, DDT is easily adsorbed to soils and sediments. It can be released gradually or suddenly during floods, heavy rainfalls etc. (Binelli et al., 2004). In the case of Lago Maggiore, the lake recovery has also been delayed by DDT releasing from sediments (Bettinetti et al., 2006). History of contamination by DDT in the lake is well documented because of hydrophobic and lipophilic properties of DDT. Due to this disposition, aquatic organisms and their predators absorb and then bioaccumulate DDT and its relatives. But as McCarthy et al. (1988) found out, the bioavailability of DDT homologues in the water column depends on the trophic status of the lake. In the peak of eutrophication of Lago Maggiore, the highest level of DDT concentration was measured. However the level could be higher,

if the DDT concentration would not be mitigated by eutrophication (Bettinetti et al., 2005).

The largest input of DDT into Lago Maggiore was caused by a manufacturing plant located next to Marmazza River, a tributary of the Toce River. The River Toce, one of the major affluents of Lago Maggiore, carried DDT into the lake for several years (Calderoni & Bernardi, 1999). Although the chemical plant was closed in 1996, an accidental industrial discharge happened in this year and also years before, which increased DDT concentration in fish tissues over the legal limit for edible fish (Ceschi et al., 1996). Due to this event commercial fishing was banned (Calderoni & Bernardi, 1999). Fish accumulate the toxic compound during their life time and seem to maintain it in their tissue (Bettinetti et al., 2006). As is well known, fish are apex predators of zooplankton and those small organisms respond faster to fluctuations of organic pollutants in the water column (Bettinetti et al., 2010). In addition, because of this food chain, faster biomagnification from zooplankton to fish is facilitated (Bettinetti et al., 2012).

#### **1.5.2.2. Zooplankton**

In Lago Maggiore, the zooplankton community underwent major changes in species composition and population density during the 20<sup>th</sup> century (Fig. 4) (de Bernardi et al., 1990). The shifts in taxon compositions have been affected mainly by environmental changes involving eutrophication and re-oligotrophication together with climate warming (Bernardi & Jørgensen, 1998; Manca et al., 2007a). In a lake, when the pelagic food web is controlled by top-down mechanisms, the zooplankton community of Lago Maggiore is mainly influenced by invertebrate and vertebrate predators (Bernardi & Jørgensen, 1998; Manca et al., 2007a). With regard to this lake, three main predator groups of zooplankton exist. The largest one is a zooplanktivorous fish (coregonid spp.) – mentioned in the following chapter. Invertebrate predators are represented by *Bythotrephes longimanus* and *Leptodora kindtii* (Manca et al., 2008). These two large (>10 mm) cladocerans can reduce the population of small zooplankton species. And when they are active in the water environment, kairomones, chemicals released by these predators proving their presence, are detectable and promote also changes in *Daphnia* phenotype, body size or life history (De Meester & Weider, 1999; Weber & Vesela, 2002). Nutrient concentration is also a significant factor affecting biomass of zooplankton (Manca et al., 2000b). The strongest response to environmental changes is from the pelagic cladoceran community, although the lake zooplankton is dominated by copepods (de Bernardi et al., 1990). Following the changes in the trophy of the lake from the early 1940s until the 21<sup>st</sup> century, we can summarize a number of shifts in zooplankton species abundance.

From the 1940s until 1970s, the cladoceran abundance in the lake was relatively low. In the water column in this decade there prevailed, together with the *Daphnia longispina* group, another cladoceran taxon – *Sida crystallina* (Manca et al., 2007a).

In 1957, *Sida crystallina* together with calanoid copepod *Heterocope saliens* disappeared from the open water until 1963, and *Eubosmina longispina* and *E. coregoni* became the dominant planktonic cladocerans (Manca et al., 2007a; Bonacina, 1977). The disappearance of some copepod species was partly outweighed by abundance increase of *Daphnia longispina* (*hyalina* morph) (Manca et al., 2007a).

The 1960s are characteristic for the appearance of *Chydorus sphaericus* in the pelagic zone in 1961 and for the moderate increase in pelagic cladoceran abundance (de Bernardi et al., 1990; Manca et al., 2007a). On the other hand, there were some reductions in copepod populations, particularly of *Eudiaptomus padanus padanus* and *Cyclops abyssorum* (Bonacina, 1977).

Rotifers started to reach the peak of their abundance in the 1970s, when species like *Conochilus unicornis*, *Keratella cochlearis* and *Kellicottia longispina* formed blooms (de Bernardi et al., 1990). Also the number of individuals of *Bosmina longirostris* slightly increased and in the late 1970s an increase in total cladoceran abundance was noticed until 1985 (Manca et al., 2007a).

Five years after the peak of eutrophication (Manca et al., 2007a), in 1985, the biomass of herbivorous zooplankton reached its maximum (de Bernardi et al., 1990). Afterwards the quantity of pelagic cladocerans overall declined until 1996. It was probably caused by two factors. The first of them was an increase of fish predation pressure and the second was a sharp increase of *Bythotrephes longimanus* in 1987. This predatory cladoceran feeds on smaller planktonic organisms, including cladocerans such as *Daphnia* (Manca et al., 2007a; Manca et al., 1992).

In the 1990s, *Bythotrephes longimanus* was still present in the water column and because of predation pressure of both, *Bythotrephes* and fish, *Daphnia* population density was decreasing and reached the lowest abundance in 1996. But despite sustained predation, *Daphnia* population started to grow until 2002, when its population became more numerous than in the early 1990s (Manca et al., 2007a).

As a result of transformations in the pelagic taxon composition, shifts in individual body length, changes in seasonality of species and other changes occurred (Manca et al., 2008). In the case of zooplankton, one of the main drivers, influencing abundance, seasonality and body size, was predation pressure. One of the invertebrate predators is *Bythotrephes longimanus* and as mentioned before, abundance of this species increased in Lago Maggiore in the late 1980s with the effect of rapid appearance as was noticed in the Great Lakes area of North America also in the 1980s (Barbiero & Tuchman, 2004). *Bythotrephes* is native in Lago Maggiore, but the most abundant is *Leptodora kindtii*, which has an important impact on its prey too. However, after an increase in *Bythotrephes longimanus* abundance, the *Leptodora* abundance gets low. This is caused foremost by a different seasonality of these two species. The peak of *Daphnia*

abundance in Lago Maggiore is usually in June and *Bythotrephes* reach high population densities also early in June. On the other hand, *Leptodora* reach the highest abundance in July - August, when it cannot affect zooplankton population as much as *Bythotrephes* can (Manca et al., 2000a).

*Daphnia* responds to the presence of invertebrate predators by alteration of body size, shifts in a vertical distribution and seasonality or changes in community structure (Barbiero & Tuchman, 2004; Manca et al., 2008). And as was measured in Lago Maggiore in 1986 by Manca & Tognota (1993), invertebrate predation pressure is responsible for 'long head morphs' or slender morph of some species from *Daphnia longispina* complex. It was the smaller size fraction of the *Daphnia* population in which the increased head lengths were noticed. Morphotypes were not distinguished into different species, but it seems, that the named 'long head' slender morph could be *Daphnia cucullata*.

Additionally to clarify the determination of *Daphnia* species in Lago Maggiore, the process of nomenclature in scientific articles must be mentioned. From 1949, the term *Daphnia hyalina* Leydig has been used to denote probably *Daphnia longispina* species (Fox et al., 1949; Manca & Tognota, 1993). In 1994, *Daphnia hyalina-galeata* s.l. term has started to indicate individuals from the *Daphnia longispina* species complex (e.g., Guilizzoni et al., 2012; Manca et al., 1994; Manca et al., 2000a; Manca, 2004). *Daphnia* species have not been sometime distinguished at all and only the genus name was used to denote the aim of interest (Visconti et al., 2008). Just in a few research papers, an effort to further the species classification was noticed. The *Daphnia longispina* group was mentioned by Manca et al. (2007a) and even *Daphnia galeata* from year 2008 was once distinguished among Cladocera of Lago Maggiore (Visconti & Manca, 2011). Despite the fact, that species from *Daphnia longispina* complex in Lago Maggiore were not distinguished, work from Marina & Tognota (1993) deals with seasonal changes in morphology of *Daphnia* from the lake, shows dissimilar morphs and there is an effort to assign the morphs to genus names. These morphotypes that were found may be the first indication of a different species or hybrids. Only Keller et al. (2008) determined the *Daphnia* species from Lago Maggiore sampled in 2004, distinguishing *Daphnia galeata* and later generation hybrids.

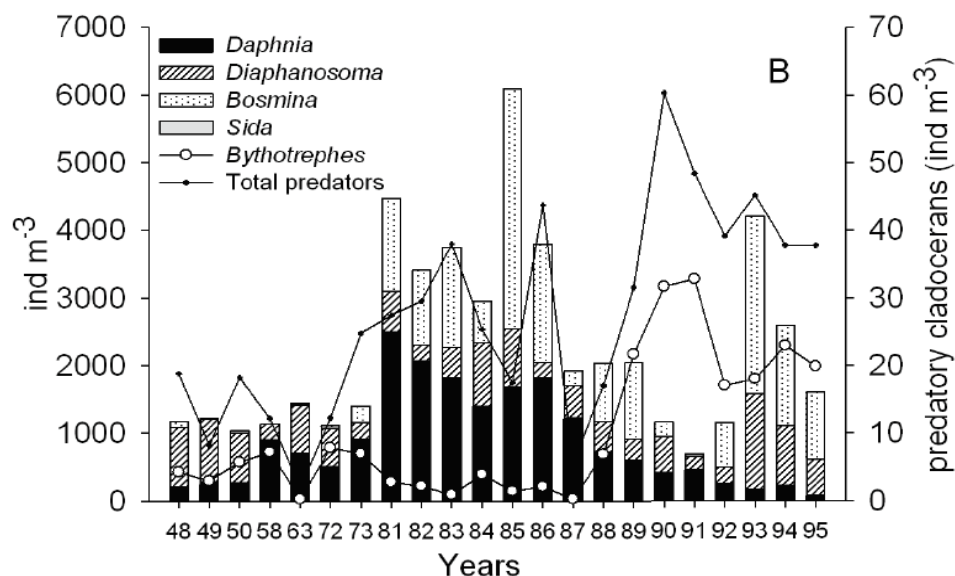


Fig. 4 Changes in cladoceran abundance and taxon composition in Lago Maggiore between 1949 and 1995 (Manca, 2011).

### 1.5.2.3 Nekton

Significant changes in the fish community in Lago Maggiore took place at the end of the 19<sup>th</sup> century and during the 20<sup>th</sup> century. There has been noticed a number of successful introductions of non-native species from other European water reservoirs, lakes or ponds. So Lago Maggiore fish stock, as well as in other European lakes, is composed of native and non-native species (Volta & Jepsen, 2008). Native zooplanktivorous ones are for example *Salmo trutta* and *Alosa agone* (known also as *Alosa fallax lacustris*). The non-native ones get into the lake by human forces. The most radical introduction was caused by the salmonid genus *Coregonus* in 1949. *Coregonus macrophthalmus*, locally named “bondella”, was introduced into the lake in the form of fry that year (Grimaldi, 1972) and became the most important species in the pelagic waters of the lake within a few decades (Manca et al., 2007a). Another local salmonid which appeared in the lake at the beginning of the 19<sup>th</sup> century is a hybrid form of two coregonid species and was named as *Coregonus lavaretus* (locally named “lavarello”) (Manca et al., 2007a). The second major fish invasion was caused by roach (*Rutilus rutilus*, Cyprinidae) in 1993. This fish species was released in Italy for recreational reasons and afterwards expanded to the Po River basin, which includes also outlets of Lago Maggiore (Volta & Jepsen, 2008).

To qualify the abundance of fish in the lake, a total catch per unit effort (CPUE) is commonly used. Because of a great deal of environmental changes in Lago Maggiore during last decades, CPUE has been substantially oscillating. In 1961, a sharp drop in CPUE occurred and salmonids could hardly be found in a water column in the following years (Manca et al., 2007a). To mention the second largest fluctuation, an overall increase

in CPUE showing high abundance of fish was noticed in 1997, probably because of a fishing ban decreed that year (de Bernardi et al., 1990; Grimaldi, 1972; Manca et al., 2007a).

Owing to the events of introductions of new species, the lake community structure changed several times. Other fish species were introduced to Lago Maggiore as well, but the events are not as examined as is the case of roach and coregonids (Volta & Jepsen, 2008).

## 1.6 Aims of the thesis

In this thesis, I focus on changes in the taxonomic composition of Lago Maggiore *Daphnia* since the mid-20<sup>th</sup> century. Local *Daphnia* have been able to overwinter in water column, thus they usually do not form dormant egg banks and may be identified from remains in cores or by studying historical samples (Manca et al., 2007a). For this reason, paleogenetic approaches, as in the cases of Lake Constance and Greifensee, are not feasible, but we may study historical plankton samples from regular monitoring. These are preserved mostly in formalin, which limits the possibilities of genetic analysis. But such preserved samples are adequate for morphological analyses, even if the phenotype is difficult to be identified in the case of hybrids (Dlouhá et al., 2010).

The first aim of this project was to evaluate the taxonomic changes in the *Daphnia longispina* complex in Lago Maggiore over past decades based on morphological features. The second aim was to quantify phenotypic variability of *Daphnia* body shape using geometric morphometrics, and analyse both seasonal and long-term temporal changes in the body shapes.

I hypothesized that the data will unambiguously demonstrate that over the period of eutrophication and re-oligotrophication, *Daphnia* in Lago Maggiore underwent similar changes as in Lake Constance (Brede et al., 2009), from *D.longispina* (“*hyalina*” morph) to *D.galeata*-dominated community with widespread hybridization. I expect that at least some of the above-discussed environmental changes in Lago Maggiore during the 20<sup>th</sup> century are reflected in changes in *Daphnia* body shapes.

For this purpose, I screened available historical samples that were collected in comparable periods of the year at the same sampling station above the deepest basin of the lake (Ghiffa) and selected those that cover the important periods of environmental changes in Lago Maggiore during the 20<sup>th</sup> century. The final selection of samples covers the period from the late 1940s and early 1950s (representing pre-eutrophication period) to 2012. *Daphnia* from these samples were screened morphologically, identified, and their shapes then characterized by geometric morphometrics.

## 2 Material and methods

### 2.1 Locality

Representative zooplankton samples from Lago Maggiore are regularly collected at station Ghiffa ( $45^{\circ}58'30''\text{N}$ ,  $8^{\circ}39'09''\text{E}$ ; Fig. 5), which is located approximately in the middle of the lake (de Bernardi et al., 1990; Manca & Ruggiu, 1998). This point represents the lake's maximum depth (370 m) and is a traditional sampling site in Lago Maggiore (Manca et al., 2007b).

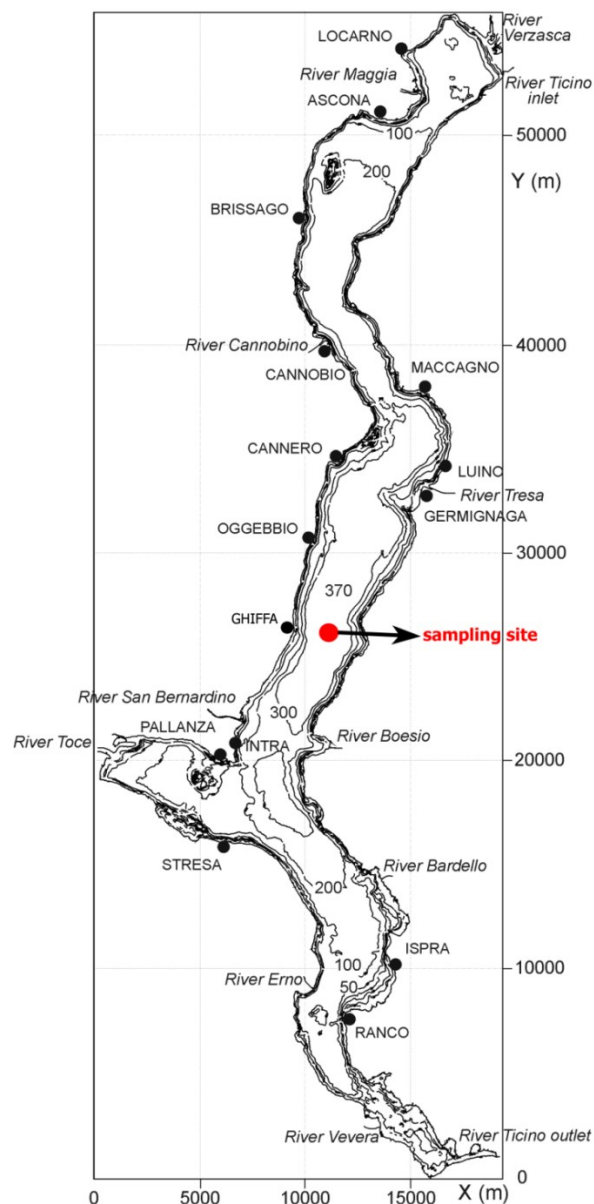


Fig.5 Bathymetric map of Lago Maggiore with tributaries, the Ghiffa sampling site is marked by red dot (Ambrosetti et al., 2012).



## 2.2 Samples

Years of samples have been chosen to cover the period from the oligotrophic stage of the lake in the late 1940s until 2012 in an effort to include as many sampled years as possible to show major changes in the lake during the last decades. To cover the season of the highest phenotypic variation (August), density peak (July) and fecundity peak (May) of *Daphnia* in Lago Maggiore (Manca et al., 2008; Manca et al., 2014), samples from May and August were analyzed whenever possible. For years 1949 and 1952, in which samples from these months were unavailable, April and June samples have been used.

Samples were stored in Museo del Plancton in the Institute of Ecosystem Study, Pallanza. As mentioned above, samples have been studied in the Ghiffa station. They were usually collected with a Clarke-Bumpus plankton sampler with a net of 76  $\mu\text{m}$  mesh size, mostly from the depth of 0-50 m either by sinusoid or vertical trajectory. The sinusoid sampling is characterized by the depth alternation during pulling of the net outwards, thus avoiding problems caused by non-homogeneous vertical and horizontal distributions of the zooplankton. For one sampled year (1996), individuals were selected from both -vertical and sinusoid draws to provide a sufficient number of individuals per season. Furthermore, for certain years, only vertically sampled individuals were available (Table 1). One sample (1948) has been collected by horizontal haul. Even in this case, however, it seems the sample is sufficiently representative, when the results are compared with samples from different years.

Undamaged mature *Daphnia* females have been randomly picked out, up to 96 specimens per sample if possible. Samples collected until 1997 are preserved in formalin, the more recent ones in ethanol. In a few cases (1948, 1949, 1952), where there were not enough specimens in one sample, individuals from different days of the same season have been put together (Tab. 1).

| Year  | Season | Day/Month | Individuals | Fixation | Sampling   |
|-------|--------|-----------|-------------|----------|------------|
| 2012  | summer | 21/08     | 96          | ethanol  |            |
| 2008  | summer | 20/08     | 96          |          |            |
| 2008  | spring | 13/05     | 96          |          |            |
| 2004  | summer | 26/08     | 96          |          |            |
| 2004  | spring | 26/05     | 96          |          |            |
| 2003  | summer | 6/08      | 96          |          |            |
| 2003  | spring | 14/05     | 96          |          |            |
| 2000  | summer | 24/08     | 96          |          |            |
| 2000  | spring | 26/05     | 96          |          |            |
| 1997  | summer | 20/08     | 96          |          |            |
| 1996s | spring | 14/05     | 21          |          |            |
| 1996q |        | 14/05     | 72          |          |            |
| 1992  | summer | 12/08     | 96          |          |            |
| 1992  | spring | 19/05     | 96          |          |            |
| 1989  | summer | 25/08     | 96          |          |            |
| 1989  | spring | 19/05     | 96          |          |            |
| 1986  | summer | 26/08     | 96          |          |            |
| 1986  | spring | 29/05     | 96          |          |            |
| 1973  | summer | 14/08     | 96          |          |            |
| 1973  | spring | 17–18/05  | 96          |          |            |
| 1952  | summer | 23/08     | 84          |          |            |
|       |        | 14/08     | 12          |          |            |
| 1952  | spring | 3/06      | 96          |          |            |
| 1949  | spring | 10/05     | 28          |          |            |
|       |        | 28/06     | 8           |          |            |
|       |        | 5/04      | 4           |          |            |
| 1948  | summer | 30/08     | 6           |          |            |
|       |        | 19/08     | 81          |          |            |
|       |        | 14/08     | 7           |          |            |
|       |        |           |             |          | vertical   |
|       |        |           |             |          | horizontal |

Tab. 1 List of analyzed samples.

### 2.2.1 Processing of samples

Initial sample processing was performed under a binocular microscope at the Institute of Ecosystem Studies in Pallanza. *Daphnia* females were picked from the sample. Subsequent sample processing took place in Prague. Using a binocular microscope, Olympus SZX12 with an objective lens Olympus DF PL 1.5x and a Canon camera, two microphotographs of every *Daphnia* individual were taken. The first photograph was of the whole body with a magnification 48x, the second with a magnification 75x was a detail of the head to document the body shape as well as potential taxonomically-relevant traits.

Representatives of all main phenotypes and individuals of transitional phenotypes of potential hybrid origin present in samples have been examined in detail under a microscope Nikon Eclipse E400. Emphasis was especially placed on the head and rostrum shape, position of antennules against rostrum, shape and size of the antennular mound and eye position. Subsequent expert assessment has been done by my supervisor, prof. Adam Petrusek.

### 2.3 Acquisition of morphometric data

Photographs of the whole *Daphnia* individuals have been used to acquire shape and size data. TpsDig program (Rohlf, 2001) for digitizing outlines and geometric morphometric analyses was used to obtain raw data on *Daphnia* body shapes. Furthermore, I measured body lengths of all individuals as the distance from the highest point of the head to the spine base (Fig. 6). Body shapes were characterized by ca 50 points roughly equidistant points along the body outline (excluding the antennae and tail spine). Points were selected manually, the starting point was located at the anterior edge of ventral carapace margin and the direction of point acquisition was clockwise (Fig 6).

Outputs in a form of set of point coordinates have been utilized as input into an R package for analyzing outlines of shapes, Momocs (Bonhomme et al., 2014). Its function for elliptic Fourier analysis (efourier) has been applied to characterize *Daphnia* shapes (normalized to size, rotation and starting point position) and 5 harmonic functions have been utilized in further analyses. This method has been used for geometric morphometrics for example also by Dlouhá et al. (2010). Afterwards, a multivariate data analysis has been performed.

Variation in the *Daphnia* body shapes was characterized in the principal component analysis (PCA) and visualized using the plot function in the package Momocs. Considering that most variation was captured in the first principal component (see Results), score values of PC1 axis were further used as a proxy for body shape. To summarize variation in *Daphnia* body shapes in relation to annual variation

and seasonality, histograms were created using a ggplot2 package with ggplot function (Wickham, 2016).

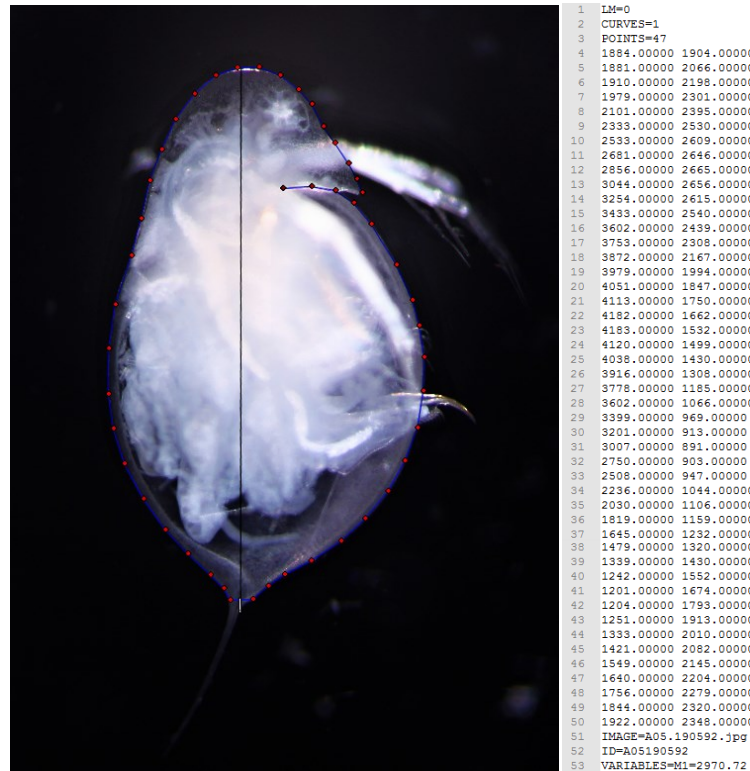


Fig. 6 Input data for morphometric analysis obtained by TpsDig2 program.

The differences in PC1 scores between taxon distribution and between years were tested by analysis of variance (ANOVA) using aov function in R program (R Development Core Team, 2008). The variation of body length and taxon distribution between years was analyzed by the use of a non-parametric Kruskal-Wallis test and a kruskal.test function.

Independently of geometric morphometric analysis, each of the 2150 *Daphnia* individuals was determined by phenotypic traits. Based on morphological features on *Daphnia* head (e.g. position of compound eye, presence of nauplius eye, head shape, rostrum shape) together with the overall body shape, each individual was assigned to a taxon from the *Daphnia longispina* complex (*D. galeata*, *D. cucullata*, *D. longispina*, or their presumed interspecific hybrids if the individuals exhibited transitional phenotypes). During this process, *Daphnia* body length was measured and using a boxplot function, the alternation of body lengths during the studied period was illustrated. *Daphnia* mean body shapes for each year are illustrated in Attachment 1.

Originally, I also counted the brood size (number of eggs or embryos) for each analyzed *Daphnia* females. Nevertheless, measured *Daphnia* fecundity is not included in the results for several reasons. Most of the samples used in my study are preserved in formalin and some of them are older than a half century, thus eggs and embryos could get lost from the brood chamber in the course of preservation or sample handling. Although there are a plenty of factors affecting the fecundity, it is not possible in my work to try to find the connectivity between them and to count the numbers of eggs and embryos. The reason is the same as has been found in the study from Manca et al. (2000a). In that study, recently sampled *Daphnia* were used and preserved in 90% alcohol. Despite the recent samples, too many eggs have been lost during the preservation and further analysis of the size-specific fecundity was not possible.

To express a relation between *Daphnia* body shapes (represented by PC1 scores), taxon assignment, seasonality and environmental variables (in detail in Attachment 2), I created regression trees using rpart package and its rpart.plot function (Therneau et al., 2003) in R. Investigated environmental factors include total phosphorus ( $\mu\text{g L}^{-1}$ ), water temperature (0-20 m; °C), chlorophyll *a* ( $\text{mg/m}^3$ ) and invertebrate predators, *Bythotrephes longimanus* ( $\text{ind/m}^3$ ) and *Leptodora kindtii* ( $\text{ind/m}^3$ ). These variables represent the annual average measured by the Institute of Ecosystem Studies at the Ghiffa sampling site. Data were provided by the Institute of Ecosystem Study in Pallanza and by CIP AIS (Commissione Internazionale per la Protezione delle Acque Italo-Svizzere). Only years 1986-2012 and 1986–2008 could be used due to lack of environmental data for other periods.

One of the original aims was to evaluate also the impact of fish predation pressure on *Daphnia* morphology or taxonomic composition. Data of the professional fishing annual catch was available but not usable for quantitative analysis because of the fishing ban for commercial fisheries from year 1996 to 2005 (Ceschi et al., 1996) and the ensuing analysis of the data would be misrepresented.

## 3 Results

### 3.1 *Daphnia* morphology and taxonomic composition

Studied samples of *Daphnia* population from Lago Maggiore are composed of diverse phenotypes (Fig. 7) showing a high variability in morphological features. The data thus suggests a substantial taxonomic turnover in Lago Maggiore during the last decades. Because of a difficulty of taxonomical assessment just by general phenotype (Dlouhá et al., 2010), it was essential to focus on particular morphological features. The following morphological characters have been examined: the head shape, ventral margin of the head, rostrum length and shape and the presence and position of the ocellus. Although the distinction of species from hybrids is difficult and not fully reliable, general trends of taxonomic changes are apparent.

The proportions of individual species and their hybrids in Lago Maggiore (as determined by phenotype) through all considered years are shown in Fig. 8. From 1948 to 1973, *D. longispina* was dominant and presumed *longispina* x *galeata* hybrid phenotypes with transitional features occurred in a pelagic zone. In this period, more precisely in 1952, the first individuals identified as *D. galeata* were found (Fig. 7 B). A few representatives of this species were characteristic with their helmet and spiky rostrum (Fig. 7 H). In 1986, a few typical *D. cucullata* phenotypes emerged having small and slender body shapes with characteristic helmets (Fig. 7 C). From 1986, *D. longispina* x *cucullata* and *D. galeata* x *cucullata* hybrids started to occasionally appear in the lake. Later, in 1989, *galeata*-like individuals appeared again. Other transitional phenotypes, as identified by detailed features on the head, lived in the lake in 1996 (Fig. 7 D). These phenotypes with rounded head, generally similar to *D. longispina*, were identified as hybrids between *D. longispina* and *D. galeata* (one interesting phenotype is illustrated in Attachment 3). The next decade (2004 and 2008) shows similar phenotypes of *D. galeata*, which occurred also in 1952 (Fig. 7 G and I). This shows that *D. longispina* and its hybrids prevailed in the water column until the end of the 20<sup>th</sup> century, when the *D. galeata* abundance started to increase and in 2012, only *D. galeata* was identified in studied samples.

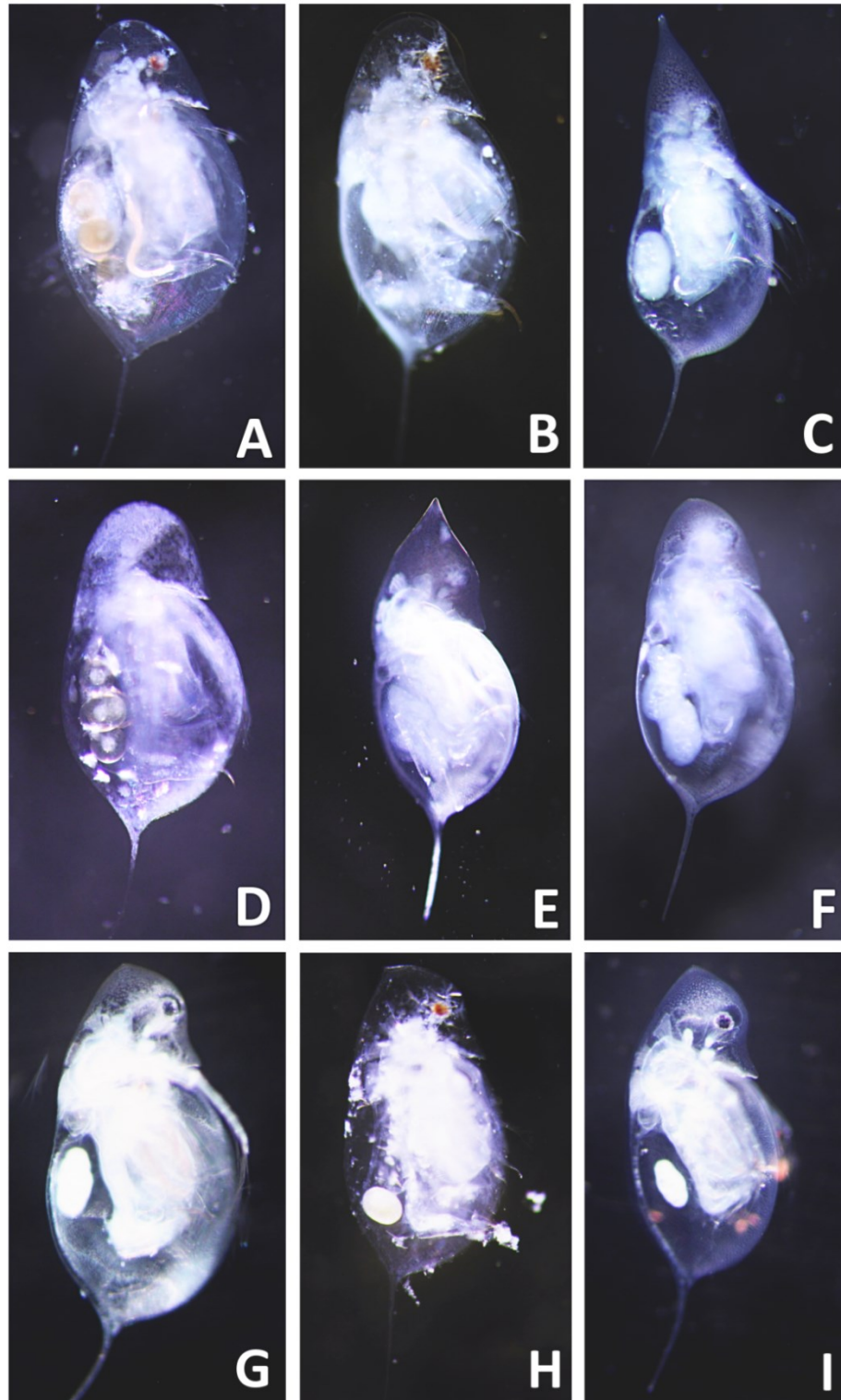


Fig.7 *Daphnia* representatives of phenotypes demonstrate fundamental morphological features. A: phenotype identified as *D. longispina* species from year 1949; B: *D. galeata*-like phenotype, 1952; C: *D. cucullata*, 1986; D: transitional phenotype identified as *longispina* x *galeata* hybrid based on detailed characteristics, 1996; E: *galeata* x *cucullata* hybrid-like phenotype, 1992; F: *longispina* x *cucullata* hybrid-like phenotype, 1986; G – I: *D. galeata*-like helmet phenotypes, 2004, 1952, 2008.

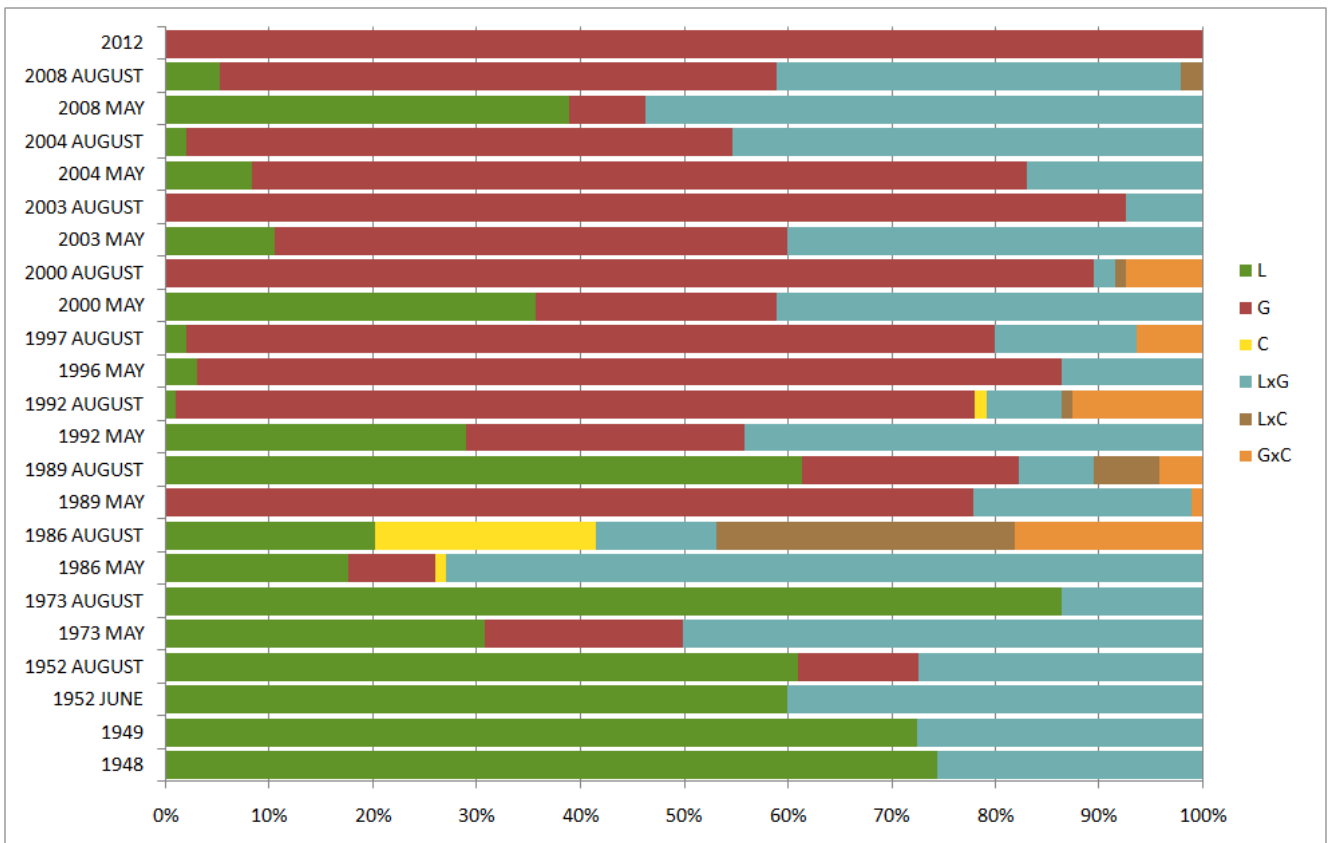


Fig. 8 Changes of taxonomic composition in *Daphnia* population from Lago Maggiore (determination based on morphological features).



### 3.2 *Daphnia* body shape variability

Principal component analysis (PCA) of all *Daphnia* individuals shows that most variation of the body shape is summarized in the first principal component (PC1) and the PC1 score values can be thus used as a single-number proxy characterizing the body shape (Fig. 9). The first PCA axis explained 93.4% of the total variance, the second axis only 2.4%. Differences in body shapes in observed years are shown in Fig. 9, when individuals with the highest score values belong to the right part of the PCA plot. They are characterized by a narrow body shape, sometimes with helmet on the top of their head. On the contrary, individuals from the left part of the PCA plot with lower score values have a wider and more rounded body shape. Individuals from some years are phenotypically similar, but a trend from a rounded to narrower body shape was detected and apparent differences in phenotypes have been also noted during the study of annual and seasonal variability (Fig. 10 and 11).

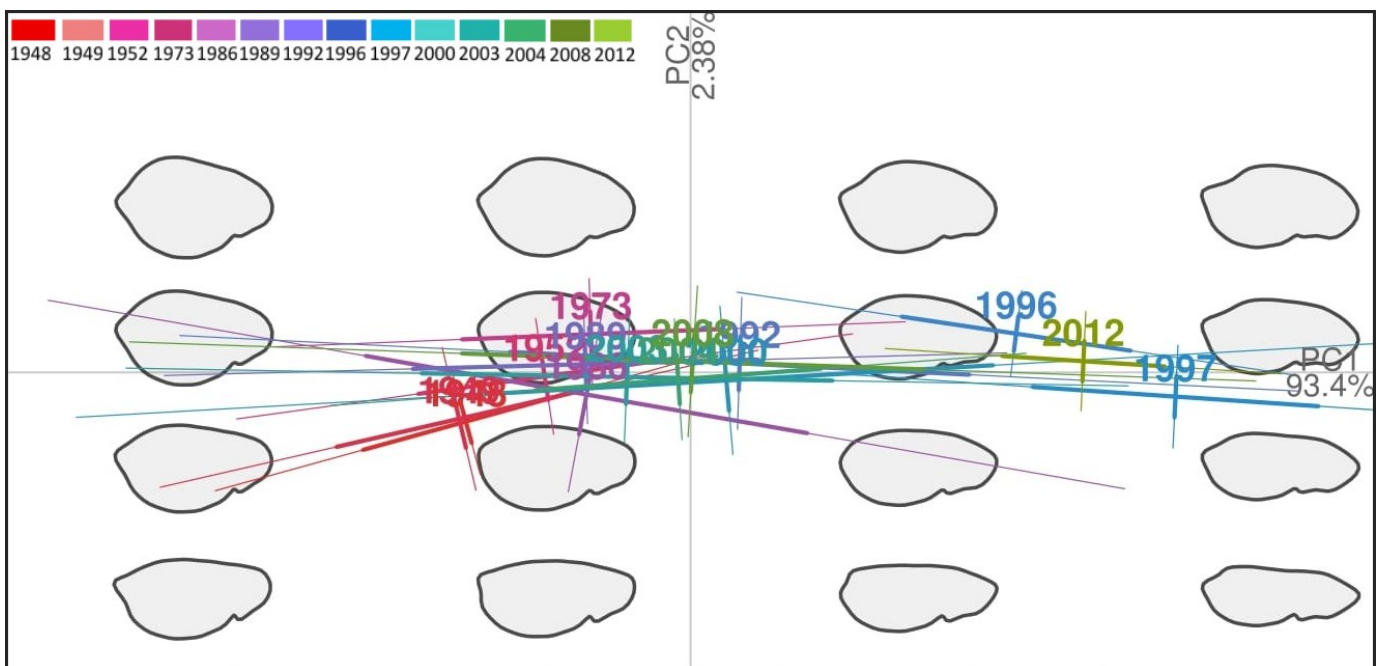


Fig. 9 Plot of principal component analysis (PCA) summarizing variation of *Daphnia* body shapes (based on elliptic Fourier analysis) in the 14 sampling years. The first two ordination axes are displayed. Each color represents a different year. Colored axes point to increasing tendency to certain body shape and outlines of representing different body shapes are illustrated.

When comparing the distribution of *Daphnia* body shapes in individual samples, distinct differences between the seasons (spring and summer), as well as changes between individual studied periods are apparent (Fig. 10). The presence of different phenotypes in a few samples is noticeable from distinctive bimodal distribution in the histograms, or from markedly outlying values. These differences often reflect the presence of more taxa, for example *D. cucullata* in 1986 (Fig. 11).

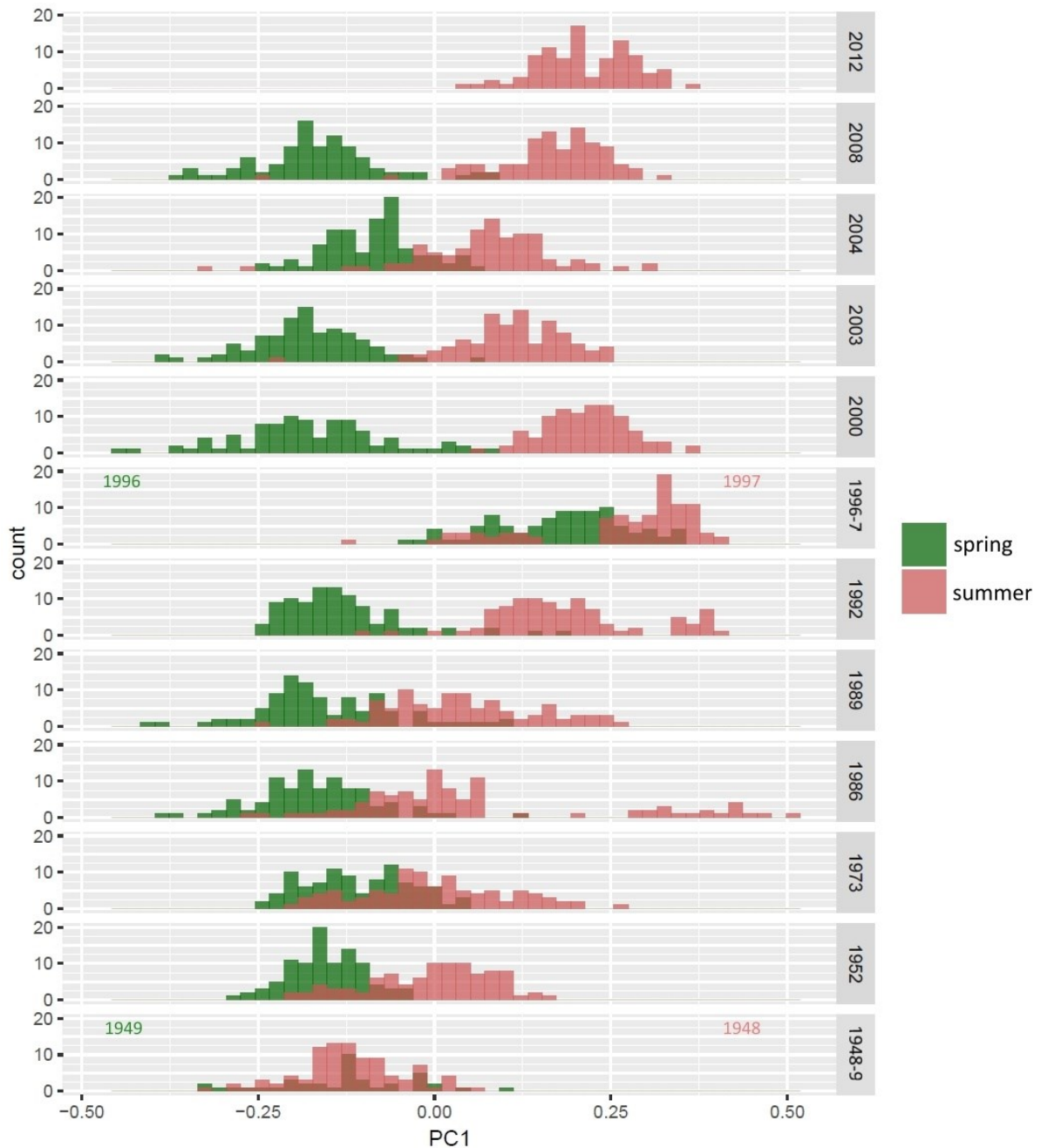


Fig. 10 Variation of body shapes (PC1 scores) in individual years and seasons (spring, summer), showing the distribution of shapes among individuals. Counts represent number of individuals.

After addition of identified taxa into the distribution of *Daphnia* body shapes, changes in taxonomical composition between years and between certain studied periods are noticeable (Fig. 11). A trend from *D. longispina* to *D. galeata* dominance in the *Daphnia* population during the 20<sup>th</sup> century is apparent. Outlying PC1 values of some individuals are explained by taxon determination. Especially the score deviation in 1986 is explained by the presence of *D. cucullata* phenotypes. PC1 score values are significantly different between the taxon distribution and even between years (ANOVA: df = 5,  $p < 2 \times 10^{-16}$ ).

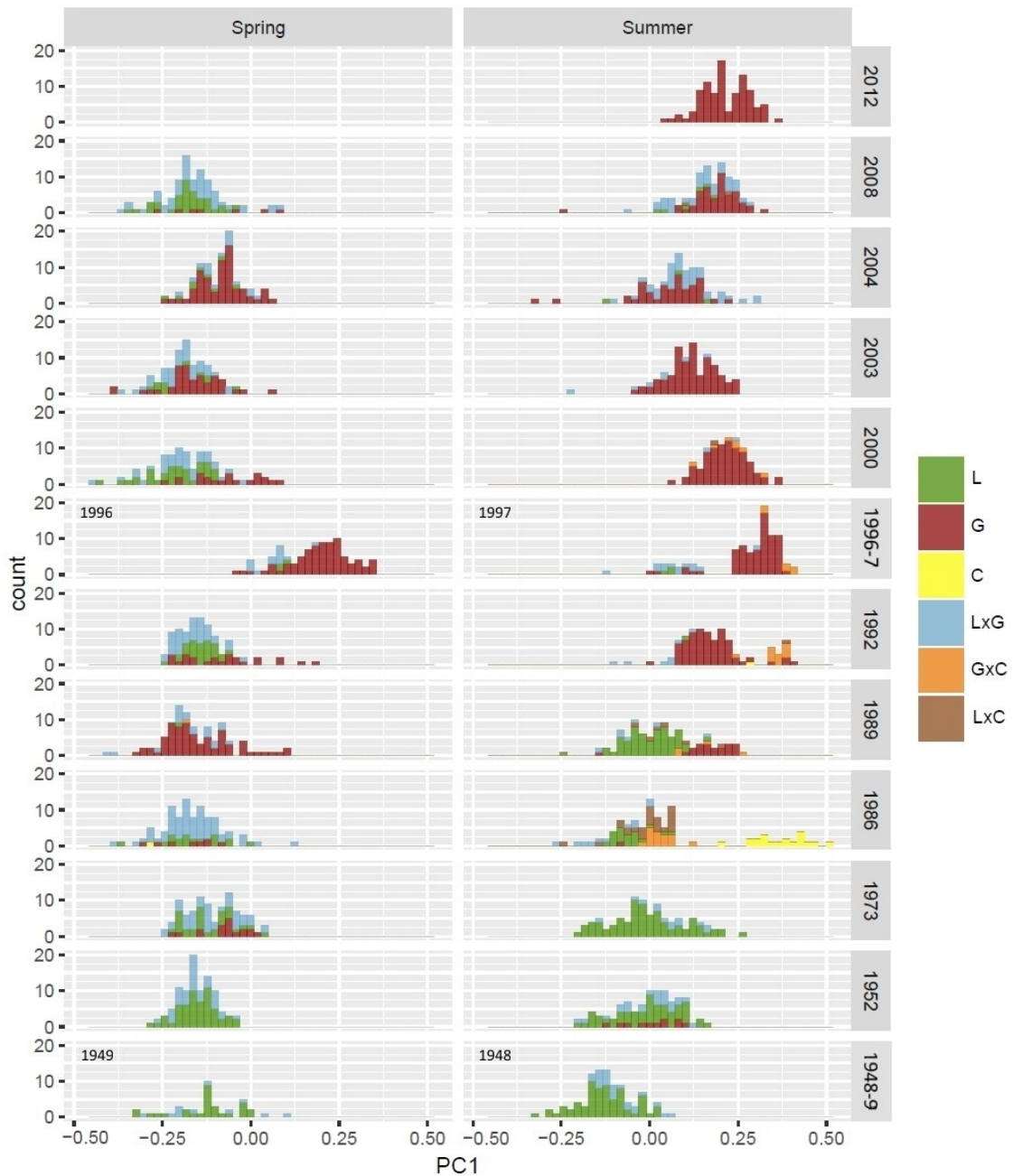


Fig. 11 Variation of body shapes (PC1 scores) in individual years and seasons (spring, summer), showing distribution of shapes among individuals of different taxa (as identified by phenotypic traits).

### 3.3 *Daphnia* body length

Another measured quantitative variable is the length of the *Daphnia* body. The body length of mature *Daphnia* individuals has been changing throughout the 20<sup>th</sup> century between studied years and often even between seasons (spring, summer) (Fig. 12). The most appreciable decrease of the body lengths was measured in 1986, when small *D. cucullata* morphs appeared. After that there was an increase in the body length from 1989 to 1992 with a peak in the summer. This was the most rapid shift and in following years the body length was almost unchanged with the exception of 2004, when the last decrease was noticed. The among-year variation had significant effects on body length and taxon distribution (Kruskal-Wallis:  $df = 22$ ,  $p < 2 \times 10^{-16}$  in all cases).

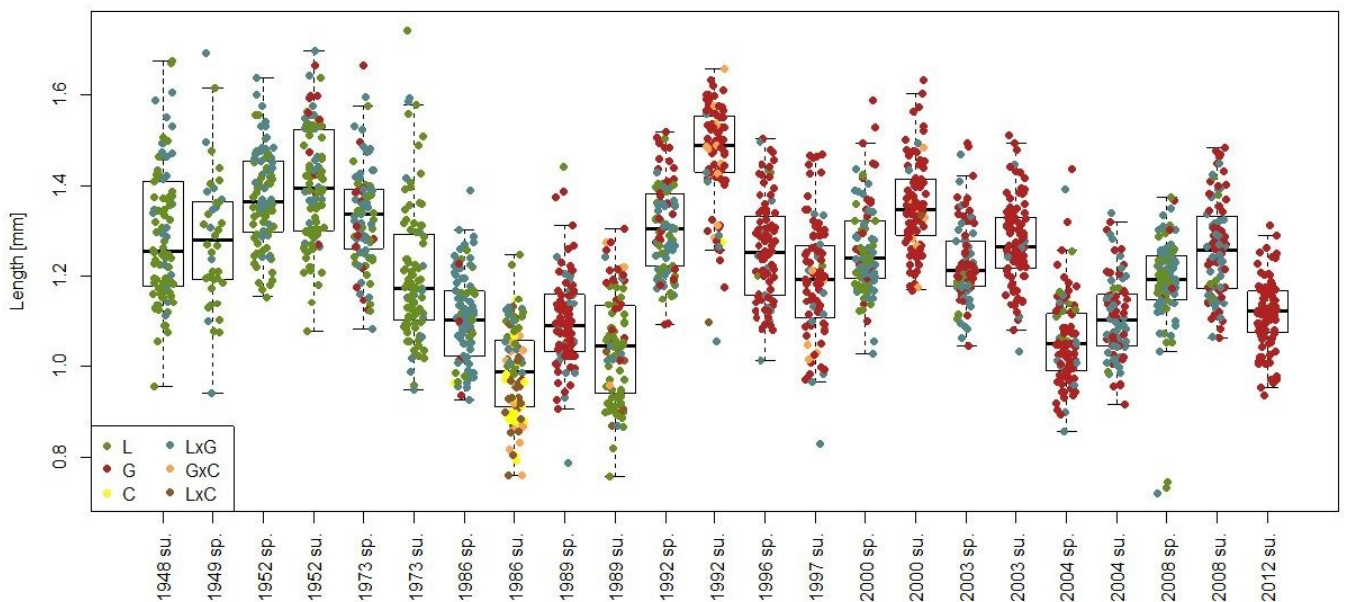


Fig. 12 Measured *Daphnia* body length variability in concerned years complemented by taxon distribution.

### 3.4 Factors explaining changes in taxonomic composition

To explain the variability in *Daphnia* body shapes (represented by PC1 scores) throughout the 20<sup>th</sup> century, different variables were used in regression tree analysis. Variables included in the first regression tree (Fig. 13) are taxon, year, season (spring, summer) and *Daphnia* body length (which was not selected in the final model). This regression tree is explaining 67.5 % variability in *Daphnia* body shapes. The most distinctive is the variability between seasons followed by the division based on taxon determination. In these nodes the division on *longispina*-like and *galeata*-like individuals is clearly seen. The last divisions in the regression tree show a difference between *Daphnia* from older and more recent years. The breaking point for this division is somewhere between 1992 and 1996, where *D. galeata*-like phenotypes begin to prevail over *D. longispina* ones.

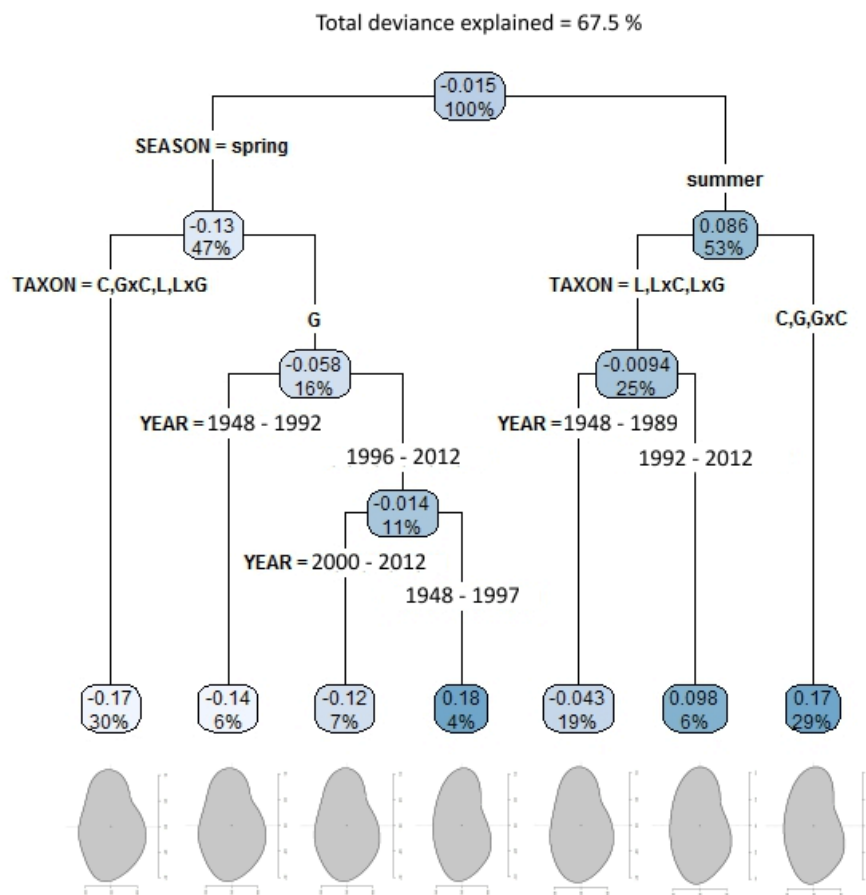


Fig. 13 Regression tree illustrating a distribution of *Daphnia* body shapes by different factors is illustrated. Predictor variable used for primary split is represented by score values from PC1 and explanatory independent variables are represented by taxon (L, G, C, LxG, GxC, LxC), year, season (spring, summer) and *Daphnia* body length. Nodes and leaves show mean values of PC1 scores. *Daphnia* mean body shapes for any target value (reconstructed from parameters of four harmonic functions) are illustrated.





When an abundance of invertebrate predators (*Bythotrephes longimanus* and *Leptodora kindtii*) was added to environmental variables used in the previous regression tree analysis, these characteristics were preferentially chosen. The phosphorus remains nevertheless selected in the first node in the tree for summer samples (Fig. 15).

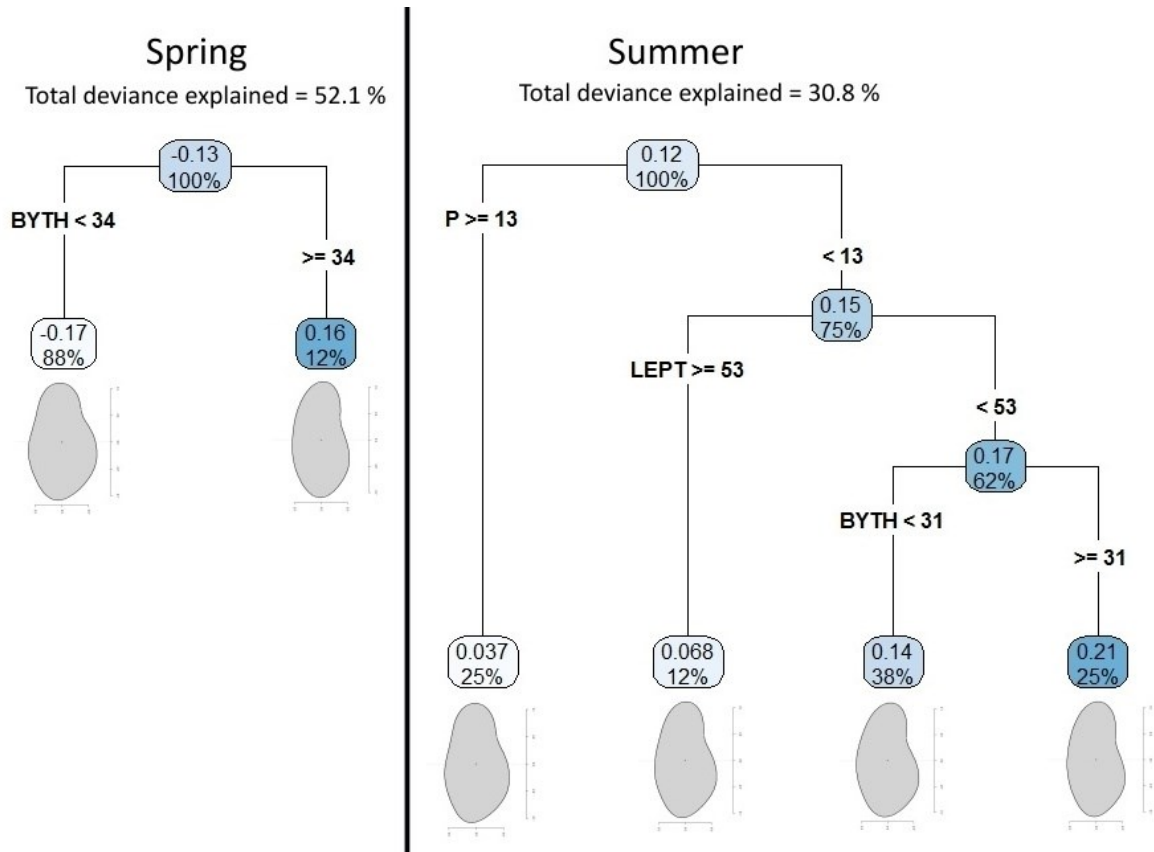


Fig. 15 Regression trees for two seasons illustrate dependent variable, which is represented by PC1 score values and explanatory independent variables represented by total phosphorus concentration ( $\mu\text{g L}^{-1}$ ), water temperature (0-20 m;  $^{\circ}\text{C}$ ) and chlorophyll a ( $\text{mg/m}^3$ ) and invertebrate predators, *Bythotrephes longimanus* ( $\text{ind/m}^3$ ) and *Leptodora kindtii* ( $\text{ind/m}^3$ ). Only years 1986 – 2008, for which relevant environmental data are available, were analyzed. Nodes and leaves show mean values of PC1 scores. *Daphnia* mean body shapes for any target value are illustrated.

## 4 Discussion

In various deep pre-alpine lakes in Central Europe, which underwent anthropogenic eutrophication, changes in the *Daphnia longispina* species complex related to changes of the environment were observed (Alric et al., 2016; Brede et al., 2009; Rellstab et al., 2011). My research was focused on the taxonomical composition of *Daphnia longispina* species complex in Lago Maggiore during the 20<sup>th</sup> century. It is, to my knowledge, the first attempt to summarize long-term changes in *Daphnia* species composition in this lake. Up to now, only short-term seasonal observations of concerned *Daphnia* species have been done. But the species were not identified or they were just labeled with one summarizing term, mostly as *Daphnia* (Manca & Tognota, 1993; Manca et al., 2008). Only Keller et al. (2008) determined *Daphnia* taxa in this lake using genetic tools (specifically, allozyme electrophoresis).

### \* *DAPHNIA* TAXONOMIC COMPOSITION

The overall scenario of taxonomical changes in the *Daphnia longispina* species complex (from “*longispina*-like” to “*galeata*-like” morphotypes) is similar to the one observed in Lake Constance and Greifensee north of the Alps (Brede et al., 2009), so it was possible to compare taxonomical shifts in those two lakes with the *Daphnia* population in Lago Maggiore. In the lake, we occasionally recorded also *Daphnia cucullata* and its hybrids with *D. galeata* and less often with *D. longispina*.

Independent data, on which our species identification can be verified, are rare. But in the studies of Keller et al. (2008) and Manca (2004), the taxonomic composition in years 2003 and 2004 was studied using genetic methods and the presence of *D. galeata* and unspecified hybrid class (with *D. longispina*) were noticed in Lago Maggiore in those years. The same *Daphnia* was recorded (based on the morphology) in my work, with the difference that a few individuals were identified as *D. longispina* (not reported by above-mentioned authors) and the hybrid group was labeled as *longispina* x *galeata* hybrids.

In August 1952, first individuals identified as *D. galeata* were recorded in our samples. After this, the trophic stage of the lake started to rising up (from 1962) (Manca et al., 1992; Manca et al., 2007a), more *D. galeata* individuals appeared. I do not assume, that its occurrence was due to eutrophication of the lake, it might have rather been a reaction to increasing predation pressure (Manca et al., 2007a; Volta & Jepsen, 2008). Some of the important changes that happened in the lake in the preceding years include the introduction of whitefish (1949), the increase of *Bythotrephes longimanus* population (1950) and the increase of fish predation pressure (1951) (Manca, 2011; Manca et al., 2007a).



Some of these changes could facilitate an increase in abundance of *D. galeata* above the detectable level. Nevertheless, there is also a probability that *D. galeata* was present in Lago Maggiore together with *D. longispina* well before 1948, because of the findings of some individuals with transitional phenotypes, which were identified as *longispina* x *galeata* hybrids, in years 1948 and 1949. Even in the cases of Greifensee and Lake Constance, *D. galeata* occurred (but did not dominate) before an increase in trophic level (Brede et al., 2009). In Greifensee, deposited ephippia of hybrids of *D. galeata* were found in sediment layers of the 1920s suggesting that *D. galeata* has been present in the lake in those periods. While in Lake Constance, the *D. galeata* appearance in the mid-1950s could be caused by a spread of the species from the southern to the northern part of the lake.

Nevertheless, designation of *D. galeata* from historical samples of the lake is accompanied by many difficulties, and it cannot be considered unambiguous. As Flößner & Kraus (1986) pointed out, the high level of phenotypic plasticity within *Daphnia longispina* complex is caused mainly by interspecific hybridization and by variable environmental conditions. By this reason, different morphs may be genetically identical individuals and vice versa (Flößner & Kraus, 1986). The accuracy of morphological and genetic determination in hybridizing species may be significantly influenced by backcrossing and gene flow, processes following interspecific hybridization (Mallet, 2005; Skage et al., 2007). This means that it is very difficult to identify introgressed individuals by their phenotypes and the taxonomy can be complicated by the occurrence of intermediate morphs (Schwenk et al., 2008). In the study of Dlouhá et al. (2010), difficulties in species determination and error rate of determination of the *Daphnia longispina* species complex are discussed. In that study (which nevertheless focused on general body shape only, not specific morphological features). It has been found that species from the complex may exhibit nearly identical body shapes (especially *D. galeata* and *D. longispina*) and just *D. cucullata* and its hybrids with *D. galeata* are the most recognizable in contrast to *longispina* x *galeata* hybrids. However, difficulties with the identification of hybrids also arose in my study, especially in the case of *longispina* x *galeata* hybrids. *Daphnia* body shapes differ between years and seasons with the most noticeable different morphotypes in 1986, where the deviation was caused by the occurrence of morphologically different *D. cucullata*.

Another potential useful method for determination of *Daphnia* species from old samples preserved in formaldehyde or denatured ethanol is based on species-diagnostic SNP (single nucleotide polymorphism) markers (Rusek et al., 2015). However, it is a destructive method, in which historical *Daphnia* samples would be homogenized for a purpose of DNA extraction (Giessler & Wolinska, 2013). The success of the analysis of formaldehyde samples is not guaranteed, so we did not use the approach for valuable samples from Lago Maggiore. However, if it was possible in the future to successfully

analyze formaldehyde samples by a non-destructive method, it would be possible to evaluate my results by the use of some genetic approach.

#### **\* ANNUAL VARIATION AND SEASONALITY**

As well as the inter-annual variation in the pelagic cladoceran abundance, the species composition altered too, similarly as Brede et al. (2009) and Manca et al. (2007a) noted. Shapes of the *Daphnia* body varied along the timescale and show mainly seasonal variability. Manca & Tognota (1993) studied in detail the environmentally induced morphology changes of *Daphnia* in Lago Maggiore in 1986. They found out that the head:carapace ratio and body length depend largely on the predation rate and different environmental factors acting during the different phases of the seasonal cycle. My results show, that not only intraspecific variation but especially seasonal changes in taxonomic composition influences these patterns: although Manca & Tognota (1993) discussed only variation within *D. hyalina*, the high helmeted individuals were most likely *D. cucullata*, which I found in summer 1986 sample from Lago Maggiore. In my dataset, it was the first record of *D. cucullata* in the lake (the species is not reported in the literature but it might have been also present earlier). Compared with lakes Greifensee and Constance, where *D. cucullata* was not recorded from sediment cores (Brede et al., 2009), the taxonomic diversity of the *Daphnia* population in Lago Maggiore in the 20<sup>th</sup> century could have been higher because of *D. cucullata* and its hybrids.

In Lago Maggiore, the inter-annual variation of *Daphnia* body shapes could be affected by events with long-term consequences. It could have started by the introduction of coregonids and thereafter by increased predation pressure (Manca et al., 2008). Afterwards, following changes in predation pressure of zooplanktivorous fish and invertebrate predators could affect population dynamics of *Daphnia*. Even seasonal variation in *Daphnia* body shapes may be affected by the abundance of a certain predator (Manca et al., 2008). According to results from the work of Manca et al. (2008), a relation between measured *Daphnia* body length from my samples and predation pressure in the lake may be noticeable in the case of year 1992, for which I measured high *Daphnia* body lengths. In 1992, *Bythotrephes* was abundant, preferring small or young *Daphnia* individuals, and thus could induce higher *Daphnia* body length.

From Fig. 10 it is evident that more rounded *longispina*-like morphs were more often present in the spring period (May). This could be explained by the highest predation of zooplanktivorous fish preferring larger invertebrates as their prey in this month. Subsequently in June, when the highest predation pressure on *Daphnia* was by *Bythotrephes* (Manca et al., 2008), long-head slender morphs usually occurred. Accordingly, the seasonality in *Daphnia* body shapes may be explained by the variation in the abundance of both invertebrate predators and fish.

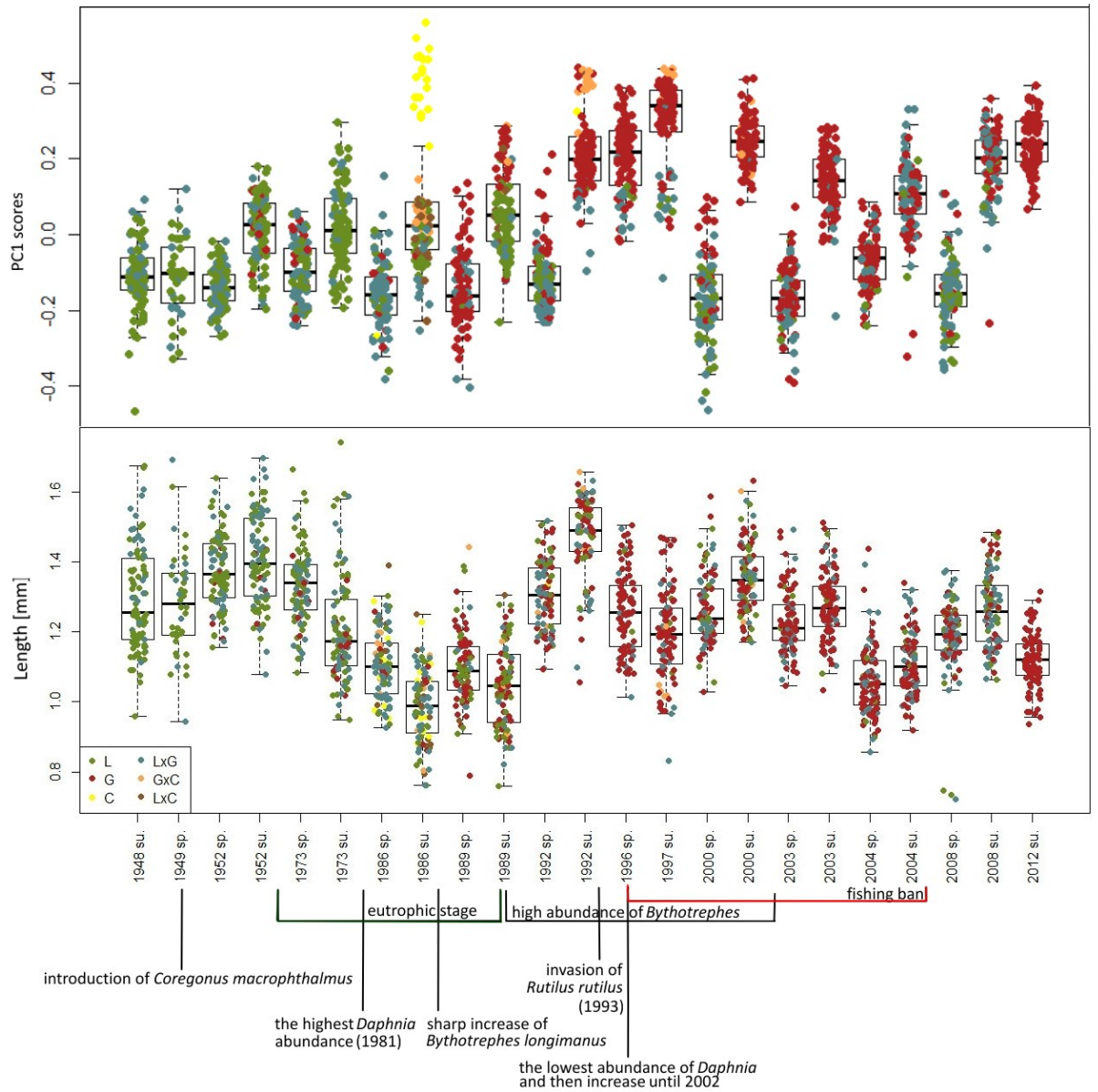


Fig. 16 Plot summarizing changes in *Daphnia* body shape (PC1 scores; above) and body length (below) complemented by taxon identification and the most significant events, that may have influenced *Daphnia* population in Lago Maggiore.

### \* *DAPHNIA* BODY LENGTH

It is well-known that one of the strongest effects on *Daphnia* body length is a size-selective predation of fish and cladoceran invertebrate predators (Stibor, 1992; Barbiero & Tuchman, 2004; Spaak et al., 2000). In the pelagic zone of Lago Maggiore, *Bythotrephes longimanus*, *Leptodora kindtii* and coregonids represent dominant feeders on zooplankton. But among the two predatory cladocerans, *Bythotrephes* usually has larger impact on *Daphnia* dynamics (de Bernardi et al., 1987; Manca et al., 2000a).

Because of the measured data, it is possible to evaluate which factors may have influenced the *Daphnia* body length. Two growths and two declines were noticed during the analyzed period. For each of the effect, hypothesis exists and will be compared with study from Manca et al. (2007a). In the years 1948 – 1952, morphs with a relatively long body occurred despite the introduction of *Coregonus macrophthalmus*, which happened in 1949 and the peak of fish abundance was in 1950 (Manca et al., 2007a). But the fish predation pressure presumably did not have a strong effect on the *Daphnia* body length. Another coregonid fish introduction took place in 1961 and probably resulted in a decrease of *Daphnia* body size within several years up to the mean body length value around 1 mm in summer 1986 (Grimaldi, 1972). In that time, small *D. cucullata* occurred in a water column. Despite the fact, that the main phase of increased fish predation pressure was during the years 1985–1997 (Manca et al., 2007a), *Daphnia* body length started to increase already after the year 1989. The reason for the body growth was probably an increased *Bythotrephes* abundance from 1987 (Manca et al., 2007a) and an observed formation of relatively high helmets of *D. galeata*, which I found to be the highest from all samples in the summer of 1992. One year later, roach invasion occurred and afterwards, in 1996, a fishing ban came into force until 2005 (Ceschi et al., 1996). As a result of the consequential increasing fish abundance, invertebrate predators decreased and more smaller *Daphnia* morphs appeared in the pelagic zone (Manca et al., 2007a). Regarding seasonal variation in the *Daphnia* body length, the seasonal differences were significantly less noticeable than in the case of seasonal changes in body shapes.

### \* INFLUENCE OF ENVIRONMENTAL CHANGES AND PREDATION

Morphology of *Daphnia* individuals is determined especially by environmental factors and predation pressure (Jacobs, 1961; Laforsch & Tollrian, 2004b). Cyclomorphosis, in a response to the seasonal variation of the environment, plays an important role as a phenotypically plastic defense against the predation (Manca & Tognota, 1993; Weber & Vesela, 2002).

Results from the analysis of the environmental impact on the *Daphnia* body shapes show the most relevant environmental factors during the studied time period. The regression tree in Fig. 13 explaining 67.5 % of total deviance indicates a high seasonal

variation in body shapes. Thus the season is the most important factor in *Daphnia* body shapes. After the season, taxonomic distribution represents another important factor, showing *galeata* and *cucullata*-like individuals on the one side and other taxa on the other side of the regression tree. It indicates the morphological differentiation between studied *Daphnia* individuals and possibly points to notable different morphotypes of small slender *Daphnia* with helmets found in several sampled years. And the distribution by years may reflect some shifts of body shapes during the re-oligotrophication stage, which initiated in Lago Maggiore in the 1990s (Manca et al., 2007a).

## 5 Conclusions

My work focused on taxonomical changes in the *Daphnia longispina* species complex from Lago Maggiore during the 20<sup>th</sup> century together with environmental factors affecting the *Daphnia* population structure. Similar studies have been done on lakes north of the Alps (Brede et al., 2009; Keller et al., 2008) and I showed a similar trend of *Daphnia* species alternation in the lake south of the Alps.

*D. longispina* was the most prevalent *Daphnia* species in Lago Maggiore in the middle of the 20<sup>th</sup> century and as has been hinted in a few former studies (e.g., Manca et al., 1997; Manca et al., 2000a), later emergence of *D. galeata* as important taxon in Lago Maggiore plankton was probably accompanied with interspecific hybridization. Both mentioned species also hybridized with *D. cucullata*. This species appeared in the lake after the peak of eutrophication in the 1980s and some individuals likely representing its hybrids have been found even 20 years after. The appearance of *D. cucullata* in the lake has not yet been recorded, although it evidently played an important role in the studied seasonal variation of phenotypes in Lago Maggiore.

In my study I have documented considerable seasonal and inter-annual changes of *Daphnia* body shapes in Lago Maggiore. The seasonal changes of morphotypes could be related to both cyclomorphosis and seasonal changes of *Daphnia* taxonomic composition, whereas both could be affected by predation pressure. Concerning noticeable inter-annual changes, they were linked to environmental factors, which affected not only the *Daphnia* population structure in the lake. However, I could not directly link any specific environmental parameter to the changes of *Daphnia* phenotypes.

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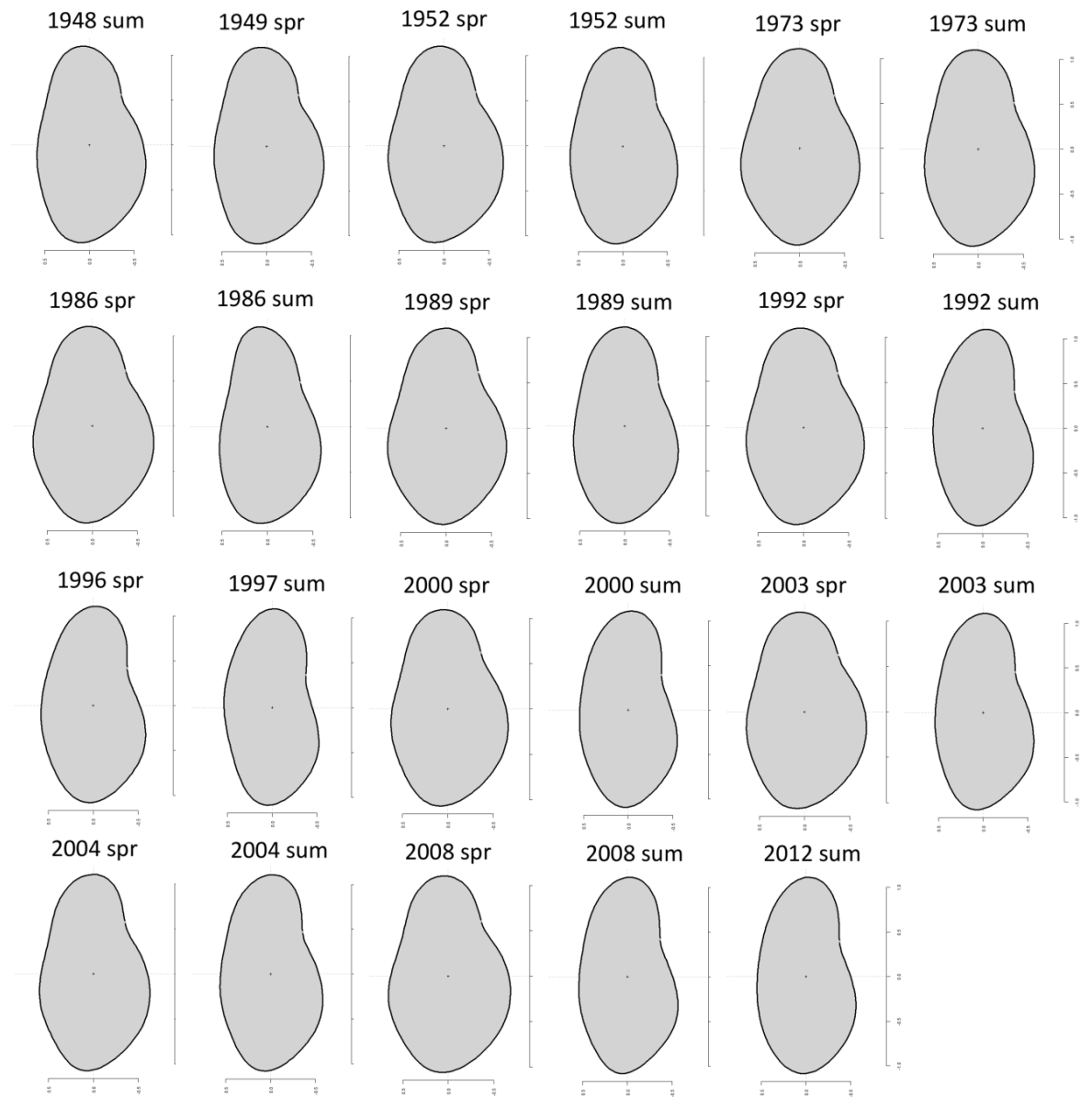
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# Appendices

## Appendix 1



Mean shapes of *Daphnia* bodies from all concerning years and periods (spr = spring, sum = summer).

## Appendix 2

| Years | Total phosphorus ( $\mu\text{g L}^{-1}$ ) | Temperature 0-20 m ( $^{\circ}\text{C}$ ) | Chlorophyll <i>a</i> ( $\text{mg/m}^3$ ) | <i>Daphnia</i> ( $\text{ind/m}^3$ ) | <i>Bythotrephes</i> ( $\text{ind/m}^3$ ) | <i>Leptodora</i> ( $\text{ind/m}^3$ ) |
|-------|---|---|--|-------------------------------------|--|---------------------------------------|
| 2012  | 11,6                                      | 14,2                                      | 3,3                                      |                                     |  |                                       |
| 2008  | 9,7                                       | 11,8                                      | 3,4                                      | 617,8                               | 16,5                                     | 35,5                                  |
| 2004  | 10,4                                      | 12,0                                      | 3,2                                      | 732,0                               | 32,7                                     | 64,6                                  |
| 2003  | 10,8                                      | 13,1                                      | 2,5                                      | 2042,1                              | 31,1                                     | 32,6                                  |
| 2000  | 11,2                                      | 12,0                                      | 2,7                                      | 583,2                               | 33,2                                     | 41,2                                  |
| 1997  | 9,5                                       | 12,6                                      | 3,6                                      | 117,5                               | 31,2                                     | 16,0                                  |
| 1996  | 9,2                                       | 12,3                                      | 4,4                                      | 34,4                                | 35,2                                     | 32,3                                  |
| 1992  | 9,7                                       | 11,3                                      | 3,4                                      | 229,8                               | 21,2                                     | 22,1                                  |
| 1989  | 15,3                                      | 12,0                                      | 3,7                                      | 639,9                               | 21,7                                     | 9,9                                   |
| 1986  | 19,2                                      | 11,4                                      | 4,5                                      | 1820,3                              | 2,1                                      | 41,6                                  |
| 1973  |   | 10,6                                      |  | 907,0                               | 7,0                                      | 17,8                                  |
| 1952  |   |   |  |                                     |  |                                       |
| 1949  |   |   |  | 268,3                               | 2,9                                      | 5,3                                   |
| 1948  |   |   |  | 209,5                               | 4,2                                      | 14,6                                  |

Table with data used for regression trees. The green area indicates the data for Fig. 14, the blue one for Fig. 15.



### Appendix 3



Interesting morphotype from *Daphnia longispina* complex, presumably *D. longispina* x *galeata* hybrid, sampled in Lago Maggiore in 1952 with rarely observed neckteeth formation. Neckteeth are known from this species complex for *D. longispina* and *D. dentifera*, but was not reported for hybrids or other species from the complex (Juračka et al., 2011). Photo by Petr Jan Juračka.