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Genetic and phenotypic differentiation of *Najas marina* L. s.l. in relation to environmental conditions

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"In the end, we will conserve only what we love; we will love only what we understand and we will understand only what we are taught."

Baba Dioum

Preface

This dissertation aims to contribute to a deeper understanding of the ecology, taxonomy, and competitiveness of the Spiny Najad, a highly diverse and common macrophyte taxon native to Europe. Research conducted for this thesis focused on investigating two different subspecies/taxa of *Najas marina* s.l. on a molecular, morphological, and ecological level. Main goals were to assess genetic variability, correlate morphological characters with genetic types and to analyze some ecological interactions of the two taxa.

The introduction of the thesis highlights the current threats for freshwater habitats due to anthropogenic use, the crucial role of macrophytes, and the importance of the correct delimitation of indicator species for ecological assessment of surface waters.

Three different research topics are described in the following chapters, presenting results on genetical, phenotypical, and ecological differences that delineate the two taxa. Each topic has been published as a separate research article in a slightly modified form according to the requirements of the respective journal. In the general discussion of this thesis, the importance of correct species recognition is emphasized stressing the application of integrative approaches combining traditional and molecular methods. The new findings on the genetic structure, morphological and ecological characteristics of two *Najas* taxa are used to discuss implications for the taxa as indicator organisms and consequences for management or conservation actions. The conclusions drawn for sampling and identifying the two cryptic taxa of *Najas* and their naturally occurring hybrids may be also effective for other similar dynamic and critical macrophyte genera that are used within monitoring procedures.

Summary

Submerged macrophytes are used in monitoring programs such as the European Water Framework Directive WFD as indicators of the effects of human pressure on lakes and rivers. Various macrophytes play a significant role as long-term Biological Quality Elements (BQEs), and among them is the red-listed *Najas marina* s.l., a taxon routinely mapped throughout Germany and Europe. Different indicator values have been assigned to the two most common taxa/subspecies *marina* and *intermedia* which are notoriously hard to differentiate due to high morphological similarity. The similarities pose a significant challenge to the use of the two taxa of *N. marina* s.l. as distinct indicator organisms because correct species identification is a prerequisite for accurate assessment of biodiversity and ecological status of waters.

This thesis aims to address this challenge by gaining a better understanding of the morphological and genetic variability of both *Najas* taxa and their hybrids including some environmental factors that might influence their ecology. The identity and spread of *Najas* plants were monitored in field surveys throughout Germany and supplemented with molecular identification of taxa from field samples and herbarium material. Molecular results were linked to phenotypical characteristics of both taxa, and ecological factors, such as light and the presence of other species, influencing the competitiveness both *Najas* taxa were simulated in a mesocosm approach.

In the first study, phylogenetic structure within the taxon was investigated using nuclear ribosomal (ITS) and chloroplast (*trn*L-F) sequence data from over one hundred *N. marina* s.l. accessions including herbarium material, representing three of the 12 subspecies and one of four varieties. The clear-cut molecular differentiation identified both lineages as distinct but cryptic due to considerable phenological resemblance. The clusters differ in 45 positions of ITS and 10 of *trn*L-F respectively, with almost no variation within. The samples grouped into two distinct clusters, which corresponded with the two different karyotypes A and B previously reported (karyotype A = *N. marina* and B = *N. major*). Hybrids were identified in four cases by the cloning of heterozygotic samples.

A second study focused on the reliable identification of both taxa and their hybrids, using an integrative approach. Six discrete and two ratio-based morphological leaf and seed characteristics were tested against restriction fragment-length polymorphism patterns (RFLP)

based on PCR of rDNA of the internal transcribed spacer (ITS) sequences 1 and 2. Leaf dimensions, especially leaf widths, were shown to be more reliable characteristics for distinguishing parental taxa, and the traditionally employed feature "number of teeth along the margin on the leaf sheaths" proved to be of low diagnostic value. Hybrids showed a mosaic or intermediate morphological pattern of parental taxa depending on the trait.

In the following study the effect of changed light conditions was tested on the growth and competition of native *Najas* taxa and other established and potentially invasive macrophyte species. Different optical active components causing turbidity events in the course of climate change such as suspended particular matter (SPM), colored dissolved organic matter (CDOM), and algal growth, induced by enhanced nutrients, were simulated in a mesocosm approach. Light conditions were maintained for 5 weeks and monitored with hyperspectral underwater radiometers and species responses were assessed using biomass production, relative growth rate (RGR) and root to shoot ratios. Native *Najas* taxa showed enhanced growth under SPM conditions, achieving two times higher RGRs (0.01 – 0.05 d^{-1}) compared to two potentially invasive macrophytes *Hydrilla verticillata* and *Lagarosiphon major*. A negative influence of the algae treatment was observed on the growth rates of almost all species used in the experiment. Establishment of invasive macrophytes *L. major* and *H. verticillata,* and the RGRs of the latter were influenced significantly by algae treatment. Thermophile and native taxa like *N. marina* s.l. might benefit from higher water temperatures and the predicted increased substance influx in the future.

The studies revealed that the two *Najas* taxa should be treated as distinct species according to their substantial genetic differentiation. Based on the new findings the further use of both *Najas* taxa within the implementation of the WFD in Germany should be routineously accompanied by identification of sample material by state-of-the-art molecular methods and genetic markers to detect cryptic spread, co-occurrence and hybridization, and to describe ecological differentiation of both taxa in more detail. A combined approach using molecular and phenotypic character traits like applied successfully in this thesis is suggested also for other morphologically cryptic macrophyte taxa that are currently part of monitoring procedures. The insights about the dynamics of these sympatrically growing taxa and their hybrids should aid to further optimize sampling and screening strategies for macrophytes to better understand how aquatic plant communities are structured and the forces driving the spread and invasion of species.

Zusammenfassung

Untergetauchte Makrophyten werden in Überwachungsprogrammen wie der Europäischen Wasserrahmenrichtlinie (WRRL) als Indikatoren für die Auswirkungen des menschlichen Handels auf Seen und Flüsse verwendet. Verschiedene Makrophyten spielen eine bedeutende Rolle als langfristige biologische Qualitätskomponenten (BQE), darunter auch das auf der roten Liste stehende Taxon *Najas marina* s.l., dass routinemäßig in ganz Deutschland und Europa kartiert wird. Den beiden häufigsten Taxa/Unterspezies *marina* und *intermedia* wurden unterschiedliche Indikatorwerte zugeordnet, die aufgrund der hohen morphologischen Ähnlichkeit bekanntermaßen schwer zu differenzieren sind. Die Ähnlichkeiten stellen eine große Herausforderung für die Verwendung der beiden Taxa von *N. marina* s.l. als unterschiedliche Indikatororganismen dar, da eine korrekte Artbestimmung eine Voraussetzung für die genaue Beurteilung der Biodiversität und des ökologischen Zustands von Gewässern ist.

Ziel dieser Arbeit ist es, diese Herausforderung anzugehen, indem ein besseres Verständnis der morphologischen und genetischen Variabilität sowohl der *Najas*-Taxa als auch ihrer Hybriden einschließlich einiger Umweltfaktoren, die ihre Ökologie beeinflussen könnten, gewonnen wird. Die Bestimmung der Identität und Verbreitung von *Najas*-Pflanzen wurde in Felduntersuchungen in ganz Deutschland beobachtet und durch die molekulare Identifizierung der Taxa aus Feldproben und aus Herbarmaterial ergänzt. Die molekularen Ergebnisse wurden mit phänotypischen Merkmalen beider Taxa verglichen, und ökologische Faktoren wie Licht und das Vorhandensein anderer Arten, die die Konkurrenzfähigkeit beider *Najas*-Taxa beeinflussen, wurden in einem Mesokosmos-Ansatz simuliert.

In der ersten Studie wurde die phylogenetische Struktur innerhalb des Taxons mit Hilfe von Sequenzdaten von nukleären ribosomalen (ITS) und chloroplastischen (*trn*L-F) Sequenzen von über hundert *N. marina* s.l. Akzessionen untersucht, einschließlich Herbar-Material, das drei der 12 Unterarten und eine von vier Sorten repräsentiert. Die klare molekulare Differenzierung identifizierte beide Linien als unterschiedlich, aber aufgrund der beträchtlichen phänologischen Ähnlichkeit kryptisch. Die Cluster unterscheiden sich in 45 Positionen der ITS und 10 der *trn*L-F Sequenzen, wobei es innerhalb der Cluster fast keine Unterschiede gibt. Die Proben wurden zu zwei verschiedenen Clustern gruppiert, die mit den

beiden zuvor bereits beschriebenen unterschiedlichen Karyotypen A und B korrespondierten (Karyotyp A = *N. marina* und B = *N. major*). Hybride wurden in vier Fällen durch das Klonen heterozygoter Proben identifiziert.

Eine zweite Studie konzentrierte sich auf die zuverlässige Identifizierung sowohl der Taxa als auch ihrer Hybriden unter Anwendung eines integrativen Ansatzes. Sechs diskrete und zwei auf Verhältnissen basierende morphologische Blatt- und Samenmerkmale wurden gegen Restriktionsfragment-Längen-Polymorphismus-Muster (RFLP) getestet, die auf der PCR von rDNS der internen transkribierten Spacer-Sequenzen (ITS) 1 und 2 basieren. Die Blattabmessungen, insbesondere die Blattbreiten, erwiesen sich als zuverlässigere Merkmale zur Unterscheidung der elterlichen Taxa, und das traditionell verwendete Merkmal "Anzahl der Zähne entlang des Randes auf den Blattscheiden" erwies sich als wenig aussagekräftig. Hybriden zeigten je nach Merkmal ein mosaikartiges oder intermediäres morphologisches Muster der elterlichen Taxa.

In der folgenden Studie wurde die Wirkung veränderter Lichtverhältnisse auf das Wachstum und die Konkurrenz der einheimischen *Najas*-Taxa und anderer etablierter und potenziell invasiver Makrophytenarten getestet. Verschiedene optisch aktive Komponenten, die im Zuge des Klimawandels Trübungsereignisse verursachen, wie z.B. Schwebstoffe (SPM), farbige gelöste organische Substanz (CDOM) und Algenwachstum, das durch erhöhte Nährstoffe induziert wird, wurden in einem Mesokosmos-Ansatz simuliert. Die Lichtbedingungen wurden fünf Wochen lang aufrechterhalten und mit hyperspektralen Unterwasser-Radiometern überwacht, und die Reaktionen der Arten wurden anhand der Biomasseproduktion, der relativen Wachstumsrate (RGR) und des Wurzel-zu-Spross-Verhältnisses untersucht. Einheimische *Najas*-Taxa zeigten unter SPM-Bedingungen ein verstärktes Wachstum und erreichten zweimal höhere RGR (0,01 - 0,05 d^{-1}) im Vergleich zu zwei potenziell invasiven Makrophyten *Hydrilla verticillata* und *Lagarosiphon major*. Ein negativer Einfluss der Algenbehandlung auf die Wachstumsraten wurde bei fast allen im Experiment verwendeten Arten beobachtet. Die Etablierung der invasiven Makrophyten *L. major* und *H. verticillata* und die RGRs der letzteren wurden durch die Algenbehandlung signifikant beeinflusst. Thermophile und einheimische Taxa wie *N. marina* s.l. könnten in Zukunft von höheren Wassertemperaturen und dem vorhergesagten erhöhten Substanzzufluss profitieren.

Die Studien ergaben, dass die beiden *Najas*-Taxa aufgrund ihrer erheblichen genetischen Differenzierung als unterschiedliche Arten behandelt werden sollten. Basierend auf den neuen Erkenntnissen sollte die weitere Verwendung der beiden *Najas*-Taxa im Rahmen der Umsetzung der WRRL in Deutschland routinemäßig von einer Identifizierung des Probenmaterials mit modernsten molekularen Methoden und genetischen Markern begleitet werden, um kryptische Ausbreitung, gemeinsames Vorkommen und Hybridisierung zu erkennen und die ökologische Differenzierung beider Taxa genauer zu beschreiben. Ein kombinierter Ansatz unter Verwendung molekularer und phänotypischer Charakteristika, wie er in dieser Arbeit erfolgreich angewandt wurde, wird auch für andere morphologisch kryptische Makrophytentaxa vorgeschlagen, die derzeit Teil von Monitoringverfahren sind.
Die Erkenntnisse über die Dynamiken dieser sympatrisch wachsenden Taxa und ihrer Hybriden sollen helfen, die Probenahme- und Screening-Strategien für Makrophyten weiter zu optimieren, um besser zu verstehen, wie aquatische Pflanzengemeinschaften strukturiert sind und welche Kräfte die Ausbreitung und Invasion von Arten vorantreiben.

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1 General Introduction

The conservation of freshwater resources is, without a doubt, one of the main challenges for humankind and our future wellbeing. To record and monitor the status and to evaluate protection measures many freshwater organisms play an important role as indicator species. Various aquatic plant species are well established long-term indicators for nutrient load and other environmental conditions. For an effective application of indicator organisms, the recognition and classification of distinct species is crucial but can be notoriously difficult for many macrophyte species. New technologies like DNA barcoding make species recognition more reliable but also raise many new questions and problems. For example, how can traditional species delimitation and taxonomic work be still involved and combined with next-generation approaches? Or what differentiates species from their hybrids morphologically, genetically, and ecologically, and what are the best DNA marker regions to be used for comprehensive analysis? Questions like these, need to be answered as we move forward and will serve as primary themes in this dissertation. Some of these questions will be discussed and answered by using *Najas marin*a L. s.l. and two of its indicative taxa currently applied in German and European lake monitoring procedures. Because plants from the different taxa display high morphological diversity, are able to form intraspecific hybrids, and are currently spreading, they were examined in this study by using thorough taxonomic analysis and an integrative approach combining molecular, phenological and ecological tools. Clear guidelines for the further use of such 'critical' indicator species and the integration of 'modern' methods and the 'old' knowledge is needed to improve current monitoring techniques and to deepen our understanding of the function of species and aquatic ecosystems in general.

1.1 Current threats to freshwaters and the role of macrophytes

Freshwater ecosystems are subject to constant change as anthropogenic pressure has intensified over the past decades (Börkey et al., 2005; Carpenter et al., 1992; Huntington, 2006). Streams, lakes, and wetlands are globally confronted with a severe loss of species diversity, habitat degradation, and invasion by non-native species (Dudgeon et al., 2006; Ricciardi & Rasmussen, 1999; Vörösmarty et al., 2000). The increased use especially of lakes for recreation and as drinking water reservoirs has negatively impacted their ecology (Carpenter et al., 1992; Oki & Kanae, 2006). Large scale pollution with organic and toxic contaminants caused by excessive land-use affects water quality and, subsequently, all organisms living in the water. The conservation and restoration of these ecosystems is, therefore, a top priority for humankind and freshwater scientists (Strayer & Dudgeon, 2010).

For the success of efforts and in order to effectively protect or restore freshwater systems a detailed understanding of the function of single organisms is crucial. Therefore the integration of knowledge on different levels of organization for example by combining molecular and ecological tools leads to the application of more holistic conservation strategies as proposed for the endangered freshwater pearl mussel e.g. *Margartifera margaritifera* L. (Geist, 2011). Such combined and holistic approaches should especially consider freshwater organisms that are important for ecosystem functioning, such as indicator, keystone, or flagship species (Geist, 2010). An important factor in this regard are macrophytes, a key structural and functional element of wetlands and the littoral of aquatic ecosystems (Feuchtmayr et al., 2009; Jeppesen et al., 1998; McKee et al., 2003). Macrophytes are among the aquatic plants which are defined as both (1) all vascular plants that can be seen with the naked eye and grow either emerged or submerged, free-floating or rooted in the sediment (Sculthorpe, 1967) and (2) the macroscopically visible algae, also called charophytes, which are submerged and anchored with rhizoids in the sediment. Thus submerged macrophytes constitute a living link between the sediment below and the water above (Carpenter & Lodge, 1986) serving as integrators of environmental conditions and as long-term indicators with high spatial resolution (Melzer 1999).

All aquatic angiosperms have evolved numerous physiological and morphological adaptations in the course of their secondary transition from a terrestrial to an aquatic environment, which took place multiple times independently in various genera (Chambers et al., 2008; Cook, 1999). Thus macrophytes are morphological, taxonomic, and ecologically diverse and can be loosely differentiated by structural properties like leaf type and overall growth form (Hutchinson, 1975; Wetzel, 2001). Most macrophytes show reduced morphological traits compared to their terrestrial counterparts, especially in their reproductive organs. Such reductions culminated in the loss of sexual reproduction and the promotion of vegetative propagation which is assumed to be the dominant mode of reproduction in water plants (Les, 1988; Philbrick & Les, 1996; Sculthorpe, 1967). The success of most invasive aquatics such as *Elodea canadensis* Michx. or *Egeria densa* Planch. can be explained by asexual reproduction (Lambertini et al., 2010). Other macrophyte taxa are still reproducing sexually and some even have developed sets of features allowing for more complex and unique mechanisms like underwater pollination, also known as hydrophily (Barrett et al., 1993; Haynes, 1988; Les, 1988). Hydrophily evolved at least nine times independently in flowering plants and 19 hydrophilous plant genera are known to exist in freshwater ecosystems (Cox, 1988; Les, 1988; Les et al., 1997; Philbrick & Les, 1996).

But what makes macrophytes such a valuable part of aquatic ecosystems? Macrophytes build the base of herbivorous and detritivorous food chains as primary producers, besides phytoplankton (Jeppesen et al., 1998; Rejmankova, 2011). In addition to providing different types of structured habitats, macrophytes can influence and considerably change the conditions of aquatic ecosystems. They have strong effects on the hydrological regime of a water body, on nutrient cycles, and sediment dynamics by preventing resuspension (Barko et al., 1991; Carpenter & Lodge, 1986; Wigand et al., 1997) and their presence is a prerequisite for maintaining clear water status (Scheffer & Jeppesen, 1998). Many free-floating, emergent, and submerged macrophyte species have the potential to improve water quality by binding and removing nutrients, organic contaminants, and even heavy metals (Dhote & Dixit, 2009). Moreover, the narrow ecological niche of certain macrophyte species makes them suitable indicator organisms for classification of the ecological quality of lakes and rivers (Melzer, 1976; Penning et al., 2008a; Schneider et al., 2000; Søndergaard et al., 2010).

A threat to freshwater ecosystems in general and to many macrophyte species that are sensitive to elevated nutrients is eutrophication (Penning et al., 2008b). In the northern hemisphere, eutrophication caused considerable shifts in the abundance and composition of underwater plant communities (Carpenter & Lodge, 1986; Hough et al., 1989; Madgwick et al., 2011). Consequent and large-scale sewage treatment has widely advanced and led to re-oligotrophication in several European inland waters (Dokulil & Teubner, 2010). Nevertheless, climate change is a fast progressing danger to aquatic ecosystems and can even intensify some effects of eutrophication (Jeppesen et al., 2014; Moss et al., 2011; Short et al., 2016). The most important factors influencing submersed plants directly in the course of climate change are changes in the physicochemical conditions in the water such as increased temperatures (Mooij et al., 2005; Short et al., 2016). Other effects driven by climate change are for example heavy precipitation, enhance nutrient loads, and suspended particular matter changing water clarity and thereby influencing macrophytes indirectly (Boyer et al., 1997; Scheffer & van Nes, 2007; van den Besselaar et al., 2013). Depending on the substance influx and resulting transparency of the water column such events can lead to the decreased abundance or even to the total loss of submerged vegetation (Feuchtmayr et al., 2009; Goldsborough & Kemp, 1988; Scheffer et al., 1993).

Further drivers of global environmental change are so-called invasive alien species (IAS), defined as non-native species that menace/threaten ecosystems, habitats, or species (United Nations, 1992). Aquatic ecosystems seem at particular risk from the spread and establishment of IAS that are often more tolerant to a wide range of environmental conditions (Havel et al., 2015; Sorte et al., 2013). Climate change is expected to facilitate the establishment of aquatic IAS and their dispersal is mostly promoted by shipping and the trade of non-indigenous species as ornamental, aquarium and garden pond plants. The establishment of many aquatic IAS can lead to the displacement of indigenous taxa and often resulting in costly management of the affected areas (Hussner, 2012; Zehnsdorf et al., 2015). Over 400 alien freshwater species (flora and fauna) are currently known to cause ecological and economic impacts on different taxonomic groups in Europe (Vilà et al., 2010). Of those alien freshwater species, 27 non-indigenous aquatic macrophyte species were alone recorded in Germany over the last decades. From those non-indigenous species, 18 macrophyte species are listed as invasive or potentially

invasive in Europe according to European and Mediterranean Plant Protection Organization (http://www.eppo.org) and 13 are already naturalized in Germany (Hussner, 2012; Hussner et al., 2010b).

Knowing all those threats to freshwaters, what should be the focus of macrophyte research? Each species, native or invasive, impacts an ecosystem by its life cycle, physiology, and behavioral and morphological traits, which in turn influence the acquisition, use, and allocation of resources (Díaz et al., 1992). Therefore, combined research on the ecology, the genetic differentiation and the analysis of their morphological and functional traits is necessary to understanding the role of specific organisms and their ecological variability. Such combined approaches will be the key to describe and predict the adaption mechanisms of species and to further maintain and manage aquatic ecosystems in the face of biodiversity loss and global change.

1.2 The importance of correct species identification in water quality assessment

Over the last few decades, submerged vegetation has commonly been used as a tool for determining ecological status and anthropogenic influence on lakes and rivers, as plants are sessile and restricted to specific habitats (Barko et al., 1986; Melzer, 1976; Schneider et al., 2000; Søndergaard et al., 2010). Monitoring programs such as the European Water Framework Directive WFD (2000/60/EC) track species composition and abundance of a variety of Biological Quality Elements (BQEs) such as fish, benthic invertebrates, macrophytes, phytobenthos, and phytoplankton. According to the WFD guidelines BQEs are regularly monitored in order to assess the ecological status of lakes and rivers. Various macrophyte species play a significant role as long-term indicator organisms and have shown strong responses to stressors like eutrophication and water level fluctuations (Kolada et al., 2012; Penning et al., 2008a; Schaumburg et al., 2004; Søndergaard et al., 2010; Stelzer et al., 2005). Results of WFD surveys are used to facilitate decision making about protection and sustainable management measures to further improve water quality and meet the ambitious goals of the WFD in Europe.

Monitoring procedures rely on correct species identification for accurate assessment of ecological states and biodiversity. Investigations of species richness, biogeography, or ecological processes rely on the "species" as biologically significant, natural units of evolution (Morard et al., 2016). Most descriptions of species are based on phenotypic characteristics, and the correct delimitation of macrophyte taxa is influenced by two major factors: (1) the morphological variability displayed by many aquatic plant species due to reduced and simple morphology and phenotypic plasticity, and (2) the expertise and experience of the investigator identifying macrophyte species. For the monitoring of macrophytes, an in-water survey by SCUBA diving is known to be the most accurate method (Jäger et al., 2004; Melzer, 1999). An added benefit is that SCUBA diving is more sustainable than destructive rake or boat sampling (Capers 2000). However, underwater investigations are expensive, time-consuming, and require that the diver has broad taxonomic knowledge (Dudley et al., 2013; Jäger et al., 2004).

What can be and has been done to overcome those difficulties in delimiting species? Over the last thirty years, rapidly evolving methods like Next Generation DNA Sequencing (NGS) and metabarcoding have facilitated effective and reliable species identification based on universal barcodes and online databases (Feliner & Rosselló, 2007; Hebert & Gregory, 2005; Will et al., 2005). Nevertheless, in an age of quick and affordable molecular analysis, field observations conducted by humans are still essential for especially when it comes to classification and correct typification which involves the designation of a nomenclatural type for a name according to the International Code of Nomenclature for algae, fungi, and plants (Turland et al., 2018). Unfortunately, the number of qualified taxonomists is constantly decreasing (Hopkins & Freckleton, 2002). Thus, the challenge for the future of species identification will be to align traditional knowledge and methods with the modern technology-based century (Boero, 2010; Figueiredo & Smith, 2015).

Further complications in macrophyte identification arise not only from the scarcity of human expertise but also from evolutionary processes like hybridization and speciation, which both can happen cryptically, meaning hidden. Cryptic species are described as species that are morphologically indistinguishable but show genetic differences, or the other way round, are characterized by the existence of different morphotypes that cannot be separated by barcoding

(Schneider et al., 2015). Hybridization occurs in 20% of all hydrophytes and is especially pronounced in aquatic plant genera, due to high vagility and low crossing barriers (Les & Philbrick, 1993). For European freshwaters, 58 macrophyte species are listed as hybridizing (Moe et al., 2013) and numerous hybrids were uncovered within the last ten years by thorough molecular surveys (Les et al., 2015; Prančl et al., 2014; Tippery & Les, 2013; Zalewska-Gałosz & Kwolek, 2014). However, the detection of aquatic plant hybridization is often hindered by several factors. First, the initial phenological uncovering of such hybrid individuals in the field is most of the times incidental, not only because hybrids often fall within the morphological range of their parents, but also because of reduced morphology and phenotypic plasticity obstructing identification of macrophyte species in general (Sculthorpe, 1967). Second, the molecular detection of hybrids can also be disguised when using common polymorphic markers, such as the nuclear ribosomal internal transcribed spacer (nrITS). Sequencing of nrITS can result in noisy unreadable nucleotide signals, which might affect phylogenetic conclusions (Bernardini & Lucchese, 2018; Les et al., 2010, 2009). For instance, such 'sequencing artifacts' or 'degraded sequences' have to be screened very carefully to allow for the molecular detection of hybrids or other associated evolutionary processes like gene flow (Rieseberg et al., 2000).

Molecular markers are without a doubt a useful tool for species delineation and the uncovering of hybridization (Adams et al., 2014). Still, the combined use of multiple loci and other phenotypic or life history traits is recommended to detect cryptic species complexes or introgression (Adams et al., 2014; Duminil & Di Michele, 2009). Introgression refers to the transfer of a small amount of the genome from one parental taxon (usually species) to another by hybridization and repeated backcrossing (Suarez-Gonzalez et al., 2018). Apart from known marker pitfalls and problems already mentioned, many longstanding taxonomic problems in macrophyte genera like *Potamogeton*, *Chara* or *Callitriche*, could only be solved with the aid of molecular data (Boegle et al., 2007; Moody & Les, 2002; Prančl et al., 2014; Whittall et al., 2004).

In any case, correct identification of organisms, including their morphological characteristics, is a prerequisite for ecological studies and for accurate biodiversity assessments (Bannar-Martin et al., 2018; Hooper et al., 2002). Due to the importance of many macrophyte species as indicator organisms within monitoring measures, a deeper

understanding of species entities and phenology is needed for decision making and successful management or conservation of freshwater habitats in the future.

1.3 *Najas marina* L. s.l. as an example of a taxonomically difficult indicator

The cosmopolitan genus *Najas* (Naiads or water nymphs) is a group of macrophytes growing completely submerged (Triest, 1988). *Najas* contains 30 - 40 species and is thereby the largest number among hydrophilous genera with its greatest diversity in tropical and subtropical regions (Haynes, 1979; Ito et al., 2017; Les, 1988). Scientific interest in this particular plant group has been driven by the challenges of taxonomic classification and the incongruences between DNA and morphological results (Li & Zhou, 2009). Recent phylogenetic analysis just confirmed the inclusion of the genus *Najas* within the Hydrocharitaceae family (Bernardini & Lucchese, 2018; Les et al., 2006). The holly-leaved Naiad or spiny water nymph *Najas marina* L. is a group within the genus traditionally considered as representing one variable species. This is frequently referred to as *N. marina* s.l. (sensu latu – in the wide sense) for several taxa are included sometimes that have been regarded as segregate taxa at different ranks ("*marina*", "*intermedia*", "*armata*" etc.). These plants grow submerged down to a depth of 4 m in a wide range of habitats from lakes, rivers, ponds, brackish to alkaline waters nearly all around the world (Haynes, 1979; Lowden, 1986; Triest, 1988). In Central Europe, *N. marina* s.l. is one of the most widespread aquatic vascular plant species (Casper & Krausch, 1980; Wiegleb, 1978).

Plants are generally described as slender, growing up to a maximum height of three meters, with shoots highly branched. Stems originate from lateral branches on the nodes of the rhizome which resembles the stem but is unbranched with one to several adventitious roots that guarantee nutrient uptake and anchoring in suitable bottom sediments (Handley & Davy, 2002; Haynes, 1979; Triest, 1988). The spiny leaves have a serrulate shape and grow in subopposite pairs and pseudowhorls of three up to seven. Minute solitary flowers occur in the axils of the lower leaf of the pseudowhorl and are 4.5 - 6 mm long (Huang et al., 2001; Triest, 1988).

The taxon is considered thermophilic, meaning that germination and florescence are both highly regulated by water temperature (Agami & Waisel, 1984; Hoffmann & Raeder, 2016). Within the annual life cycle, plants reproduce mostly by seeds, which require periods with water temperatures of more than 15 °C for germination and more than 20 °C for maturation (Agami et al., 1984; Hoffmann et al., 2014a, 2013b). Seeds overwinter and after a mandatory dormancy period of 1 - 3 months, a temperature range from 12 - 16 °C is needed for successful germination (Agami & Waisel, 1984; Forsberg, 1965; Van Vierssen, 1982). Under favorable conditions seeds have been reported to survive up to three years in lake sediments (Handley & Davy, 2005). Other modes of vegetative reproduction like extensive lateral growth, fragmentation, apomixis, or the occasional formations of turions were reported for *N. marina* s.l. (Agami et al., 1986; Handley & Davy, 2000; Sculthorpe, 1967).

N. marina s.l. is hydrophilous and plants are dioecious with female and male flowers on different individuals exhibiting a strong sexual dimorphism (Hoffmann et al., 2014a). Large differences in growth were observed based on the dioecious nature, and male plants of *N. marina* s.l. could be shown to grow 20 - 40% faster than female specimens in German lakes (Hoffmann et al., 2014a). Male plants produce extensive amounts of pollen and after releasing, the pollen is transported by water movement and currents. When female plants start bearing flowers, male plants have been observed to show already signs of senescence (Hoffmann et al., 2014a). After successful fertilization by pollen, female plants grow further and persist until the seeds are fully mature, leading to a predominance of female plants which reduces the competition between the sexes (Hoffmann et al., 2014a; Triest, 1989). These differences in phenology and flowering time are assumed to be the reason for the overlooking of male plants for many years in Britain (Handley & Davy, 2000) and the maintenance of species boundaries (Triest, 1988).

The plants' vegetative characteristics, like dimension, shape, and number of spines of the leaves, are used in taxonomy but exhibit large morphological variability (Haynes, 1979; Triest, 1991) which makes delimitation of taxa notoriously difficult and gives rise to ongoing taxonomic discussions (Bernardini & Lucchese, 2018; Braun, 1864; Casper, 1979; Ito et al., 2017; Lowden, 1986; Rendle, 1899). *N. marina* s.l. as circumscribed by Triest (1988) comprises twelve different subspecies and four varieties, although segregates are sometimes considered either

as varieties or distinct species. The two most common and widespread subspecies in Europe and Germany are *N. marina* L. subsp. *marina* and *N. marina* L. subsp. *intermedia* (Wolfg. ex Gorski) Casper (Casper, 1979). In this thesis, the two subspecies are further referred to as *N. marina* L. subsp. *major* (All.) Viinikka (= *N. marina* subsp. *marina*) and *N. marina* L. subsp. *marina* (= *N. marina* subsp. *intermedia*) following the description of Viinikka (1976) and based on the uncovering of the wrong typification by Bräuchler (2015).

Both are considered subspecies of *N. marina* L. (Viinikka, 1976) and were often merged under the *Najas marina* complex (Bräuchler, 2015) due to high phenotypic variability in both taxa (Haynes, 1979; Triest, 1988). The most recent taxonomic treatment of the genus by Ito et al. (2017) recovered two clades under *N. marina* using plastid DNA (*matK, rbcL, rpoB, rpoC1*) and nuclear DNA markers (ITS). But the authors reject any morphological, karyological, and geographical division of *N. marin*a s.l. as proposed by other taxonomists, and continue to refer to the taxon as the "*Najas marina* complex" (Ito et al., 2017).

Both taxa possess 2n = 12 chromosomes, though tetraploid individuals were reported from the African continent (Tischler, 1917; Triest et al., 1989; Winge, 1927). The diploid taxa are well-differentiated in their karyotypes, named type A (subsp. *major* = subsp. *marina* auct.) and type B (subsp. *marina* = subsp. *intermedia* auct.) (Viinikka, 1977, 1976). Although artificial crosses between the two subspecies were reported already by Viinikka (1976) no evidence for the existence of natural hybrids could be given on molecular or chromosomal levels at that time. The first naturally occurring hybrid plants, collected from Sempachersee located in Switzerland, where both taxa were known to coexist since the year 1976, were confirmed by analysis of enzyme polymorphisms (Triest, 1991, 1989). Morphologically the hybrids corresponded to A type plants and electrophoretic studies of isoenzymes of hybrid leaves showed intermediate patterns in the POD (peroxidase) and SkDH (shikimate dehydrogenase) markers compared to the parental taxa (Triest, 1989). The same analysis also consolidated the genetic differentiation of the two karyotypes A and B and indicated a possible greater genetic diversity in the genus (Triest, 1991; Triest et al., 1986). Data from electrophoretic studies suggests monomorphism and weak population differentiation in many hydrophilous species, although isozymes markers often provide meaningful estimates of population structure and outcrossing rates (Barrett et al., 1993; Les, 1988).

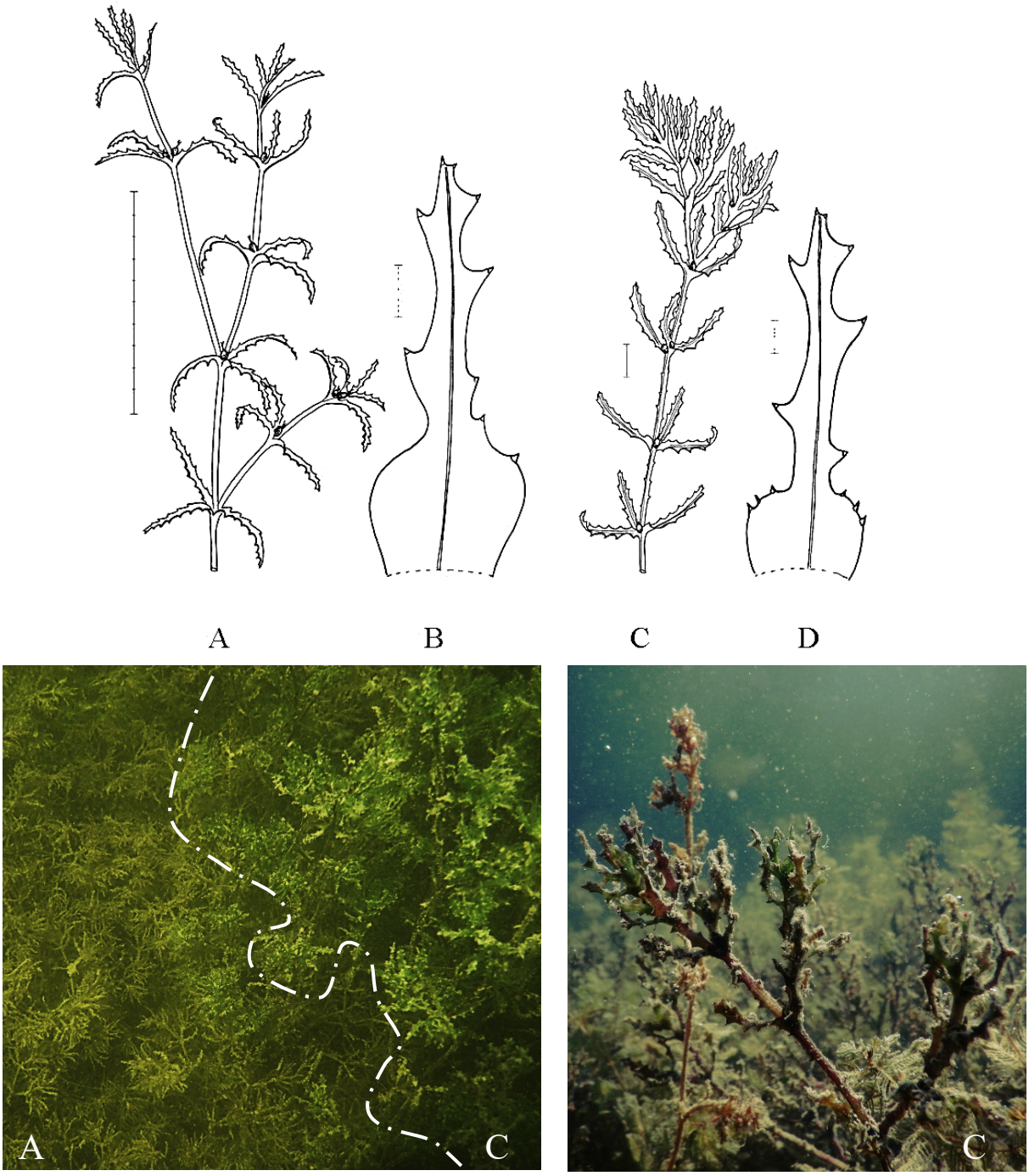


Figure 1.1: Pictures of morphology and habitus of both *Najas* taxa. TOP Botanical illustration obtained from van de Weyer et al., (2007): A) Habitus of *N. m.* subsp. *marina* (scale 10 cm), B) leaf and leaf sheath of *N. m.* subsp. *marina* (scale 1 mm), C) habitus of *N. m.* subsp. *major* (scale 1 cm), D) leaf and leaf sheath of *N. m.* subsp. *major* (scale 1 mm). BOTTOM Photographs of different extensive populations of *N. marina* s.l. underwater in Lake Staffelsee (left) and in Lake Waging (right). In Lake Staffelsee (left) both taxa can be seen growing next to each other, to better distinguish populations a white dot-dash line was added. Capital letters in the pictures represent the two taxa depicted in the line drawings above.

N. m. subsp. *major* is distributed from Europe to Central Asia, in temperate and warm temperate areas. *N. m.* subsp. *marina* is recorded more often in cold to warm temperate areas but both taxa show considerable geographical overlap (Triest, 1989) with numerous sympatrically distributed populations being recorded in Germany (Doll & Pankow, 1989; Pietsch, 1981; Triest, 1991). When growing together plants of both taxa are hard to delineate, nevertheless, A-type plants are morphologically described as 'dark green to purplish, crispy and coarse', while B type plants are described as 'olive green and more slender' (Triest, 1991) (Fig. 1.1).

Historical references of habitat preferences of the genus could be given by Paleolimnology using the seeds of *N. marina* s.l. which were found in high abundance in early Holocene deposits in Europe and those records indicate that plants were commonly present in eutrophic, alkaline lakes during that era (Bennike et al., 2001). A subsequent decline of *N. marina* s.l. is supposed to be due to the so-called Little Ice-Age, followed by pollution of lakes as well as draining in the past decades (Bennike et al., 2001; Zhu et al., 2007).

More recent studies from Doll (1981) and Pietsch (1981) examined ecology and habitat preferences of *N. marina* s.l. but focussed mainly on plant communities in Central Europe and northern Germany without distinguishing between *N. m.* subsp. *marina* and *N. m.* subsp. *major*. Most recent ecological descriptions by Triest (1988) affirmed differences between the taxa based on isozyme polymorphism, and mention distinct habitat preferences, especially for abiotic factors such as conductivity and nutrient concentrations. *N. m.* subsp. *marina* appears to cover a larger range of habitats but seems to be absent in calcium and calcium carbonate-rich waters (Triest, 1988).

Due to its ecological preferences the broader taxon *N. marina* s.l. is currently used as biological indicator organism under the Water Framework Directive (WFD) in Germany and other European countries (Leyssen et al., 2005; Penning et al., 2008a; Poikane et al., 2018; Schaumburg et al., 2014, 2004; Søndergaard et al., 2010; Willby et al., 2009; Zervas et al., 2018). According to the German WFD assessment method, both taxa are designated distinct indicator values that are based on previous studies on macrophyte abundance and the implementation of a macrophyte reference index (Stelzer, 2003; Stelzer et al., 2005). Because of its proposed higher tolerance to nutrient-rich waters *N. m.* subsp. *major* is considered an

indicator of disturbance within WFD guidelines, whereas *N. m.* subsp. *marina* is given a neutral or even good indicative value, depending on the type of lake. When occurring in dominant stands, *N. m.* subsp. *marina* can decrease the ecological rating of the afflicted site significantly (Schaumburg et al., 2007). However, the execution of regular surveys and enhanced mapping in the course of WFD legal framework has uncovered reasons for eutrophication and has facilitated a significant improvement of the status of European and German lakes over the last decades (Dokulil & Teubner, 2010; Nixdorf et al., 2004). Moreover, the surveys helped in gaining information on the occurrence of rare macrophytes species in general and the increasing spread of *N. marina* s.l. plants in German lakes over the last few decades in particular (Hoffmann et al., 2013b; Knösche, 2008; Korsch, 2011; Leske et al., 2005; Poschlod, 2015). These results also question the current endangered status of *N. marina* s.l. in some European countries (Cheffings et al., 2005; Korneck et al., 1996; Oppel, 2010). Great efforts are taken to intercalibrate the different ecological assessment methods involving various macrophytes species used in Europe (Poikane et al., 2011).

Besides all the taxonomic uncertainties *N. marina* s.l. is a suitable indicator for rising temperatures induced by climate change. Due to the species' thermophilic nature, it can be used as an indicator for the potential spread of invasive neophytes in southern Germany (Handley & Davy, 2005; Hoffmann & Raeder, 2016). It is though still unclear whether to further distinguish both taxa in German or other European WFD procedures firstly because of inconsistencies in morphological treatment and secondly because the ecological drivers for the current increase of *N. marina* s.l. populations are still poorly understood. One of the main challenges for using the two taxa as indicator species is that discrimination between the two taxa is still difficult due to persisting morphological identification problems arising from polymorphous characters like the number of spines on the leaf sheaths (Fig. 1: B, C). Consequences of misidentification are not only distorted ecological assessments but also inaccurate records of newly introduced or re-established populations of *N. marina* s.l. resulting in cryptic species distribution (Gutte et al., 2008; Knösche, 2008; Korsch, 2011; Oppel, 2010). Furthermore, these taxonomic uncertainties impede a more detailed description of the taxas' autecological niches, their actual spread, and possibly distinct adaptation mechanisms.

1.4 Objectives

The unambiguous identification of indicator species is a crucial prerequisite for the reliability and accuracy of the assessment of water quality and species richness in biological monitoring. Dealing with morphological reduction and phenotypic plasticity are two major challenges that impair correct identification in aquatic macrophyte species in the field. Concerning existing uncertainties and contradictions in identifying both *Najas* taxa, *N. marina* subsp. *major* and *N. marina* subsp. *marina* and the current spread of their populations in Germany, a thorough genetic and morphological analysis is urgently needed. Furthermore, the ecological characterization of these taxa needs to be revised based on their molecular identification in order to assess their actual distribution and recent spread correctly.

This thesis aimed at analyzing the genetic structure and linking it to morphological as well as ecological peculiarities of German *Najas* populations. The main objectives of this thesis were:

1. Assess the genotypes and their variability within and between populations of the two *Najas* taxa from European lakes based on two different markers.
2. Compare the morphological characteristics currently used to distinguish between the two taxa with the results of the molecular studies and establish a method for correct and quick delimitation of taxa.
3. Analyze the interactions of the two *Najas* taxa with other invasive macrophyte species under variable ecological parameters, especially light.

2 General Methodology

2.1 Study area and sampling of plant material

The lakes chosen for the sampling of *Najas* plants cover the widest possible range of types of waters that can be found in South Germany (Bavaria). An essential basis for the selection of the lakes were multiple studies conducted for the development of the Macrophyte Index by Melzer, (1988). Occurrences of *Najas* within some of those lakes date back to 1988 and have been regularly mapped since 2000 according to WFD requirements. In a preliminary study conducted by Wutz (2011), morphological inconsistencies were observed in the delimitation the two different taxa of *Najas* plants derived from known or new locations. To confirm the identity of plants in the pre-study, molecular analysis was applied to the fresh samples as well as on reference herbarium material obtained from the Botanische Staatssammlung München (M). The results of the preliminary study indicated that both *Najas* taxa with their pronounced phenological polymorphism could have adapted to altered environmental factors, such as higher temperatures. Further is was assumed that hybridization may have obscured clear-cut morphological characteristics of *Najas* plants hampering proper identification.

In the vegetation periods of 2010, 2011 and 2012, 54 lakes located in Bavaria were screened for the presence of *Najas* by SCUBA diving or snorkeling (Fig. 2.1). When present, whole plants were sampled along a point or strip transect, put in plastic bags and stored cool until further processed. For molecular analysis, at least 30 different shoots per lake from different plants and populations were collected, which were stored in Eppendorf tubes filled with 96 % (v/v) ethanol. One representative herbarium specimen per lake or transect was collected and is stored as a reference at the TUM collection. Within this thesis, plant material from altogether 46 different lakes was used, 30 of those lakes are located in Bavaria (areas 1-15, Fig. 2.1) including one of them lying partly in the adjacent state of Hesse, four lakes in Baden-Württemberg (16) and two lakes in neighboring Austria (17). Another ten lakes from the Mecklenburg-Brandenburg Lake District (18) were added to the study area. The selected lakes can be grouped into the regional aspects as seen in Figure 2.1. Additional plant material was collected from different herbarium specimens by Dr. Christian Bräuchler made available by various botanists from other institutes all over Europe and beyond (Appendix A1).

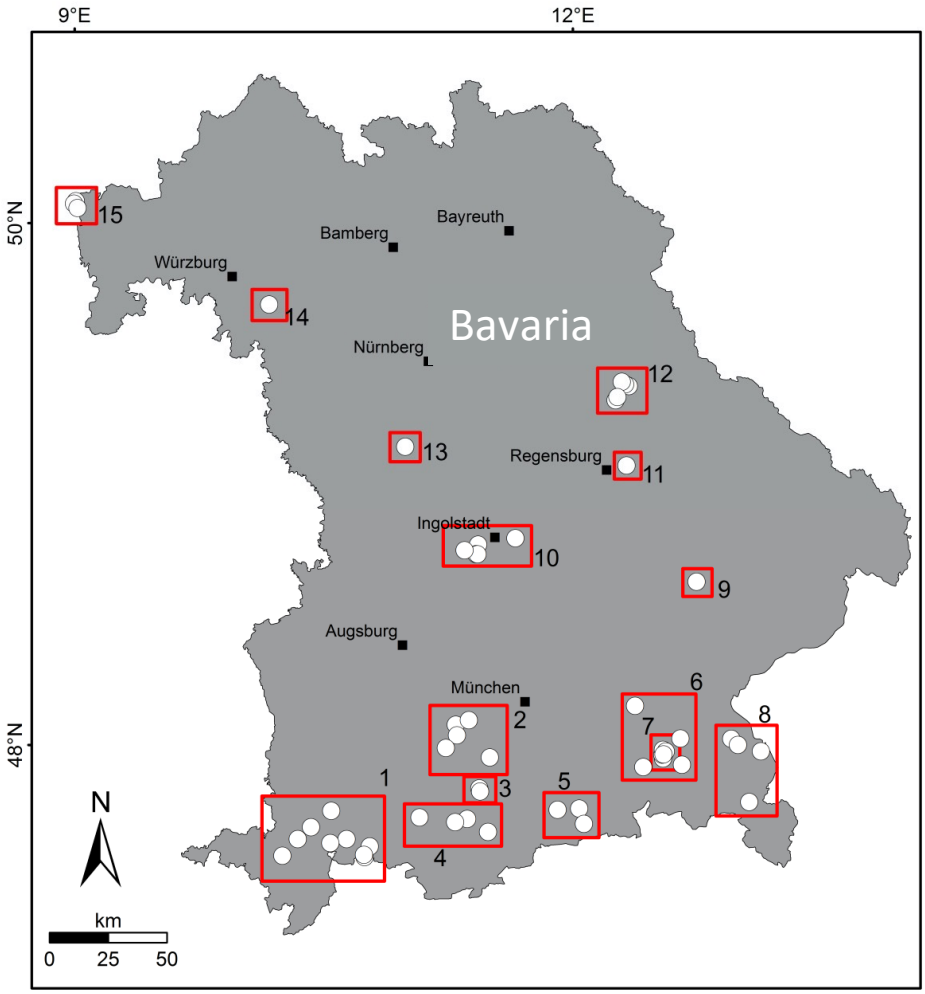


Figure 2.1: Locations of the selected lakes for prescreening and sampling of plants. Lakes that were part of the screening but without presence of *Najas* plants are underlined.
(1) Lakes of the Allgäu: Alatsee, Attlesee, Großer Alpsee, Grüntensee, Hopfensee, Niedersonthofener See, Notzenweiher, Öschlesee, Schwaigsee, Weißensee **(2) „Fünfseenland"**: Ammersee, Pilsensee, Starnberger See, Weßlinger See, Wörthsee **(3) Osterseen Lake District**: Gröbensee, Großer Ostersee, Westlicher Breitenauer See **(4) „Blaues Land"**: Kochelsee, Riegsee, Staffelsee **(5) Lakes of Miesbach county**: Tegernsee, Schliersee, Spitzingsee **(6) Chiemgau**: Chiemsee, Klostersee/Seeon, Simssee, Soyensee **(7) Eggstätt–Hemhofer Lake District**: Hartsee, Kesselsee, Langbürgener See, Pelhamer See, Schloßsee **(8) Berchtesgadener Land and Rupertiwinkel**: Abtsdorfer See, Waginger-Tachinger See, Thumsee, **(9) Reservoir in Niederbayern**: Vilstalsee **(10) Quarry ponds near Ingolstadt**: Aberlsee, Großer und Kleiner Weicheringer See, Schafirrsee, Ulrichsee **(11) Oxbow lake of the Danube**: Donaustauf **(12) Upper Palatinate Lake District**: Ausee, Brückelsee, Knappensee, Murnersee, Steinberger See **(13) Franconian Lake District**: Großer Brombachsee **(14) Quarry pond in Lower Franconia**: Wüffertsee, **(15) Kahl am Main**: See Freigericht Ost, Großwelzheimer See, Kahler Waldseebad, See Emma Nord (partly in Hesse)
Lakes not in Fig. 2.1: **(16) Baden-Württemberg:** Lake Constance (Bodensee), Degersee, Mindelsee, Muttelsee **(17) Austria**: Mattsee, Obertrumer See **(18) Mecklenburg-Brandenburg Lake District**: Brodowinsee, Großer Kelpinsee, Großer Peetzigsee, Jakobsdorfer See, Lehstsee, Lützlower See, Pinnower See, Sabinensee, Weißer See, Werlsee **(19) Inntal:** Hödenauersee

2.2 Morphological measurements

Najas plants were sampled for morphological analysis from 25 different lakes in 2010 for a preliminary study (Wutz, 2011). In consecutive years those populations were re-sampled and various other populations that were found in 11 additional lakes were also morphologically analyzed (Fig 2.1, Appendix A1). Plant material was always examined in fresh condition immediately after sampling for morphological characteristics as described here. After the preliminary study, numerous populations distributed all over Lake Staffelsee were chosen for a more thorough sampling in 2012 and 2015, due to high morphological variabilities of plants in this lake. Reference populations for each taxon were derived from Lake Abtsdorfer See (*N. marina* subsp. *marina*; ITS1) and Lake Starnberger See and Lake Constance (Bodensee) (*N. marina* subsp. *major;* ITS2). Those reference populations were chosen based on the previous genetic results (Wutz, 2011). In the presented study measurements of a total of 475 adult and flowering plant individuals were analyzed. All plants were examined with a stereo magnifying glass (6.5 - 40 x magnification, Wild Heerbrugg, Herrbrugg, Switzerland) for the following features:

- Sex based on inflorescences over the entire plant
- Blade widths in mm excluding and including the spines, at two to three different measuring points per leaf (Fig. 2.2)
- Blade length in mm (Fig. 2.2)
- Number of spines on the margins of the leaf sheaths with the number of teeth on the left and right side of the leaf sheath (Fig. 2.2)

From one plant individual at least three different leaf sheaths were analyzed for the number of teeth on each side of the sheaths, being recorded separately. Particularly rare morphological features were recorded photographically with a Kappa DX20 camera in conjunction with the Kappa program package KappaImage Base 2.7.2 (Kappa optronics GmbH, Gleichen, Germany).

More detailed leaf dimensions (blade widths and lengths) were determined for all samples from 2012 and those from the Staffelsee and the other reference lakes. Five leaves were randomly selected from each plant and used for the measurement.

For this, the moist leaves were pressed between two microscope slides. Two to three measurements were taken per leaf for each characteristic. The leaf width including the spines is referred to as "leaf width broad" (LWB), while the leaf width without the spines is named "leaf width narrow" (LWN).

The blade length was determined by two measurements along the blade, one of which was drawn freehand (black line, Fig. 2.2) and the other by a linear distance measurement (red line, Fig. 2.2). The biometric measurements were performed using the Metreo planimetry program of the Kappa program package KappaImage Base 2.7.2 (Kappa optronics GmbH).

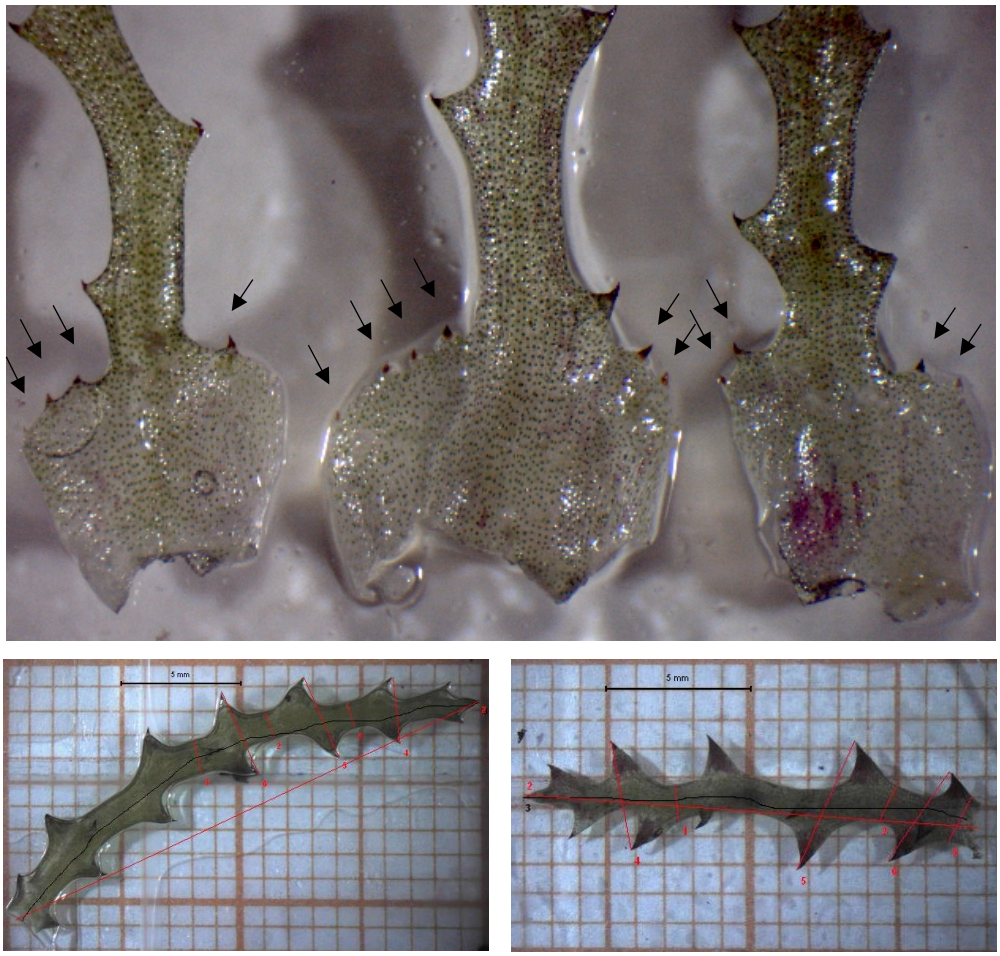


Figure 2.2: Pictures of *N. marina* s.l. leaves magnified 6.5 times under a stereo microscope. TOP Plant samples (*N. marina* subsp. *major*) from Lake Starnberg, collected in July 2011. All three leaves were derived from the same plant, the number of spines on the left and right side of the margin of the leaf sheaths were noted as follows: 3/1 4/2 2/2. BOTTOM Photographs used for measurements of two leaves from different plants both collected at Lake Staffelsee in 2012. Red orthogonal lines indicate the distance of the leaf width, for leave length the black irregular line was used.

2.3 Molecular analysis

2.3.1 DNA extraction

The DNA was isolated from the plant material which was fixed and stored in 96 % (v/v) ethanol for the molecular analysis. The plant material was removed from seed residues and combined with zirconium oxide beads (ZrO_2, Si- Libeads Typ ZY, Ø 1.8 – 2.0 mm Yttrium stabilized, Sigmund Lindner GmbH, Warmensteinach, Germany) and the lysis buffer which was contained in the DNA extraction kit DNeasy® Plant Mini Kit (Qiagen, Venlo, Netherlands) in 2 mL Eppendorf containers with a collecting tray. These containers were then shaken in a Micro-Dismembrator II (Bachofer GmbH, Reutlingen, Germany) for three minutes at a deflection of 7 mm to mechanically crush and chemically break up the plant cells. The further steps for the isolation of *Najas* DNA essentially followed the manufacturer's instructions for the DNeasy® Plant Mini Kit, which is based on several simple centrifugation steps.
These are in detail:

1. Cell lysis and precipitation of cell components (of proteins and polysaccharides) by RNase treatment for the removal of RNA residues
2. Separation of cell residues by several centrifugation steps
3. Hydrogenation of DNA
4. Binding of DNA to a silica gel membrane which is embedded in the centrifugation columns
5. Washing of DNA by several centrifugation steps
6. Eluting DNA in AE buffer included in the kit

To estimate the quantity and quality of the isolated DNA, 10 µL of the isolated samples were mixed with application buffer and electrophoretically separated into an agarose gel together with a defined DNA standard (λ DNA/ HindIII *Eco*RI, Fermentas, Waltham, MA, U.S.A.). The rest of the isolated DNA solutions were frozen at - 20° C until further use.

2.3.2 PCR and Sequencing

Amplification and sequencing of certain DNA marker regions provide characteristic base sequences and are used for phylogenetic analysis, in which those homologous/same sequence segments are compared to each other in an alignment. In general, differences in homologous areas can be caused by mutations such as deletions, insertions (summarized as indels) or point mutations, etc. The comparisons and its evaluation can then be used to infer evolutionary relationships between and within taxa. To obtain more accurate results a nuclear (nDNA) and a chloroplast DNA (cpDNA) marker, two different regions with distinct cellular origin and genesis, were chosen for *Najas* DNA samples and amplified using universal primers. The internal transcribed spacer region (ITS) within the ribosomal DNA operon contains the genetic information for the construction of ribosomes and is present multiple times in tandem repeats within the nuclear DNA. This ribosomal DNA (rDNA) is divided into different areas: the genetic information of the 18S subunit of the ribosomes, the first transcribed internal spacer (ITS1), the genetic information of the 5.8S subunit of the ribosomes, the second transcribed internal spacer (ITS2) and the genetic information of the 28S subunit of the ribosomes (Fig. 2.3). The two non-coding ITS regions 1 and 2 may both show mutations and are therefore very well suited for comparative sequencing of fungi, plants, and animals (White et al., 1990; Yao et al., 2010).

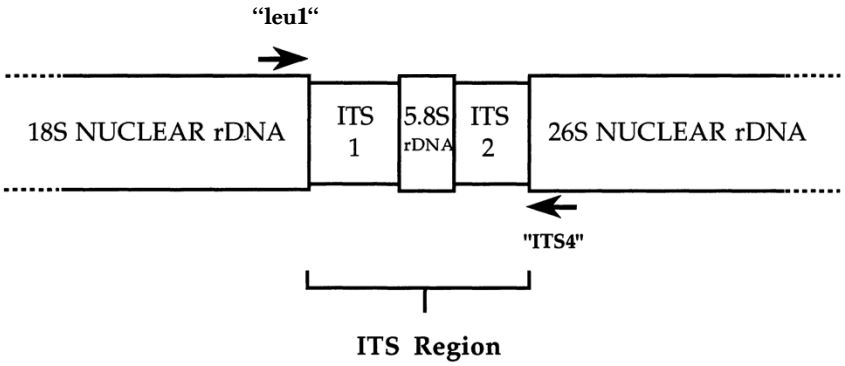


Figure 2.3: Organization of the ITS region, reprinted and modified from Baldwin et al. (1995). Arrows indicate orientation and approximate position of primer sites. Primer names (in quotation marks) and sequences are from White et al. (1990).

For the PCR, a Taq polymerase (Boehringer, Ingelheim am Rhein, Germany) and the primer pairs **leu1** (5′-GTC CAC TGA ACC TTA TCA TTT AG-3′, Vargas et al. (1998)) and **ITS4** (5'-TCC TCC GCT TAT TGA TAT GC-3', White et al. (1990)) were used.

The second marker is a spacer region that originates from the chloroplast genome and is located between the lysine tRNA exon (*trn*L) and the tRNA gene for the amino acid phenylalanine (*trn*F). The structure of the marker is shown in Figure 2.4. The grey marked coding regions of the DNA are highly conserved, whereas the intermediate "spacers" or introns can be highly variable, similar to the ITS region of the ribosomal DNA. Primer pairs **C/F** or **C/D** and **E/F** of Taberlet et al. (1991) were used for the amplification of the *trn*L-F region, which was done in one or two parts following Bräuchler et al. (2004).

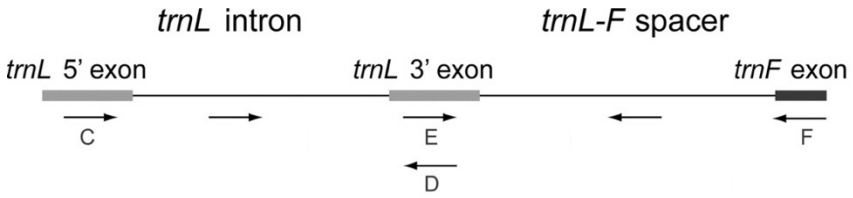


Figure 2.4: Scheme of *trn*L-F region and primers used to amplify and sequence copies. Reprinted and modified from Pirie et al. (2007).

PCR was carried out in a thermal cycler (Primus, MWG Biotech, Ebersberg, Germany) with the following program settings: (1) preheating at 110°C for 2 m 30 sec., (2) 40 cycles at 94°C for 30/45 sec., 53°C for 30/45 sec., 72°C for 1 min. 15/30 sec. and (3) a final extension phase at 72°C for 10 minutes. The longer times were always used for the *trn*L-F amplification reaction. The entire volume of the PCR reaction was always 50 µL, including 2 µL of template DNA (20 - 100 ng/µL; 1.5 - 7.7 nM DNA) and with either 0.1 µL, (ITS) or 0.125 µL, (*trn*L-F) of each primer with a concentration of 100 pmol/µL. After a successful PCR run the PCR products were checked on an agarose gel and subsequently cleaned using the NucleoSpin® Extract Kit (Machery Nagel GmbH, Düren, Germany). Thus, impurities such as primer residues and other nucleotide compounds could be removed for sequencing. The procedure corresponded to the standard protocol of the kit and was carried out unchanged except for an extension of the centrifugation steps by one minute each. The same primers were used for the sequencing of the ITS and the *trn*L-F regions. Sequencing of the two DNA regions was performed on an ABI PRISM 3730 in combination with the use of the BigDye® Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, U.S.A.) according to the protocols provided by the sequencing service of the Faculty of Biology (LMU). Both amplification and sequencing were carried out at the former Chair of Systematic Botany (LMU) in collaboration with Prof. Günther Heubl (retired) and Dr. Christian Bräuchler (today Naturhistorisches Museum Wien).

2.3.3 PCR - RFLP

To quickly identify the ITS genotype of a *N. marina* s.l. PCR product, a rapid detection method using restriction digestion and gel electrophoresis was developed. The already known ITS sections of both ribotypes were examined for restriction interfaces within their variable ranges. If one or more of these restriction enzyme sites is specific only for one of the respective ITS types, that site serves as a distinguishing feature. The restriction sites were checked by using the built-in application tool in BioEdit v7.2.5 (Hall, 1999). Similar molecular identification methods were also used for unclear morphotypes of *Elodea* spp. and adequately tested (Gross et al. 2003). After PCR of the desired region, the amplified DNA was digested by the ITS type 2 specific enzyme Hind III. After the cleavage of the DNA fragments, electrophoresis was performed to control the success of digestion. The following gel image (Fig. 2.5) shows one band for ITS type 1 (red frame, band size approx. 770 bp) and two bands for ITS type 2 (blue frame, approx. 290 bp and 480 bp). The enzyme Hind III therefore only cuts at the specific DNA sequence that corresponds to ITS type 2 DNA. Amplified DNA of ITS type 1 does not have an interface and therefore remains undigested. This technique can also be used to identify hybrid individuals. Their PCR products contain both ITS ribotypes and the restriction patterns are discernable with three bands (Fig. 2.5) and are therefore genetically unambiguous.

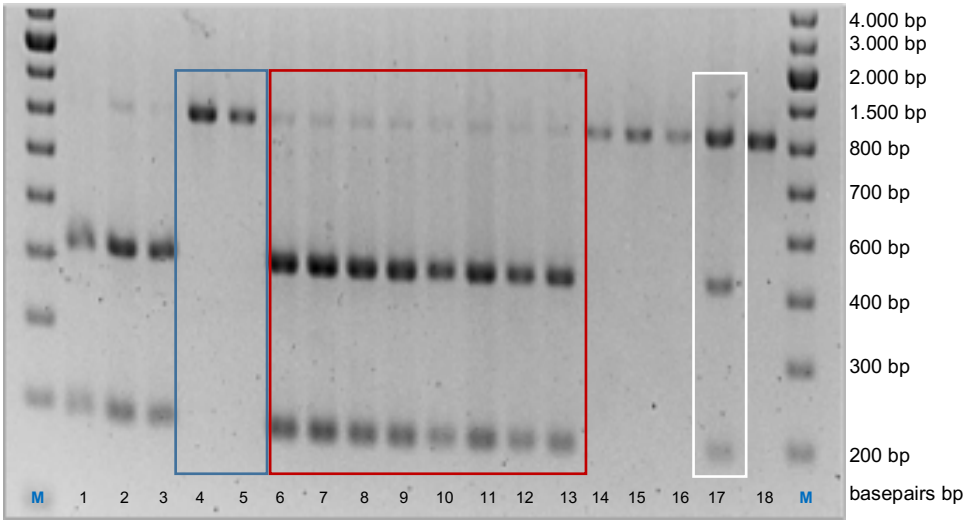


Figure 2.5: Agarose gel picture of extracted *Najas* DNA stained with ethidium bromid, after the digestion with HindIII; M: marker, blue: (samples 4, 5) ITS type 1, red: (samples 6 - 13) ITS type 2, white: hybrids

2.4 Ecological experiments and mesocosm setup

A mesocosm system was developed to create conditions as close to nature as possible for the investigation of native and invasive macrophyte species without taking the risk of unintentionally introducing these species into natural waters (Fig. 2.6). This closed system consisted of twelve identical 1000-liter Intermediate Bulk Containers (IBCs) which served as mesocosms. They allowed experiments to be carried out almost under field conditions, as the containers could be exposed outside under natural light conditions. To screen off incident light through the transparent walls of the tanks and to prevent unwanted heating, they were wrapped in a white translucent film. The mesocosms stood next to each other in a west-south-westerly direction, the experiment was carried out between April and October 2016.

The experiment is divided into four phases: Running-in period of the system (9 weeks), growth of native and near-native species (7 weeks), invasion by neophytes (2 weeks) and development of macrophytes under the different simulated turbid conditions (5 weeks). The harvesting of the plants for analysis took place towards the end of the vegetation period at the beginning of October. Experimental factors essential for the experiments, such as water and sediment, were determined or standardized for each ICB in advance and regularly monitored during the course of the experiment.

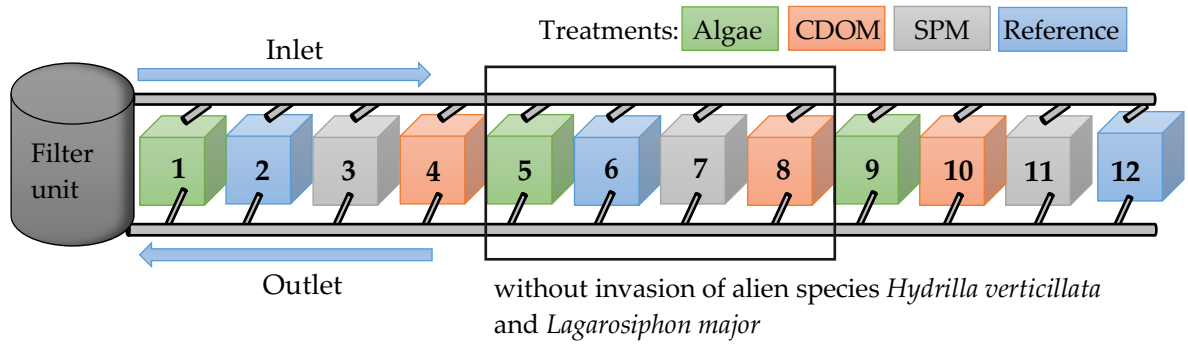
The water used for the experiment was drawn from Lake Starnberg. Each tank was filled with 800 liters of lake water in spring. Fluctuations in the water level during the course of the experiment were compensated with water from Lake Starnberg from a storage tank. After the turbidity had started, tap water was used to maintain the water levels and to avoid the unwanted introduction of algae. From a collecting tank with a filter unit, the water was pumped with the aid of a submersible circulation pump with a capacity up to 10,000 L/h. The water was distributed via a pipe system over all mesocosms and circulated between them (Fig. 2.6). A coarse dirt filter and an ultraviolet treatment with 18-Watt UVC lamp (UVC 18, Osaga, Glandorf, Germany) were built into the drain to reduce the planktonic algae and an unwanted algal bloom in the water.

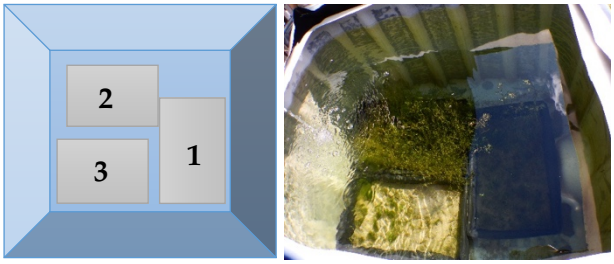
Three identical plastic boxes were installed at the bottom of each mesocosm containing the plant substrate (Fig. 2.6). For the plants to root deeply, a coarse and a fine gravel layer were placed in the boxes, followed by an 0.08 m thick layer of sifted sediment, which was sampled

from Lake Starnberg in early spring. The sediment can be described as a very fine, nutrient-rich substrate consisting of lime sludge with organic, undecomposed parts (10 P_2O_5-P_{tot} [mg $100g^{-1}$ soil]). The water, as well as the boxes filled with the substrate, were already inserted into the mesocosms in May 2016. After nine weeks of equilibration, the macrophyte cuttings were implanted into the boxes in June/July 2016. The three different boxes in each ICB were arranged randomly to compensate for position effects.

In each mesocosm, box 1 contained only sediment without macrophytes at the beginning of the experiment. During the summer, however, the oospores contained in the sediment formed lush *Chara contraria* A. Braun ex Kützing populations. Box 2 was half planted with *N. m.* subsp. *intermedia* seedlings from Chiemsee and half with *N. m.* subsp. *marina* seedlings from Lake Staffelsee. In each case, 15 young *Najas* rooted individuals with a length of about 7 cm were planted, which were collected at the beginning of June in the respective lakes. Similarly, box 3 was equipped with 15 *Myriophyllum verticillatum* L. seedlings from the surrounding Lakes Osterseen and 15 *Elodea nuttallii* (Planch.) H.ST.John seedlings from Lake Chiemsee. The seedlings of all plants, except for the two *Najas* species had a length of ten nodes.

In August 2016, after seven weeks, the invasive neophytes *Lagarosiphon major* (Ridl.) Moss and *Hydrilla verticillata* (L.f.) Royle were introduced into a part of the mesocosms in addition to the plants that had already been planted and grown. The alien species originated from the online shop extraplant.de and were cultivated in aquaria half a year before the experiment at the Limnological Research Station in Iffeldorf. They were acclimatized for five days in a climatic chamber at a water temperature of 20 °C and a light/dark period of 10/14 hours before being introduced into the mesocosms. In mid-August, ten fragments of *L. major* and *H. verticillata* with a length of ten nodes were added to each of the three boxes in the mesocosms by simply placing the shoots on the existing plant stands. The two alien species were not introduced into mesocosms 5 - 8 (Fig. 2.6), as these approaches were used as a reference without invasion.





Planting scheme and picture of the 3 boxes with native and near-native species

1 Sediment (*Chara contraria*)
2 *Najas marina* + *N. major*
3 *Myriopyhllum vertcillatum* + *Elodea nuttallii*

Picture of the IBCs setup with the collecting tank including the filter unit

Figure 2.6: Outlines and pictures of the experimental setup for the ecological study in the mesocosms CDOM: colored organic matter, SPM: suspended particulate matter.

As soon as the turbidity of the individual tanks was initiated, the circulation of water between the mesocosms was stopped and each tank received an aerator. Using a hyperspectral underwater radiometer (RAMSES ACC-Vis, TriOS GmbH, Rastede, Germany, 320 - 950 nm as m W m^{-2}, 180° detection field) to measure photosynthetically active radiation (PAR) and a turbidity probe from WTW (VisoTurb 900-P IDS, WTW, Weilheim, Germany), it was ensured that the turbidity intensity within the treated mesocosms was almost identical and constant over the turbidity phase. The turbidity intensity was expressed in NTU (Nephelometric Turbidity Unit) and was in the range 0.2 ± 0.43 NTU before turbidity, whereas during turbidity it was 1.16 ± 0.60 NTU and thus above the limit of 1.0 NTU for drinking water.

3 Hybridization and cryptic invasion in *Najas marina* L. (Hydrocharitaceae)?

A similar version of this chapter was published: Rüegg, S., Raeder, U., Melzer, A., Heubl, G., Bräuchler, C., 2017. Hybridisation and cryptic invasion in *Najas marina* L. (Hydrocharitaceae)? Hydrobiologia 784, 381-395. Published online: DOI: 10.1007/s10750-016-2899-z

Candidate's contribution:
Selection of sample material and conduction of additional sampling of plants, performance of molecular analysis (DNA extraction and PCR), preparation and analysis of molecular and sequence data, writing and revision of the complete manuscript including Maps and Tables as well as the Supporting Information.

3.1 Abstract

Macrophytes have been used as bioindicators for eutrophication assessment in freshwaters required by the European Water Framework Directive (WFD). The red listed *Najas marina* s.l. is routinely mapped in Germany. Different indicator values have been assigned to the subspecies *marina* and *intermedia* which are, however, frequently hard to tell apart due to morphological similarity. Therefore, phylogenetic structure within *N. marina* s.l. was investigated using nuclear ribosomal (ITS) and chloroplast (*trn*L-F) DNA sequence data from over a hundred accessions, representing three of the 12 subspecies and one of four varieties in *N. marina.* The samples group in two distinct clusters, which could be correlated to the two karyotypes previously reported. The clusters differ in 45 positions of ITS and 10 of *trn*L-F respectively, with almost no variation within. Conflicting placement in the nuclear and chloroplast tree supported by cloning of heterozygotic samples identified hybrids in four cases. The clear-cut molecular differentiation in spite of morphological similarity identifies both lineages as distinct but cryptic species (*N. marina* and *N. major*). Based on our modified concept and the uncertainty introduced by former misidentification, the use of the two taxa for the purpose of the WFD and regional red list status needs re-evaluation.

3.2 Introduction

Availability and preservation of high quality freshwater resources is one of the major challenges for the future of our planet (Hering et al., 2015). Large percentages of freshwater lakes have been polluted at a global scale in the past. In order to monitor pollution and the effects of water quality improvements the European Water Framework Directive (2000/60/EC) requires consistent monitoring of surface water bodies with the objective of reaching a good ecological state or potential (European Commission, 2005; Geist, 2014). Occurrence and abundance of certain macrophytes are considered good indicators in this context (Melzer, 1999; Schaumburg et al., 2004; Schneider et al., 2000). Classification of ecological status is expressed by a reference index value (RI) that describes the deviation of the observed submersed plant community (i.e. certain indicator species) from a reference condition (Stelzer et al., 2005). The reference condition (equal to high ecological status) is defined as "natural, undisturbed/minor human impacted" and differs according to lake type (Wallin et al., 2003). Deviations in species composition and abundance from the respective reference are used to quantify the level of degradation. Also, dominant stands of single species (e.g. *Ceratophyllum demersum*) can result in lower ranking of the ecological status (Schaumburg et al., 2011).

Najas marina L. is one of the species used for implementation of the WFD (Schaumburg et al., 2004; Stelzer et al., 2005). The subcosmopolitan species is one of the most widespread aquatic vascular plants in Central Europe (Wiegleb, 1978), comprising 12 different subspecies and four varieties (Triest, 1988). The plants are dioecious annuals, growing completely submerged in shallow lakes (1-3 m deep) and rooting extensively in sediments. Two subspecies, *Najas marina* subsp. *marina* and *Najas marina* subsp. *intermedia* (Wolfg. ex Gorski) Casper are distributed from Europe to Central Asia, in temperate and warm temperate areas (subsp. *marina*) and in cold to warm temperate areas (subsp. *intermedia*) (Triest, 1989). Currently they are ranked differently within the WFD assessment system for German lakes (Pietsch, 1981; Schaumburg et al., 2011). In consequence, ambiguities in identification may affect classification of a lakes ecological status. *N. marina* subsp. *marina* is consistently influencing the status negatively, because it is rarely found at undisturbed reference sites. *N. marina* subsp. *intermedia* is assessed mostly neutral (or even positive depending on water depth and type of lake), except for mass occurrences which result in a more negative ranking (Schaumburg et al., 2011). In other

European countries eutrophication assessment for lakes is often performed without distinguishing those two taxa (Penning et al., 2008b).

High morphological variability and/or phenotypic plasticity makes correct identification notoriously difficult not only in *Najas* L. (Les et al., 2015; Triest, 1988) but also in various other aquatic plant groups (Barrett et al., 1993; Ito et al., 2010; Les & Philbrick, 1993; Sculthorpe, 1967; Simpson, 1988). In *N. marina* s.l. states of key characters used for delimitation of the two taxa, like seed or leaf dimensions and teeth on margins of leaf sheaths, are frequently overlapping (Casper & Krausch, 1980; Triest et al., 1986; van de Weyer et al., 2011; Viinikka, 1976) (Tab. 3.1). Especially in the field identification of plants is difficult, rendering the two taxa morphologically cryptic. Misinterpretation of nomenclatural types and subsequent misapplication of names further added to the complexity of the situation (Bräuchler, 2015).

Table 3.1: Measurements of leaf characters (width and length) in mm cited from literature.

Character	*Najas marina* subsp. *marina*	*Najas marina* subsp. *intermedia*	Citation
Width of leaves	**1.1 – 1.5** (0.8 -) **1 – 1.5** (- 2.5) (2.7 -) **3.5 – 5.1** (- 6.1)	**0.5 – 0.9** (- 1) (0.2 -) **0.5 – 0.9** (- 1.1) (1.5 -) **1.8 – 2.8** (- 3.3)	Viinikka (1976) Casper & Krausch (1980) Triest et al. (1986)
Length of leaves	**16 – 31** (10 -) **19 – 34** (- 45) (11.6 -) **15.1 – 23.3** (- 28.9)	**7 – 19** (- 24) (4 -) **9 – 26** (- 38) (4.4 -) **6.5 – 11.1** (- 12)	Viinikka (1976) Casper & Krausch (1980) Triest et al. (1986)
Number of teeth on margin of leaf sheath	Without, rarely one	**1 – 3** (4) on each side	Casper & Krausch (1980)

In the past, karyological studies (Viinikka, 1976; Winge, 1927) and isozyme analysis (Triest et al., 1986) helped addressing this taxonomic problem and identifying doubtful specimens. In recent years, DNA analysis enhanced our general understanding of *Najas* in various ways and finally placed it in Hydrocharitaceae (Chen et al., 2012; Les et al., 2006). In this family, taxonomy could be resolved in some genera (Les et al., 2006) and species complexes (e.g. *N. flexilis* s.l. and *N. guadalupensis* s.l.; Les et al., 2015, 2010) by applying standard molecular phylogenetic markers like the nuclear ribosomal internal transcribed spacer region (ITS1-5.8S-ITS2) and the chloroplast *trnK* region. These analyses allowed for detection of interspecific hybrid plants (Les et al., 2010) and cryptic species identification (Les et al., 2015). When used as ecological indicator, groups containing cryptic species as defined in Geller et al. (2010) could

lead to inaccuracy of results from monitoring (Geller, 1999; Lobel et al., 1990). With its subspecies ranked differently, *N. marina* s.l. is a good example: while still included in the red lists of several countries (Cheffings et al., 2005; Korneck et al., 1996) it seems to be generally spreading (Hoffmann et al., 2013b) and is regarded as invasive in some places (Hoffmann et al., 2013a). Due to the taxonomic uncertainty, it is however not possible to reliably name new or old records based on morphology alone. Some records are definitely new (Buch et al., 2012), but some simply have been overlooked (Bräuchler, 2010). Thus, it remains cryptic, whether both or just one of the subspecies are spreading or how far one may invade the territory of the other unnoticed. Geller et al. (2010) coined the term cryptic invasion for such cases, where the invasion itself remains unrecognized. The different forms of these cryptic invasions, as defined by them, are characterised by novelty and taxonomic rank of the invader. Both factors may play an important role in the spread of *N. marina* s.l. in German lakes. New genotypes invading some lakes cryptically could gradually eliminate the native ones, like reported for North American *Phragmites australis* (Cav.) Trin. ex Steud. (Saltonstall, 2002).

To examine existing and historical populations of *N. marina* s.l. we applied two different molecular markers (ITS, *trn*L-F). Based on these data we aimed at the following: 1) provide data of nuclear ribosomal and cpDNA variation in *N. marina* s.l. in Europe, 2) name samples taxonomically correct, 3) trace new populations and monitor those recently recorded by regular mapping and sampling (using southern Germany as major focal point), 4) check if any type of cryptic invasion occurred 5) reconsider the use of *N. marina* s.l. as indicator for further implementation of the WFD.

3.3 Materials and Methods

3.3.1 Study species

In spite of the existing morphological ambiguities within *N. marina* s.l. described above, both taxa (subsp. *marina* and subsp. *intermedia*) were accepted as subspecies confirmed by karyotype and isozyme analysis (Triest et al., 1986; Viinikka, 1976; Winge, 1927). In contrast to Viinikka (1976), who published a formally correct and thus binding treatment on the nomenclature naming the two taxa subsp. *major* (All.) Viinikka and subsp. *marina*, they have been referred to as *N. marina* subsp. *marina* (= *major*) and subsp. *intermedia* (= *marina*), respectively (e.g. Casper, 1979; Triest, 1988). A detailed overview on the history of this confusion and typifications are provided by Bräuchler (2015). For the purpose of this study, when addressing the two distinct groups, we refer to them as *N. marina* and *N. major* as defined by Viinikka (1976) and Bräuchler (2015). *N. marina* s.l. refers to the broadly defined taxon. When using names of taxa without indication of rank, they are put in quotation marks e.g. "*intermedia*".

3.3.2 Sampling strategy and herbarium material

Fresh plant material was obtained while mapping macrophytes in numerous lakes for implementation of WFD (particular northern lakes) and during a three year state-funded project monitoring the distribution of *N. marina* s.l. predominantly in Bavarian lakes. 66 lakes were initially screened for presence of *N. marina* s.l. and their respective ribotypes within the scope of the project, specifically focusing upon smaller lakes (<0.5 km^2) as they are not covered by WFD-related monitoring. Lake choice was guided by enquiries of local fisheries and lake owners about the presence of *Najas* plants. Fresh leaf material of *N. marina* s.l. could be collected from 46 lakes throughout Germany, most of them located in Bavaria (29) or adjacent states (Baden-Württemberg (4) and Hessen (1)) and two in neighboring Austria (Appendix A1). Additional material was obtained from ten lakes from the Mecklenburg-Brandenburg Lake District, which are mapped regularly according to the requirements of the WFD (Schaumburg et al., 2011).

Fully developed individuals were sampled +/- every 2 meters along transects during growing season. Depending on population size, a maximum of 30 samples per transect was collected

and subsequently dried in silica gel. Plants were identified using standard taxonomic keys (Casper & Krausch, 1980; Haeupler & Muer, 2007; van de Weyer et al., 2011). Populations in Bavarian lakes were documented by one representative specimen each. All specimens are preserved at the herbarium TUM (Holmgren & Holmgren, 1998), located at the Limnological Research Station Iffeldorf as a special collection. Populations located in potential hybrid-zones, which could be identified by a first round of sequencing, were sampled more densely in order to test for presence of unnoticed hybrids. Here, a specimen was prepared for each individual sample. Due to a denser sampling in these areas, some individuals not morphologically distinct, have been proven to be of hybrid origin (e.g. Lake Staffelsee). Dried leaf fragments were removed from specimens obtained from various international herbaria (marked with * in Appendix A1) to increase geographic coverage outside of Germany and to include reference material of previous studies on karyology and isozyme patterns (Triest et al., 1986; Viinikka, 1976). To allow for more general conclusions, herbarium material from throughout Europe and beyond was included in the study as well (Appendix A1).

Point distribution maps were generated using QGIS (v2.6.1-Brighton) (QGIS, 2014) and sampled localities were geo-referenced (WGS 84, UTM zone 32 northern hemisphere) and mapped with data from Natural Earth (free vector and raster map data @ naturalearthdata.com, (Kelso & Patterson, 2010). Coordinates of sampling sites are listed in Appendix A1.

3.3.3 DNA extraction, amplification, sequencing and cloning

Total DNA was extracted from 40 - 80 mg of fresh or 20 - 50 mg of dried plant tissue using two different extraction kits: DNeasy Plant Kit (Qiagen, Venlo, Netherlands) and NucleoSpin Plant-Kit (Macherey-Nagel, Düren, Germany) following the manufacturer's protocols. A list of sampled populations, voucher information and kit used is provided in Appendix A1. DNA was dissolved in 50-100 µL elution buffer. After initial checks for quality and concentration on a 0.8% (w/v) agarose-gel, a standard amount of 1 - 2 µL of template solution (20 - 100 ng/µL; 1,5 - 7,7 nM DNA) was used for PCR. Amplification and sequencing of the nuclear ribosomal ITS region (including the complete sequence of ITS-1, 5.8S and ITS-2, and partial sequences of the flanking 18S and 26S) was conducted using the primers leu1 (Vargas et al., 1998) and its4 (White et al., 1990) as described in Bräuchler et al. (2010). For the plastid *trn*L-*trn*F region this was done in one or two parts following Bräuchler et al. (2004) using the primer pairs C/F or

C/D and E/F of Taberlet et al. (1991). The chloroplast marker was analysed for a reduced sampling (56 out of 189 samples) to check for overall consistency with ITS results. Only in the potential hybrid zones the *trn*L-F chloroplast marker was sequenced additionally for various samples to test for introgression. PCR products were purified using the NucleoSpin Gel and PCR Clean up Kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's protocol. Products were sequenced on an ABI PRISM 3730 (Applied Biosystems, Waltham, USA) automated DNA Analyzer. All sequences have been deposited in GenBank (Acc.-Nr. KT596444 - KT596674).

To determine whether superimposed sequence electropherograms are an indication of heterozygosity and hybridisation or due to coexistence of both ITS types in all populations, cloning was performed on newly amplified ITS products of four representative accessions: Two of the putative hybrid accessions with heterozygotic signals from Lake Staffelsee (I = STF 8.3 and II = 10_Aquarium) and two individuals each with unambiguous sequences, representing the two different ITS types as references (a = Lake Staffelsee 2.1, (KT596497); b = Lake Ammer 2011, (KT596460)). Cloning was performed using the pGEM®-T Vector System II A3610 (Promega, Madison, USA) following manufacturer's protocol. For ligation, 3 µL of the PCR products were used (1 hour at RT); after transformation colonies were grown on LB medium plates (1 L containing: 10 g tryptone, 5 g yeast extract, 10 g NaCl, and 15 g agar). Recombinant bacteria colonies were selected, re-suspended in 50 µL of bdH_2O and heat-inactivated for 10 minutes at 94 °C. 1 µL of the suspension was used for PCR. Samples were amplified, cleaned up, and sequenced as mentioned above. The resulting 114 sequences were submitted as a separate alignment (KT596331 - KT596443).

3.3.4 Alignment and phylogenetic analysis

ITS sequences were generated for a total of 175 samples (excluding subcloned sequences), obtained from 114 extant populations and 61 herbarium specimens. Due to the mentioned taxonomic problems most of them have not been identified to subspecies level. Nonetheless, we were able to include at least some verified samples for most infraspecific taxa found in Central Europe. 15 additional sequences were retrieved from GenBank (** marked accessions in Appendix A1). The outgroup, chosen according to Chen et al. (2012), consisted of 12 species

of Hydrocharitaceae (*Najas* and *Hydrocharis* L., Appendix A1) and was represented by 28 accessions.

All sequences obtained were aligned using the online version of MAFFT v7 (Katoh & Standley, 2013) and optimized manually using the multi-sequence alignment editor BioEdit v7.2.5 (Hall, 1999). To test for monophyly of and genetic structure within *N. marina* s.l. the dataset for each marker was analysed separately using Bayesian inference and the Markov-chain-Monte-Carlo (MCMC) algorithm implemented in MrBayes v3.2.3 (Ronquist & Huelsenbeck, 2003). Appropriate sequence evolution models were chosen using MrModeltest v2.3 (Nylander, 2008; Posada & Crandall, 1998). Best fitting substitution models (Rodríguez et al., 1990) were selected by the Akaike information criterion (Posada & Buckley, 2004) for both markers (ITS: GTR+I+G and *trn*L-F: GTR+G). Settings for the analysis were as follows: 2.000.000 generations, infinite-gamma distribution (ITS) or gamma distribution (*trn*L-F), 4 chains, with every 100th tree sampled, average standard deviation of split frequencies below 0.01. After discarding trees yielded before likelihood stationary (burnin = 60000), the remaining trees were summarized in a 50% majority rule consensus tree, using posterior probabilities (PP) as a measure of clade support. Trees were visualized using Dendroscope v2.7.4 (Huson et al., 2007) and edited in Adobe Illustrator CS 5.

3.4 Results

3.4.1 Sequence data and tree topologies

The ITS alignment included 189 accessions (ingroup 162), from which 174 were newly obtained for this study (Appendix A1). ITS sequences were ranging from 350 to 773 bp. Differences in length are due to missing parts of the flanking 18S and 26S rDNA regions. 26 samples were incomplete in ITS-1 or ITS-2 (marked with [1] in Appendix A1). Complete 5.8 S (159 bp) and (in-) complete ITS- 1 (279-281 bp) and ITS-2 components (238-250 bp) were part of all sequences. The alignment consisted of 932 positions, 353 being parsimony informative. For the ingroup, three indels (1 -2 bp) had to be inserted in the alignment (ITS-1: 2, ITS-2: 1), all being phylogenetically uninformative (data not shown). Two major ribotypes, named type A and B, could be identified, differing in 45 positions (Tab. 3.2). Differences were calculated using two representative sequences of each type: ITS-A (Lake Abtsdorf, KT596458) and ITS-B (Lake Ammer, KT596460).

In the ITS tree topology (Fig. 3.2) accessions of *Najas* are split in two major clades. Posterior probability (pp) for all nodes is 1.000 in case not specified differently. Branching pattern in clade I is as follows: 1) a subclade of *N. orientalis* Triest & Uotila, *N. flexilis* (Willd.) Rostk. & W.L.E. Schmidt and *N. conferta* (A.Braun) A. Braun as subsequent sisters; 2) a lineage of *N. gracillima* (A.Braun ex Engelm.) Magnus, *N. minor* All. and *N. oguraensis* Miki (largely unresolved); 3) a group of *N. gracillima* and *N. tenuissima* (A. Braun ex Magnus); 4) a cluster containing an unidentified *Najas* accession from Namibia, a lineage (pp = 0.999) of *N. madagascariensis* Rendle and *N. tenuifolia* R. Br. and 5) the four accessions of *N. graminea* Delile. Number of outgroup samples was more extensive in the ITS matrix. Clade II comprises all accessions of *N. marina* s.l., with a deep split into two subclades representing the two ribotypes. Within type B (Fig. 3.2), the samples from Guatemala (KT596572) and USA (KT596592) formed a separate cluster characterized by only one synapomorphy (position 777 of the ITS alignment).

Table 3.2: DNA variation in nrITS (751 nt) sequences among ITS types A and B inferred from reference sequences. Numbers indicate quantity of differences in each region, and flanking parts of 18S and 26S did not show any variation.

Region	**internal transcribed spacer 1**																								
Number of substitutions	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
ITS-A (KT596458) Abs1	T	C	T	A	A	-	-	T	T	A	A	T	C	T	T	G	A	C	C	C	A	A	G	T	G
ITS-B (KT596460) Amm1	C	T	G	C	C	G	T	G	C	C	T	C	T	A	C	A	C	T	T	T	G	G	T	C	T

Region	**5.8 S**		**internal transcribed spacer 2**																
Number of substitutions	1	2	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
ITS-A (KT596458) Abs1	G	C	C	C	C	C	A	C	A	T	C	C	T	T	G	A	T	-	T	C
ITS-B (KT596460) Amm1	A	T	A	T	T	T	C	T	T	A	T	T	A	C	A	C	C	A	C	T

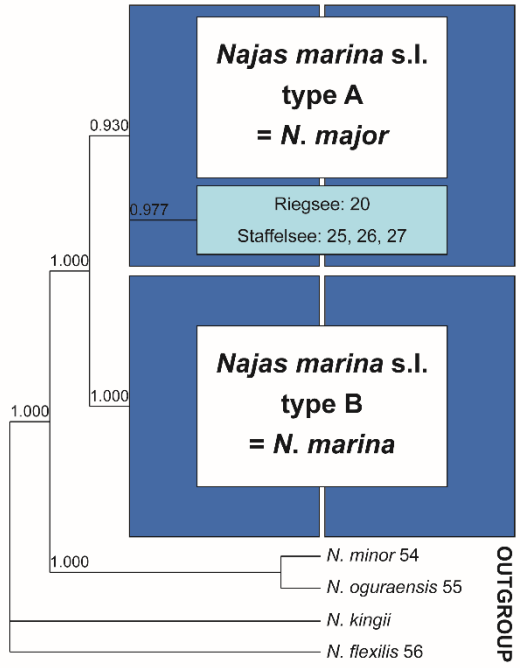


Figure 3.1: 50%-majority-rule consensus tree inferred from Bayesian analysis based on *trn*L-F sequence data of *Najas marina* and outgroup accessions. Posterior probabilities are provided as measure of clade credibility above branches. The subclades containing accessions of *N. marina* are summarized (*Najas marina* type A = *N. major* and *Najas marina* type B = *N. marina*) and indicated by boxes. Terminal numbers indicate running numbers in the *trn*L-F alignment (see Appendix A1) where more than one accession has been included for a given taxon.

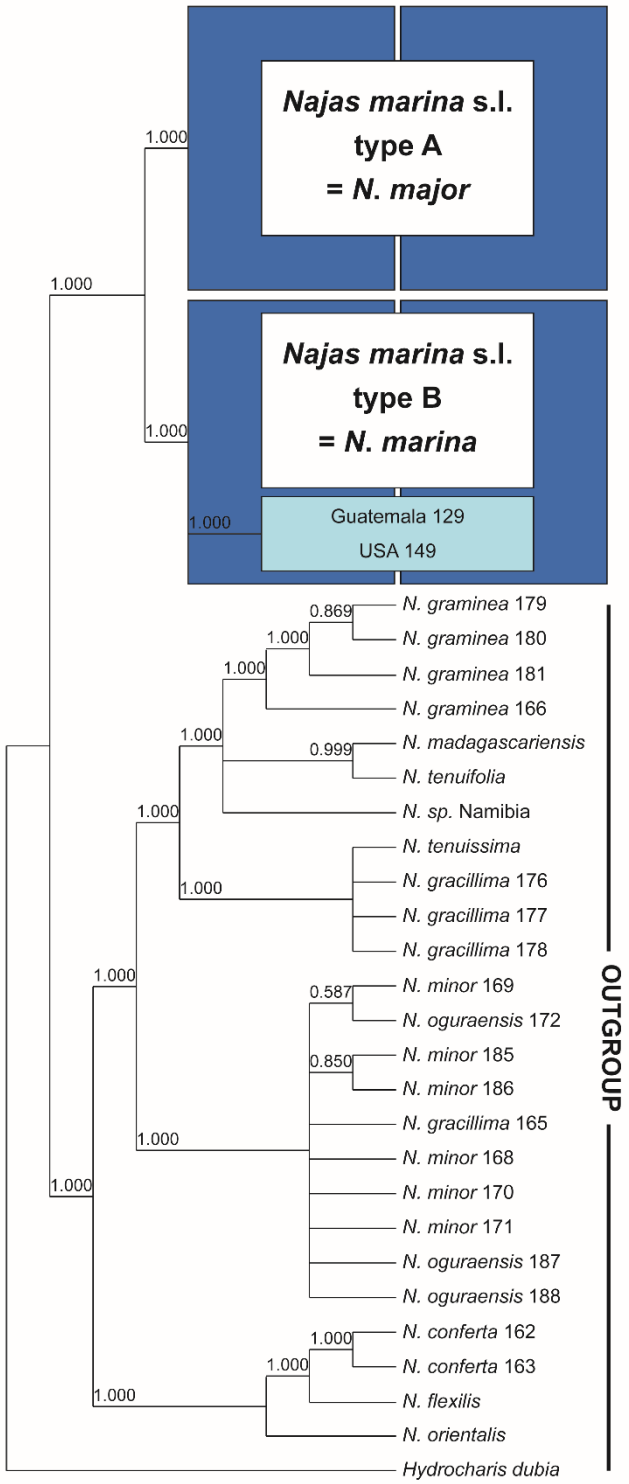


Figure 3.2: 50%-majority-rule consensus tree inferred from Bayesian analysis based on ITS sequence data of *Najas marina* and outgroup accessions. Posterior probabilities are provided as measure of clade credibility above branches. Clade II and its subclades are summarized (*Najas marina* type A = *N. major* and *Najas marina* type B = *N. marina*) and indicated by boxes. Terminal numbers indicate running numbers in the ITS alignment (App. A1).

For *trn*L-F analysis, 52 sequences of *N. marina* s.l. formed the ingroup (Appendix A1), while the outgroup consisted of four further *Najas* species (including position 50946 to 51973 of the *N. flexilis* plastid genome (Peredo et al., 2013). The outgroup was reduced here, since the marker was sequenced primarily to test for congruent branching pattern within *N. marina* s.l. The alignment consisted of 1363 positions, 75 of which were parsimony informative; sequences ranged from 883 to 1023 bp. For the ingroup, the alignment contained 5 indels (5 - 15 bp). In the *trn*L-F dataset the same two major haplotypes were obtained as in the ITS analysis, accordingly, named *trn*L-F-A and *trn*L-F-B. These haplotypes differed in 10 positions. Differences were calculated using two representative accessions of each type: *trnL-F*-A (Lake Abtsdorf, KT596624) and *trn*L-F-B (Lake Ammer, KT596625). Sampling for the *trn*L-F subset included the sample from USA (KT596666, Appendix A1), which did not show any differences to the other accessions grouped in A. Sequences derived from one sample of Lake Riegsee (KT596639) and three from Lake Staffelsee (KT596644, KT596645, KT596646 – the latter two of hybrid origin) form a subgroup (Fig. 3.1) not inferred in the ITS tree, but supported by one synapomorphy (position 170 of the *trn*L-F alignment).

For both alignments none of the indels were phylogenetically informative or affected tree topology (data not shown). In both the ITS and *trn*L-F tree topology, samples of *N. marina* formed a monophyletic lineage (Fig. 3.1), consistently split in two major groups (type A and B, Fig. 3.1; 3.2). Based on samples of reference populations used for isozyme and karyological studies (Triest et al., 1986; Viinikka, 1976), it was possible to correlate ITS ribotypes - and respective *trn*L-F haplotypes - with karyotypes as follows: *"major"* = karyotype A = ITS type A, *"marina"* = karyotype B = ITS type B.

3.4.2 Hybrids and cloning

Two samples from Lake Staffelsee (# 87 and 97, Appendix A1) showed differences in clustering in the ITS and *trn*L-F analysis (ITS-B: KT596530, KT596540; *trn*L-F-A: KT596645, KT596646). ITS sequences of numerous samples (7 samples from Lake Staffelsee and 3 from Lake Pelham) showed double peak signals at multiple nucleotide sites (not listed in Appendix A1 due to illegibility of sequences). Such heterozygotic positions, as described in Soltis et al. (2008) can be a used for tracing back hybrids (in diploid or allopolyploid groups), when copies from both parents are retained. Repeated PCR and sequencing of those 12 accessions did not improve

results. Therefore, PCR products have been cloned for 4 individuals: a = control ITS ribotype A, b = control ITS ribotype B, I = putative hybrid 1 from Lake Staffelsee, II = putative hybrid 2 from Lake Staffelsee. Altogether we generated 114 subcloned sequences: 35 for a, 36 for b, 10 for I and 33 for II. The control samples had sequences identical throughout for the respective ITS ribotype (a: KT596331 - KT596366; b: KT596367 - KT596401). ITS sequences from each putative hybrid accession (I and II) were a mix of both ribotypes, with an unequal ratio (ITS- A: ITS-B) in both hybrid individuals as follows: I = 2:6, (KT596435 - KT596443); II = 19:14 (KT596402 - KT596434).

Table 3.3: Overview of mutations detected in nrITS regions (18S, ITS 1, 5.8S, ITS 2) of the subcloned sequences (750 nt) from individuals a,b, I, II. Numbers indicate total amount of point mutations in each sequence set compared to the reference sequences, excluding differences listed in Table 3.2)

Individual	a	b	I	II
18S	1	8	3	5
ITS 1	27	16	8	30
5.8S	11	11	4	16
ITS 2	27	18	6	21
% of sequences with mutations	71	75	75	64,3

a = Lake Staffelsee 2.1, b = Lake Ammersee 2011, I = STF 8.3, II =10_Aquarium

The sequences inferred from non-hybrid individuals of other populations by direct sequencing without cloning, are almost identical for each type throughout the distribution range. All subcloned sequences of hybrid individuals in contrast, show a considerable number of point mutations compared to the reference sequences (Tab. 3.2). These mutations were evenly distributed along the sequences, including the normally highly conserved 5.8S rDNA (Tab. 3.3). For each hybrid individual (I and II) one subcloned sequence of chimeric origin was generated, partially consisting of ITS A and ITS B (KT596437, KT596415).

3.4.3 Monitoring and distribution of populations

When the monitoring of 46 lakes within the scope of this project began in 2011, new records of *N. marina* s.l. were made for 19 of those lakes (**bold** accessions in Appendix A1). Simultaneous presence of both individuals (type A and type B) could be verified in one hydrologically isolated lake (Lake Staffelsee) and three lake systems (Lake Waging - Lake Taching, Lake

Obertrum -Lake Mattsee, Eggstätt-Hemhof Lake District) (Fig. 3.3 C). In the latter, two or more lakes are connected by (natural or artificial) channels, but maintain ecologically distinct features. Detection of hybrids was only possible for Lake Staffelsee. No clear geographic clustering could be observed in Europe, although *N. marina* within Germany tends to be restricted to the North and the edge of the Alps in the South (Fig. 3.3 B, C).

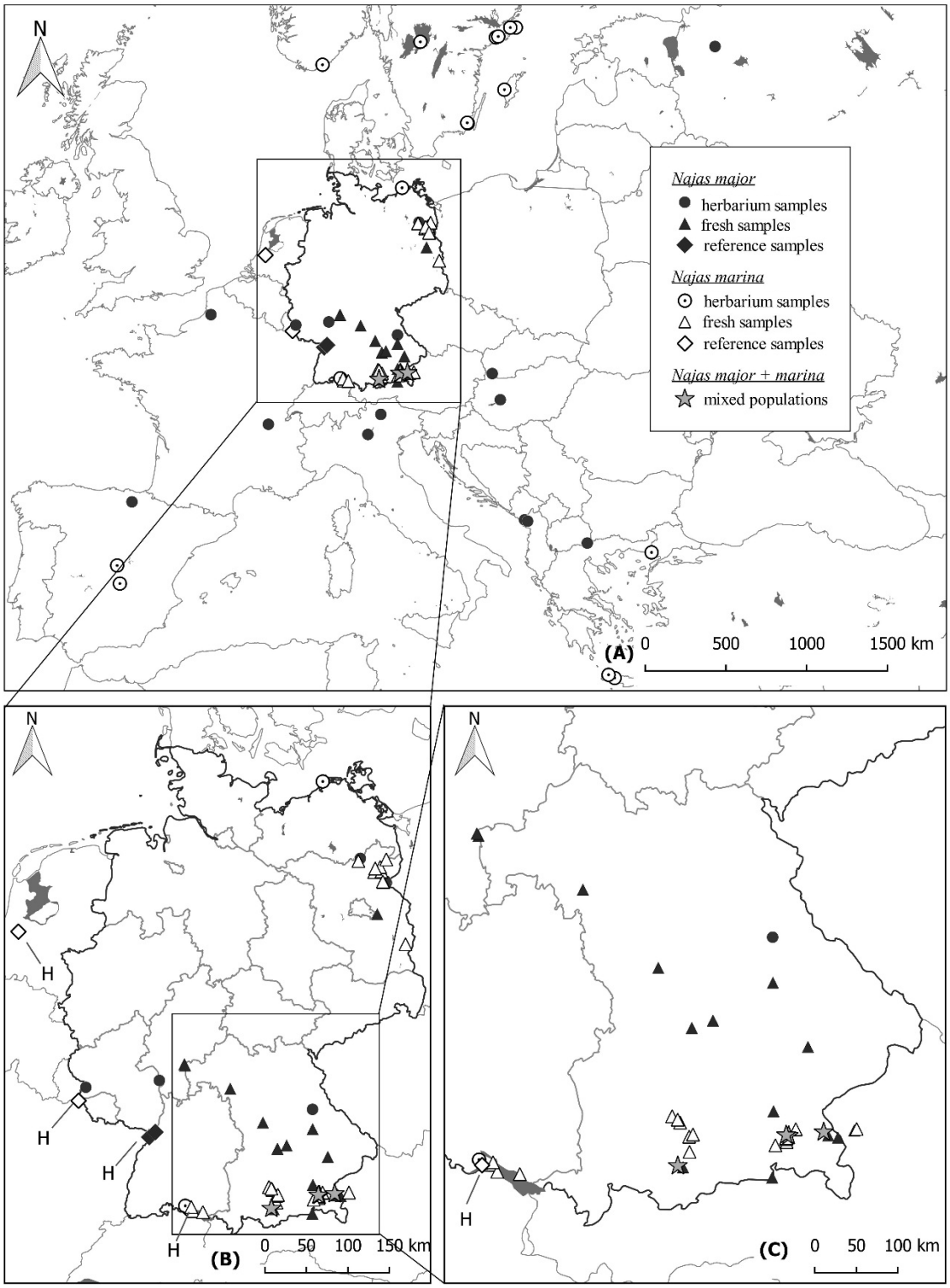


Figure 3.3: A – C Maps indicating origin of *Najas* samples.Each mark refers to one collecting site. A Map of Europe showing herbarium (circle and diamond), fresh (triangle) and hybrid (stars) samples based on DNA verified accessions. B Map of Germany. Reference accessions (diamond) assessed for karyotype in previous studies (Triest et al. 1986) are labelled with H. C Map of Southern Germany (Bavaria). Legend supplement: *Najas major* = *Najas marina* subsp. *marina* auct., *Najas marina* = *Najas marina* subsp. *intermedia* auct. (auct. = auctorum).

3.5 Discussion

3.5.1 DNA Barcoding in *N. marina* s.l. and correlation of lineages with karyology

Using reference populations included in isozyme and karyological studies (Triest, 1989; Triest et al., 1986; Viinikka, 1976), we were able to correlate ITS types with karyotypes as described above. No sequences could be matched with the tetraploid type ("BB") reported from Israel (Triest et al., 1989; Viinikka et al., 1987), since it was not covered by our sampling.

In spite of known pitfalls in employing ITS data for phylogenetic reconstruction (Álvarez & Wendel, 2003), the marker proved useful for sample identification in *N. marina* s.l. in our analysis, as it did also for other aquatics. Problematic samples could also be clearly identified with this DNA barcode in *Ranunculus* L. (Telford et al., 2011) and other members of Hydrocharitaceae such as *Elodea canadensis* Michx. and *E. nuttallii* (Planch.) H.St.John (Huotari & Korpelainen, 2013). It was further suggested for barcoding other vascular plant groups by Gemeinholzer et al. (2006) and the China Plant BOL Group (2011). For sample identification in *N. marina* s.l. we recommend the use of one of the ITS markers (ITS-1 or ITS- 2), since variable positions were detected predominantly there (Tab. 3.2). Like in various other aquatics (Chen et al., 2013; Les et al., 2006), the plastid *trn*L-F region represents an alternative or additional DNA barcode in *N. marina* s.l. Using only one of these two barcodes would be sufficient in places where only one of the cryptic species occurs. Potential hybrids in areas of sympatry will mostly show double peaks in ITS sequences, giving a first hint on hybridisation. Only a very small fraction may show true introgression of a plastid, as indicated for two samples (# 87 and 97) from Lakes Staffelsee here (Appendix A1). For proper sample identification, use of specific restriction enzymes may be a cheap and quick alternative to sequencing.

As outlined, both markers applied in this study support the presence of two distinct taxa within *N. marina* s.l., as did the studies by Triest (1989) and Viinikka (1976). Given the considerable divergence at the DNA sequence level revealed here, it seems more appropriate to consider the taxa as separate species, who do not show a morphological differentiation that is normally caused by effects as selection or drift. We propose that the two lineages, A and B, from now on should be known by the names *N. major* All. (including *Najas marina* var. *ohwii* Triest, here represented by an isotype) and *N. marina* L. (including "*intermedia*" and "*armata*"). The split of *Najas* samples in two clades in the ITS topology (Fig. 3.2) corresponds well with

the two sections currently recognized, i.e. *Caulinia* (Willd.) A. Braun and *Najas*. The strong structuring among samples of section *Caulinia* (Fig. 3.2, marked as "OUTGROUP") may indicate some geographical signal, but the focus of this study was on *N. marina* s.l. Given their placement in the subgroups of clade I, however, some of the accessions of *N. gracillima*, *N. minor* and *N. oguraensis* may have been misidentified.

3.5.2 Diversity of genetic lineages

The substantial divergence between sequences of lineages A and B in both markers used here was unexpected for samples that had been considered to belong to the same species. Judging from previous studies on other plant groups dealing with genetic variation at the infraspecific level (Bräuchler et al., 2010, 2004; Gehrke et al., 2008), much less variation was expected. Whereas the taxa described in *N. marina* s.l. have sometimes even been regarded as mere varieties of one polymorphic species (Magnus, 1870), our results confirm that taxonomic rank has been underestimated. This underlines the importance and necessity of molecular screening, especially of common and widespread taxa. The deep split among samples of *N. marina* s.l. observed here is best explained by an ancient division into two major evolutionary lineages with complete lineage sorting, resulting from or accompanied by chromosomal rearrangements (Viinikka, 1976; Winge, 1927).

The genetic homogeneity detected within lineages and populations is in contrast to the high molecular evolutionary rates in nuclear and chloroplast genes shown for other annuals (Andreasen & Baldwin, 2001; Yue et al., 2010). The genetic homogeneity detected within lineages and populations was also unexpected. We assumed the obligate sexual life cycle of the taxa would indicate higher evolutionary rates especially since Triest (1991, 1989) showed high genetic variability based on ADH enzyme polymorphism among populations of *N. marina* s.l. In other monoecious taxa of *Najas,* like *N. canadensis* 14 different ribotypes could be revealed based on ITS, whereas *N. flexilis* accessions were almost identical throughout their North American range (Les et al., 2015). Dioecy as outcrossing mechanism should promote broader genetic exchange (Barrett et al., 1993; Tippery & Les, 2013) and is likely to occur in closely related and sympatric species (Les et al., 2010). Such gene flow could also lead to low levels of genetic variation and has been suggested to be rather normal among and within populations of aquatic vascular plants (Barrett et al., 1993; Lambertini et al., 2010).

This is supported by the findings for *Aldrovanda vesiculosa* L. (Hoshi et al., 2006), *Hygrophila polysperma* (Roxb.) T.Anderson (Mukherjee et al., 2016), *Halophila ovalis* (R.Br.) Hook.f. (Nguyen et al., 2014; Waycott et al., 2002) and others.

We suggest that genetic uniformity as observed in this marker region is enforced by concerted evolution (Baldwin et al., 1995) leading to homogenization of the ITS tandem repeats in each lineage, which has also been assumed for other aquatic species (Hoshi et al., 2006; Ito et al., 2013; Les et al., 2015). High levels of connectivity and resulting high gene flow between lakes or lake systems could further enhance this effect. Strong founder effects (Austerlitz et al., 2000) and/or genetic bottlenecks followed by few introductions of similar haplotypes could also account for loss of genetic diversity during the colonization process in invasive and hydrophilous plant species (Huotari & Korpelainen, 2013; Lambertini et al., 2010; Riis et al., 2010; Sakai et al., 2001).

Clustering of two New World samples (Fig. 3.2, Guatemala 129 and USA 149) in the ITS dataset indicate that some differentiation occurred, nonetheless. An additional accession from the US (HM240442) included, did not share the synapomorphy (position 75: T for a C) supporting that clade, whereas the relevant position in other sequences of the set from a previous study (HM240444, HM240443; Les et al. (2010) could not be identified clearly (Y=T/C). This differentiation possibly indicates a regional ribotype, but denser sampling is necessary to test how far this synapomorphy reflects a natural group. The same is true for the samples from Lakes Staffelsee and Lake Riegsee, clustering in the *trn*L-F dataset. Given the great homogeneity of all other sequences grouped in type A, it seems unlikely that a private plastid haplotype evolved in the comparatively young environment of these lakes. Since *N. marina* s.l. is recorded and analysed genetically for Lake Staffelsee and Lake Riegsee for the first time here, it may stem from a recent introduction of seeds from an unknown distant population not covered by our sampling. Both lakes are located very close to each other and there is high risk of introduction due to intense leisure and fishery activities.

3.5.3 Cloning and hybrids

Chimeric sequence types, as detected within the subcloned hybrid samples, indicate the existence of two ribotypes in one individual, but are typical artefacts when cloning mixed homologous products (Brakenhoff et al., 1991) and cannot hold as evidence for introgression.

Variation among ITS copies within one individual, as indicated by the numerous point mutations in subcloned sequences, may be obscured by a certain predominant ITS type. In our case we consider them polymerase errors or spontaneous mutations during the process of cloning rather than true polymorphisms (McInerney et al., 2014), since the highly conserved 5.8 S region also contains multiple mutations. The rationale for this assumption is the almost unaltered persistence of both major ribotypes throughout all samples sequenced directly.

Since cloning of samples with heterozygous signals confirmed the existence of hybrids for Lake Staffelsee, such can be assumed to exist also in other lakes (e.g. Lake Pelham, data not shown), from which further heterozygous sequences were obtained. Incongruences in clustering of samples in ITS versus *trn*L-F analyses, as detected in two individuals from Lake Staffelsee (KT596530, KT596540), could be an indication of introgressive hybridisation between maternal *N. major* (ITS A, *trn*L-F A) and paternal *N. marina* (ITS B, *trn*L-F B) plants, and was also described by Triest (1989). Capture of plastid haplotypes following such introgressive hybridisation events has been reported for some other aquatic species, like *Elodea canadensis* (Huotari & Korpelainen, 2013), *Ruppia maritima* (Ito et al., 2013; Triest & Sierens, 2010) as well as numerous other angiosperm groups (e.g. Bräuchler et al., 2010; Yuan and Olmstead, 2008). Such plastid introgression is not uncommon in sympatric species with reproductive compatibility (Acosta & Premoli, 2010). To test the hypothesis of chloroplast capture, further analysis with codominant DNA markers would be the method of choice (Álvarez & Wendel, 2003).

In other critical groups like *Najas flexilis* (Les et al., 2015, 2010) hybrid detection was accomplished by applying nuclear (ITS) and plastid (*trnK, matK, rbcL*) markers, indicating that hybrid formation in other *Najas* taxa with similar genetic differences seems to be possible and that standard markers/barcodes are sufficient for their detection. Due to karyological differences, formation of hybrids between different karyotypes in *N. marina* s.l. seems unlikely. Nevertheless, it has been reported by Viinikka (1976) and Triest (1989), although plants bearing fruits and producing fertile hybrid offspring are rare. The hybrid individuals are detected rather on accident by thorough sampling and molecular screening, confirming the results of Les et al. (2010). Consequently, hybridization may occur more frequently than expected and extent of introgression is hard to assess. In areas, where both types are present,

differences in flowering time can form a barrier (Triest, 1989) and may support persistence of cryptic lineages in sympatry, as observed for *Juncus effusus* L. (Michalski & Durka, 2015).

3.5.4 Distribution and spread of cryptic species

Given the wide geographic distribution of *N. marina* s.l., some geographical clustering was expected. There is, however, hardly any divergence that could be correlated to biogeography. Both ITS ribotypes are distributed over Europe without any large-scale spatial structure. At a smaller scale it seems that *N. marina* (ITS-type-B) is more frequent in the North of Germany and at the edge of the Alps. This is in accordance with (Triest, 1989) who attributes the taxon a large range of habitats and a preference to chloride- and sulphate-rich waters. With the primary focus on southern German lakes, no samples could be obtained from Central Germany and only a few from the northern part (Fig. 3.3 B). A denser sampling may allow drawing conclusions on biogeography.

The spread of both taxa and co-occurrence as revealed by our study, remained largely unnoticed until now (e.g. Eggstätt-Hemhof Lake District or Lake Staffelsee, where only *N. marina* was reported before (Melzer, unpublished data)). This underlines the necessity of vouchering in course of inventories as has been postulated for ecological studies in general (Schilthuizen et al., 2015). In absence of hard evidence (i.e. herbarium specimens) it may be speculative which ITS type is native or actually new to a given lake because of possible taxonomic confusion and misidentification in the past. Similar problems are reported by Les et al. (2015) for American *N. flexilis* and *N. canadensis*, which are regarded as sibling species. Their long-persistent sympatric distribution could be revealed by fossil records, but their morphological distinction remains difficult to assess unless data of seed morphology are compared statistically. Whether cryptic invasion is really taking place in or among *N. marina* and *N. major*, can best be inferred by phylogeographic reconstruction, based on herbar and fossil records. Such cryptic invasions are difficult to counteract (*Glyceria* R.Br., Gerlach et al., 2009), especially when the possible invader is listed as rare (e.g. *Najas gracillima*, Les et al., 2013) or endangered, as is currently the case for *N. marina* s.l. in some European countries. Judging from our results, conservation status has to be reconsidered.

Our study points out the importance of molecular genetic analyses for detection of cryptically invading populations. Incorrect classification during field work using existing keys restricts

accurate assessment of the taxa's actual distributions as well as their ecological characterization. Both need to be revised in light of our findings to avoid further spread for both taxa remaining cryptic. Although our study provides a powerful tool to name samples properly, differing WFD indicator values for each of the taxa may need modification in the future.

3.6 Conclusions and Outlook

This study of *N. marina* s.l. populations confirms presence of two morphologically similar and closely related taxa. They show considerable genetic differences in the nuclear and plastid markers ITS and *trn*L-F, which can be clearly correlated with different karyotypes and isozyme patterns detected previously. We propose that an ancient split of lineages combined with reproductive isolation due to genomic rearrangements led to parallel morphological evolution and in consequence to cryptic speciation. We have shown that nuclear (ITS) and chloroplast (*trn*L-F) markers can be used for clear sample identification and detection of hybrids, nonetheless. The possibility of cryptic invasion of either *N. marina* and/or *N. major* is discussed here for the first time but could not be reliably verified based on the data available. New records and the revised application of correct names for the two species as proposed by Bräuchler (2015), underline the necessity of further research based on more extensive sampling. Seed bank analysis from sediment cores could help reconstructing colonization history. In addition, the effect of annual dynamics on overall population structure and range expansion should be investigated more closely using microsatellite or SNP based genotyping.

4 Phenotypic variation disguises genetic differences among *Najas major* and *N. marina*, and their hybrids

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Candidate's contribution:
Conduction of sampling of plants, implementation and execution of standardized morphological measurements of plant material, planning of experiments and selection of the ligase enzymes for RFLP analysis, conduction of statistical analysis and interpretation of data, writing and revision of the complete manuscript including Figures and Tables.

4.1 Abstract

In this study we examined morphological variation in two macrophyte species (*Najas major* All., *N. marina* L. and their hybrids) obtained from German fresh water systems. Clear-cut delimitation of these two taxa is notoriously difficult but important as they are used as indicator organisms for water quality within the European Water Framework Directive (WFD) and have recently been revealed as two genetically separate species. To reliably identify both taxa and their hybrids, we used an integrative approach testing six discrete and two ratio-based morphological leaf and seed characteristics against restriction fragment-length polymorphism patterns (RFLP) based on PCR of rDNA internal transcribed spacer (ITS) sequences. Morphometric data of 475 plant individuals from 25 different German lakes showed basic correlation with the species delimitation suggested by molecular data, but revealed considerable overlap for characteristic state ranges, which can lead to misidentification of species if a low number of observations is made on these traits. Hybrids showed a mosaic of both parental and intermediate morphological traits. Notably, the traditionally employed feature "number of teeth along the margin on the leaf sheaths" proved to be of low diagnostic value. Leaf dimensions, especially leaf widths, were shown to be more

reliable characteristics for distinguishing parental taxa. In practice, further use of both *Najas* species within the implementation of the WFD should be accompanied by molecular genetic testing to detect both cryptic co-occurrence and hybridization. This study points out the importance of thorough sampling and molecular screening in widespread and taxonomically difficult groups.

4.2 Introduction

Global environmental change, degradation of natural habitats and the resulting loss in biodiversity are major anthropogenic factors affecting freshwater ecosystems (Geist, 2011). To mitigate these processes, efforts to protect and document biodiversity by applying classical taxonomic and modern molecular techniques are made (Duminil & Di Michele, 2009; Steele & Pires, 2011). However, cryptic species and hybridization often impede the identification and correct distribution mapping of plant species. This problematic phenomenon is specifically widespread within macrophytes due to their simplified anatomy (e.g. reduced leaves) and considerable morphological variability in the few diagnostic traits available to identify species. In addition, low crossing barriers result in high rates of hybridization (Les & Philbrick, 1993; Sculthorpe, 1967). Basic molecular techniques such as DNA sequencing or amplified fragment length polymorphism (AFLP) analysis provided new approaches to detect hybridization and previously unrecognized cryptic diversity in various notoriously challenging genera of macrophytes i.e. *Potamogeton* (Kaplan & Fehrer, 2013; Whittall et al., 2004); *Ranunculus* section *Batrachium* (Hörandl & Emadzade, 2012; Zalewska-Gałosz et al., 2015); *Najas* (Les et al., 2010); *Callitriche* (Prančl et al., 2014), and *Chara* (Boegle et al., 2007).

Among the problems still not readily addressed in many groups are hybrids and their confounding effect on morphological distinction of taxa (Rieseberg et al., 1993). Species misidentification and errors in delineation of their spatial distribution may have severe consequences on applied practice, especially if species are used as indicators. Within the implementation of the European Water Framework Directive (WFD) various aquatic macrophyte species are applied as biological control elements for ecological assessment and monitoring of water quality (Penning et al., 2008b; Stelzer et al., 2005). For European freshwaters, 58 macrophyte species are listed as hybridizing (Moe et al., 2013) and

approximately ten of them are indicator organisms of eutrophication within WFD guidelines for Germany (Schaumburg et al., 2014) and other European countries (Penning et al., 2008b). Hybrid species frequently exhibit a mosaic of parental and intermediate characteristics (Rieseberg et al., 1993) and recognizing them in the field is further impeded by overall shrinking taxonomic expertise (Figueiredo & Smith, 2015).

One of the most popular molecular markers for analyzing plant groups is the internal transcribed spacer region (ITS) of the nuclear ribosomal 18S–5.8S–26S cistron (Baldwin et al., 1995). Despite the known drawbacks for this marker (Álvarez & Wendel, 2003), its use with subsequent cloning and in combination with plastid sequence data has proven to be sufficient for tracing hybridization in multiple studies (Kaplan & Fehrer, 2013; Les et al., 2010; Tippery & Les, 2013). Although molecular methods allow and facilitate identification of hybrids and cryptic species, delimitation problems in the field prevail due to lack of comprehensive data for most critical plant groups. Detection of hybrids happens mostly incidentally and sometimes stays unrecognized until molecular data is collected and screened (Les et al., 2010; Rüegg et al., 2017). Even then, data examination has to be done carefully with respect to the chosen target region, since artefacts such as superimposed and illegible sequences can complicate recognition of their hybrid nature (Tippery & Les, 2013; Whittall et al., 2004).

In this study, we use *Najas major* All. and *N. marina* L., two subcosmopolitan, annual, dioecious, submerged macrophyte species to demonstrate the possibility of re-examining and testing morphological concepts with the aid of molecular genetic techniques. Both were considered subspecies of *N. marina* (Viinikka 1976) or were often merged under the *Najas marina* L. s.l. (see Bräuchler, 2015 for discussion). The species thereby serve as an example for a critical group with confounding taxonomy and the potential for cryptic divergence, including both taxa mentioned. Both taxa show reduced and convergent morphological traits, which exhibit broad morphological variation, that often overlap (Triest, 1988; Viinikka, 1976). This leads to persistent morphological and taxonomic confusion as well as inaccurate recording of species distribution in Europe and Germany (Bettinger et al., 2013; Lansdown, 2016). Nonetheless, several studies were able to show that the taxa are differentiated in their karyotype (Viinikka, 1976) and isozyme patterns (Triest et al., 1986) and molecular data suggest treating them as separate species (Rüegg et al., 2017). Due to phenological differences, the two taxa have been shown to be able to hybridize so far only unidirectionally when male *N. marina* pollinate

female *N. major* plants, which results in the formation of mostly infertile offspring (Triest, 1989; Viinikka, 1976). Only a few naturally occurring hybrids have been identified in Europe (Triest, 1989), but for given reasons many more may have remained undetected. The possibility of hybrid formation and overlap of morphological variation is obscuring the accurate identification of species in the field and is limiting utility of both taxa as currently distinct indicator organisms according to the requirements of the WFD for German lakes (Schaumburg et al., 2014, 2004). This emphasizes the need for an integrative approach using a combination of multiple independent sets of characteristics (Duminil & Di Michele, 2009) including molecular and morphological data, in order to facilitate proper species identification in this problematic taxon not only for assessing actual specific spread but also for planning conservation and/or management strategies.

The core objectives of our study were to (1) overcome persisting identification problems by testing diagnostic morphological characteristics on a genetic background including parent species and hybrids and to compare these results to measurements known from literature. (2) To develop a simple and rapid PCR-RFLP method for monitoring genetic variation using the ITS marker region that allows an accurate species identification without sequencing and using morphology. (3) To assess and discuss the consequences of our findings for further mapping procedures involving possibly cryptic macrophyte species in general and with regard to the usage of *Najas* within WFD procedures in particular. *Najas marina* and *N. major* should thereby serve as examples of widespread species in which thorough sampling helps to understand morphological implications of hybridization and to show how insufficient morphological data influences species detection.

4.3 Materials and Methods

4.3.1 Study area and sampling strategy

A total of 475 adult and flowering plant individuals were sampled from populations of *N. marina* and *N. major* in 25 lakes throughout Germany (Appendix A2). All specimens were collected by diving along a point or strip transect from August to October. 315 plants were collected as described in 2010 (125 from *N. major*, 190 from *N. marina*), 160 samples were gathered in 2012 and 2015 (71 from *N. major*, from 76 *N. marina*, and 13 hybrids). In several cases, multiple transects per lake were sampled (Appendix A2). Depending on density of the plant stands, three to ten individuals per transect were collected. One representative DNA sample and voucher specimen was prepared per transect for the survey in 2010, whereas DNA samples and voucher specimen were taken from each individual collected in 2012 and 2015 (Appendix A2). All vouchered individuals were analyzed molecularly as described in Rüegg et al. (2017). Morphological measurements were taken from all individuals collected throughout these years. Herbarium vouchers were deposited at TUM herbarium (Thiers, 2017) in the macrophyte reference collection at the Limnological Research Station Iffeldorf. Of the sites previously described (Rüegg et al., 2017), 17 plants were collected at Lake Abtsdorf in 2015 as a reference for *N. major*, 50 plants were obtained from Lakes Starnberg (38) in 2012 and Lake Constance (12) in 2015, as a reference for *N. marina*. All three lakes show minimum risk of introduction of the other species from neighboring lakes due to the distance among them and to the next lakes housing the respective other species. In Lake Staffelsee, identified as hybrid zone in our previous study (Rüegg et al., 2017), 110 individuals of both taxa and their hybrids (54 from *N. major*, 26 from *N. marina*, and 13 hybrids) were collected in 2012 and 2015 at the same locations using GPS devices and data points. Standard taxonomic keys were used to identify plants prior to further morphological and molecular analysis (Casper & Krausch, 1980; van de Weyer et al., 2011).

4.3.2 Morphometrics

For this study a total number of 1737 leaves was analyzed: *N. major* n = 724, *N. marina* n = 948, hybrids n= 65. Quantitative morphological characteristics were measured from 475 plants (*N. major* n = 196, *N. marina* n = 266, hybrids n = 13) and 408 seeds (*N. major* n = 159, *N. marina*

n = 239, hybrids n = 10) using a dissecting microscope (6.5 - 40·x magnification) linked to a digital camera (Kappa PS20H), which was controlled by interactive software (Kappa imageBASE v2.7). In a preliminary study from 2010, 8-10 plants per transect were collected and leaf measurements were taken from three leaves per plant by hand with the aid of graph paper. Overall, 315 plants (*N. major* n = 125, *N. marina* n = 190, number of plants included in the measurements mentioned above) were measured that way. From 160 plants that were collected in 2012 and 2015 (*N. major* n = 71; *N. marina* n = 76, hybrids= 13), three to five representative leaves and five seeds (when present) per individual were randomly chosen for measurements. Characteristics examined were: length and width of seed (SL and SW) as well as leaf length (LL) and two different widths on each leaf: one width at teeth (= leaf widths broad: LWB) and at sinuses (= leaf widths narrow: LWN), all measured in mm. Each leaf width was measured at up to three points, depending on leaf length. LWB and LWN were both measured vertical to the midrib of the leaf. For both measuring methods (graph paper and digital), fresh leaves were prepared equally by placing them between two microscopic glass slides. Moreover, the total number of marginal teeth on the leaf sheath was noted for each leaf. Though this characteristic was considered to be of no true diagnostic value (Triest, 1988; Viinikka, 1976), its perpetuated employment within recent taxonomic keys (Casper & Krausch, 1980; van de Weyer et al., 2011). emphasizes the need of a thorough re-examination. For each plant, sex was determined as a non-quantitative characteristic. Due to seasonal sex ratio patterns (Hoffmann et al., 2014a) and the late sampling dates, more female individuals (89%) were collected, though sex-related differences were not significant for any of the traits examined (Wilcox test, $p > 0.05$).

4.3.3 PCR – RFLP analysis

Restriction site analysis of PCR amplified ribosomal ITS fragments was performed for a representative number of both taxa and included samples collected for a previous study (Rüegg et al., 2017) in order to verify type of specimens characterized earlier by genetic or morphological analysis (see Appendix A2). RFLP analysis was performed overall on 35 specimens of *N. major*, 38 specimens of *N. marina*, and 11 hybrids. For each individual, DNA extraction and PCR amplification were carried out using the primer pair leu1 (Vargas et al., 1998) and its4 (White et al., 1990). Purification of products and cloning followed by sequencing

was performed as reported by Rüegg et al. (2017). Between 1 - 4 µL of the purified PCR products (1 - 20 ng/µL; 0.1 - 1.5 nM) were digested overnight (app. 16 h) in a total volume of 20 µL containing 2 µL, of enzyme (1 U/ µL) (Thermo Fisher Scientific, Massachusetts, USA), 2 µL of 10x BSA Buffer R that is provided with the enzyme (1 mM Tris HCl; 10 mM KCl; 0.02 mg/mL BSA, 0.1 mM EDTA, 0.1 mM DTT) and bdH_2O. Hind III was chosen as a restriction enzyme based on the restrictions map tool implemented in BioEdit (Hall, 1999) and double checked with the virtual digestion tool available online at restrictionmapper.org. Hind III cuts once at position 278 (5′ A↓AGT_T 3′) within the ITS2 of the reference sequence of *N. marina* (KT596460) but not in *N. major*. Therefore, we expected the method to allow for a distinction between the two taxa and their hybrids, which consequently could be identified by undigested PCR products (one band) and digested DNA fragments (two bands). Digestion efficiency and length of resulting fragments were checked on a 2% (w/v) agarose gel, using ethidium bromide staining and a 100kb ladder (Thermo Fisher Scientific, Massachusetts, USA). Doubtful RFLP results were repeated and double-checked.

4.3.4 Data analysis

Statistical analysis of cumulative data was compiled using the open source software R v. 3.4.4 (R Development Core Team, 2013). Taxa were identified based on genetic markers (PCR-RFLP) as described before and in all statistical analyses, one grouping variable (taxa) was used. Due to the nested design of the study (multiple measurements per leaf and individual, randomly chosen leaves and plants) standard deviation, standard error, and means were calculated for each individual. Means were then pooled over the two different taxa and their hybrids for further analysis. Number of plants or individuals is given for each statistic. The different datasets where inspected for normality of residuals (*shapiro.test*) and homogeneity of variances (*var.test*) via the given functions and diagnostic plots. If values were not normally distributed, non-parametric tests were conducted (*wilcox.test*). Otherwise, an ANOVA (*aov* function) with default setting was performed. Tukey's HSD was conducted as posthoc test for unequal sample frequencies to assess group specific differences between means of quantitative characteristics. Count data (number of teeth) were tested using a generalized linear model (*glm*, *family= poisson (link = "log")*) followed by a multiple comparison (package *multcomp*) with adjusted p values (*bonferroni*). Linear Discriminant Analysis (LDA) and Canonical Variate

Analysis (CVA) was conducted on quantitative morphological data to determine which characteristics best discriminate the studied species using the functions *lda* with default settings (package *MASS*) or the function *CVAbipl.pred.regions* (package *UBbipl*). The CVA is a form of multivariate analysis, which minimizes within group (replicate) variation and maximizes the between-group variance (Gower et al., 2010). Afterward, a leave-one-out cross-validation (*loocv*) method was applied to validate the models and calculate error rates for reclassification. Plots were generated using the *ggplot2* or *ggpubr* package.

4.4 Results

4.4.1 PCR - RFLP analysis

RFLP analysis using Hind III showed one undigested band at approximately 750 bp for *N. major* samples whereas the digestion of *N. marina* resulted in two fragments shown as two bands at approximately 475 bp and 270 bp in the agarose gel. A molecular substitution from C to A at site 284 within the ITS2 region causes a loss of the Hind III recognition site in *N. major*. The hybrid nature of 13 samples could be confirmed by additive RFLP banding patterns from both parents showing a combination of all three bands. The identity of various hybrid specimens (Appendix A2) was previously confirmed by cloning and sequencing of the ITS region, resulting in the presence of both parental sequences as reported in Rüegg et al. (2017). All 34 samples of *N. major* displayed the described RFLP pattern, though two samples had to be re-examined due to possible contamination. For *N. marina* specimens 38 samples showed the expected pattern. RFLP analysis results revealing hybrids by additive RFLP banding patterns was confirmed by a second analysis for those 13 samples.

4.4.2 Monitoring and identification of plants

PCR - RFLP helped in delimitation of 13 hybrid specimens, which would have been otherwise identified as *N. major* based on morphology (Tab. 4.1, 4.2). By identifying plant material genetically, it was shown that hybrids occur naturally in mixed populations at Lake Staffelsee. Due to higher sampling density and a better understanding of co-occurrence of the two different taxa, 13 hybrid specimens were collected from Lake Staffelsee in two different years, 2012 and 2015. General occurrence of hybrid individuals was persistent, but their frequency differed depending on the abundance and mixed growth of parental taxa. Apparently, F1 hybrids develop anew each year, since no fertile seeds have been reported or detected on hybrid individuals so far. Nonetheless, we chose to include hybrid seeds in this study since they did not show any signs of deformation or abnormal growth.

4.4.3 Morphological variation

The results of the morphometric measurements on leaves and seeds demonstrated high variability within each taxon, depicted also by a high number of outliers (Fig. 4.1 a-d; Fig. 4.3 b, c). Character state ranges measured in this study showed considerable overlap with

interquartile ranges for both taxa and their hybrids (Tab. 4.1; Fig. 4.1 a-d). Standard deviations and errors were relatively low due to high number of measurements and accuracy of tools used (Tab. 4.1). Significant among-group differences between *N. marina* and *N. major* were calculated for mean values pooled from individuals for almost all leaf and seed characters measured using ANOVA: LWN ($F_{2, 472} = 843$, $p < 0.001$), LWB ($F_{2, 472} = 696$, $p < 0.001$), and length ($F_{2, 472} = 13.82$, $p < 0.001$), as well as seed width ($F_{2/72} = 69.31$; $p < 0.001$), seed length ($F_{2/80} = 52.99$; $p < 0.001$) and ratio length: width ($F_{2/75} = 29$; $p < 0.001$). Only the characteristic ratio narrow: broad did not obtain such high among-group difference ($F_{2, 471} = 0.337$, $p = 0.71$), which could also be seen in comparing mean values (as described below). LWN and LWB were shown to correlate highly with each other (function *rcorr* 0.94).

Mean values of all traits measured showed significant differences between *N. marina* and *N. major* (Tukey test $p \leq 0.001$) but not between each of them and the hybrids, depending upon trait (Fig. 4.1 c, d). With regard to LWN and LWB, hybrids did also differ significantly from the two taxa (Tukey test $p < 0.001$). Leaf widths (LWN, LWB) measured from the 13 hybrid plants showed to be intermediate between the parental taxa (Tab. 4.1). Measurements taken from hybrid seeds otherwise, showed closer resemblance to characteristic states taken for *N. major* plants (Fig. 4.3 a-c; Tab. 4.1). Except for the traits of leaf length (Tukey test $p = 0.256$) and number of the teeth on leaf sheaths (Tukey test $p < 0.038$), hybrid individuals are more likely to appear like *N. marina* plants (Fig 4.1 d; Fig. 4.2 b). For the trait leaf ratio narrow: broad no significant difference could be detected between *N. major* and hybrid individuals (Wilxoc test $p = 0.181$), nor between *N. marina* and hybrids (Wilcox-test $p = 0.438$). Seed width (Tukey test: $p = 0.778$), seed length (Tukey test: $p = 0.450$) and seed ratio length: width (Tukey test: $p = 0.834$) did not show any significant differences between *N. major* and hybrids either. *N. marina* and hybrid seed differed significantly from each other in width and length (Tukey test: $p < 0.001$), but not in ratios of seed length: width (Tukey test: $p = 0.187$) (Fig. 4.3 c). Notably, only five seeds each could be measured from two of the overall 13 as hybrids-identified individuals.

LDA of four quantitative morphological characteristics indicated good separation between *N. major* and *N. marina* (Fig. 4.4; 4.5). The first linear discriminant LD1 explains more than 99 % of the between-group variance. From all four morphological characteristics (LWN, LWB, length and teeth) used in the model, the variables that provided most effective discrimination between *N. marina* and *N. major* were LWN (coefficient 2.636) and LBW (coefficient 0.709). The

number of teeth on the leaf margins and leaf length showed weaker influence on the first Linear Discriminant LD1 (teeth: coefficient -0.3993, length: coefficient 0.0337) and therefore do not exert such a strong influence on the discrimination of the taxa and their hybrids. Values for hybrid samples show overlap with *N. major* samples but are for the most part located between the two taxa (Fig. 4.5).

The CVA misclassified more individuals from *N. major* than from *N. marina.* Sample means for individuals from *N. major* show broader ranges for measurements of leaf widths and the number of teeth, whereas sample means from *N. marina* plants varied more in leaf length. Both plots (LDA and CVA) show that hybrid samples are intermediate between both parental taxa but overlap more with *N. major* than with *N. marina* (Fig. 4.4; 4.5). Four *N. major* plants are misclassified according to CVA analysis as *N. marina*, and were drawn from Lakes Staffelsee, Mindelsee and Muttelsee. Eight samples of *N. marina* that are misclassified as hybrids and lie within the 90% bag of *N. major* samples were obtained from Lakes Staffelsee, Starnberg, Wörthsee and Pelham. All of these lakes lie within sympatric ranges, except for Lake Starnberg and Lake Wörthsee.

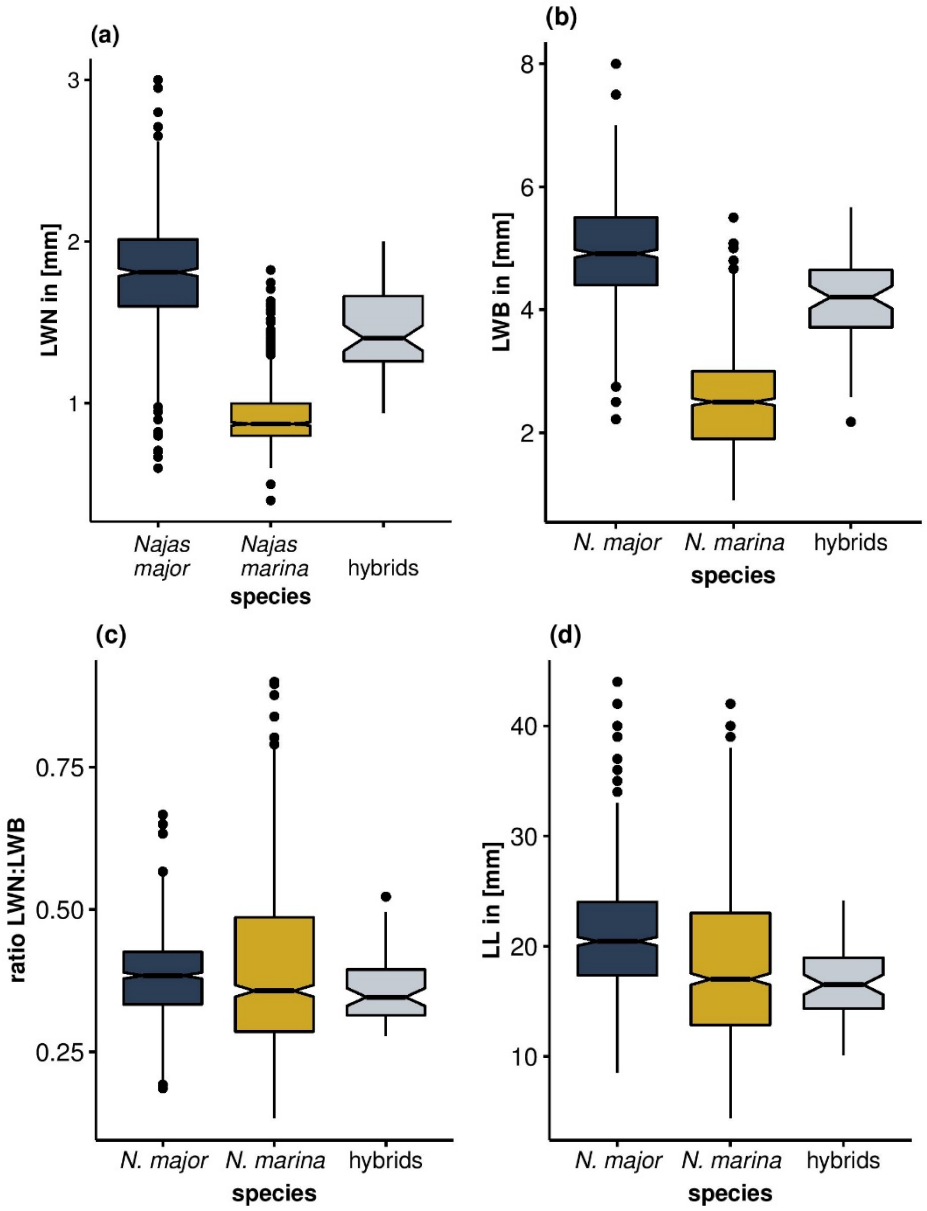

Figure 4.1: (a) - (d): Leaf characteristics among *N. major* (n is the number of leaves measured, n = 724), *N. marina* (n = 948), and their hybrids (n = 65). Boxplots indicate interquartile ranges with median values (heavy lines), "whiskers" are extended to a maximum of 1.5 × interquartile range, and outliers are shown as black circles. Notches were added approximating a 95% confidence interval (CI) for the median. Comparison of mean values for the characteristics were pooled over individuals: *N. major* n = 196, *N. marina* n = 266, hybrids n = 13. Characteristic LWN = leaf width narrow (a) and LWB = width broad (b) differed significantly between *N. major* (blue boxes), *N. marina* (yellow boxes), and their hybrids (gray boxes). No differences were detected between parental taxa and hybrids in the characteristic leaf width ratio narrow: broad (c) between *N. major* and hybrids nor between *N. marina* and hybrids. For *N. major* and hybrids, mean values of the characteristic leaf length (d) showed significant differences (Tukey test: p = 0.006), but mean values of *N. marina* and hybrid plants did not.

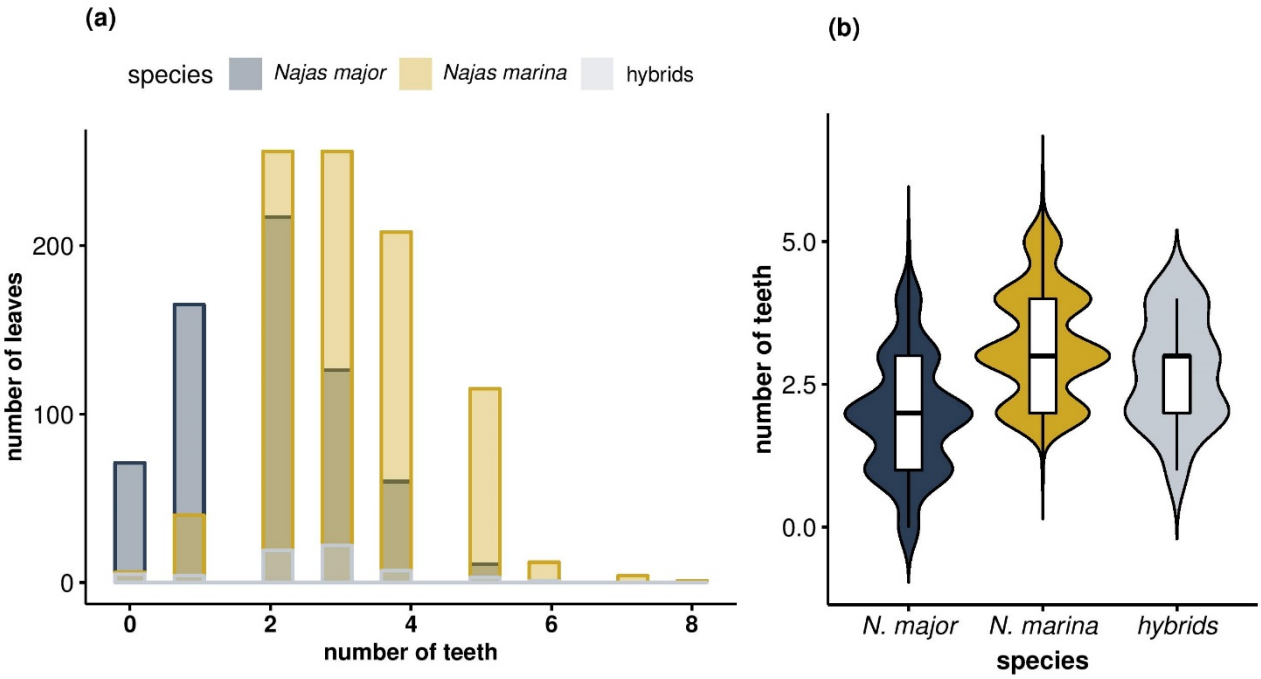

Figure 4.2: (a): Histogram showing the counts for the number of teeth on the leaf sheaths for different taxa and the hybrids; (b): Violin plot for the characteristic "number of teeth on the leaf sheaths" shown for different taxa and the hybrids. (a) *N. major* (n is the number of leaves measured, n = 725), *N. marina* (n = 948) and hybrids (n = 64). Highest number of counts for teeth on the leaf sheaths was observed for both species *N. major* (blue bars) and *N. marina* (yellow bars) at "2". No teeth (0) were observed on leaf sheaths form 69 leaves from 47 different *N. major* plants but were also observed on 6 leaves from 6 different *N. marina* plants, and on five leaves of three different hybrid plants (gray bars).
(b) *N. major* (n is the number of plants measured, n = 196), *N. marina* (n = 266) and hybrids (n = 13). Significant differences for the characteristic number of teeth on the leaf sheaths were observed between *N. marina* (yellow violin) and *N. major* (blue violin), *N. major* and hybrids (gray violin) but not between *N. marina* and hybrids. Plots show the density or distribution shape of the data. The box inside the violin indicates the interquartile ranges with mean values (heavy lines, 'whiskers' = 1.5 × interquartile range).

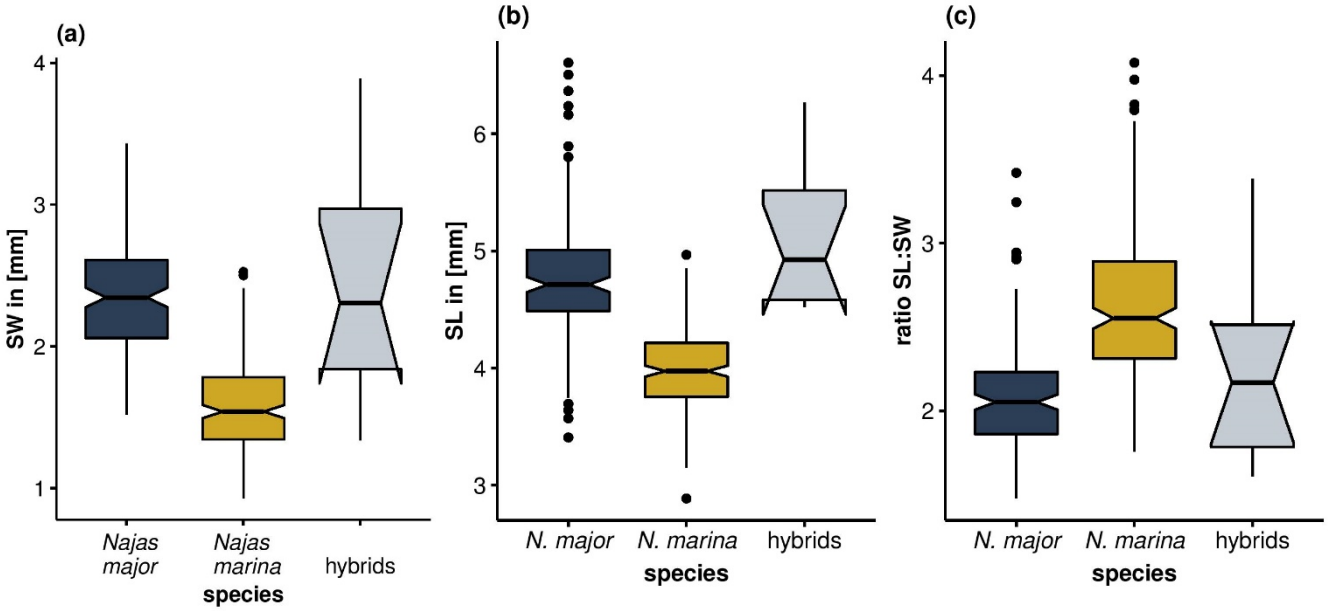

Figure 4.3: (a) - (c): Boxplots of seed characteristics among different taxa. *N. major* (n is the number of seeds measured = 159), *N. marina* (n = 219) and hybrids (n = 10). Taxa were delimited based on distinct RFLP ITS patterns. Boxplots indicate interquartile ranges with median values (heavy lines), 'whiskers' are extended to a maximum of 1.5 × interquartile range, and outliers are shown as black circles. Notches were added approximating a 95% confidence interval (CI) for the median. Notches are outside for hybrids due to low sample size. Mean values pooled over individuals (n) differed for all characteristics significantly between *N. major* (blue boxes, n = 32) and *N. marina* (n = 49, yellow boxes) (Tukey test: $p < 0.001$). For *N. major* and hybrids (n = 2, gray boxes) mean values did not differ significantly for any characteristic tested, whereas *N. marina* seeds differed significantly from hybrid seeds with regard to width (a) and length (b) but did not differ in ratios of seed length: width (c). *N. major* seeds were generally "bigger" than those from *N. marina*, though showing a lower length to width ratio. Hybrid seed appear morphological like *N. major* seeds.

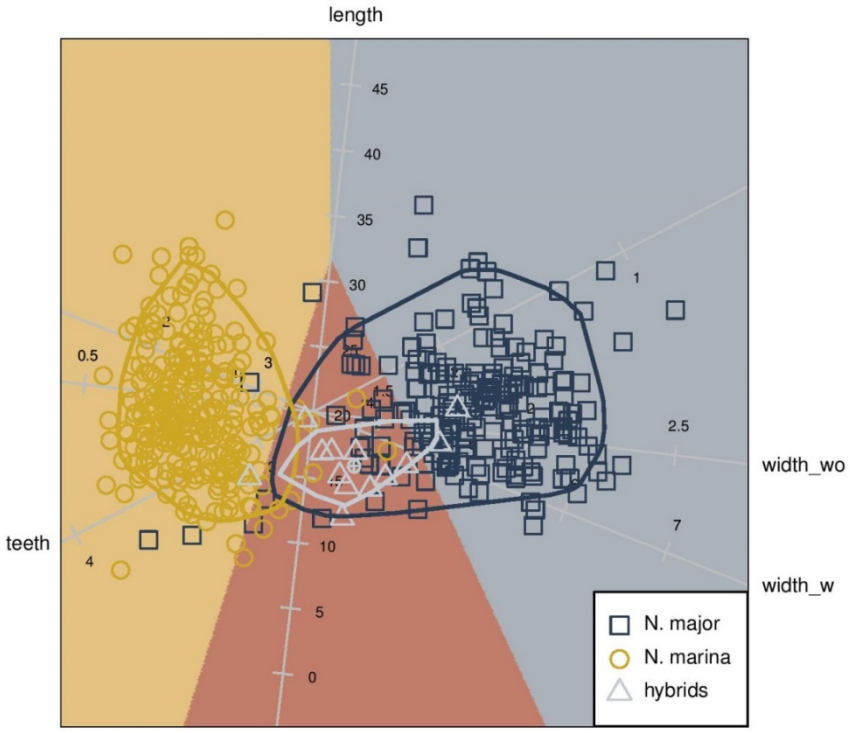

Figure 4.4: Biplot of the canonical variate analysis (CVA) of all samples.
N. major (n = number of plant individuals = 196), *N. marina* (n = 265), and hybrids (n = 13) using all four morphological variables (LWN = width_wo, LWB = width_w, length and teeth). Classification regions were added (shown as background colors) and 0.9 bags were drawn. A bag approximates the box in a boxplot, where 90% of the data points lie within the polygon. The amount of separation obtained between the species is shown using the biplot as a graphical display in the classification process. Samples that were misclassified (symbols that are not within their corresponding classification region in the plot) were all obtained from different lakes. Misclassification rates according to the CVA analysis are given in Table 4.3.

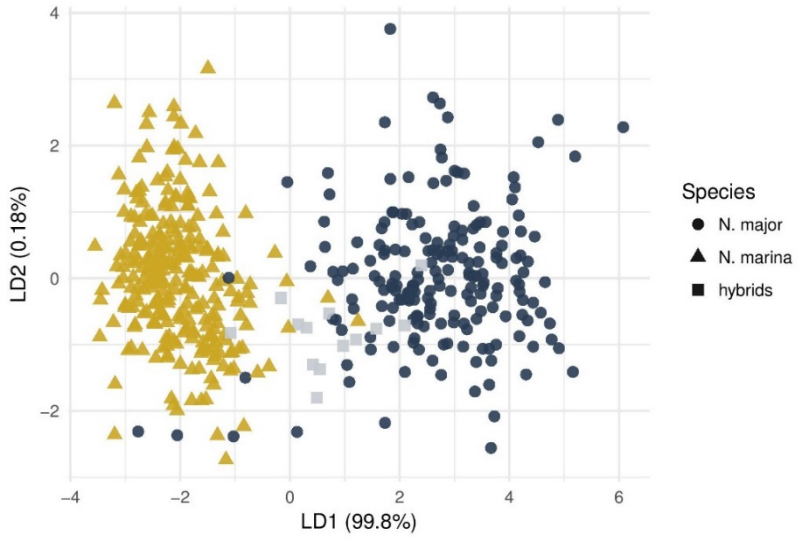

Figure 4.5: Plot of the linear discriminant analysis (LDA) of the same datasets as shown in Figure 4.4. The first discriminant axis (LD1) separates the taxa including hybrids describing 99.82% of the variation expressed in the data. The second discriminant axis (LD2) contributes 0.18% of the variation to further distinguish the taxa and their hybrids from each other.

Table 4.1: Mean value, standard deviation (SD) and standard error (SE) for morphological characteristics measured (n is the number of leaves or seeds measured; leaves: *N. marina*, n = 725, *N. major*, n = 948, hybrids, n = 64; seeds: *N. major*, n = 159 *N. marina*, n = 219, hybrids, n = 10).

Species	Statistics	LWB leaf width broad (mm)	LWN leaf width narrow (mm)	Leaf length (mm)	Number of teeth on margin of leaf	Seed width (mm)	Seed length (mm)
***N. major* All.**	Mean	**4.83**	**1.82**	**21.20**	**1.87**	**2.35**	**4.79**
	SD	0.91	0.37	5.31	1.22	0.40	0.54
	SE	0.03	0.01	0.19	0.05	0.03	0.04
***N. marina* L.**	Mean	**2.54**	**0.92**	**18.37**	**3.01**	**1.57**	**4.00**
	SD	0.83	0.30	6.91	1.29	0.31	0.34
	SE	0.03	0.01	0.22	0.04	0.02	0.02
Hybrids	Mean	**4.10**	**1.44**	**16.55**	**2.57**	**2.47**	**5.11**
	SD	0.68	0.25	3.23	1.27	0.85	0.62
	SE	0.08	0.03	0.40	0.27	0.20	0.18

Table 4.2: Measurements of leaf and seed characteristics in mm from literature and calculated in this study. Values are given as mean values ± SD. Full ranges of observed traits are given in brackets. All measurements were taken from fresh plant material. Citation index: 1) Viinikka (1976), 2) Casper and Krausch (1980), 3) Triest et al. (1986), in studies 1) and 3) dried leaves were measured.

Character	*Najas major* **All.**	*Najas marina* **L.**	**Hybrids**	**Citation**
Leaf width (mm)	(2.7 -) **3.5 - 5.1** (- 6.1)	(1.5 -) **1.8 - 2.8** (- 3.3)		3)
	(0.8 -) **1.0 - 1.5** (- 2.5)	(0.2 -) **0.5 - 0.9** (- 1.1)		1) + 2)
broad (LWB)	(2.2 -) **3.9 - 5.7** (- 8.0)	(0.9 -) **1.7 - 3.4** (- 5.5)	(2.1 -) **3.4 - 4.8** (- 5.7)	**This study**
narrow (LWN)	(0.6 -) **1.5 - 2.2** (- 3.0)	(0.4 -) **0.7 - 1.1** (- 2.8)	(0.9 -) **1.1 - 1.7** (- 2.0)	
Leaf length (mm)	(10 -) **19 - 34** (- 45)	(4 -) **9 - 26** (- 38)		1) + 2)
	(11.6 -) **15.1 - 23.3** (- 28.9)	(4.4 -) **6.5 - 11.1** (- 12.0)		3)
	(8.5 -) **15.9 - 26.5** (- 44.0)	(4.4 -) **11.3 - 24.7** (- 42.0)	(10.1 -) **13.3 - 19.8** (- 24.1)	**This study**
Number of teeth on margin of leaf sheath	Without, rarely one	**1 - 3** (4) on each side		2)
	(0 -) **1 - 3** (- 6)	(0 -) **2 - 4** (- 8)	(0 -) **1 - 4** (- 6)	**This study**
Seed length (mm)	(3.5 -) **4.5 - 6.4** (- 8.0)	(2.3 -) **3.0 - 4.0** (- 4.8)		1) + 2)
	(3.3 -) **4.2 - 5.4** (- 6.6)	(2.6 -) **3.4 - 4.2** (- 5.1)		3)
	(3.4 -) **4.2 - 5.3** (- 6.6)	(2.9 -) **3.7 - 4.3** (- 5.0)		**This study**
Seed width (mm)	(2.7 -) **3.5 - 5.1** (- 6.1)	(1.5 -) **1.8 - 2.8** (- 3.3)		1)
	(1.3 -) **2.1 - 2.8** (- 4.1)	(0.9 -) **1.2 - 2.0** (- 2.7)		3)
	(1.5 -) **1.9 -2.7** (- 3.4)	(0.9 -) **1.3 - 1.9** (- 2.5)	(1.3 -) **1.6 - 3.3** (- 3.9)	**This study**

Table 4.3: Smallest CVA cross validation error rates calculated using the leave-one-out (loocv) method for different sized subsets of the characteristics used to describe the different taxa and their hybrids. Mean values over the individuals are used calculating the error rate.

Subset size	**Cross validation error rate**	**Associated characteristics**
1	0.129	LWN (leaf width narrow)
1	0.175	LWB (leaf width broad)
2	0.129	LWN + LWB
3	0.103	LWN + LWB + teeth
4	0.092	LWN + LWB + teeth + length

4.5 Discussion

4.5.1 Identification of taxa and their hybrids

Samples of *N. major* and *N. marina* could be accurately discriminated based on molecular markers and afterwards morphological traits were critically verified within and among molecularly defined groups. Morphological results are in accordance with measurements for karyotype A and B conducted in studies by Viinikka (1976) and Triest (1988) (Tab. 4.2). Both *N. marina* and *N. major* used in this and previous studies correspond thereby not only genetically (Rüegg et al., 2017) but also morphologically to karyotypes A and B.

Detection of hybrids as well as a more detailed morphological characterization of those plants was only possible based on molecular results. The sole morphological identification of hybrid plants is almost impossible in the field because different quantitative traits of hybrids in this and other studies (Triest, 1989) appeared either phenotypically intermediate or resembled one of the parents. In this study leaf measurements, (LWB and LWN) of hybrids are intermediate between both parental taxa whereas seed measurements of hybrids resemble rather *N. major* plants. Some hybrid traits like teeth on the leaf sheaths, leaf or seed ratios were mosaically distributed between both taxa. Other aquatic plant hybrids like *Nymphaea* are also known for such limitations and representing a mosaic of both parental and intermediate characters rather than strictly intermediate ones (Les et al., 2004; Rieseberg et al., 1993). Another example for the concealment of hybrids in *Najas* was described by Les et al. (2010), where identification of *N. flexilis* × *N. guadalupensis* subsp. *olivacea* hybrids was hampered by their strong resemblance to one of the parental plant species (*N. guadalupensis*).

Only certain leaf (i.e. LWN) and mainly seed (i.e. SW and SL) characteristics could be shown to correlate with genetic types and can be considered useful for a morphological distinction between the two taxa (without hybrids). Results are in accordance with a previous study by Peredo et al. (2011), who arrived at the same conclusion that *N. marina* can be regarded as an "aggregate taxon of two cryptic species". Except for minor seed characters, no consistent pattern of morphological variations could be detected to delimit infraspecific taxa reliably.

However, the applicability of seed for delimitation of the two taxa is limited by their availability due to dioecy of plants as well as sampling date and can therefore only be used as additional characteristics when present.

Further problematic vegetative characteristics are the length of leaves and the number of teeth on the leaf sheaths, which were already considered of low diagnostic value by Triest et al. (1986). Viinikka (1976) suggested that peculiarity of teeth and the length of leaves is closely dependent on the stage of development due to their slow growth. Based on our results, we strongly recommend not using just the spines/teeth on the margins of the leaf sheaths, as is done in most standard keys for final delimitation of the two taxa. Measurements of leaf widths as given in Table 4.1 should be used and included in those keys instead. In cases of doubt or a high probability that hybrids are present, only molecular analysis will help to make a clear-cut decision.

The integrative approach used in this study to distinguish between the two *Najas* taxa and their hybrids can be considered successful because we gained a more accurate understanding of the distribution patterns of *N. marina* and *N. major*, identified reasons for misidentification of plants, and proposed a fast method for clear cut identification. Morphology-based identification methods can be useful if several characteristics are combined, as shown in this study, although they reach their limits because measurements are time consuming and have to be made in sufficiently great numbers and detail using digital imaging. In the case of *Najas,* prior recognition of the presence of cryptic species within various regional databases (Bettinger et al., 2013) would have been advantageous also for other plant surveys e.g. Hoffmann & Raeder (2016). The correction of geographic range maps should be considered, and accurate information should be given for distribution of *N. marina* and *N. major* underlying sympatric ranges. Further expansions of (sympatric) ranges for both taxa seem very likely due to the supposed cryptic (Rüegg et al., 2017) and invasive spread reported for some German lakes (Hoffmann & Raeder, 2016).

The development of a PCR-based RFLP method for identification is considered useful and can be recommended as a quicker and cheaper alternative to DNA barcoding by sequencing and cloning of doubtful sample material in *Najas*. Molecular tools like DNA barcoding should be used with caution and in conjunction with other methods (Duminil & Di Michele, 2009; Steele & Pires, 2011). Nevertheless, these methods helped substantially in uncovering enduring mistakes and have already revealed cryptic introductions or invasions for other aquatic species (Les et al., 2013; Whittall et al., 2004). Only by generating the molecular datasets is was possible to detect hybrids and reliably assesses the distribution of taxa in this study, but linkage to

multiple morphological characteristics that can be measured and documented readily is required to re-evaluate and overcome persistent identification problems. Combined approaches will aid in establishing reliable molecular markers to identify plants with reduced morphology and high phenotypic plasticity based on thorough taxonomic work, especially when disagreement between both traditional morphological and molecular methods still prevails (Duminil & Di Michele, 2009).

4.5.2 Ecology of taxa and their hybrids - implications and recommendations for further mapping and studies

Hybrids of the two *Najas* taxa are expected to appear spontaneously in other lakes as well, since both taxa propagate each year exclusively by sexual reproduction via underwater pollination (Triest, 1988). The only molecularly verified hybrids so far have been proven to exist in Lakes Sempach and Pfäffikon, located in Switzerland (Triest, 1989), but more lakes located in Germany with the co-occurrence of both taxa are already known, e.g. Lake Waging-Taching and some lakes of the Eggstätt-Hemhofer Lake district (all in Bavaria; Rüegg et al. (2017)), Lakes Weutschsee and Oberucker (both in Mecklenburg-Pomerania; Doll & Pankow (1989), Lakes Nemitz and Tegel (Brandenburg and Berlin; Viinikka (1976)).

Besides the restriction of co-occurrence of the species based on abiotic factors such as biogeographic history, also biotic factors such as temperature can be assumed to play a key role for the hybridization of both taxa. The life cycle of *Najas* plants is known to depend heavily on temperature, by influencing germination (Handley & Davy, 2005) as well as florescence. Triest (1991) reported that differences in flowering time form a hybridization barrier in populations observed in the Swiss Alp region. Gender-related differences in flowering times for *N. marina* plants were also described in southern German regions (Hoffmann et al., 2014a). Formation of unidirectional hybrids resulting in hybrid plants that partly resemble *N. major* plants seems plausible for hybrid specimens detected in this study. Future investigations should determine whether lakes with both taxa and hybrids have significantly different temperature profiles in comparison to lakes containing only one of the two taxa. Sufficient sites exist to pursue additional ecological, morphological and molecular investigations.

Since misidentification of plants from both taxa is most likely to happen in areas with sympatric distribution, the detection and mapping of co-occurrence and spread of either taxa is more important in the course of WFD related monitoring than simply confirming the identity of doubtful specimens and potential hybrids with the aid of molecular methods. Thresholds of measurements of leaf characteristics as given in this study (Tab. 4.2) can be useful for assessing if one of the taxa is present in a lake, but have to be applied carefully and in sufficiently high numbers. We also recommend deriving multiple measurement from distinct plants and various leaves as done in this study.

Highly experienced taxonomists may be able to delimitate specimens accurately, but this expertise is currently very scarce (Figueiredo & Smith, 2015). Training for mapping procedures in the course of WFD-related monitoring is time consuming and difficult, and availability of verified herbarium material is limited. By bundling all the plant material from one mapping season to process them together is one way to achieve more accurate and faster species and hybrid delimitation. This approach is surely not necessary for all species but may be useful for those involving taxonomically problematic groups. Since we cannot solve taxonomic differentiation problems in the field with the aid of molecular methods at present, vouchering of plants as herbarium material becomes crucial, which should be done routinely during mapping, and should not create any additional expense.

A consequence of not recognizing species richness or sympatric distribution in macrophyte surveys can be the misinterpretation of ecological states when confounding species are used as biological indicators, as it is the case for the two *Najas* taxa. In general, macrophyte surveys evaluating European water quality are based on identification of individual species and uncertainty measurements are more sensitive when quantitative data is collected (Dudley et al., 2013). Hybrids are rarely included in assessing macrophyte species richness (Rørslett, 1991) because they are overlooked and their identifications requires molecular tools (Kaplan & Fehrer, 2013). In consequence, hybrids are not recorded at all in areas with sympatric co-occurrence of widespread species like *N. major* and *N. marina* within regular, morphologically based macrophyte surveys.

Our results show that under natural conditions, hybridization between taxa in places of co-occurrence is common and hybrid origin was able to be confirmed for 13 out of the 90 plants collected (~14%). The occurrence of hybrid plants in Lake Staffelsee was verified for two

vegetative periods in 2012 and 2015. In general, it is still unclear how common and stable hybrid populations are because thorough molecular screening as done in this study is not part of the standard WFD mapping procedures. Apparently, co-occurrence of taxa and their hybrids is sustained, and hybrid sterility is undoubtedly a function of the extensively rearranged genomes of the two taxa (Viinikka, 1976; Peredo et al., 2011, D. Les pers. com.). This assumption raises more questions on the hybrids ecological and evolutionary significance and should be addressed in future studies.

4.6 Outlook

To gain deeper knowledge about the hybrids and their genesis, it would be worth the effort to perform additional molecular analyses with more polymorphic markers. Techniques like amplified length polymorphism (AFLP) have been used for species identification in a variety of other aquatic genera such as *Chara* (Boegle et al., 2007) or *Potamogeton* (Whittall et al., 2004). Other markers such as simple sequence repeats (SSR i.e. microsatellites) can even detect recent hybridization events (Duminil & Di Michele, 2009) and have already been used to evaluate genetic diversity in species like *Halophila* (Nguyen et al., 2014). A list of aquatic species from taxonomic and morphologically confounding groups should be developed, thus emphasizing the need of molecular identification. Furthermore, an herbarium reference collection of problematic taxa should be established for each lake, e.g. in the course of regular monitoring according to WFD guidelines. Species selection should be based on their importance as indicators, their vulnerability status, or their role as invasive species in order to facilitate identification and further decision making.

5 *Najas marina* and *N. major* benefit from low light conditions caused by climate change in competition with native and alien invasive macrophytes

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Candidate's contribution:

Conception and execution of experiments, statistical analysis and interpretation of data, writing and revision of the complete manuscript including Figures and Tables.

5.1 Abstract

Climate change can result in locally increased precipitation and alter the amount and quality of substances that get washed into freshwater systems from their catchments. This is relevant for suspended particulate matter (SPM), colored dissolved organic matter (CDOM) as well as for nutrients, all potentially affecting primary producers. Additionally, changing environmental conditions can affect competition between native and invasive macrophyte species. In this study, the influx of the three optically active substances altering the photosynthetically active radiation (PAR) was simulated in mesocosm experiments with a focus on the response of native macrophyte communities (*Najas marina, N. major, Myriophyllum verticillatum, Eloda nuttallii*) to the immigration of two potentially invasive macrophytes (*Hydrilla verticillata, Lagarosiphon major*). Light conditions were monitored with hyperspectral underwater radiometers and species responses were assessed using biomass production, relative growth rate (RGR) and root to shoot ratios. Native *Najas* taxa showed enhanced growth under turbid conditions, achieving two times higher RGRs (0.01 – 0.05 d^{-1}) compared to invasive species. Overall, algal turbidity had a negative but not significant effect on most

RGRs of the species. Significant negative influence of changed light conditions was observed on the growth rates of invasive macrophytes *H. verticillata* (-0.002 – -0.03 d^{-1}) and root to shoot ratios of *L. major* (0.002 – 0.014 d^{-1}). In future, the native *Najas* taxa will likely benefit from climate change, which will lead to higher water temperatures and increased turbidity. Depending on the timing and duration of future warming and turbidity events native species might have an advantage over invasive macrophytes.

5.2 Introduction

Ongoing and future climate changes will alter the environmental conditions in aquatic ecosystems within the next decades and consequently affect the underwater vegetation (Ejankowski & Lenard, 2015; Mooij et al., 2005; Schep et al., 2008). Other important consequences of global change are invasions of non-native species into aquatic habitats (Keller et al., 2011). Many invasive aquatic species have already caused significant detrimental economic impacts which resulted in a loss of biodiversity and even ecosystem functioning (Hussner et al., 2010b). Climate change, apart from its direct effects like increasing water temperature, can have indirect effects on the aquatic ecosystems as well. More precisely, longer, more frequent droughts and heavy rain events can alter the influx of substances like suspended particulate matter (SPM), colored organic matter (CDOM), and nutrients from the catchments into lakes and rivers (Chambers, 1987; Mormul et al., 2012). On the one hand, droughts and heavy rain events facilitate soil erosion that results especially in agricultural areas in higher concentrations of suspended particulate matter in receiving waters (Denic & Geist, 2015; Lummer et al., 2016). On the other hand, local flood and heavy rain events can raise the discharge of humic-rich water from wetlands especially in the northern hemisphere (Kritzberg et al., 2014). Additionally, nutrient-rich outflow from the land side can potentially increase the growth of algae, the main light competitor for submerged macrophytes (Ejankowski & Lenard, 2015; Middelboe & Markager, 1997). Overall, the shifts in precipitation lead to an increase in SPM, in CDOM and, as a result of nutrient input, in algae, which as optically active substances determine the light available to plants in the water column by absorbing, scattering or reflecting certain parts of the light spectrum (Kritzberg et al., 2014). Many studies have proven light intensity and availability to be a crucial factor for macrophyte

growth, biomass and species distribution in general (Barko et al., 1982; Chambers, 1987; Middelboe & Markager, 1997). Further it was shown that increased humic substances impact the ability of alien macrophyte species to invade aquatic habitats (Mormul et al., 2012). It is therefore expected that changes in the quality, i.e. the spectrum, of plant available light have significant effects on functional traits like plant growth and consequently on the composition of the submerged plant community.

Non-native macrophyte species, especially potentially invasive water plants, possess greater potential for phenotypic plasticity or increased growth rates and are therefore seen as more resilient to changes in environmental conditions such as light (Eller et al., 2015; Riis et al., 2012) or temperature (Hyldgaard & Brix, 2012; Riis et al., 2012). *Elodea nuttallii* (Planch) H.St.John (Szabó *et al*., 2019); *Lagarosiphon major* (Ridl.) Moss, (Riis *et al*., 2012); *Hydrilla verticillata* (L.f.) Royle, (Eller *et al*., 2015), and many of the non-native invaders also represent low light adapted species (Hussner et al., 2010a; Riis et al., 2012) and are able to outcompete other macrophytes under low light conditions (Mormul et al., 2012; Szabó et al., 2019). Invasive species such as *L. major* and *H. verticillata* are especially successful during establishment and known for rapid growth rates, enhanced length growth, and dense canopy formation (Herb & Stefan, 2006; Riis et al., 2012).

Overall, native macrophytes are expected to be outcompeted by invasive species in combination with proceeding climate change in the future (Hyldgaard & Brix, 2012; Lukács et al., 2017; Vilà & Weiner, 2004). Despite this, we suppose that native macrophytes are capable of competing with invasive species under certain circumstances. Dynamics of species abundance and assemblage of growth form types could not be related to environmental variables in a long-term study and single disturbance events such as weather extremes must be assumed to be an important factor in the competition and dominance of certain species, also stressing the importance of the growth form of the species (Chambers, 1987; Lukács et al., 2017; Wiegleb et al., 2014).

The adaptive strategy of species native to Europe, especially to Germany, such as *Najas major* All. (= *N. marina* L. subsp. *marina*), and *Najas marina* L. (= *Najas marina* subsp. *intermedia* (Wolfg. ex Gorski) Casper) has been described in previous studies with regard to growth (Agami et al., 1980), distribution (Agami et al., 1984), and physiology (Agami & Waisel, 1985). Few recent studies exist on the competitive potential of the two taxa which were shown to differ

substantially on a genetic (Rüegg et al., 2017) and a karyological level (Triest, 1988). Allelopathic activity makes *N. marina* a successful competitor, not only against phytoplankton but also against other macrophyte species (Agami & Waisel, 1985; Gross et al., 2003). In more shallow water and at high irradiation levels *Najas* can achieve high growth rates and is able to build rich plant stocks (Agami et al., 1984; Pietsch, 1981).

Based on detailed ecological characterization (Doll, 1981; Pietsch, 1981), the two taxa were assigned different indicator values and both are used within the assessment of water quality according to the European Water Framework Directive (WFD) (Schaumburg et al., 2004). Lake sites that are dominated by the more tolerant *N. major* are ranked a lower quality than sites prevailed by the sensitive *N. marina,* indicating *N. major* is less common at reference lakes (Schaumburg et al., 2014). Both *Najas* taxa serve as an example of native species common in Europe and are well adapted to rising temperatures (Handley & Davy, 2005; Hoffmann et al., 2013a; Hoffmann and Raeder, 2016). Both *Najas* taxa have formed mass occurrences in Bavarian lakes, comparable to the spread of invasive neophytes (Hoffmann & Raeder, 2016; Rüegg et al., 2017). The extensive spread of *Najas* taxa often downgraded the ecological rating of afflicted lakes (Schaumburg et al., 2014) which causes severe problems, i.e. restricting leisure activities, fishery and inland navigation. Cryptic phenological differences make it hard to distinguish the morphological traits of taxa and their adaptive strategies (Rüegg et al., 2019; Triest, 1988), which are of interest for drawing conclusions on their current and future spread.

The present study used a mesocosm experiment to simulate and maintain turbid conditions and test the competitive strength of native *Najas* taxa compared to native and to alien species under changing light quality. To achieve this objective, the growth of the two native species *N. marina* and *N. major* in competition with two non-native and potentially invasive macrophytes species, *H. verticillatum* and *L. major*, was determined under sufficient and near-natural light conditions but increased concentrations of optically actives substances. *Myriophyllum verticillatum* L. and the in Germany naturalized invasive neophyte *E. nuttallii* were also included in the study to compare the reactions of *Najas* to native and a naturalized species. In detail, the following hypotheses were tested: (1) Light conditions resembling different turbid conditions can artificially be induced and significantly influence the light quality of the photosynthetically used light. (2) Macrophyte growth of native and establishment of invasive species is significantly impacted by the changed light conditions.

5.3 Materials and Methods

5.3.1 Design of the study and the mesocosms, plant material

The experiments were conducted from April to October 2016 (169 days, 23 weeks) in an outdoor mesocosm system located in the Bavarian Prealps next to the Limnological Research Station of the Technical University of Munich 50 km south of Munich (Germany). An overview of the four phases of the experiment is given in Figure 5.1. The system consisted of twelve identical rigid intermediate bulk containers (IBCs) made of polyethylene with a volume of 1000 L (1x1x1 m). The tanks were coated with opaque silage film to prevent penetration and scattering of light from the outside. In early April, the IBCs were filled with lake water from the pre-alpine, oligo-mesotrophic Lake Starnberg (phase1, Fig. 5.1).

Scientific divers collected the sediment for the experiment from Lake Starnberg in a depth of 2 to 3 m in early spring. The grain sizes of the sediment were analyzed by a commercial soil laboratory (AGROLAB GmbH, Landshut, Germany) and comprised sand 45%, silt 54%, and clay <0.5%, whereby the total organic carbon (TOC) accounted for 3.4 %. Firstly, the bottoms of polyethylene boxes (0.21x0.13x0.15 m) were covered with a 0.04 m thick layer of coarse substrate (gravel > 0.01 m) to ensure drainage and then a 0.06 m thick layer of the lake sediment was added.

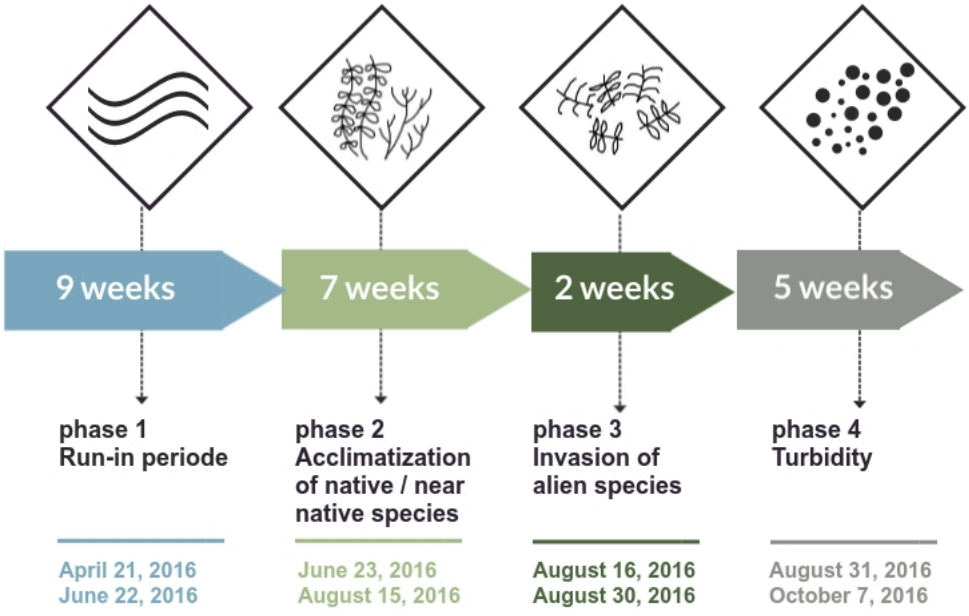

Figure 5.1: Timeline of the experimental run.

On June 23rd, 2016 each mesocosm was equipped with three boxes containing the sediment (phase 2, Fig. 5.1). Subsequently, the macrophytes were planted into the sediment in the boxes. The selection of the macrophytes used in the experiment was based on previous surveys at lake Starnberg, where all native and established species naturally occur (Melzer, 1999; Stelzer et al., 2005). The sediment and water were also derived from lake Starnberg to ensure all species had similar good growing conditions. The three boxes within each IBC were set up with different plant material and the boxes were then randomly arranged. One box contained only sediment. Another box was filled with 15 rooted individuals of *N. major* and *N. marina* between about 7 cm long. Each species was planted on one side of the box. In the same way, a third box was planted with 15 individual fragments of *M. verticillatum* and *E. nuttallii* each. Fragments of both species were ten internodes long and similar in size and weight to rooted individuals of *Najas*. The number of 30 plants per box (according to 600 - 900 individuals m^{-2}) was based on plant densities found in the field (Chick & Mlvor, 1997) or densities tested in other studies (Ciurli et al., 2009) in order to imitate competition at high densities. The plant material was collected two weeks prior to planting from different nearby locations. *E. nuttallii* and *N. marina* originated from Lake Chiemsee. *M. verticillatum* was obtained from Lake Fohnsee that belongs to the Lake Osterseen district. *N. major* was collected at Lake Staffelsee.

After an adaptation and growth period of altogether 53 days (one and a half weeks floating in a bucket, six weeks planted in the boxes), all plants were well developed. In the course of the experiment, small stands of stoneworts, identified as *Chara contraria* A.Braun ex Kützing, developed evenly in all boxes from propagules that were stored in the sediment. The stoneworts were not included in the measurements because they germinated at different times and developed with a time shift compared to the macrophytes selected for the experiment.

The collection of the model species used for the experiment was not possible before the end of June, as they were only found in respective lakes comparatively late in the season. The reasons for this were unusually low temperatures in May and June 2016; in these months the mean temperature was 2 - 3 °C below the long-term monthly average temperatures (Deutscher Wetterdienst, DWD). Consequently, the start of phase 2 of the experiment was delayed.

On August 15th, 2016, fifteen plant fragments of *L. major* and *H. verticillata* each-were added to the three different boxes in all but four mesocosms (one of each treatment including a reference tank) (phase 3, Fig. 5.1). Both alien species were obtained from a water plant nursery

(extraplant.de) and cultivated in aquaria (18 - 20 °C, 10 h day/14 h night) half a year before the experiment. The fragments comprised of five internodes and were placed in the boxes onto the developed plant stands to simulate (auto-)fragmentation of invasive species and a subsequent establishment of detached and floating fragments.

To suppress microalgae growth and to ensure identical initial preconditions, all containers were connected to a central aeration and common filtration system during the nine-week run-in period of the system, during the seven-week acclimation phase of the native and near-native plants, and during the two-week period of invasion by non-native species (Fig. 5.1).

5.3.2 Turbidity experiment and physio-chemical measurements

For the experimental run, the containers were separated from the central aeration and filtration system and fitted with individual circulating pumps for aerating and for carefully mixing the water column. The experiment consisted of three treatments and one untreated reference in one tank each. This setup was replicated two times, to twelve mesocosms in all (phase 4, Fig. 5.1). The treatments simulated either an increase in suspended particulate matter (SPM), colored dissolved organic matter (CDOM) or algae. The different types of turbidity were artificially created in randomly chosen mesocosms. SPM-turbidity was simulated by adding in total 250 g dried, sieved and homogenized lake sediment from Lake Starnberg with a defined composition of three particle sizes (38, 20, 10µm, mass ratio 7:1:2) to three mesocosms. 150 g of the sediment mix was added at the beginning of the experiment, subsequently, 50 g were added two times in weekly intervals to maintain constant turbidity. An algae bloom was induced in three containers by adding 50 L of a mixed algae suspension (optical density at 730 nm; OD_{730} = 0.7 - 1.1) from green algae and cyanobacteria which was cultivated at the limnological research station four weeks prior to the experiment. The green algae suspension (OD_{750} = 0.5 - 0.8) contained mostly coccal planktic green algae (dominated by *Scenedesmus* spec, *Oocystis parva, Monoraphidium* cf *contortum*) and was cultivated in an aerated 10 L aquarium filled with water from Lake Starnberg (P_{ges} = 0.005 - 0.007 mg L^{-1}) at room temperature with a 10 h day/14 h night rhythm. Evaporation losses were compensated with tab water when necessary. The cyanobacteria *Synechocystis sp.* (PCC 6803) was derived from SAG Culture Collection of Algae at Goettingen University, Germany, and the suspension was cultured for

more than four weeks before the experiment, according to Rippka et al. (1979). Brownification by CDOM treatment was simulated by adding a mixture of equal volumes of the three following humic concentrates to the three tanks: ToruMin (TETRA GmbH, Melle, Germany), Hobby Humin Fit (Dohse Aquaristik GmbH & Co. KG, Grafschaft-Gelsdorf, Germany) and Sera bio humin (sera GmbH, Heinsberg, Germany). Initially, 150 mL of the concentrate were added to the water, four weeks later another 50 mL of the mix were added to preserve the turbidity.

The different types of turbidity were initiated at the end of August and maintained for 6 weeks (42 days) to simulate a summer flood event. During this time, the physical and chemical parameters of the water in the mesocosms were characterized. Every week temperature [°C], pH, conductivity [µS], O_2 concentration [mg L^{-1}] and turbidity [NTU] were measured in situ with a multi probe sensor (Multi 3430, WTW, Weilheim, Germany) and a turbidity meter (VisoTurb 900-P IDS, WTW, Weilheim, Germany). Additionally, each IBC was fitted with a temperature logger (Pendant UA-001, HOBO onset, Bourne, MA, USA) to monitor hourly the water temperature. Water samples were taken biweekly and analyzed immediately in the laboratory according to the following standard methods: soluble reactive phosphorus (SRP) and total phosphorus (EN1189), nitrate-nitrogen (DIN ISO EN 13395-D28, and ammonium nitrogen (DIN ISO EN 38406-E5).

5.3.3 Light measurements

The spectral property and the intensity of the downwelling light in the treatments were determined with hyperspectral underwater radiometers (RAMSES ACC-Vis, TriOS GmbH, Rastede, Germany, 320 - 950 nm as mW m^{-2}, 180° detection field). In one of each treatment type and one reference, the radiometers were placed in the center of randomly selected mesocosms at a depth of 0.5 m to measure the downwelling light ($E_{d0.5}$). An additional sensor was installed in an untreated mesocosm just below the water surface to determine the incident light (E_{0-}) as reference for the light transmission. To ensure stable measurements, the devices were exposed in the mesocosms prior to the application of the optically active substances. All sensors were programmed to measure the diurnal light variations every 15 minutes. At these times, the

average from five consecutive measurements with an integration time of 10 seconds was calculated.

The measurements aimed to determine the daily light intensity and the light transmission for each treatment after the turbidity had been induced. Therefore, all data from phase 4 were collected (31.8.-7.10.2016). Based on these data the daily means of the photosynthetically active radiation (PAR, 400 - 700nm as W $m^{-2}d^{-1}$) and the light transmission rates ($E_{d0.5}$ / E_{0-}) were calculated for each treatment. Furthermore, the relative difference between the maximum and minimum irradiance (ε) was computed for PAR as:

$$\varepsilon = \frac{E_{d,max} - E_{d,min}}{E_{d,mean}} \quad \text{(eq. 1)}$$

to estimate the fluctuations in the plant-available light.

5.3.4 Plant growth

Plants were harvested and examined at study termination. Roots were separated from the sediment by gentle washing. All plant materials were weighed after drying at 80 °C in a forced-air oven to constant mass. The dry weight (DW) of shoots and roots was weighted initially and at the end of the experiment to the nearest 0.01 g, either of single plants or individuals were weighted together, and a mean value was used for calculating the Relative Growth Rate (RGR; [d^{-1}]) according to Hoffmann and Poorter (2002):

$$\mathrm{RGR} = \frac{[\ln(\mathrm{DW}_{final}) - \ln(\mathrm{DW}_{initial})]}{t} \quad \text{(eq. 2)}$$

where DW_{final} was the dry weight of the total over ground biomass and t was the number of days of growth. $DW_{initial}$ was determined by calculating mean values using the dry masses of 6 - 12 representative non-planted shoots of each species that were similar in size and fresh weight, compared to the shoots planted in the mesocosms. This approach is according to conventional methods found in the literature and described in several other studies working with macrophytes (Gross et al., 2001; Hussner, 2009; Riis et al., 2012). *N. marina* initial mean dry weight ±1 SE: 0.017 ± 0.008 g, n = 12; *N. major*: 0.03 ± 0.01 g, n = 12; *E. nuttallii*: 0.004 ± 0.003 g, n = 12; *M. verticillatum*: 0.016 ± 0.03 g, n = 6; *H. verticillatum*: 0.02 ± 0.01 g, n = 12; *L. major:* 0.039 ± 0.017 g, n = 12.

5.3.5 Statistics

Statistical analysis was conducted using the software R v. 3.5.2 (R Development Core Team, 2013). Data from the light measurements were analyzed with analysis of variance (ANOVA). A paired t-test with Bonferroni correction was used to determine differences in the light intensities when comparing the treatments.

Growth data and physicochemical conditions were analyzed using one-way ANOVA and the relationship between values was analyzed using the Pearson's correlation (rcorr). Data were tested for normality (Shapiro test) and variance homogeneity (Barlett's test) to meet the assumptions of the statistical analysis. Significant differences between means were identified by the *post hoc* Tukey's honestly significant difference (HSD.test) multiple range test at the 0.05 significance level. If assumptions of ANOVA were not met, differences among means were tested using the Kruskal Wallis rank sum test. In case of significance, the Kruskal Wallis rank sum test was followed by a Dunn's test (*post hoc)* for multiple comparisons of groups (dunn.Test) at the 0.05 significance level.

5.4 Results

5.4.1 Environmental conditions

5.4.1.1 Physiochemical conditions

Physical parameters of water in the twelve different mesocosms did not differ significantly before the treatment (ANOVA, $p = 0.96 - 0.99$). The following physical parameters showed strong correlation in the course of the experiment: pH-value and conductivity [µS] ($r(72) = -0.85$, $p < 0.000$), temperature and O_2 content in [mg L^{-1}] ($r(96) = 0.73$, $p < 0.000$).

No significant differences in temperature were detected between the tanks throughout the entire duration of the experiment (Kruskal, $\chi^2(11) = 4.14$, $p = 0.97$) nor did the different turbidity treatment influence the temperature of the mesocosms significantly (Kruskal, $\chi^2(3) = 0.81$, $p = 0.85$). During the experiment, the temperature in all IBCs ranged between 9.2 °C and 24.7 °C (min - max), whereby mean temperatures were 17.6 - 18.4 °C (± 4.1 - 4.6). Mean pH-values in the mesocosm with humic substances were significantly lower compared to those with other treatments (Tab. 5.1). The pH-values of the mesocosms with the algae and the sediment supply did not differ from the untreated reference, but the CDOM treatment differed from the others with slightly lower values. Compared to the other treatments and the untreated reference, only the CDOM supply caused significantly increasing conductivities (ANOVA, $F_{(3, 128)} = 7.93$, $p = 6.85e^{-05}$).

Within the three untreated mesocosms and the three equally treated with algae or CDOM, no significant differences were found for any of the physical parameters (ANOVA, $p > 0.1$). Within the three IBCs treated by SPM only the pH values differed significantly (ANOVA, $F_{(2, 21)} = 5.79$, $p = 0.01$). The pH-value in one mesocosm was significantly lower 8.8 (± 0.2) than in the two others in which 9.2 - 9.3 (± 0.3) were measured. The turbidity values in treated mesocosms were significantly different from those of the reference containers (ANOVA, $F_{(3, 68)} = 3.49$, $p = 0.02$). The highest NTU-values were measured in the algae treatment, the largest standard deviation occurred in the containers with the SPM supply (Tab. 5.1).

Before the treatment, the mean values of the water chemical parameters were highly similar in all mesocosms due to the connected setup (Tab. 5.2). After turbidity was induced, the treatments differed most in nitrate-nitrogen (NO_3-N) and total phosphorus (total P) content, whereby the IBCs with the algae supply showed the highest values compared to the reference.

Highest ammonia-nitrogen (NH_4- N) concentrations were measured in the SPM tanks. In general, chemical conditions were slightly altered by each treatment induced by the addition of nutrients either by sediment, algae suspension or humic substances. Nevertheless, the trophic state of the initially connected mesocosm system and later separated tanks can always be characterized as oligo-mesotrophic.

Table 5.1: Mean values of physical parameters of the water in the different mesocosms and treatments (n = 18 – 49). Brackets indicate standard deviations (SDs). Different superscript letters indicate statistical differences at the 0.05 significance level (*post hoc* Tukey's test). CDOM: colored organic matter, SPM: suspended particulate matter, NTU: nephelometric turbidity unit.

	Means and standard deviations (SDs)			
	Temperature [°C]	**pH**	**Conductivity [µS]**	**NTU**
Before treatment	18.4 (4.3)	8.6 (0.2)	201 (9)	0.03 - 0.16 (0.1)
Reference	18.2 (4.6)	9.2 (0.3) ^{ab}	215 (27) ^{a}	0.08 (0.3) ^{b}
Algae treatment	17.6 (4.1)	9.4 (0.2) ^{a}	216 (24) ^{a}	0.53 (0.5) ^{a}
CDOM treatment	17.8 (4.1)	8.7 (0.3) ^{c}	267 (20) ^{b}	0.24 (0.3) ^{ab}
SPM treatment	17.8 (4.2)	9.1 (0.3) ^{b}	230 (32) ^{a}	0.35 (0.7) ^{ab}

Table 5.2: Measurements of chemical parameters in the of the water used in the different mesocosms and treatments (n = 1 - 4). CDOM: colored organic matter, SPM: suspended particulate matter, SRP: soluble reactive phosphorus.

	NO^3-N [mg L^{-1}]	**NH^4-N [mg L^{-1}]**	**SRP [mg L^{-1}]**	**Total P [mg L^{-1}]]**
Before treatment	0.203	0.021	0.001	0.006
Reference	0.241	0.098	0.004	0.009
Algae treatment	1.255	0.096	0.000	0.013
CDOM treatment	0.678	0.035	0.009	0.015
SPM treatment	0.449	0.193	0.004	0.008

5.4.1.2 Light conditions

The induced turbidity affected the light intensity in the treated mesocosms significantly compared to the reference. The photosynthetically active radiation available for the plants per day was distinctively lower in the treated mesocosms compared to the reference (ANOVA, $F_{(3,42)} = 3.86$ $p = 0.016$) (Tab. 5.3). The algae supply caused an attenuation of PAR to 70 % and the SPM supply to 74 % compared to the reference. Furthermore, the induced turbidity affected the overall fluctuation of the irradiation (Tab. 5.3). The data indicate that the increase in light fluctuations depended on the type of turbidity. In the mesocosms with added humic substances, the light fluctuations were 25 % greater than in the reference. In contrast to this, the elevated SPM concentrations increased the light fluctuations only by 13 %. The greatest fluctuations in the plant available light were measured in mesocosms with the induced algae bloom, in which the variations in the incident light was 55 % greater than in the reference.

Table 5.3: Mean values of the plant available light per day and the relative differences between maximum and minimum irradiance (ε). Standard deviation shown in brackets. Superscripts in the same column indicate significant differences ($p < 0.05$). CDOM: colored organic matter, SPM: suspended particular matter, PAR: photosynthetically active radiation.

	PAR [1] [W m^{-2} d^{-1}]	ε PAR	% of PAR incident light [2]
Reference	836.1 (367.9) [a]	1.19	89.2
Algae treatment	584.1 (295.8) [b]	1.84	62.3
CDOM treatment	619.4 (243.3) [b]	1.49	66.1
SPM treatment	631.9 (249.1) [b]	1.35	67.4

[1] Measured 50 cm below water surface

[2] Incident light was measured 5 cm below water surface

The increased concentrations of optical substances had distinct effects on the light quality (Fig. 5.2). In the untreated reference mesocosms, the rate of light transmission was lowest at the beginning of the blue light waveband between 400 to 450 nm and highest at wavelengths between 450 and 600 nm. Subsequently, it constantly decreased in the red waveband to 0.6 at 700 nm. The addition of humic substances changed the light characteristics in the treated mesocosms significantly. Great light absorption was apparent in the blue light waveband compared to the reference condition, resulting in a brownish watercolor (Fig. 5.2). The

transmission per meter increased in the blue light band form only 20 % (400 nm) to about 70 % (500 nm) and reached about 90 % toward the red light band (600 - 700 nm). The induced algae bloom decreased the light transmission most in the blue and red light wavebands (400 - 500 / 600 - 700 nm) but had almost no impact on the green light waveband (500 - 600 nm). In contrast to this, the higher concentration of suspended particulate matter had only little effect on light quality. Apart from a slightly higher absorption rate between 400 and 425 nm and greater fluctuations of the transparence rate in the red wave range, the light quality in the SPM treated tanks was almost identical to the reference mesocosm (Fig. 5.2).

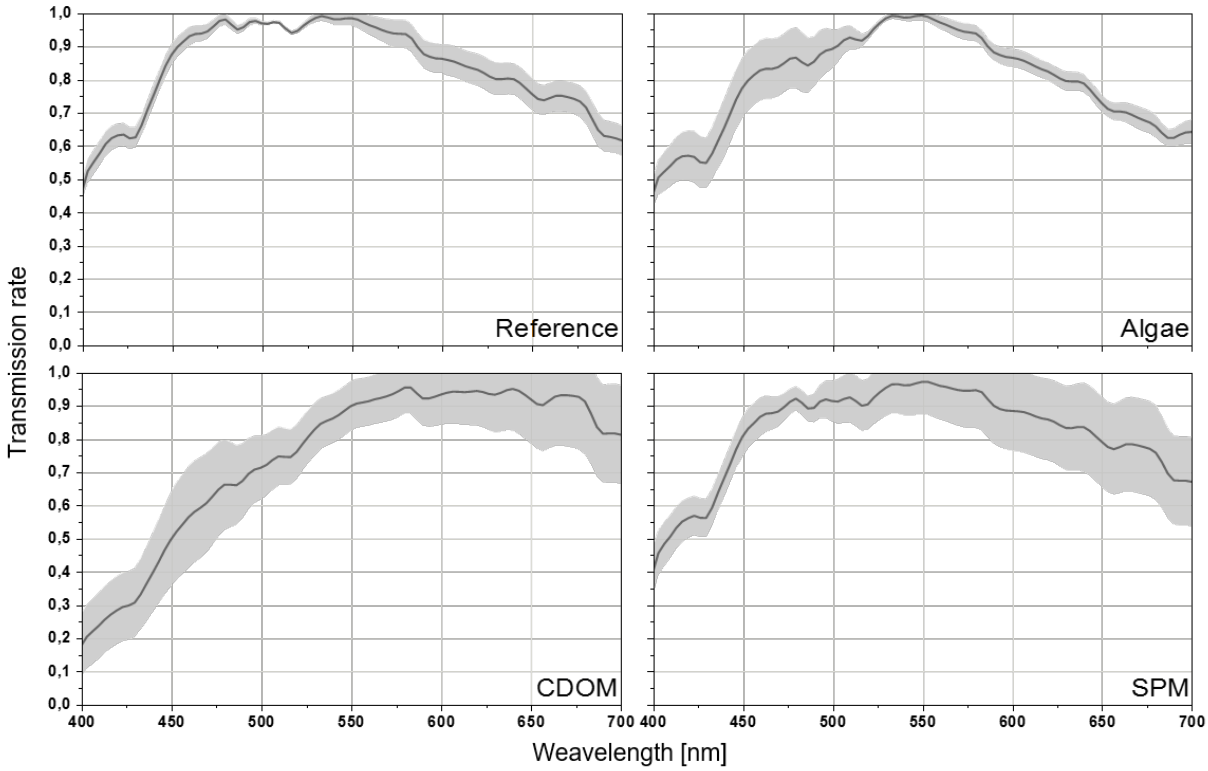

Figure 5.2: Light transmission rates per meter for the incident light in mesocosms with different treatments. Based on daily measurements between 31.08. and 12.09. (phase 4). Grey area indicates the standard deviation.

5.4.2 Macrophyte growth and development

Significant differences in the RGRs and other growth parameters were observed mostly between different macrophyte species. The only significant effect of the treatment when comparing the RGRs and other growth parameters within one species was calculated for *H. verticillata*. The intensity of the turbidity measured in NTUs had no consistent effect on the overall growth of the different macrophyte species.

In contrast, the quality of turbidity affected the development of the macrophytes. The SPM treatment favored the growth of both *Najas* taxa, of which the calculated RGRs accounted to 0.05 d^{-1}; whereas these values were only 0.04 d^{-1} for *N. marina* and 0.03 d^{-1} for *N. major* under reference conditions (Fig. 5.3 A, Appendix A3).

Under algae and CDOM treatment the RGRs of *N. major* decreased by 0.01 d^{-1} compared to the reference, although not significantly. In the CDOM mesocosms, *N. marina* showed with about 0.03 d^{-1} approximately the same RGRs as in the reference, whereas in algae treatments RGRs of only 0.01 were reached. Corresponding to the enhanced RGRs under the SPM treatments *N. major* developed around 4 g biomass, about 2 g more, under SPM turbidity compared to the reference (Fig 5.3 B, Appendix A3).

As well, *N marina* produced more biomass in the SPM mesocosms, but the difference to the reference was with 0.6 g less pronounced. The biomass gain of *N. major* under the algae and the CDOM treatments was similar or even higher than under reference conditions but was not significantly different. In contrast, *N. marina* developed considerably less biomass under these conditions. Especially in the CDOM mesocosms, *N. major* built ten times more biomass than *N. marina*. The CDOM treatment had a slightly increasing effect on the root to shoot ratio of *N. major*, whereas lower root to shoot ratios were measured under SPM treatment and under reference conditions in both *Najas* taxa (Fig. 5.3 C, Appendix A3).

The naturalized *E. nuttallii* reached with 0.03 - 0.04 d^{-1} comparable RGRs to the two *Najas* taxa. *E. nuttallii* is the only species with increased RGRs and biomasses under algae turbidity in comparison to all other conditions. Lowest RGRs were calculated under SPM conditions. The ratio of root to shoot biomass was highest under reference and CDOM conditions and lowest under algae and SPM treatment (Fig. 5.3 C, Appendix A3).

With -0.002 d^{-1} the lowest RGR was calculated for *M. verticillatum* plants grown under algae treatment, the highest RGR values were obtained under SPM treatment. The shoot and root

DW of *M. verticillatum* were very low in general and not significantly affected by the different treatments (Kruskal, $\chi^2(3) = 3.36$, $p = 0.34$). The root to shoot ratios of *M. verticillatum* were higher compared to all other species, and highest under CDOM treatment (Fig. 5.3 C, Appendix A3).

For *H. verticillata* the treatments had a significant effect on RGRs (ANOVA, $F_{(3, 20)} = 3.9$, $p = 0.02$) and root and shoot biomass (Kruskal, $\chi^2(3) = 8.15$, $p = 0.04$). The highest RGRs and root and shoot dry weights were obtained for *H. verticillata* plants growing under reference conditions (Appendix A3). Negative RGRs were detected for all plants grown under the different turbidities, and the lowest RGRs were calculated under algae treatment. In all treatments, *H. verticillata* showed similar root to shoot biomass ratios. All three turbidity treatments had a negative but not significant effect on the RGRs and the root and shoot dry weights of *L. major*. The species root to shoot ratios were slightly higher, but not significant, under reference conditions than under CDOM and algae treatment (ANOVA, $F_{(3, 20)} = 2.7$, $p = 0.07$).

Overall, the highest RGRs were obtained by the two *Najas* taxa, *N. major* 0.04 d^{-1} (± 0.01) and *N. marina* 0.03 d^{-1} (± 0.02) (means ± SD, pooled over all treatments). *E. nuttallii* showed equally high RGRs with 0.03 d^{-1} (± 0.01). The species with lowest growth rate and the only plant deteriorating was *H. verticillata* with -0.004 d^{-1} (± 0.02). *L. major* and *M. verticillatum* showed similar low RGRs of 0.003 - 0.006 d^{-1}, (± 0.001).

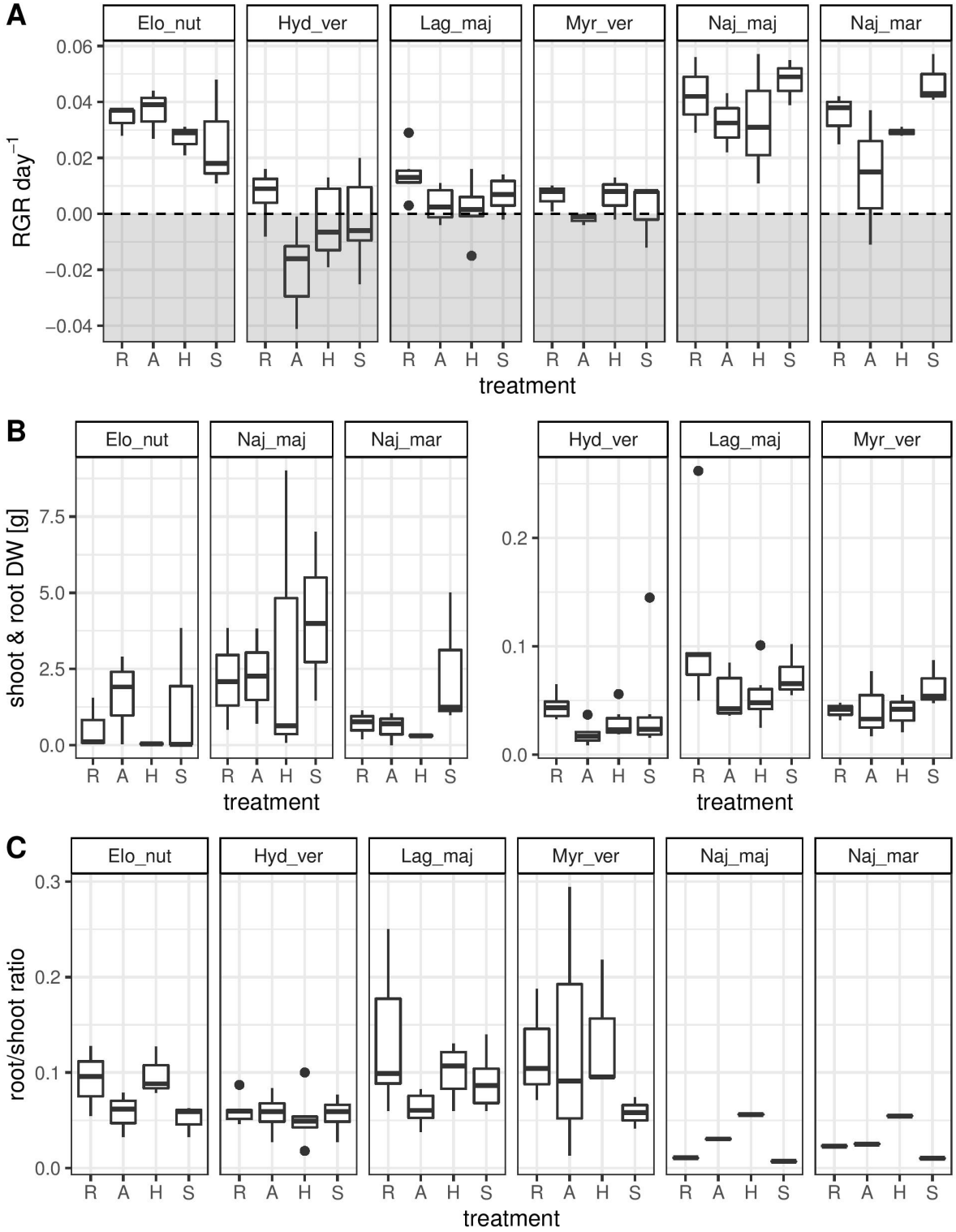

Figure 5.3. A - C: Boxplots of plant growth parameters of six different macrophyte species in the mesocosms under the influence of the four different treatments R: reference condition, A: algae, H: humic substances = CDOM (colored organic matter), S: SPM (suspended particulate matter). Species abbreviations: Elo_nut = *E. nuttallii*; Hyd_ver = *H. verticillata;* Lag_maj = *L. major*; Myr_ver = *M. verticillatum*; Naj_maj = *N. major;* Naj_mar = *N. marina*. Points depict outliers that lie 1.5 times outside the interquartile range. **A)** Relative growth rates (RGR) in [d^{-1}]; (n = 3 - 6). **B)** Shoot and root biomass dry weight (DW) in [g], (n = 3 - 6). **C)** Root / shoot DW biomass ratio in [g g^{-1}], (n = 1 - 6).

5.5 Discussion

For the first time, artificially induced turbidity conditions, which resemble the light alterations caused by climate change, were tested in an experiment using multiple species. The study focused on the effects and competitive growth of the native and invasive macrophyte species under the influence of SPM, CDOM, and algae bloom as a response to increased nutrients. The results of this mesocosm study provide novel information on the competitiveness of native, naturalized and alien macrophytes in the perspective of realistic climate change scenarios. Invasive macrophytes are supposed to perform better under conditions followed by climate change and thereby compete successfully with or even exceed native species (Hyldgaard & Brix, 2012; Mormul et al., 2012; Riis et al., 2012; van Kleunen et al., 2010). Our findings suggest that light scenarios under increased turbidity levels and meso-oligotrophic nutrient conditions do not necessarily result in a competitive advantage of invasive macrophytes over native ones. The influx of different substances changed light quality significantly as proven by spectral measurements showing that the light quality was more impacted than the light quantity. The simulated algae and CDOM light conditions were still sufficient for plants to grow and develop but caused distinct light regimes as well as fluctuating conditions for the macrophytes. In this experiment performance-related traits of native macrophytes like *Najas* were affected only moderately. Relative growth rates and establishment of invasive species were in general more impacted by the turbid conditions. This could to some extent be due to the timing of substance influx during the vegetation period, which effected establishment and growth of invasive macrophytes more than native ones. Growing conditions like light and nutrient availability were sufficient for all species to develop equally good, but it is known that under competition the sensitivity of seasonal biomass production to basic physical lake parameters can be higher for some macrophytes (Herb & Stefan, 2006; Mormul et al., 2012).

Both *Najas* taxa were characterized by high RGRs and biomass values in the untreated reference conditions and under the simulated turbidities. The calculated RGRs and the measured biomasses of *N. marina* plants corresponded to the values of the study of Agami et al., (1984) but they were lower than the results described by Hoffmann et al., (2013a). The importance of the influence of light and temperature on the growth of *N. marina* was pointed out in their experiments (Agami et al., 1984; Hoffmann et al., 2013a). To complete their life

cycle, *Najas* plants have a minimal light requirement of 250 µE m^{-2} s^{-1} and need temperatures of at least 15° C for growth and of 20° C for seed formation (Agami et al., 1984; Hoffmann et al., 2013a). These conditions were satisfied in the untreated as well as in mesocosms with the different artificial turbidities and even led to high growth rates compared to the reference conditions.

In this study, both *Najas* taxa reached RGRs similar to those of *E. nuttallii* but higher RGRs than *M. verticillatum* and the two invasive species *H. verticillata* and *L. major*. This result indicates the highly competitive potential of both *Najas* taxa. In particular, *N. major* always achieved higher RGRs and biomasses than *N. marina.* This is consistent with the known fact that *N. major* is more successful than *N. marina* in warmer waters and more tolerant of turbid and eutrophic conditions (Doll, 1981; Pietsch, 1981). Both *Najas* taxa can compete with other macrophyte species under different nutrient conditions and temperature regimes (Agami and Waisel, 1985; Gross et al., 2003; Hoffmann et al., 2013a). The high RGRs measured under artificial turbidites, showed that both *Najas* taxa have an advantage under these conditions compared to the other macrophytes in the experiment.

Current, as well as the predicted distribution of *N. marina,* emphasizes the species potential for mass development (Hoffmann et al., 2013b; Hoffmann and Raeder, 2016) despite its non-clonal growth. In contrast to the other species used in the study, male and female plants of both *Najas* taxa exhibit different growth forms, and dense, female-dominated stands persist towards the end of the vegetation period (Hoffmann et al., 2014a). The high RGRs and biomasses of *Najas* plants detected in this study could be a result of the survival of mostly female, seed-bearing plants at the end of the experiment. This strategy and the sexual dimorphism as displayed in both *Najas* taxa have to be taken into account when conducting further experiments.

It is often suggested that alien invasive species might take advantage of high resource availability and thereby outcompete initially co-existing natives (Dukes & Mooney, 1999; Eller et al., 2015; Lukács et al., 2017). TOC content of the lake sediment, which was the same in all boxes due to the design of the experiment, provided sufficient growth properties for all macrophytes (Barko & Smart, 1986). Nutrient uptake (N, P) was mostly limited to absorption by the roots since water nutrient conditions were constantly oligo- mesotrophic. Carbon or nutrient limitations can be excluded because (1) water was added regularly (2) the amount of

water per individual was relatively large (>10L per plant) and (3) no positive effect of the added carbon sources (humic substances) occurred in the CDOM basins. Under DIC limitation, the CDOM fertilization should have had a detectable positive effect due to the photochemical and organic decomposition of the humic substances and the release of active carbon.

Due to other studies, we assumed that an increased shoot allocation would occur under turbid conditions resulting in lower root to shoot ratios (Barko & Smart, 1986; Riis et al., 2012). However, the different species did not show significant effects in root allocation to simulated light conditions, except for *L. major*.

For *L. major* high root growth was observed only under reference and CDOM conditions. Under SPM and algae treatments root to shoot ratios were lower in *L. major* plants compared to reference conditions. The same was true for *E. nuttallii* and *M. verticillatum*, whereas *H. verticillatum* did not show any tendencies. *H. verticillatum* has a comparable small root system and is limited in its nutrient uptake from low-density, high organic sediments (Barko & Smart, 1986).

Both *Najas* taxa showed smaller root to shoot ratios under SPM and reference conditions. On the other hand, larger or equally high root to shoot ratios were measured for both *Najas* taxa under algae and CDOM treatment, which is typical for some macrophyte species when light conditions or sediment fertility is low (Barko and Smart, 1981). Since no significant differences in root to shoot ratios or biomass allocation could be observed for the two *Najas* taxa, other factors may also be important such as root surface area, internal anatomy, and physiological transport mechanisms (Barko & Smart, 1981) which were not included in the analysis of this study.

For all aquatic plants except *E. nuttallii*, growth and development under the algae treatment decreased and RGRs and biomass were reduced by 40% compared to the reference. *E. nuttallii* is known to be sensitive to high irradiances (Hussner et al., 2010a) and low-temperature conditions (Hoffmann et al., 2014b). This fact explains the high RGRs achieved by this species in the course of the experiment. However, the growth of *E. nuttallii* was not supported by higher DOC concentrations as reported in previous studies (Mormul et al., 2012). *N. major* and *N. marina* were also negatively affected by the algae treatment, but in contrast to the other macrophytes, both *Najas* taxa showed at least some growth and no signs of degradation.

However, a significant influence of the algae bloom on RGRs could only be shown for *H. verticillata*.

The general limited or inhibited growth of most macrophytes can be explained by the fact that planktic algae compete with the submerged macrophytes for the same wavelengths ranges of light due to similar photosynthesis systems (Kirk, 2010). Furthermore, certain algae such as cyanobacteria or diatoms have a competitive advantage compared to macrophytes, as they can use additional ranges of the light spectrum for photosynthesis due to special additional pigments (Kirk, 2010; Yentsch, 1980). Spectral measurements showed that phytoplankton caused a decrease of the photosynthetically usable light for the macrophytes in the algae treatment. As a result, under algae turbidity the macrophyte growth rates were decreased, even though the total phosphorus concentration in the algae treatments was slightly higher than in the references.

Future climate scenarios predict higher probabilities for flood events followed by increased run offs from catchment areas (Mooij et al., 2005; Schep et al., 2008). Such extreme events have been shown to influence planktonic communities and will favor algae/cyanobacterial blooms (Ejankowski & Lenard, 2015). Consequently, light conditions for submersed macrophytes could become less favorable in the future, especially in shallow lakes (Mooij et al., 2005). The results of the study imply that, even under direct competition with algae, native species like *Najas* will be able to compete with alien invasive macrophytes, provided that light conditions stay above the minimum requirements.

Spectral measurements showed that the light quality of the SPM turbidity was similar to reference conditions and the addition of the suspended matter did not change the light transmission rates as much as other added optical substances. Overall radiation was even enhanced in the SPM treatment. Through dispersion and reflection of light by solved particles, less fluctuation in PAR was achieved. Enhanced plant growth resulting from this effect was though only observable in *Najas*, and we suppose plants gained an advantage over other macrophytes due to rapid growth rates and the growth form.

Resource availability and specific disturbance regimes are most commonly influencing the performance of co-occurring invaders and native species, whereby alien invasive species can be favored under low levels of resources, such as light (Daehler, 2003). To overcome unfavorable light conditions *Najas* and *Elodea* can form dense canopies by abundant and tall

plants to suppress competitors (Agami & Waisel, 1985; Chambers, 1987; Herb & Stefan, 2006; Szabó et al., 2019). Due to faster growth rates and the production of larger leaves those species can gain an advantage under low light conditions and dominate underwater vegetation with high biomass (Barko et al., 1982; Lukács et al., 2017).

Since *Najas* and *Elodea* appear to have similar niches and adaptation mechanisms, it can be assumed that their potential to compete with other plants is equally pronounced. When growing together under disturbance regimes such as increased substance influx and temperatures both species will be in direct competition. The success of single species will depend on the type, duration and timing of such disturbance events. We assume that *Elodea* will dominate under algae turbid conditions, whereas *Najas* will benefit from the influence of SPM.

5.6 Conclusion

The native *Najas* taxa can form mass occurrences like the naturalized invasive species *E. nuttallii*. It could be shown that the two native macrophytes *N. marina* and *N. major* can compete with potentially invasive species like *L. major* and *H. verticillata* under the tested low light and oligo-mesotrophic conditions. Overall, the growth and distribution of *Najas* plants in summer-warm lakes with rising temperatures will likely be more robust in the future to low light conditions than previously assumed. Consequences of climate change, such as increased surface water temperatures and changes in light conditions due to extreme flooding, will influence species composition and growth of macrophytes in favor of more adaptive species like the two *Najas* taxa native to Germany/Europe.

6 General Discussion

The three case studies described in chapters 1 - 2 give detailed insight into the genetic and morphological structure of two different taxa of populations of *Najas marina* s.l. Chapter 3 highlights the basic ecological requirements of the two taxa regarding their potential to adapt to different light conditions and compete with other potentially invasive species.

Based on the results presented in this thesis, morphological species delimitation is impaired by high variability of polymorphic characters currently used for identification and species determination. To overcome misidentification, updates should be made to the keys that identify and differentiate both taxa, as well as the nomenclature for the two taxa *N. marina* and *N. major* according to their ranks in the evolutionary line, which are still often summarized under *N. marina* s.l. This research shows for the first time that both taxa display substantial genetic differentiation in the respective marker regions but are able to hybridize. Hybrid and parental plants can grow intermixed where species co-occur but cannot be distinguished based on morphology alone. An integrative approach that includes molecular methods is needed for hybrid detection and species assignment of ambiguous sampled plant material. The results from the third case study showed that both taxa have great potential to compete with each other and with other potentially invasive species. Ecological requirements of both taxa are overlapping in some lakes that were mapped and described throughout this thesis, which suggests that sympatric distribution and cryptic spread is likely in unknown areas. Due to the taxas' invasive growth and indicative properties, understanding this cryptic spread and hybridization of both taxa is crucial, but each have received little attention in current research. The following sections assess the power of the integrative approach and discusses the implications of results with regard to phylogenetics, ecological monitoring, and conservation or management strategies for *Najas marina* s.l.

6.1 Reasons for dynamics - high differences among but low diversity within the two taxa of *Najas marina* s.l.

Macrophytes are known for their spatiotemporal dynamics in species distribution and a major challenge for aquatic botanists is understanding the ecological and evolutionary processes driving adaptation and speciation in aquatic plants (Eckert et al., 2016; García-Girón et al., 2019; Wolfer & Straile, 2004).

The results of this thesis strongly support treating the two taxa as different species due to high genetic diversity in their ITS and *trn*L-F markers (Chapter 1). To date, taxonomic ranks have been underestimated by most researchers and plant identification keys and we thereby propose to refer to the two taxa as *N. major* (= *N. m.* subsp. *major*) and *N. marina* (= *N. m.* subsp. *marina*). It is likely that an ancient split between the two *Najas* lineages exists, given the substantial genetic differences found in the conservative nrDNA and chloroplast marker regions. This assumption is further supported by the fact that both taxa display distinct karyotypes that differ extensively (Viinikka, 1976). The new findings of the genetic division, demonstrated within this thesis, are not yet supported by most recent phylogenetic treatments (Bernardini & Lucchese, 2018; Ito et al., 2017). This fact indicates that the new species concept will take more time to be fully realized and established.

Another main finding of the study was the low infraspecific genetic variability observed within each taxon. Compared to the genetic distinctiveness between the taxa, the genetic uniformity of populations and individuals within each taxon was unexpectedly high for the ITS and chloroplast marker regions (Chapter 1). The assumption was that infraspecific variation in ITS would be similarly small as observed for groups that are often not distinguished from each other or only at the level of varieties or subspecies (Bräuchler et al., 2010, 2004; Gehrke et al., 2008). In general, the level of genetic variation can vary even between closely related taxa depending on the life-history traits, reproduction, dispersal, establishment, and survival requirements of a species (Barrett et al., 1993). For example, asexual reproduction in aquatic plants is considered to perpetuate genetic uniformity and to drive the potential of many widespread clonal invasive species to adapt to uniform and stable environments (Lambertini et al., 2010; Les, 1988; Les & Philbrick, 1993; Philbrick & Les, 1996). Therefore, the observed

genetic patterns could be related to the spread, hydrophily, dioecy, and the annual, sexual life cycle of both *Najas* taxa.

Both *Najas* taxa are known as obligate annuals and reproduce exclusively by sexually matured seeds (Triest, 1988). Sexual reproduction as a source of variation via genetic recombination is considered to promote genetic differences and should counteract genetic pauperization in aquatic plants (Les, 1988; Philbrick & Les, 1996). The dioecious life form and annual sexual reproduction followed by seed formation in *Najas* should theoretically lead to high levels of diversity within populations and low inter- populational differentiation (Barrett et al., 1993; Les, 1988; Les et al., 1997; Triest, 1991). This assumption was strengthened by significant isozyme polymorphism observed between populations and also between individuals in each of the taxa of *Najas* (Triest, 1989). Isozyme polymorphism does not always automatically indicate the presence of quantitative genetic variation, or vice versa (Barrett et al., 1993; Barrett & Shore, 1989), but usually DNA-based markers detect higher polymorphism than isozyme methods (Fernando & Cass, 1996; Hofstra et al., 2000). Still, for the clonal macrophyte *Potamogeton pectinatus* L. considerable variation on a genetic level was described compared to the monomorphism detected by isozyme studies (Mader et al., 1998). Recent evidence suggests that somatic mutations can help explain the occurrence of genetic diversity in sterile clonal populations (Barrett, 2015). When interpreting the genetic homogeneity observed within the two sexually reproducing *Najas* taxa, other evolutionary mechanisms such as founder effects, gene flow, outcrossing and inbreeding have to be taken into account.

For example, another factor associated with the annual, reproductive cycle of *Najas* promoting higher genetic diversity is dioecy. Dioecy prevents self-pollination and inbreeding and is typically associated with high outcrossing rates (Les, 1988). *Najas* is already known as an obligate outcrosser (Triest, 1991) which means that due to dioecy the transfer of pollen to a genetically different individual should lead to the broader exchange of genetic material and the production of genetically variable offspring (Barrett et al., 1993; Les, 1988; Tippery & Les, 2013). High levels of genetic diversity, such as isozyme polymorphism in outcrossing species, have been revealed in contrast to extensive areas of genetic uniformity in selfing, apomictic, and clonal species (Barrett & Shore, 1989). The comparison of genetic variation between dioecious species and other closely related taxa of *Najas* with different breeding systems could provide a better understanding of the consequences of dioecy and outcrossing on the genetic

variety of populations and individuals as done for the genus *Arabidopsis* (Wright et al., 2003). However, it is possible that the effects that outcrossing rates and inbreeding have on the genetic diversity in *Najas* are not being reflected in the ITS and chloroplast markers analyzed in this thesis, and thus other chloroplast or mitochondrial DNA markers may have to be examined.

What other factors could be responsible for the observed low genetic variation between and among the populations of the two *Najas* taxa? Gene flow is known to influence genetic variability, by the transfer of genetic material from one population to another which is possible within or also between taxa by the migration of individuals, dispersal of gametes, or recolonization after extinction (Barrett et al., 1993). High levels of connectivity can facilitate higher gene flow between lakes or lake systems and if gene flow is high, it can result in reduced genetic differentiation between populations by the homogenization of genomes. Local adaptation and the geographically isolated nature of habitats and populations are supposed to restrict gene flow and promote genetic differences between aquatic plant populations (Barrett et al., 1993; De Meester et al., 2002; Santamaría, 2002; Triest, 1991). Isozyme and DNA studies have indicated that the general mechanism of water pollination in hydrophilous species like *Najas* results in a more limited gene flow compared to wind pollination. This is because pollen or seed flow is prevented outside of the immediate surroundings of a water body and because of the directional transport of propagules in running waters (Barrett et al., 1993; Laushman, 1993; Les, 1988). Population differentiation within and among the two *Najas* taxa would theoretically be possible due to potentially low gene flow, the fact that the plants are not easily distributed, and that many populations are highly isolated (Triest, 1989). Molecular mechanisms like concerted evolution responsible for the homogenization of ITS tandem repeats could prevent detection of genetic divergence; so other methods like microsatellites or isozyme analysis might give better estimates of gene flow or historical changes in populations structure (Alice et al., 2001; Neigel, 1997).

Other potential reasons for the commonly observed low genetic variation within aquatic angiosperm populations are founder effects that can be associated with the long-distance dispersal of seeds or shoots of aquatic plants (Kliber & Eckert, 2005; Santamaría, 2002). Founder effects are defined as the loss of genetic diversity within a population resulting from the establishment of a small number of individuals (Roman & Darling, 2007). We know that the rapid colonization of *N. marina* s.l. plants at new localities is based on a small number of seeds

and determined by the genetic composition of those first immigrants (Triest, 1989). Invasion and colonization processes can involve the severe reduction in the demographic size of a population, also known as bottleneck, which results from habitat fragmentation and isolation, further reducing the genetic variability of the population (Roman & Darling, 2007). The observed patterns of genetic homogeneity within and among populations in each taxa of *Najas* could probably be the result of such bottlenecks and the founder principle. The negative effects of low population size and associated genetic losses can be overwhelmed by large propagule pools and multiple introduction events as observed for some invasive aquatic species (Roman & Darling, 2007). This can't be ruled out for the dispersal of *Najas* seeds since the continuous, annual repopulation of *Najas* plants at persisting locations is based on a large amount of seed (Triest, 1989). To draw legitimate conclusions about the role of bottlenecks or other supposed mechanisms responsible for low genetic variability, introduced populations (populations that spread to novel areas by humans) should be compared to native ones (Bossdorf et al., 2005). To show if *Najas* taxa have undergone a significant loss of genetic diversity, DNA from introduced, invasive like populations could be compared to 'native', historical or fossilized material (Roman & Darling, 2007).

What could be the molecular reasons for the low genetic differences in the ITS marker region analyzed in the studies? An explanation for the uniformity at the molecular level is, that concerted evolution could have resulted in homogenization of ITS tandem repeats within each taxon (Arnheim, 1983) through genomic mechanisms of turnover like gene conversion and unequal crossing over (Dover, 1994). Low genetic variation within ITS can also result if the rate of mutation among the copies is slower than the molecular forces driving the concerted evolution. In the ribosomal ITS marker, genetic uniformity is a consequence of the homogenization of the tandem repeats, followed by slow mutations rates, which was proposed to be the reason for observed low genetic diversity in several other aquatic plants such as *Aldrovanda* (Hoshi et al., 2006); *Ruppia* (Ito et al., 2013), or *Najas* (Les et al., 2013). The process of concerted evolution is known but still not fully understood and often confounds the interpretation of sequence polymorphism in the ITS marker (Álvarez & Wendel, 2003). Only by cloning efforts, is it possible to unveil divergent intragenomic copies and decomposing the alluded consensus sequence in ITS markers (Feliner & Rosselló, 2007). However, the ITS marker was sufficient for distinguishing the two *Najas* taxa and their hybrids in our case.

Diverse ecological factors have to be considered to play a role in shaping population genetic structure, and observed diversity in neutral molecular markers might not always reflect the genetic variation relevant to the ecological success of introduced populations (Bossdorf et al., 2005). To make any further assumptions about the genetic structure in *Najas* more information is required regarding the interactions among life history, ecology and mating system and how these govern genetic parameters.

6.2 Combining traditional and modern identification tools in macrophyte research - a useful approach?

The cryptic spread of species and hybridization are common in many aquatic plant genera (Kaplan & Fehrer, 2004; Les et al., 2009, 2004; Moody & Les, 2002; Prančl et al., 2014) and have been revealed for *Najas marina* s.l. in this study. Differences based on seed morphology are most significant as shown in Chapter 2 and can be used to distinguish the two taxa from each other. But differences in seed morphology are not adequate as a single distinguishing characteristic since reproductive traits are fully developed at the end of the vegetation period and only female individuals can be distinguished when bearing fruit. An easier and more reliable determination of taxa, especially when ambiguous and polymorphic specimens are collected, is the use of simple PCR-RFLP methods that target specific key sites of variation between the two *Najas* ITS ribotypes (Chapter 2).

One of the main reasons for the need of molecular identification methods for macrophyte species is phenotypic plasticity, which is the capacity of a given genotype to express different phenotypes in different environments due to ecological parameters (Sultan, 2000). Such morphological plastic responses were observed in response to mainly abiotic parameters such as temperature and light intensity (Eller et al., 2015; Hyldgaard & Brix, 2012) for instance in clonal, invasive species (Riis et al., 2010) and many other macrophyte species complexes, such as *Posidonia* (Campey et al., 2000); *Potamogeton* (Kaplan, 2002); *Chara* (Schneider et al., 2016), and *Ranunculus* (Garbey et al., 2004). Moreover, several studies showed that high phenotypic plasticity, not local adaption, such as adaptive genetic changes, are promoting the colonization success of macrophytes groups with low genetic diversity (Hoshi et al., 2006; Riis et al., 2010; Schneider et al., 2016; Szabó et al., 2019; Telford et al., 2011; Wolfer & Straile, 2004). Thus,

quantitative morphological traits might most likely be controlled by the combination of phenotypic plasticity and genetic differentiation (Gao et al., 2018). For this reason, macrophyte taxa require systematic morphological and genetic studies to assess the drivers of such morphological plastic responses.

There is no doubt about the significant role and usefulness of molecular data in the assessment of biodiversity, as they allow for correct species delimitation in cases of high phenotypic plasticity, detection of 'cryptic' diversity, and the recognition of hybridization events in the first place. The nuclear ribosomal transcribed spacer is a common marker used for phylogenetic studies (Baldwin et al., 1995) and can be used in combination with morphological data (Duminil & Di Michele, 2009). Besides known pitfalls, such as concerted evolution (Álvarez & Wendel, 2003), the ITS marker region has been proven effective in detecting taxon-specific differences in *Najas* and various other macrophyte genera (Ito et al., 2017; Les et al., 2006; Nguyen et al., 2014; Nowak et al., 2016).

Results demonstrated that, through subcloning interspecific hybrids (*N. major* × *N. marina*) retained both copies of ITS sequences, so-called ribotypes, inherited from their parental species (Chapter 1). Hybridization events have already been described for other taxa in the genus *Najas.* For example, the detection of the *N. flexilis* × *N. guadalupensis* subsp. *olivacea* hybrids by Les et al. (2010) was only possible by applying cloning techniques on chimeric sequences that often appear as 'noisy' sequencing artefacts (Les et al., 2015; Moody & Les, 2002). For the analysis of sequences and for the detection of possible hybrids, I want to emphasize the importance of cloning efforts, which, unfortunately, are infrequently applied in routine phylogenetic projects. Cloning is the most effective way to uncover intragenomic copies that might be concealed by the consensus sequence in the ITS marker(s) driven by concerted evolution and homogenization (Feliner & Rosselló, 2007).

One could argue that traditional taxonomic work and the use of morphological measurements to group and classify organisms are outdated by more accurate and reliable molecular methods. While NGS techniques are becoming more cost-efficient, and molecular-based classification is increasing, traditional taxonomy is in serious distress and the knowledge of experts and taxonomists is continuously disappearing (Boero, 2010). Traditional taxonomy involves type specimens and the designation and referencing of types specimens (typification) by the author who describes the taxon. It means that the type of name of the organism being studied is

referenced to certain physical specimen(s) (or illustration), known as the holotype (McNeill, 2014). This typification is crucial because it serves as a link between the information accumulated on the organisms and the organisms' correct names (Figueiredo & Smith 2015). In the course of this study, it was possible to combine morphological and molecular methods with taxonomic work to unravel the correct typification of the two *Najas* taxa as *N. major* and *N. marina* (see detailed discussion in Bräuchler, 2015). Any further research on the two *Najas* taxa will profit from the integrative approach presented in this thesis; and we recommend that future research should adopt the revised and correct typification of names.

Furthermore integrative taxonomy should intend to refer to a multi-character approach using a large number of characters including DNA sequences and other types of data such as morphology to delimit, discover, and identify meaningful, natural species and taxa at all levels (Will et al., 2005). The use of single genes as a "universal barcode" such as the ITS marker region (Li et al., 2011; Yao et al., 2010) corresponds to the return to an ancient, typological, single-character-system approach for identifying and describing species (Will et al., 2005). Especially in macrophyte research, classical phenotypic identification must be consistent with modern molecular analysis tools and identification of species to achieve the greatest taxonomic value out of an often-limited set of morphological characters (Barrett et al., 1993; Santamaría, 2002; Schneider et al., 2015). The usage of combined methods is necessary to overcome morphological reduction and uncover (hyper-) cryptic, or sibling species often present in even well-known (aquatic) plant genera (Adams et al., 2014; Les et al., 2013).

6.3 Consequences for *Najas marina* L. s.l. as an indicator species within WFD guidelines

The use of many widely distributed macrophytes as indicator organisms is limited because of the natural variability in their abundance and high phenotypic plasticity. Consequently, the determination of taxa often relies on expert judgment (Poikane et al., 2018; Schneider et al., 2016). The genetic division that was observed in the current thesis, is in contrast to the morphological and ecological similarities between the two *Najas* taxa. Both *Najas* taxa co-occur and thereby obviously share ecological niches, consequently ecological similarities combined

with high variability in morphological traits among populations and individuals are hampering the value of both taxa as indicator organisms.

The correct identification of taxa does becomes even more crucial when it comes to macrophyte species that are used as bioindicators and that are able to form natural hybrids because the presence of morphologically often intermediate and indistinguishable hybrids among their parental taxa further blurs species boundaries and complicates recognition in the field (Kaplan & Fehrer, 2004; Zalewska-Gałosz et al., 2015) as reported for example for *Callitriche* and *Potamogeton* (Martinsson, 1991; Prančl et al., 2014). The delimitation of indicative macrophyte taxa from each other and their hybrids is important since some of the taxa can be meaningful for monitoring ecological states of freshwaters and changes driven by climate change (Ejankowski & Lenard, 2015; Penning et al., 2008a) and their identification should be straightforward and reliable through observation of robust morphological and/or molecular characteristics.

N. marina s.l. serves as an example of a commonly distributed taxon, displaying high variability in morphology and ecological requirements (Chapters 2 & 3). Thus, the findings of this study call into question the current indicator classification of both taxa according to German WFD mapping procedures. Cryptic spread and hybridization, overlapping ecological ranges, as well as confounding morphological characters make it difficult to make a clear-cut delimitation of taxa in the field, especially when taxa grow sympatrically. Uncertainties in the identification of both *Najas* taxa have to be solved, and ecological factors influencing the spread have to be analyzed, for the further use of both *Najas* taxa as indicator organisms.

Wide geographical distribution and a high abundance are given for both taxa (Triest, 1989) which makes them theoretically good indicators, besides other assets (Diekmann, 2003; Ellenberg et al., 1992). However, any bias in the dataset used for the estimation of a species original indicator value could have resulted in a distorted analysis (Diekmann, 2003). Judging from the results presented here on the close morphological resemblance of both taxa, former studies (Doll, 1981; Pietsch, 1981) could have misidentified populations and wrongly estimated their niches and associated values. Ecological and biodiversity studies still rely on correct species identification and only molecular-based identification facilitates the correct delineation of *Najas* taxa when sample material is ambiguous at the moment. Great efforts are being made to achieve standardized and straightforward monitoring procedures.

In this context the comparability of survey results is crucial within the intercalibration process of the WFD concerning selected species, sampling techniques, data analysis, and evaluation (Moss, 2007; Poikane et al., 2018; Søndergaard et al., 2010). Consequences of potential errors in identification and recognition of individual species can have a larger impact when it comes to the collection of presence/absence data and when macrophytes are scarce or taxa richness is low. Assessment methods should use quantitative data where possible, but errors in the estimation of species abundance affect the metrics of macrophyte-based indices even more significantly (Dudley et al., 2013).

Most WFD implementation guidelines only address the broader taxon *N. marina* s.l. as an indicator (Greece, Zervas et al., (2018); EU in general, Poikane et al., (2018); UK, Willby et al., (2009); Belgium, Leyssen et al., (2005)) although both taxa are native in most European countries (Triest (1988), http://www.plantsoftheworldonline.org). Germany (Schaumburg et al., 2014, 2004) and Finland (Leka et al., 2008) are the only countries with more specific delineation of the indicative taxon. Even the WISER database, which aims to and intercalibrate Europe-wide monitoring procedures, only mentions *N. marina* as the indicative taxon (Kolada et al., 2012). Within the WISER intercalibration study, *N. marina* is assigned a good indicative value (Poikane et al., 2018), whereas within German WFD guidelines the presence of *N. marina* (referred to as *N. marina* subsp. *intermedia*) is influencing the ecological assessment more negatively (Schaumburg et al., 2014). As a consequence, these differing evaluations within WFD guidelines make it difficult to determine the influence that each *Najas* taxa has on the ecological assessment of lakes.

Further thorough monitoring accompanied by genetic analysis is needed in German lakes to elucidate the current spread and identify the drivers of the rapid radiation and formation of dominant plant stands of the two *Najas* taxa. The establishment and invasive spread of European *N. marina* in Ohio have been reported by Wentz & Stuckey (1971) due to eutrophication, rising water temperature, and anthropogenic influence. A combination of multiple causes is most likely, but temperature is one of the main factors driving the spread of this strongly thermophile macrophyte considering the temperature-dependent life cycle of the taxa (Hoffmann et al., 2013b; Hoffmann & Raeder, 2016). The spread of *N. marina* s.l. by temperature is supported also by macrorests found in sediment deposits from the Neolithic

Age which show a more northerly distribution of *Najas* during the climate optimum of the Atlantic period (Poschlod, 2015).

The known niches and the indicative values/status of both taxa (Doll & Pankow, 1989; Ellenberg et al., 1992; Pietsch, 1981) need a thorough revision and re-evaluation supported by molecular analysis including the analysis of their hybrids. The following approach is suggested in the case of mapping of ambiguous plant material and until the indicative status of both taxa can be confirmed:

1. Establishment of a standard molecular PCR-RFLP based identification within the German WFD mapping procedures, including vouchering of all samples as herbarium material and building a specimen library for each lake.
2. Genetic and morphological analysis of historic plant material from herbarium collections including lectotypes for different *Najas* taxa.
3. Collection of ecological parameters for each lake (e.g.: temperature, conductivity, nutrient loads, pH, etc.) and correlation of habitat preferences with sampled *Najas* populations and resulting genetic type i.e. ribotypes.
4. Adjustment of indicative values of taxa used for different European WFD guidelines based on the correlation of genetic and ecological data. Standardize WFD monitoring guidelines Europe-wide and regularly update results by publishing on online databases such as WISER (http://freshwaterecology.info).
5. Decide based on mapping of genotypes and their abundance if dominant stands have to be managed or if occurrences of both taxa and their hybrids have to be protected/conserved in respective lakes.

In case the identification and mapping procedures are not being standardized within the European WFD monitoring guidelines, the results cannot be compared between countries, and the indicative value of both taxa cannot be linked correctly to the ITS ribotypes/karyotypes. The collection of herbarium vouchers is therefore crucial to facilitate retrieval and verification of specimens as well as the reuse of the associated metadata (Schilthuizen et al., 2015) such as abundance, autecology and morphological variation of both taxa. Based on the mapping of genotypes and their abundance it must be decided whether dominant *Najas* stocks need to be managed or whether the occurrences of both taxa and their hybrids have to be protected to conserve rare genotypes.

6.4 Implications of hybridization for genetics, ecology and the spread of taxa

Results show a strong genetic differentiation coupled with incomplete reproductive isolation for the two lineages of *Najas.* Another main finding from this thesis is the molecular detection of naturally occurring hybrids between the two *Najas* taxa in German lakes and the genetic structure of ITS and *trn*L-F markers in those hybrids. Hybridization has a major role in evolution, and among related taxa it can range from the production of sterile offspring, through introgression of alleles into populations, to the formation of new species. Introgression involves the transfer of genes between species, mediated primarily by backcrossing (Rieseberg et al., 1993; Twyford & Ennos, 2012). Potential adaptive effects can result from introgression such as the transfer of important phenotypic traits between species, which may even lead to positive fitness in the recipient species (Suarez-Gonzalez et al., 2018). But no signs of any introgression or positive adaptive traits could be detected for the molecular or morphological markers in *Najas* plants analyzed in the studies. The probability for backcrossing of *Najas* parental species to their hybrids is low, due to hybrid infertility, but can't be ruled out so far.

Hybridization often leads to uncertainties in taxonomy and the blurring of species boundaries, which is common also in other macrophyte taxa given their variable breeding systems and life histories (Ito et al., 2010; Kabatova et al., 2014; Schneider et al., 2016; Whittall et al., 2004). Incomplete reproductive isolation could be an indication of an incomplete or still ongoing speciation mechanism and/or a lack of ecological speciation, a process where natural selection drives the evolution of reproductive incompatibility (Nosil et al., 2009). In the case of *N. marina* s.l. it is assumed that taxa are stable, and hybridization results rather in reduced fitness of parental taxa, caused by the infertility of F_1 progenitors/hybrids. The reproductive isolation of both taxa is further assumed to result from, or is accompanied by, extensive chromosomal rearrangements (Peredo et al., 2011; Viinikka, 1976; Winge, 1927).

Spontaneous hybridization is rare among hydrophilic species (Les & Philbrick, 1993) and is not ubiquitous among plant families (Ellstrand et al., 1996). Notably, the frequency of spontaneous natural hybridization is most common among outcrossing species because outcrossing mechanisms favor the formation of hybrids (Rieseberg, 1997). When vegetative reproduction, permanent odd polyploidy, or apomixes follow such hybridization events, hybrids can thereby

be stabilized in aquatic plant populations (Ellstrand et al., 1996; Les & Philbrick, 1993). In other aquatic plants, differences in flowering time prevent hybridization and maintain reproductive isolation of sympatric cryptic lineages as described for *Juncus effusus* L. (Michalski & Durka, 2015). In the case of the two *Najas* taxa, differences in flowering time could not be maintained in some lakes, which is likely attributable to ecological factors like temperature. It can, therefore, be concluded that the combination of enforced outcrossing through dioecy, range overlap, and similar floral morphology contributed to hybridization between *Najas* taxa similar to North American *Nymphoides* (Tippery & Les, 2013).

Ecological preferences for both *Najas* species are overlapping for some lakes in the Bavarian Alpine Foreland, such as Lake Staffelsee. In that lake, both *Najas* taxa can populate the same niches, proliferate, and even hybridize as demonstrated in this thesis (Chapters 1 & 2). Other hybrids that originate from a lake in the pre-alpine region, Lake Sempach in Switzerland, have already been described by Triest (1991). He observed that *N. marina* flowers, yields fruit, and decays earlier than *N. major* and concluded from isozyme patterns that the detected hybrids were formed by male *N. marina* plants pollinating female *N. major* plants, although the reverse crossing was not ruled out. Triest concluded that a shift in the flowering period promotes a one-way gene flow from *N. marina* to *N. major* plants (Triest, 1991). Not all populations that grow sympatrically generate hybrids; the Nemitzsee and the Tegelersee in the region north of Berlin (Triest, 1989) and the Lakes Waginger-Tachinger in the South of Bavaria (Rüegg et al., 2017) are salient examples where mixed populations are known to exist. Regarding the latter, although molecular detection methods are usually sufficient for hybrid identification, morphological overlap and the lack of knowledge about frequency and distribution of naturally occurring *Najas* hybrids makes the sampling stage a game of chance. To avoid the omission of hybrids plants that truly exist in sympatric regions, a more systematic procedure has to be developed to optimize sampling patterns and techniques.

Regarding the notion that the formation of hybrids was not possible, Triest proposed that different flowering times cause reproductive isolation and form a barrier to hybridization for the two *Najas* taxa in the Alp region (Triest, 1991). We also propose that environmental conditions, and primarily water temperature, play a significant role in the life cycle of both *Najas* taxa and in facilitating simultaneous flowering and hybridization. Judging from the results of this thesis and of other studies (Hoffmann et al., 2014a; Hoffmann & Raeder, 2016;

Poschlod, 2015) it seems probable that the spread of both taxa is promoted mostly by rising temperatures. However, other abiotic factors such as light and nutrient conditions might also be influencing the life cycle, spread, and hybridization of *Najas* taxa. Presently, we can be sure that the two *Najas* taxa co-exist and that they differ from each other extensively in terms of their karyotype (A and B karyotypes); these karyological differences most probably result in sterility of hybrid caused by chromosomal structural rearrangements that prevent normal meiosis (Peredo et al., 2011; Triest, 1989). Further ecological and chromosomal analysis of the two taxa and hybrid plant populations in *Najas* would be necessary to evaluate if ecological factors are more important in speciation and the maintenance of hybrid zones than genomic incompatibility *per se* (Jiggins & Mallet, 2000).

Other possible mechanisms of dispersal, such as apomixis, have been discussed for *Najas* in Britain (Handley & Davy, 2005), but this mode of reproduction is likely not the cause for the maintenance of the detected annual hybrid populations in Lake Staffelsee. Seed formation was highly frequent in almost every female plant, and both flowering male and female plants were always present in surveys at Lake Staffelsee. Agami et al. (1986) described autotetraploid *N. marina* plants from Israel building shoots that carry dormant, perennating, vegetative buds, which overwinter at temperatures below 13 °C and should thereby be regarded as turions. It is unclear if reproduction and dispersal by turions or apomixis are facultative in *Najas*, but it could become more frequent if seed maturity and dormancy can no longer be achieved due to changing climatic conditions, and when milder, shorter, and ice-free winters are becoming more frequent (Carpenter et al., 1992; Mooij et al., 2005).

More information on natural occurring hybrid populations of *Najas* has to be collected first to clarify the fertility of hybrid plants and whether they are able to interbreed or backcross with parents, which could lead to introgressive hybridization and/or an advanced vigor of hybrid plants (Ellstrand & Schierenbeck, 2006). Aggressively growing populations of *Najas* hybrids have been reported for American *Najas flexilis* × *N. guadalupensis* by Les et al. (2010) leading to a potential conservation threat. Currently, an invasive spread of hybrid plants does not seem possible, based on their low detection rate and due to their infertility. Although hybridization of the two *Najas* taxa might occur more often than recognized so far as small lentic ecosystems (≤10 ha) are still under-represented in WFD surveys (Bolpagni et al., 2019). No germination experiments were carried out in this study with hybrid seeds discovered in the Staffelsee, but

it is known from previous studies that hybrid seeds are normally infertile (Triest, 1991; Viinikka, 1976). Distribution of any possible fertile hybrid seed by carp, waterfowl, or by other natural long-distance dispersal outside a water body cannot be ruled out (Agami & Waisel, 1988, 1986). However, molecularly proven hybrid plants discovered in Staffelsee and those reported by Triest (1989, 1991) in Gnadensee are assumed to result from two different hybridization events. Hybrid plants seem to form newly each year in each lake and grow in the same niches as parental plants.

Since both taxa are currently spreading, the endangered redlist status of *N. marina* s.l. in Germany (Korneck et al., 1996) should be reconsidered, at least at a regional level (e.g., in Bavaria). If dominant plant stocks of either one of the taxa are detected, the management of dominant and invasive-like populations can be achieved by jute matting as proposed by Hoffmann et al. (2013a). Known sympatric sites should also be monitored consistently on a genetic level to facilitate detection, and correct determination of possible dominant, invasive like, or hybrid populations of *Najas*. A combined monitoring approach using integrative methods is a necessary prerequisite for assessing the consequences of hybridization and cryptic spread of plants and for making informed decisions about conservation or management decisions. Further this approach will also aid in achieving a holistic view of *Najas marina* s.l., including its phylogeny, functional morphology, behavior, and ecology in order to better understand the role of certain macrophyte species within the aquatic ecosystem in general.

6.5 Outlook

By using an integrative approach, genetic, morphological, and ecological differences of the two macrophyte taxa *N. marina* and *N. major* were described in more detail in this study and the approach described here could serve as a model for other critical or cryptic macrophyte genera/groups that are used for monitoring purposes within the WFD and elsewhere. The results of this thesis also give first insights on the occurrences and dynamics of sympatrically growing macrophyte populations with possible hybrid formation and should aid to further optimize sampling and screening strategies within monitoring programs. The two *Najas* taxa serve as rare examples of annual, hydrophilous and dioecious macrophytes and the integration of knowledge about biotic and abiotic factors controlling ecosystem properties is required to understand how aquatic plant communities are structured and the forces driving the spread and invasion of species.

The RFLP-PCR analysis presented in this thesis, helped substantially to identify problematic or doubtful *Najas* plant material either from newly discovered habitats or from historic herbarium collection. All physical *Najas* specimens collected are therefore primary data and should be properly labelled and stored in a publicly-accessible collection for example at a natural history museum for retrieval, verification, and adding additional information (Schilthuizen et al., 2015). For future research on *Najas* or other phenotypically plastic macrophyte taxa, are state-of-the-art molecular identification techniques and genetic markers will have to be considered to discriminate and annotate ambiguous plant material. Other genetic markers like microsatellites or "Simple Sequence Repeats" (SSR) provide higher genome coverage and can be applied in further molecular research on *Najas* to find out more about evolutionary mechanisms in both taxa. In particular, intragenomic variation within multigene families other than ITS could give a better understanding of genetic differences among and within populations and/or individuals of *Najas*. Moreover, quantitative trait locus (QTL) mapping can be used as a first step to identify broad genomic regions that contribute to phenotypic differences (Stern, 2013).

Results gained from mesocosm experiments and WFD mapping indicated that both taxa have a broader ecological niche and adaptive potential than initially expected. This indicates that basic experiments with thresholds on candidate ecological factors such as nutrient loads or

light quality should be performed. Future mesocosm studies should simulate different timings of disturbances events within the vegetation period (spring vs. summer) and the duration and intensity of such turbidity events could be manipulated.

The indicative value of both taxa should be approached with caution until the ecological niches of both species have been confirmed and matched with molecular results. Additional studies on biogeography and the evolutionary origin of both taxa could be helpful to understand the current spread and the future role of those hybridizing species. Analysis of other hybrid populations should be extended to the locations cited throughout this thesis. Experiments on the fertility of hybrids will contribute to a better understanding of their role within the aquatic ecosystem and the consequences of their persistence. The possibilities offered by environmental DNA sampling tools as used for the detection and surveillance of invasive aquatic plant species such as *E. densa* (Scriver et al., 2015) could offer a significant advantage to the pre-screening of lakes for possible cryptic co-occurrence of both taxa and hybrid plants of *Najas*.

7 Publication list and author contributions

The following peer-reviewed publications were included in this thesis:

Rüegg, S., Raeder, U., Melzer, A., Heubl, G., Bräuchler, C., 2017. Hybridisation and cryptic invasion in *Najas marina* L. (Hydrocharitaceae)? Hydrobiologia 784, 381-395. DOI: 10.1007/s10750-016-2899-z

U.R. and A.M. conceived and designed the analysis and choose the study area, S.R., G.H. and C.B. performed the molecular analysis, contributed data and helped interpret the analysis, S.R., U.R., A.M., G.H. and C.B. wrote the paper.

Rüegg S., Bräuchler C., Geist J., Heubl G., Melzer A., Raeder U., 2019. Phenotypic variation disguises genetic differences among *Najas major* and *N. marina*, and their hybrids. Aquatic Botany 153:15-23. DOI: 10.1016/j.aquabot.2018.11.005

S.R., C.B., U.R., A.M., and J.G. conceived and designed the analysis, S.R., G.H. and C.B. performed the molecular analysis, S.R performed the statistical analysis and interpreted the data, S.R., C.B., J.G., G.H., A.M. and U.R. wrote the paper.

Rüegg, S.*, Hoffmann, M.*, Geist, J., Raeder, U. 2019. *Najas marina* and *N. major* benefit from low light conditions caused by climate change in competition with native and alien invasive macrophytes; in prep., *Equal contributing authors

M.H., S.R, J.G. and U.R. conceived the study; M.H. and S.R. designed and performed the experiments, including the collection of growth data; M.H. and S.R. performed the statistical analysis and interpreted the data; M.H., S.R., U.R. and J.G. wrote the paper

Oral contributions related to the PhD thesis:

Rüegg, S., Raeder, U. Bräuchler, C., 2017. Aktuelle Makrophytenentwicklung in deutschen Gewässern Prognosen und Handlungsbedarf. Einsatz molekularbiologischer Methoden für das Monitoring, Makrophytenworkshop April 26-28, Limnologische Station Iffeldorf, Germany.

Rüegg, S., Raeder, U., Bräuchler, C., 2016. Möglichkeiten und Grenzen der Makrophytenbestimmung mit klassischen und molekular-genetischen Methoden am Beispiel von *Najas marina* und *Najas major*. Jahrestagung der Deutschen Gesellschaft für Limnologie und der deutschsprachigen Sektionen der SIL, September 26-30, Vienna, Austria.

Rüegg, S., Raeder, U., Bräuchler, C., 2016. Ausbreitung des Großen Nixkrautes (*Najas marina*) in Deutschland – ein Erfolg für die WRRL? Jahrestagung der Deutschen Gesellschaft für Limnologie und der deutschsprachigen Sektionen der SIL, September 21-25, University Duisburg-Essen, Germany.

Rüegg, S., Hoffmann, M., Raeder, U., Fresh Blood for and Fresh Water Meeting 2015. Lakes during climate change - How enhanced flooding changes light conditions and its' influence on macrophyte vegetation. Research Institute for Limnology in Mondsee & University of Innsbruck, April 15-17, Mondsee, Austria.

Poster presentations:

Rüegg, S., Sebald, S., Hoffmann, M., 2017. How changed light conditions influence certain macrophyte species, 3rd International Conference, Water resources and wetlands (WRW), September 8-10, Tulcea, Romania.

8 Abbreviations

Comment: Abbreviations of plant name authors and journal titles as well as herbarium acronymes are not listed because, in accordance with common practice, they follow the standards given in IPNI (2020) and Index Herbariorum (Holmgren & Holmgren, 1998).

auct.	*auctorum*, Latin for 'of authors'
AFLP	amplified fragment length polymorphism
ANOVA	analysis of variance
App.	Appendix
app.	approximately
bdH2O	bi(double)distilled water
bp	base pairs
BQEs	biological quality elements
BSA	bis(trimethylsilyl)acetamide
cpDNA	chloroplast DNA
CVA	canonical variate analysis
DNA	deoxyribonucleic acid
DOC	dissolved organic carbon
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
e.g.	*exempli gratia*, Latin for 'for example'
EPPO	European and Mediterranean Plant Protection Organization
et al.	*et alia*, Latin for 'the others'; abbreviation for author citation
etc.	*et cetera*, Latin for 'and others'
HCl	hydrogen chloride
HindIII	restriction endonuclease isolated from *Haemophilus influenzae*
HSD	honestly significant difference
IAS	invasive alien species
IBC	intermediate bulk container
i.e.	*id est*, Latin for 'that is to say'
KCl	potassium chloride
km	kilometer, 1000 m
L	liter
LB	lysogeny broth
LD	linear discriminant
LDA	linear discriminant analysis
LMU	Ludwig-Maximilian-Universität (München)
LWB	leave width broad

LWN	leave width narrow
MAFFT	multiple alignment using fast Fourier transform (multiple sequence alignment program)
MCMC	Markov-chain-Monte-Carlo
mm	millimeter, 10^{-3} m
matK	coding region of the chloroplast genome
NaCl	sodium chloride
NGS	next generation sequencing
nM	nanomolar, 10^{-9} mol/L
nrITS	nuclear ribosomal internal transcribed spacer
OD	optical density
POD	peroxidase, group of enzymes that break up peroxides
PCR	polymerase chain reaction
pp	posterior probability
Pp.	pages
rbcL	coding region of the chloroplast genome
RFLP	restriction fragment length polymorphism
rpoB/*rpoC1*	coding regions of the chloroplast genome
RT	room temperature
s.l.	*sensu lato*, Latin meaning 'in the broad sense'
SCUBA	self-contained underwater breathing apparatus
SD	standard deviation
SE	standard error
SkDH	shikimate dehydrogenase, enzyme that catalyzes one step of the shikimate pathway
sp./spp.	species, singular/plural, Latin for 'species'
ssp./subsp.	subspecies, alternative abbreviations used, Latin for 'subspecies'
SPM	suspended particulate matter
Tab.	Table
Tris	tris(hydroxymethyl)-aminomethane
*trn*L-F	intergenic spacer region of the chloroplast genome
TUM	Technical University Munich
UVC	ultraviolet C (100-280 nm)
WFD	water framework directive
µL	microliter, 10^{-6} L

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Appendix

All appendices (A1-A3) given here are also available online as electronic supplementary material with the respective publications/chapters.

Appendix A1

List of Taxa included in this study. Samples are sorted by taxa (1st *N. marina*, 2nd subspecies of *N. marina*, 3rd other *Najas* species (alphabetical), within each taxon samples are sorted alphabetical by country. Herbarium abbreviations are according to Holmgren & Holmgren (1998). Running numbers are as follows: (1) Peredo et al. (2013), (2) Na, H.R. & Choi, H.-K. published in GenBank, (3) Les et al. (2010). Generic concept according to Triest (1988), Data entry for each sample follows the following scheme: number of sequence in *trn*L-F/ ITS alignment, taxon/species, collection data information, ITS type (A/B/X= outgroup sequences), *trn*L-F type (A/B/X), GenBank accession numbers

Bold accessions are marked as localities were *Najas* populations have not been monitored before,
grey shading indicates fresh obtained plant material treated with the DNeasy® Plant Kit (Qiagen, Venlo, Netherlands) following the manufacturer's protocol. Additional steps included homogenization of plant material using ZrO_2-beads and a micro-dismembrator II (Bachofer, Reutlingen, Germany).

* Herbarium plant material was treated with the NucleoSpin® Plant-Kit (Macherey-Nagel, Düren, Germany) following the manufacturer's protocol.

** GenBank accessions.

*** Sequence submitted, but not included in alignment

[1] ITS-1 missing: 160, 137;

[1*] ITS-1 incomplete: 4, 38, 119, 123, 127, 133, 135, 138, 140, 142, 143, 144, 145, 149, 154, 156, 157, 161, 163, 173, 174

[2] ITS-2 incomplete: 1, 6, 96

# in *trn* L-F alignment	# in ITS alignment	Taxon / species	Collection data (country, district, lake/site; date; collector; voucher number; collection number; acronym)	ITS type	*trn* L-F type	GenBank accession number ITS	GenBank accession number *trn* L-F	Georeference (lat)	Georeference (long)
	1	*Najas marina* L.	Albania, Skutari Lake; 1928-07-21; Schütt, B.; B-10-0113347; B * [2]	A		KT596444		42.138983°	19.457516°
1	2	*N. marina*	Austria, Salzburg Land, Mattsee; 2012-08-06; Rüegg S.; 26-01-03.61; TUM	A	A	KT596445	KT596620	47.968289°	13.080669°
2	3	*N. marina*	Austria, Salzburg Land, Obertrumer See; 2012-08-06; Rüegg S.; 26-01-02.51; TUM	B	B	KT596446	KT596621	47.977356°	13.106586°
	4	*N. marina*	Finnland, Aland Islands, Ramsholmcu-Möckelö; 1961-08-27; Scholz, H.; B-10-0306876; 2653b; B *	A		KT596447		60.166473°	19.911094°
	5	*N. marina*	France, Dombes, Pond near Villars-es-Dombes; 1986-08-14; Angerer, O.; M-0158736; M *	A		KT596448		46.002203°	5.017698°
3	6	*N. marina*	France, Mareuil-Caubert; 1988-09-11; Lambinon, J.; 88/308; Soc Ech. 14720; B * [2]	A	A	KT596449	KT596622	50.078356°	1.821615°
	7	*N. marina*	Germany, Baden-Württemberg, Bodensee; 2010-07-23; Wutz K. ; 26-01-02.5; TUM	B		KT596450		47.696606°	9.073502°
4	8	*N. marina*	Germany, Baden-Württemberg, Bodensee; 2012-08-30; Rüegg S., Hippich M.; 26-01-02.52; TUM [1*]	B	B	KT596451	KT596623	47.666611°	9.218261°
	9	*N. marina*	Germany, Baden-Württemberg / Bavaria, Degersee; 2010-08-31; Wutz K.; 26-01-03.2; TUM	A		KT596452		47.610045°	9.656357°
	10	*N. marina*	Germany, Baden-Württemberg, Mindelsee; 2010-08-23; Wutz K.; 26-01-03.6; TUM	A		KT596453		47.753548°	9.016367°
	11	*N. marina*	Germany, Baden-Württemberg, Muttelsee; 2010-08-31; Wutz K.; TUM	A		KT596454		47.618584°	9.668209°
	12	***N. marina***	**Germany, Bavaria / Hesse, See Emma Nord; 2010-08-24; Wutz K.; TUM**	**A**		**KT596455**		**50.075660°**	**8.998755°**
5	13	*N. marina*	Germany, Bavaria / Hesse, See Emma Nord; 2011-08-04; Schümann M.; 26-01-03.20; TUM	A	A	KT596456	KT596624	50.075103°	8.997347°
	14	***N. marina***	**Germany, Bavaria, Abtsdorfer See; 2010-09-08; Wutz K.; 26-01-03.1; TUM**	**A**		**KT596457**		**47.916798°**	**12.902125°**
6	15	*N. marina*	Germany, Bavaria, Abtsdorfer See; 2011-07-28; Schümann M.; 26-01-03.13; TUM	A	A	KT596458	KT596625	47.914986°	12.904111°
	16	*N. marina*	Germany, Bavaria, Ammersee; 2010-08-26; Wutz K.; 26-01-02.3; TUM	B		KT596459		48.001752°	11.099192°
7	17	*N. marina*	Germany, Bavaria, Ammersee; 2011-08-16; Schümann M.; 26-01-02.22; TUM	B	B	KT596460	KT596626	48.069474°	11.110245°
	18	*N. marina*	Germany, Bavaria, Chiemsee, Aiterbacher Winkel; 2010-08-28; Wutz K. ; 26-01-02.6; TUM	B		KT596461		47.875782°	12.354595°
8	19	*N. marina*	Germany, Bavaria, Chiemsee; 2011-08-23; Schümann M. ; 26-01-02.23; TUM	B	B	KT596462	KT596627	47.882633°	12.349392°
	20	***N. marina***	**Germany, Bavaria, Donaustauf; 2010-09-28; Wutz K.; 26-01-03.3; TUM**	**A**		**KT596463**		**48.939661°**	**12.518887°**
9	21	*N. marina*	Germany, Bavaria, Donaustauf ; 2011-08-16; Schümann M. ; 26-01-03.14; TUM	A	A	KT596464	KT596628	49.025919°	12.200375°
10	22	*N. marina*	Germany, Bavaria, Eggstätt-Hemhofer Seenplatte, Hartsee; 2011-07-11; Schümann M. ; 26-01-02.24; TUM	A	A	KT596465	KT596629	47.929064°	12.371114°
	23	*N. marina*	Germany, Bavaria, Eggstätt-Hemhofer Seenplatte, Langbürgner See; 2010-09-01; Wutz K. ; 26-01-02.7; TUM	B		KT596466		47.910346°	12.347376°
11	24	*N. marina*	Germany, Bavaria, Eggstätt-Hemhofer Seenplatte, Langbürgner See; 2011-07-11; Schümann M.; 26-01-02.27; TUM	B	B	KT596467	KT596630	47.904647°	12.344878°
12	25	*N. marina*	Germany, Bavaria, Eggstätt-Hemhofer Seenplatte, Kesselsee; 2011-07-14; Schümann M.; 26-01-02.25; TUM	B	B	KT596468	KT596631	47.916111°	12.353625°
13	**26**	***N. marina***	**Germany, Bavaria, Eggstätt-Hemhofer Seenplatte, Pelhamersee; 2011-07-05; Schümann M.; TUM**	**B**	**B**	**KT596469**	**KT596632**	**47.933228°**	**12.343783°**
	27	*N. marina*	Germany, Bavaria, Eggstätt-Hemhofer Seenplatte, Pilsensee; 2011-07-22; Schümann M. ; 26-01-02.28; TUM	B		KT596470		48.024019°	11.196553°
	28	*N. marina*	Germany, Bavaria, Eggstätt-Hemhofer Seenplatte, Pilsensee; 2010-08-16; Wutz K. ; 26-01-02.8; TUM	B		KT596471		47.929431°	12.345086°
14	29	*N. marina*	Germany, Bavaria, Eggstätt-Hemhofer Seenplatte, Schloßsee; 2011-07-14; Schümann M. ; 26-01-02.29; TUM	B	B	KT596472	KT596633	47.913297°	12.343394°
15	**30**	***N. marina***	**Germany, Bavaria, Groß Weicheringer See; 2011-08-29; Schümann M.; 26-01-03.17; TUM**	**A**	**A**	**KT596473**	**KT596634**	**48.702861°**	**11.322433°**
	31	***N. marina***	**Germany, Bavaria, Großer Brombachsee; 2010-09-28; Wutz K.; 26-01-03.5; TUM**	**A**		**KT596474**		49.133584°	10.961295°
16	32	*N. marina*	Germany, Bavaria, Großer Brombachsee; 2011-08-10; Schümann M. ; 26-01-03.15; TUM	A	A	KT596475	KT596635	49.132189°	10.960203°
	33	*N. marina*	Germany, Bavaria, Großwelzheimer Badesee; 2010-08-24; Wutz K.; TUM	A		KT596476		50.058403°	9.014778°
17	**34**	***N. marina***	**Germany, Bavaria, Großwelzheimer Badesee; 2011-08-04; Schümann M.; 26-01-03.16; TUM**	**A**	**A**	**KT596477**	**KT596636**	**50.058389°**	**9.015919°**
	35	***N. marina***	**Germany, Bavaria, Hödenauersee; 2012-08-25; Rüegg S.; 26-01-02.64; TUM**	**A**		**KT596478**		**47.625242°**	**12.192633°**
	36	***N. marina***	**Germany, Bavaria, Klein Weicheringer See; 2011-08-29; Schümann M.; 26-01-03.18; TUM**	**A**		**KT596479**		**48.704844°**	**11.323031°**
18	37	*N. marina*	Germany, Bavaria, Klostersee bei Seeon; 2011-07-25; Schümann M.; 26-01-02.26; TUM	B	B	KT596480	KT596637	47.975447°	12.447653°

# in *trn* L-F alignment	# in ITS alignment	Taxon / species	Collection data (country, district, lake/site; date; collector; voucher number; collection number; acronym)	ITS type	*trn* L-F type	GenBank accession number ITS	GenBank accession number *trn* L-F	Georeference (lat)	Georeference (long)
	42	***N. marina***	**Germany, Bavaria, See Freigericht-Ost; 2010-08-24; Wutz K.; 26-01-03.7; TUM**	**A**		**KT596485**		50.082776°	9.008973°
	43	*N. marina*	Germany, Bavaria, Simssee; 2010-09-01; Wutz K.; 26-01-02.11; TUM	B		KT596486		47.887072°	12.245457°
21	44	*N. marina*	Germany, Bavaria, Simssee; 2011-07-28; Schümann M.; 26-01-02.30; TUM	B	B	KT596487	KT596640	47.858761°	12.226958°
	45	*N. marina*	Germany, Bavaria, Soyensee; 2010-09-01; Wutz K.; 26-01-03.8; TUM	A		KT596488		48.106138°	12.206744°
22	46	*N. marina*	Germany, Bavaria, Soyensee; 2011-07-25; Schümann M.; 26-01-03.21; TUM	A	A	KT596489	KT596641	48.106139°	12.206744°
	47	*N. marina*	Germany, Bavaria, Staffelsee; 2010-09-05; Wutz K.; T 1.2; 26-01-03.10, TUM	A		KT596490		47.683376°	11.184469°
	48	*N. marina*	Germany, Bavaria, Staffelsee; 2010-09-05; Wutz K.; T 2; 26-01-02.12, TUM	B		KT596491		47.701471°	11.159210°
	49	*N. marina*	Germany, Bavaria, Staffelsee; 2011-08-17; Schümann M.; A1; 26-01-05.8, TUM	A		KT596492		47.709936°	11.171094°
23	50	*N. marina*	Germany, Bavaria, Staffelsee; 2011-08-18; Schümann M.; B1; 26-01-05.8, TUM	B	B	KT596493	KT596642	47.709936°	11.171094°
	51	*N. marina*	Germany, Bavaria, Staffelsee, 1.3; 2012-08-23; Rüegg S.; 26-01-03.25; TUM	A		KT596494		47.678614°	11.176433°
	52	*N. marina*	Germany, Bavaria, Staffelsee, 1.4; 2012-08-23; Rüegg S.; 26-01-03.26; TUM	A		KT596495		47.678614°	11.176433°
	53	*N. marina*	Germany, Bavaria, Staffelsee, 1.5; 2012-08-23; Rüegg S.; 26-01-03.27; TUM	A		KT596496		47.678614°	11.176433°
	54	*N. marina*	Germany, Bavaria, Staffelsee, 2.1; 2012-08-23; Rüegg S.; 26-01-03.28; TUM	A		KT596497		47.678064°	11.155497°
	55	*N. marina*	Germany, Bavaria, Staffelsee, 2.2; 2012-08-23; Rüegg S.; 26-01-03.29; TUM	A		KT596498		47.678064°	11.155497°
	56	*N. marina*	Germany, Bavaria, Staffelsee, 2.3; 2012-08-23; Rüegg S.; 26-01-03.30; TUM	A		KT596499		47.678064°	11.155497°
	57	*N. marina*	Germany, Bavaria, Staffelsee, 2.4; 2012-08-23; Rüegg S.; 26-01-03.31; TUM	A		KT596500		47.678064°	11.155497°
	58	*N. marina*	Germany, Bavaria, Staffelsee, 2.5; 2012-08-23; Rüegg S.; 26-01-03.32; TUM	A		KT596501		47.678064°	11.155497°
	59	*N. marina*	Germany, Bavaria, Staffelsee, 3.1; 2012-08-23; Rüegg S.; 26-01-03.33; TUM	A		KT596502		47.682334°	11.159234°
	60	*N. marina*	Germany, Bavaria, Staffelsee, 3.2; 2012-08-23; Rüegg S.; 26-01-03.34; TUM	A		KT596503		47.682334°	11.159234°
	61	*N. marina*	Germany, Bavaria, Staffelsee, 3.3; 2012-08-23; Rüegg S.; 26-01-03.35; TUM	A		KT596504		47.682334°	11.159234°
	62	*N. marina*	Germany, Bavaria, Staffelsee, 3.4; 2012-08-23; Rüegg S.; 26-01-03.36; TUM	A		KT596505		47.682334°	11.159234°
	63	*N. marina*	Germany, Bavaria, Staffelsee, 3.5; 2012-08-23; Rüegg S.; TUM	A		KT596506		47.682334°	11.159234°
	64	*N. marina*	Germany, Bavaria, Staffelsee, 4.1; 2012-08-23; Rüegg S.; 26-01-02.41; TUM	B		KT596507		47.687064°	11.16427°
24	65	*N. marina*	Germany, Bavaria, Staffelsee, 4.2; 2012-08-23; Rüegg S.; 26-01-02.42; TUM	B	B	KT596508	KT596643	47.687064°	11.16427°
	66	*N. marina*	Germany, Bavaria, Staffelsee, 4.3; 2012-08-23; Rüegg S.; 26-01-03.37; TUM	A		KT596509		47.687064°	11.16427°
	67	*N. marina*	Germany, Bavaria, Staffelsee, 4.4; 2012-08-23; Rüegg S.; 26-01-03.38; TUM	A		KT596510		47.687064°	11.16427°
	68	*N. marina*	Germany, Bavaria, Staffelsee, 5.1; 2012-08-23; Rüegg S.; 26-01-03.39; TUM	A		KT596511		47.688088°	11.132762°
	69	*N. marina*	Germany, Bavaria, Staffelsee, 5.2; 2012-08-23; Rüegg S.; 26-01-03.40; TUM	A		KT596512		47.688088°	11.132762°
	70	*N. marina*	Germany, Bavaria, Staffelsee, 5.3; 2012-08-23; Rüegg S.; 26-01-03.41; TUM	A		KT596513		47.688088°	11.132762°
	71	*N. marina*	Germany, Bavaria, Staffelsee, 5.4; 2012-08-23; Rüegg S.; 26-01-03.42; TUM	A		KT596514		47.688088°	11.132762°
	72	*N. marina*	Germany, Bavaria, Staffelsee, 5.5; 2012-08-23; Rüegg S.; 26-01-03.43; TUM	A		KT596515		47.688088°	11.132762°
	73	*N. marina*	Germany, Bavaria, Staffelsee, 6.1; 2012-08-23; Rüegg S.; 26-01-03.44; TUM	A		KT596516		47.697183°	11.140881°
	74	*N. marina*	Germany, Bavaria, Staffelsee, 6.2; 2012-08-23; Rüegg S.; 26-01-03.45; TUM	A		KT596517		47.697183°	11.140881°
	75	*N. marina*	Germany, Bavaria, Staffelsee, 6.3; 2012-08-23; Rüegg S.; 26-01-02.43; TUM	B		KT596518		47.697183°	11.140881°
	76	*N. marina*	Germany, Bavaria, Staffelsee, 6.4; 2012-08-23; Rüegg S.; 26-01-02.44; TUM	B		KT596519		47.697183°	11.140881°
	77	*N. marina*	Germany, Bavaria, Staffelsee, 6.5; 2012-08-23; Rüegg S.; 26-01-03.46; TUM	A		KT596520		47.697183°	11.140881°
	78	*N. marina*	Germany, Bavaria, Staffelsee, 7.1; 2012-08-23; Rüegg S.; 26-01-03.47; TUM	A		KT596521		47.693161°	11.154067°
	79	*N. marina*	Germany, Bavaria, Staffelsee, 7.3; 2012-08-23; Rüegg S.; 26-01-02.45; TUM	A		KT596522		47.693161°	11.154067°
25	80	*N. marina*	Germany, Bavaria, Staffelsee, 7.4; 2012-08-23; Rüegg S.; 26-01-03.48; TUM	A	A	KT596523	KT596644	47.693161°	11.154067°
	81	*N. marina*	Germany, Bavaria, Staffelsee, 7.5; 2012-08-23; Rüegg S.; 26-01-02.46; TUM	B		KT596524		47.693161°	11.154067°
	82	*N. marina*	Germany, Bavaria, Staffelsee, 8.1; 2012-08-23; Rüegg S.; 26-01-03.49; TUM	A		KT596525		47.699197°	11.161717°
	83	*N. marina*	Germany, Bavaria, Staffelsee, 8.2; 2012-08-23; Rüegg S.; 26-01-03.50; TUM	A		KT596526		47.699197°	11.161717°

# in *trn* L-F alignment	# in ITS alignment	Taxon / species	Collection data (country, district, lake/site; date; collector; voucher number; collection number; acronym)	ITS type	*trn* L-F type	GenBank accession number ITS	GenBank accession number *trn* L-F	Georeference (lat)	Georeference (long)
	-	*N. marina*	Germany, Bavaria, Staffelsee, 8.3; 2012-08-23; Rüegg S.; 26-01-05.20; TUM***	-	-	KT596619		47.699197°	11.161717°
	84	*N. marina*	Germany, Bavaria, Staffelsee, 9.1; 2012-08-23; Rüegg S.; 26-01-03.51; TUM	A		KT596527		47.710441°	11.169561°
	85	*N. marina*	Germany, Bavaria, Staffelsee, 9.2; 2012-08-23; Rüegg S.; 26-01-03.53; TUM	A		KT596528		47.710441°	11.169561°
	86	*N. marina*	Germany, Bavaria, Staffelsee, 9.3; 2012-08-23; Rüegg S.; 26-01-02.47; TUM	B		KT596529		47.710441°	11.169561°
26	87	*N. marina*	Germany, Bavaria, Staffelsee, 9.4; 2012-08-23; Rüegg S.; 26-01-05.21; TUM	B	A	KT596530	KT596645	47.710441°	11.169561°
	88	*N. marina*	Germany, Bavaria, Staffelsee, 9.5; 2012-08-23; Rüegg S.; 26-01-03.54; TUM	A		KT596531		47.710441°	11.169561°
	89	*N. marina*	Germany, Bavaria, Staffelsee, 10.1; 2012-08-23; Rüegg S.; 26-01-02.48; TUM	B		KT596532		47.704025°	11.174819°
	90	*N. marina*	Germany, Bavaria, Staffelsee, 10.2; 2012-08-23; Rüegg S.; 26-01-03.55; TUM	A		KT596533		47.704025°	11.174819°
	91	*N. marina*	Germany, Bavaria, Staffelsee, 10.3; 2012-08-23; Rüegg S.; 26-01-03.56; TUM	A		KT596534		47.704025°	11.174819°
	92	*N. marina*	Germany, Bavaria, Staffelsee, 10.4; 2012-08-23; Rüegg S.; 26-01-02.49; TUM	B		KT596535		47.704025°	11.174819°
	93	*N. marina*	Germany, Bavaria, Staffelsee, 10.5; 2012-08-23; Rüegg S.; 26-01-03.57; TUM	A		KT596536		47.704025°	11.174819°
	94	*N. marina*	Germany, Bavaria, Staffelsee, 11.2; 2012-08-23; Rüegg S.; 26-01-02.50; TUM	B		KT596537		47.697802°	11.177683°
	95	*N. marina*	Germany, Bavaria, Staffelsee, 11.3; 2012-08-23; Rüegg S.; 26-01-03.58; TUM	A		KT596538		47.697802°	11.177683°
	96	*N. marina*	Germany, Bavaria, Staffelsee, 11.5; 2012-08-23; Rüegg S.; 26-01-03.59; TUM [2]	A		KT596539		47.697802°	11.177683°
27	97	*N. marina*	Germany, Bavaria, Staffelsee, 12.1; 2012-08-23; Rüegg S.; 26-01-05.24; TUM	B	A	KT596540	KT596646	47.68975°	11.178956°
	98	*N. marina*	Germany, Bavaria, Staffelsee, 12.3; 2012-08-23; Rüegg S.; 26-01-03.60; TUM	A		KT596541		47.68975°	11.178956°
	99	*N. marina*	Germany, Bavaria, Staffelsee, 12.4; 2012-08-23; Rüegg S.; 26-01-02.51; TUM	B		KT596542		47.68975°	11.178956°
	100	*N. marina*	Germany, Bavaria, Staffelsee, 12.5; 2012-08-23; Rüegg S.; 26-01-05.27; TUM	A		KT596543		47.68975°	11.178956°
	-	*N. marina*	Germany, Bavaria, Staffelsee, 10_Aquarium; 2013-08-15; Rüegg S.; 26-01-05.30; TUM	-	-				
	101	*N. marina*	Germany, Bavaria, Starnberger See ; 2010-08-18; Wutz K.; T 4; 26-01-02.16; TUM	B		KT596544		47.858883°	11.302090°
	102	*N. marina*	Germany, Bavaria, Starnberger See; 2011-08-09; Schümann M.; 26-01-02.31; TUM	B		KT596545		47.921239°	11.293892°
28	103	*N. marina*	Germany, Bavaria, Starnberger See, Allmannshausen; 2011-08-08; Schümann M.; TUM	B	B	KT596546	KT596647	47.921239°	11.293892°
	104	*N. marina*	Germany, Bavaria, Tachinger See, 2010-09-08, Wutz K., 26-01-02.18, T 2; TUM	B		KT596547		47.987217°	12.748589°
29	105	*N. marina*	Germany, Bavaria, Tachinger See; 2011-07-27; Schümann M. ; 26-01-05.9; TUM	A	A	KT596548	KT596648	47.954922°	12.749778°
	106	*N. marina*	Germany, Bavaria, Waginger See; 2010-09-08; Wutz K.; 26-01-03.12; TUM	A		KT596549		47.929279°	12.800611°
30	107	*N. marina*	Germany, Bavaria, Waginger See; 2011-07-26; Schümann M.; 26-01-03.24; TUM	A	A	KT596550	KT596649	47.931422	12.7978
31	108	*N. marina*	Germany, Bavaria, Wörthsee, 2010-08-16, Wutz K., 26-01-02.19, T 1; TUM	B	B	KT596551	KT596650	48.053692°	11.160887°
	109	*N. marina*	Germany, Berlin, Müggelsee; 2006-07-10; Weigend, M.; ex BSB; B*	A		KT596552		52.502599°	13.201386°
32	**110**	***N. marina***	**Germany, Brandenburg, Brodowinsee; 2011-07-28; Beck B., Gridling T.; 26-01-02.33; TUM**	B	**B**	**KT596553**	**KT596651**	**52.906856°**	**13.966017°**
33	111	*N. marina*	Germany, Brandenburg, Eberswalde, Parsteiner See; 1979-06-30; Konczak, P.; B *	A	A	KT596554	KT596652	52.920766°	14.022984°
	112	*N. marina*	Germany, Brandenburg, Gr. Kelpinsee; 1994-07-18; Konczak & Kromsch; B *	B		KT596555		53.055609°	13.743263°
34	**113**	***N. marina***	**Germany, Brandenburg, Großer Kelpinsee; 2011-07-29; Leidholdt J.; 26-01-02.34; TUM**	**B**	**B**	**KT596556**	**KT596653**	**53.057308°**	**13.737181°**
35	114	*N. marina*	Germany, Brandenburg, Großer Peetzigsee; 2011-07-31; Beck B., Gridling T; 26-01-02.35.; TUM	B	B	KT596557	**KT596654**	**53.067022°**	**13.919547°**
36	**115**	***N. marina***	**Germany, Brandenburg, Jakobsdorfer See; 2011-07-25; Beck B., Gridling T; 26-01-02.36.; TUM**	**B**	**B**	**KT596558**	**KT596655**	**53.131764°**	**13.885231°**
37	**116**	***N. marina***	**Germany, Brandenburg, Lehstsee; 2011-07-19; Leidholdt J.; TUM**	**B**	**B**	**KT596559**	**KT596656**	**53.22325°**	**13.34215°**
38	**117**	***N. marina***	**Germany, Brandenburg, Lützlower See; 2011-07-25; Beck B., Gridling T.; 26-01-02.37; TUM**	**B**	**B**	**KT596560**	**KT596657**	**53.247675°**	**14.037939°**
39	118	*N. marina*	Germany, Brandenburg, Pinnower See; 2011-08-06; Kronseder K.; 26-01-02.38; TUM	B	B	KT596561	**KT596658**	**51.962697°**	**14.518939°**
	119	*N. marina*	Germany, Brandenburg, Großer Seddin-See; 1979-08-x; Rinza, H.; B * [1*]	B		KT596562		52.271481°	13.030311°
40	**120**	**N. marina**	**Germany, Brandenburg, Sabinensee; 2011-07-26; Galm M., Gridling T.; TUM**	**B**	**B**	**KT596563**	**KT596659**	**53.111297°**	**13.761903°**
41	**121**	**N. marina**	**Germany, Brandenburg, Weißer See; 2011-07-28; Beck B., Gridling T.; TUM**	**B**	**B**	**KT596564**	**KT596660**	**52.913922°**	**13.951908°**
	122	*N. marina*	Germany, Brandenburg, Werlsee; 2010-08-01; Wutz K.; TUM	A		KT596565		52.421186°	13.813587°

# in *trn* L-F alignment	# in ITS alignment	Taxon / species	Collection data (country, district, lake/site; date; collector; voucher number; collection number; acronym)	ITS type	*trn* L-F type	GenBank accession number ITS	GenBank accession number *trn* L-F	Georeference (lat)	Georeference (long)
49	156	*N. m.* subsp. *intermedia*	Netherlands, Slikkendam, Pond (Nieuwekoopse plassen); 1982-09-09; Triest L; 71.; BRVU * [1*]	B	B	KT596599	KT596668	52.155432°	4.857700°
50	157	*N. m.* subsp. *intermedia*	Italy, Alto Adige, Kalterer See; 1985-08-17; Angerer, O.; M-0158738; M * [1*]	A	A	KT596600	KT596669	46.382924°	11.268892°
51		*Najas marina* subsp. *marina*	France, Seltz, near river Rhine; 1982-09-13; Triest L.; 107; BRVU *		A		KT596670	48.894296°	8.121548°
	158	*N. m.* subsp. *marina*	Germany, Rheinland-Pfalz, Neuburgweiher; 1982-09-13; Triest L.; 93; BRVU *	A		KT596601		48.978954°	8.274120°
52		*N. m.* subsp. *marina*	Luxembourg, Remerschen, near river Mosel; 1982-08-28; 69; BRVU *		A		KT596671	49.488813°	6.360988°
	159	*N. m.* subsp. *marina*	Sweden, Uppland, Lidingö, Gråviken,Västra; 2001-08-29; Peterson, T.; S-N0786-260; S *	B		KT596602		59.354222°	59.354222°
	160	*N. marina* susp. *marina* var. *ohwii Triest**	Japan, Hondo, Loaidamo in Tokyo; x-Sep-1953; Ohwi, J.; TSM.976; S *[1]	A		KT596603		-	-
	161	*Najas conferta* (A.Braun) A. Braun	Ecuador, Napo, Laguna Grande de Cuyabeno; 1984-08-12; Laegaard, S.; 52537; B * [1*]	X		KT596604		79°12´W 00° 00´N	
	162	*N. conferta*	Brazil, Ceará, Rio Sao Joao; 1976-11-20; Bogner, J.; M-0158741; 1225; M *	X		KT596605		-22.827474°	-43.357947°
	163	*N. conferta*	Peru, Puerto Maldonado, Laguna Cococha; 1992-07-x; Kasselmann, C.; 194 * [1*]	X		KT596606		-10.127778°	-77.283889°
56	164	*Najas flexilis* (Willd.) Rostk. & Schmidt	Germany, Baden-Württemberg: Bodensee; Untersee; 1973-09-02; Angerer, O.; M-0158753; M *	X		KT596607		47.705086°	9.060642°
	165	*Najas gracillima* (Engehn.) Magn	Greece, Thessaloniki, Kalohori; 1989-09-10; Raus, Th., Schiers, Ch.; MSB-005158; 14271; Soc. Ech. 16620; MSB *	X		KT596608		35.476818°	23.933502°
	166	*Najas graminea* Delile	France, Corse, Barrage de Codole; 1995-07-27; Lambinon, J.; M-0158750; 95/525; Soc. Ech. 17638; M *	X		KT596609		42.587496°	8.951647°
53		*Najas kingii* Rendle	ZZ, cultivated at Munich Botanical Garden; 1958-03-x; Heine, H.; M-0158730; M *		X		KT596672		
	167	*Najas madagascariensis*	Madagascar, Antsiranana; 1900-01-00; Tomey, W.; M-0158728; M *	X		KT596610		-12.329593°	49.296430°
	168	*Najas minor* All.	France, Territoire de Belfort, Fourchu Lake; 1989-09-08; Rastetter, V.; MSB-005160; Soc. Ech. 14721; MSB *	X		KT596611		47.541885°	7.057535°
	169	*N. minor*	Germany, Bavaria, Schwandorf; 2004-08-29; Dunkel, F.G.; M-0158742; DU-10474-3; M *	X		KT596612		49.370675°	12.158242°
	170	*N. minor*	Spanien, País Vasco y Alto Ebro, Alava: Marieta; 1984-09-13; Morante, G.; M-0158748; VIT-2028.84 (69); M *	X		KT596613		42.923795°	-2.571569°
54	171	*N. minor*	USA, Louisiana, Parish: Claiborne; 1985-07-31; Thomas, R.D., Aquatic Plant Class; M-0158743; 92980; M *	X	X	KT596614	KT596673	32.768530°	-93.004641°
55	172	*Najas oguraensis* Miki	India, Kashmir, Dal-See; 1983-09-10; Casimir, M.; MSB-005156; MSB *	X	X	KT596615	KT596674	34.115218°	74.867191°
	173	*Najas tenuifolia* R. Br.	Australia, Queensland, Townsville; 1967-10-19; den Hartog, C.; M-0158744; 83836; M * [1*]	X		KT596616		-19.263054°	146.817969°
	174	*Najas tenuissima* (A.Braun ex Magnus) Magnus	Finland, Karjaa, Lapinjärvi Lake; 1984-08-16; Kurtto, A.; MSB-005155; 4515, Soc. Ech. 12735; MSB * [1*]	X		KT596617		60.622780°	26.187559°

# in *trn* L-F alignment	# in ITS alignment	Taxon / species	Collection data (country, district, lake/site; date; collector; voucher number; collection number; acronym)	ITS type	*trn* L-F type	GenBank accession number ITS	GenBank accession number *trn* L-F	Georeference (lat)	Georeference (long)
	175	*Hydrocharis dubia* (Blume) Backer	(2)** Republic of Korea	X		HQ687166			
56		*N. flexilis*	(1)** USA: Connecticut		X		JX978472		
	176	*N. gracillima*	(2)** Republic of Korea	X		HQ687138			
	177	*N. gracillima*	(2)** Republic of Korea	X		HQ687140			
	178	*N. gracillima*	(2)** Republic of Korea	X		HQ687142			
	179	*N. graminea*	(2)** Republic of Korea	X		HQ687144			
	180	*N. graminea*	(2)** Republic of Korea	X		HQ687146			
	181	*N. graminea*	(2)** Republic of Korea	X		HQ687148			
	182	*N. marina*	(3)** USA: Florida	X		HM240442			
	183	*N. marina*	(2)** Republic of Korea	A		HQ687150			
	184	*N. marina*	(2)** Republic of Korea	A		HQ687152			
	185	*N. minor*	(2)** Republic of Korea	X		HQ687154			
	186	*N. minor*	(2)** Republic of Korea	X		HQ687156			
	187	*N. oguraensis*	(2)** Republic of Korea	X		HQ687160			
	188	*N. oguraensis*	(2)** Republic of Korea	X		HQ687158			
	189	*N. orientalis* Triest & Uotila	(2)** Republic of Korea	X		HQ687162			

Appendix A2

List of specimens included in this study. Herbarium vouchers were deposited in the Technical University of Munich (TUM) herbarium (Thiers, 2017) samples marked with * one representative voucher specimen was prepared per transect, ** cloned sequence

Number of individuals meassured	Number of leaves meassured per individual	*Taxon / species*	Collection data (country, district, lake/site; date; collector; voucher number; collection number; acronym)	ITS type	*trn* L-F type	GenBank accession number ITS	GenBank accession number *trn* L-F	Geo-reference (lat)	Geo-reference (long)	Digestion with Hind III
1	5	*N. marina*	Germany, Bavaria, Staffelsee, 1.2; 2012-08-23; Rüegg S.; 26-01-03.26; TUM	B	B	MH492979	MH513875	47.678614°	11.176433°	-
1	5	*N. marina*	Germany, Bavaria, Staffelsee, 1.3; 2012-08-23; Rüegg S.; 26-01-03.27; TUM	A	A	MH492980	MH513888	47.678614°	11.176433°	-
1	4	*N. major*	Germany, Bavaria, Staffelsee, 1.4; 2012-08-23; Rüegg S.; 26-01-03.28; TUM	A	A	KT596495	MH513889	47.678614°	11.176433°	-
1	5	*N. major*	Germany, Bavaria, Staffelsee, 1.5; 2012-08-23; Rüegg S.; 26-01-03.27; TUM	A	A	KT596496	MH513924	47.678614°	11.176433°	-
1	5	*N. major*	Germany, Bavaria, Staffelsee, 2.1; 2012-08-23; Rüegg S.; 26-01-03.28; TUM *'	A	A	KT596497	MH513890	47.678064°	11.155497°	-
1	5	*N. major*	Germany, Bavaria, Staffelsee, 2.2; 2012-08-23; Rüegg S.; 26-01-03.29; TUM	A	A	KT596498	MH513891	47.678064°	11.155497°	-
1	5	*N. major*	Germany, Bavaria, Staffelsee, 2.3; 2012-08-23; Rüegg S.; 26-01-03.30; TUM	A	A	KT596499	MH513892	47.678064°	11.155497°	-
1	5	*N. major*	Germany, Bavaria, Staffelsee, 2.4; 2012-08-23; Rüegg S.; 26-01-03.31; TUM	A	A	KT596500	MH513893	47.678064°	11.155497°	-
1	5	*N. major*	Germany, Bavaria, Staffelsee, 2.5; 2012-08-23; Rüegg S.; 26-01-03.32; TUM	A	A	KT596501	MH513894	47.678064°	11.155497°	-
1	5	*N. major*	Germany, Bavaria, Staffelsee, 3.1; 2012-08-23; Rüegg S.; 26-01-03.33; TUM	A	A	KT596502	MH513895	47.682334°	11.159234°	-
1	5	*N. major*	Germany, Bavaria, Staffelsee, 3.2; 2012-08-23; Rüegg S.; 26-01-03.34; TUM	A	A	KT596503	MH513922	47.682334°	11.159234°	-
1	5	*N. major*	Germany, Bavaria, Staffelsee, 3.3; 2012-08-23; Rüegg S.; 26-01-03.35; TUM	A	A	KT596504	MH513896	47.682334°	11.159234°	-
1	5	*N. major*	Germany, Bavaria, Staffelsee, 3.4; 2012-08-23; Rüegg S.; 26-01-03.36; TUM	A	-	KT596505	-	47.682334°	11.159234°	-
1	5	*N. major*	Germany, Bavaria, Staffelsee, 3.5; 2012-08-23; Rüegg S.; TUM	A	A	KT596506	MH513897	47.682334°	11.159234°	-
1	5	*N. marina*	Germany, Bavaria, Staffelsee, 4.1; 2012-08-23; Rüegg S.; 26-01-02.41; TUM	B	B	KT596507	MH513876	47.687064°	11.16427°	-
1	5	*N. marina*	Germany, Bavaria, Staffelsee, 4.2; 2012-08-23; Rüegg S.; 26-01-02.42; TUM	B	B	KT596508	KT596643	47.687064°	11.16427°	-
1	5	*N. major*	Germany, Bavaria, Staffelsee, 4.3; 2012-08-23; Rüegg S.; 26-01-03.37; TUM	A	A	KT596509	MH513898	47.687064°	11.16427°	-
1	5	*N. major*	Germany, Bavaria, Staffelsee, 4.4; 2012-08-23; Rüegg S.; 26-01-03.38; TUM	A	A	KT596510	MH513899	47.687064°	11.16427°	-
1	5	*N. major*	Germany, Bavaria, Staffelsee, 5.1; 2012-08-23; Rüegg S.; 26-01-03.39; TUM	A	A	KT596511	MH513900	47.688088°	11.132762°	-
1	5	*N. major*	Germany, Bavaria, Staffelsee, 5.2; 2012-08-23; Rüegg S.; 26-01-03.40; TUM	A	A	KT596512	MH513901	47.688088°	11.132762°	-
1	5	*N. major*	Germany, Bavaria, Staffelsee, 5.3; 2012-08-23; Rüegg S.; 26-01-03.41; TUM	A	A	KT596513	-	47.688088°	11.132762°	-
1	5	*N. major*	Germany, Bavaria, Staffelsee, 5.4; 2012-08-23; Rüegg S.; 26-01-03.42; TUM	A	A	KT596514	MH513902	47.688088°	11.132762°	-
1	5	*N. major*	Germany, Bavaria, Staffelsee, 5.5; 2012-08-23; Rüegg S.; 26-01-03.43; TUM	A	A	KT596515	MH513903	47.688088°	11.132762°	-
1	5	*N. major*	Germany, Bavaria, Staffelsee, 6.1; 2012-08-23; Rüegg S.; 26-01-03.44; TUM	A	A	KT596516	MH513904	47.697183°	11.140881°	-
1	5	*N. major*	Germany, Bavaria, Staffelsee, 6.2; 2012-08-23; Rüegg S.; 26-01-03.45; TUM	A	A	KT596517	MH513905	47.697183°	11.140881°	-
1	5	*N. marina*	Germany, Bavaria, Staffelsee, 6.3; 2012-08-23; Rüegg S.; 26-01-02.43; TUM	B	B	KT596518	MH513877	47.697183°	11.140881°	-
1	5	*N. marina*	Germany, Bavaria, Staffelsee, 6.4; 2012-08-23; Rüegg S.; 26-01-02.44; TUM	B	B	KT596519	MH513878	47.697183°	11.140881°	-
1	5	*N. major*	Germany, Bavaria, Staffelsee, 6.5; 2012-08-23; Rüegg S.; 26-01-03.46; TUM	A	A	KT596520	MH513906	47.697183°	11.140881°	-
1	5	*N. major*	Germany, Bavaria, Staffelsee, 7.1; 2012-08-23; Rüegg S.; 26-01-03.47; TUM	A	A	KT596521	MH513907	47.693161°	11.154067°	-
1	5	*N. marina*	Germany, Bavaria, Staffelsee, 7.3; 2012-08-23; Rüegg S.; 26-01-02.45; TUM	B	B	KT596522	MH513879	47.693161°	11.154067°	-
1	5	*N. major*	Germany, Bavaria, Staffelsee, 7.4; 2012-08-23; Rüegg S.; 26-01-03.48; TUM	A	A	KT596523	KT596644 MH513908	47.693161°	11.154067°	-

Number of individuals meassured	Number of leaves meassured per individual	*Taxon / species*	Collection data (country, district, lake/site; date; collector; voucher number; collection number; acronym)	ITS type	*trn*L-F type	GenBank accession number ITS	GenBank accession number *trn*L-F	Geo-reference (lat)	Geo-reference (long)	Digestion with Hind III
1	5	*N. marina*	Germany, Bavaria, Staffelsee, 7.5; 2012-08-23; Rüegg S.; 26-01-02.46; TUM	B	B	KT596524	MH513880	47.693161°	11.154067°	-
1	5	*N. major*	Germany, Bavaria, Staffelsee, 8.1; 2012-08-23; Rüegg S.; 26-01-03.49; TUM	A	A	KT596525	MH513909	47.699197°	11.161717°	-
1	5	*N. major*	Germany, Bavaria, Staffelsee, 8.2; 2012-08-23; Rüegg S.; 26-01-03.50; TUM	A	A	KT596526	MH513910	47.699197°	11.161717°	-
-	-	*N. marina s.l.*	Germany, Bavaria, Staffelsee, 8.3; 2012-08-23; Rüegg S.;26-01-05.22; TUM **	hybrid	A	KT596619 KT596618	MH513911	47.699197°	11.161717°	x
-	-	*N. marina s.l.*	Germany, Bavaria, Staffelsee, 8.4; 2012-08-23; Rüegg S.;26-01-05.22; TUM	hybrid	B	MH492981	MH513881	47.699197°	11.161717°	-
1	5	*N. marina s.l.*	Germany, Bavaria, Staffelsee, 8.5; 2012-08-23; Rüegg S.;26-01-05.22; TUM	B	B	MH492982	MH513882	47.699197°	11.161717°	-
1	5	*N. major*	Germany, Bavaria, Staffelsee, 9.1; 2012-08-23; Rüegg S.; 26-01-03.51; TUM	A	A	KT596527	MH513912	47.710441°	11.169561°	-
1	5	*N. major*	Germany, Bavaria, Staffelsee, 9.2; 2012-08-23; Rüegg S.; 26-01-03.53; TUM	A	A	KT596528	MH513913	47.710441°	11.169561°	-
1	5	*N. marina*	Germany, Bavaria, Staffelsee, 9.3; 2012-08-23; Rüegg S.; 26-01-02.47; TUM	B	B	KT596529	MH513883	47.710441°	11.169561°	x
1	5	*N. marina s.l.*	Germany, Bavaria, Staffelsee, 9.4; 2012-08-23; Rüegg S.; 26-01-05.21; TUM	**B**	**A**	KT596530	KT596645	47.710441°	11.169561°	-
1	5	*N. major*	Germany, Bavaria, Staffelsee, 9.5; 2012-08-23; Rüegg S.; 26-01-03.54; TUM	A	A	KT596531	MH513914	47.710441°	11.169561°	x
1	5	*N. marina*	Germany, Bavaria, Staffelsee, 10.1; 2012-08-23; Rüegg S.; 26-01-02.48; TUM	B	B	KT596532	MH513884	47.704025°	11.174819°	-
1	5	*N. marina*	Germany, Bavaria, Staffelsee, 10.2; 2012-08-23; Rüegg S.; 26-01-03.55; TUM	A	A	KT596533	MH513915	47.704025°	11.174819°	-
1	5	*N. marina*	Germany, Bavaria, Staffelsee, 10.3; 2012-08-23; Rüegg S.; 26-01-03.56; TUM	A	A	KT596534	MH513916	47.704025°	11.174819°	-
1	5	*N. marina*	Germany, Bavaria, Staffelsee, 10.4; 2012-08-23; Rüegg S.; 26-01-02.49; TUM	B	B	KT596535	MH513885	47.704025°	11.174819°	-
1	5	*N. major*	Germany, Bavaria, Staffelsee, 10.5; 2012-08-23; Rüegg S.; 26-01-03.57; TUM	A	A	KT596536	MH513917	47.704025°	11.174819°	-
1	5	*N. marina*	Germany, Bavaria, Staffelsee, 11.2; 2012-08-23; Rüegg S.; 26-01-02.50; TUM	B	B	KT596537	MH513886	47.697802°	11.177683°	-
1	5	*N. major*	Germany, Bavaria, Staffelsee, 11.3; 2012-08-23; Rüegg S.; 26-01-03.58; TUM	A	A	KT596538	MH513918	47.697802°	11.177683°	-
1	5	*N. major*	Germany, Bavaria, Staffelsee, 11.5; 2012-08-23; Rüegg S.; 26-01-03.59; TUM	A	A	KT596539	MH513919	47.697802°	11.177683°	-
1	5	*N. marina s.l.*	Germany, Bavaria, Staffelsee, 12.1; 2012-08-23; Rüegg S.; 26-01-05.24; TUM	**B**	**A**	KT596540	KT596646	47.68975°	11.178956°	-
1	5	*N. marina*	Germany, Bavaria, Staffelsee, 12.3; 2012-08-23; Rüegg S.; 26-01-03.60; TUM	A	A	KT596541	MH513920	47.68975°	11.178956°	-
1	5	*N. marina*	Germany, Bavaria, Staffelsee, 12.4; 2012-08-23; Rüegg S.; 26-01-02.51; TUM	B	B	KT596542	MH513887	47.68975°	11.178956°	-
1	5	*N. major*	Germany, Bavaria, Staffelsee, 12.5; 2012-08-23; Rüegg S.; 26-01-05.27; TUM	A	A	KT596543	MH513921	47.68975°	11.178956°	-
5	5	*N. marina*	Germany, Baden-Württemberg, Bodensee, A; 2012-08-30; Rüegg S., Hippich M.; 26-01-02.52; TUM	B	B	KT596451	KT596623	47.666611°	9.218261°	-
4	5	*N. marina*	Germany, Baden-Württemberg, Bodensee, B; 2012-08-30; Rüegg S., Hippich M.; 26-01-02.52; TUM	B	B	-	-	47.735114°	9.172839°	-
3	5	*N. marina*	Germany, Baden-Württemberg, Bodensee, C; 2012-08-30; Rüegg S., Hippich M.; 26-01-02.52; TUM	B	B	-	-	47.649042°	9.462161°	-
2	5	*N. marina*	Germany, Bavaria, Starnberger See, A1; 2015-09-15; Sebald, S.; 26-01-02.68; TUM	B	-	-	-	47.921482°	11.294181°	x
2	5	*N. marina*	Germany, Bavaria, Starnberger See, A3; 2015-09-15; Sebald, S.; 26-01-02.69; TUM	B	-	-	-	47.921482°	11.294181°	x
2	5	*N. marina*	Germany, Bavaria, Starnberger See, A4; 2015-09-15; Sebald, S.; 26-01-02.70; TUM	B	-	-	-	47.921482°	11.294181°	x
2	5	*N. marina*	Germany, Bavaria, Starnberger See, A5; 2015-09-15; Sebald, S.; 26-01-02.71; TUM	B	-	-	-	47.921482°	11.294181°	x
2	5	*N. marina*	Germany, Bavaria, Starnberger See, B1; 2015-09-15; Dobler, A.; 26-01-02.72; TUM	B	-	-	-	47.921482°	11.294181°	x

Number of individuals meassured	Number of leaves meassured per individual	*Taxon / species*	Collection data (country, district, lake/site; date; collector; voucher number; collection number; acronym)	ITS type	*trn* L-F type	GenBank accession number ITS	GenBank accession number *trn* L-F	Geo-reference (lat)	Geo-reference (long)	Digestion with Hind III
2	5	*N. marina*	Germany, Bavaria, Starnberger See, B2; 2015-09-15; Dobler, A.; 26-01-02.73; TUM	B	-	-	_	47.921482°	11.294181°	x
2	5	*N. marina*	Germany, Bavaria, Starnberger See, B3; 2015-09-15; Dobler, A.; 26-01-02.74; TUM	B	-	-	_	47.921482°	11.294181°	x
2	5	*N. marina*	Germany, Bavaria, Starnberger See, B4; 2015-09-15; Dobler, A.; 26-01-02.75; TUM	B	-	-	_	47.921482°	11.294181°	x
2	5	*N. marina*	Germany, Bavaria, Starnberger See, B5; 2015-09-15; Dobler, A.; 26-01-02.76; TUM	B	-	-	_	47.921482°	11.294181°	x
2	5	*N. marina*	Germany, Bavaria, Starnberger See, C1; 2015-09-15; Fritz, C.; 26-01-02.77; TUM	B	-	-	_	47.921482°	11.294181°	x
2	5	*N. marina*	Germany, Bavaria, Starnberger See, C2; 2015-09-15; Fritz, C.; 26-01-02.78; TUM	B	-	-	_	47.921482°	11.294181°	x
2	5	*N. marina*	Germany, Bavaria, Starnberger See, C3; 2015-09-15; Fritz, C.; 26-01-02.79; TUM	B	-	-	_	47.921482°	11.294181°	x
2	5	*N. marina*	Germany, Bavaria, Starnberger See, C4; 2015-09-15; Fritz, C.; 26-01-02.80; TUM	B	-	-	_	47.921482°	11.294181°	x
2	5	*N. marina*	Germany, Bavaria, Starnberger See, C5; 2015-09-15; Fritz, C.; 26-01-02.81; TUM	B	-	-	_	47.921482°	11.294181°	x
2	5	*N. marina*	Germany, Bavaria, Starnberger See, D1; 2015-09-15; Heinrich, L.; 26-01-02.82; TUM	B	-	-	_	47.921482°	11.294181°	x
2	5	*N. marina*	Germany, Bavaria, Starnberger See, D2; 2015-09-15; Heinrich, L.; 26-01-02.83; TUM	B	-	-	_	47.921482°	11.294181°	x
2	5	*N. marina*	Germany, Bavaria, Starnberger See, D3; 2015-09-15; Heinrich, L.; 26-01-02.84; TUM	B	-	-	_	47.921482°	11.294181°	x
2	5	*N. marina*	Germany, Bavaria, Starnberger See, D4; 2015-09-15; Heinrich, L.; 26-01-02.85; TUM	B	-	-	_	47.921482°	11.294181°	x
2	5	*N. marina*	Germany, Bavaria, Starnberger See, D5; 2015-09-15; Heinrich, L.; 26-01-02.86; TUM	B	-	-	_	47.921482°	11.294181°	x
1	5	*N. marina*	Germany, Bavaria, Staffelsee, 1.1; 2015-09-17; Rüegg S., A. Schmitz, C. Böhme; 26-01-02.87; TUM	B	-	-	-	47.678614°	11.176433°	x
1	5	*N. major*	Germany, Bavaria, Staffelsee, 1.2; 2015-09-17; Rüegg S., A. Schmitz, C. Böhme; 26-01-03.81; TUM	A	-	-	-	47.678614°	11.176433°	x
1	5	*N. major*	Germany, Bavaria, Staffelsee, 1.3; 2015-09-17; Rüegg S., A. Schmitz, C. Böhme; 26-01-03.82; TUM	A	-	-	-	47.678614°	11.176433°	x
1	5	*N. marina*	Germany, Bavaria, Staffelsee, 1.4; 2015-09-17; Rüegg S., A. Schmitz, C. Böhme; 26-01-02.88; TUM	B	-	-	-	47.678614°	11.176433°	x
1	5	*N. major*	Germany, Bavaria, Staffelsee, 2.9; 2015-09-17; Rüegg S., A. Schmitz, C. Böhme; 26-01-03.83; TUM	A	-	-	-	47.678614°	11.176433°	x
1	4	*N. marina*	Germany, Bavaria, Staffelsee, 2.10; 2015-09-17; Rüegg S., A. Schmitz, C. Böhme; 26-01-02.89; TUM	B	-	-	-	47.678064°	11.155497°	x
1	5	*N. major*	Germany, Bavaria, Staffelsee, 2.11; 2015-09-17; Rüegg S., A. Schmitz, C. Böhme; 26-01-03.84; TUM	A	-	-	-	47.678064°	11.155497°	x
1	5	*N. marina*	Germany, Bavaria, Staffelsee, 2.12; 2015-09-17; Rüegg S., A. Schmitz, C. Böhme; 26-01-02.90; TUM	B	-	-	-	47.678064°	11.155497°	x
1	5	*N. marina s.l.*	Germany, Bavaria, Staffelsee, 3.5; 2015-09-17; Rüegg S., A. Schmitz, C. Böhme; 26-01-05.30; TUM	hybrid	-	-	-	47.682334°	11.159234°	x
1	5	*N. major*	Germany, Bavaria, Staffelsee, 3.6; 2015-09-17; Rüegg S., A. Schmitz, C. Böhme; 26-01-03.85; TUM	A	-	-	-	47.682334°	11.159234°	x
1	5	*N. major*	Germany, Bavaria, Staffelsee, 3.7; 2015-09-17; Rüegg S., A. Schmitz, C. Böhme; 26-01-03.86; TUM	A	-	-	-	47.682334°	11.159234°	x
1	5	*N. major*	Germany, Bavaria, Staffelsee, 3.8; 2015-09-17; Rüegg S., A. Schmitz, C. Böhme; 26-01-03.87; TUM	A	-	-	-	47.682334°	11.159234°	x

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1	5	*N. marina*	Germany, Bavaria, Staffelsee, 4.1; 2015-10-05; Rüegg S.; 26-01-02.91; TUM	B	-	-	-	47.687064°	11.16427°	x
1	5	*N. marina*	Germany, Bavaria, Staffelsee, 4.2; 2015-10-05; Rüegg S.; 26-01-02.92; TUM	B	-	-	-	47.687064°	11.16427°	x
1	5	*N. marina s.l.*	Germany, Bavaria, Staffelsee, 4.3; 2015-10-05; Rüegg S.; 26-01-05.31; TUM	hybrid	-	-	-	47.687064°	11.16427°	x
1	5	*N. marina s.l.*	Germany, Bavaria, Staffelsee, 4.4; 2015-10-05; Rüegg S.; 26-01-05.32; TUM	hybrid	-	-	-	47.687064°	11.16427°	x
1	5	*N. major*	Germany, Bavaria, Staffelsee, 4.5; 2015-10-05; Rüegg S.; 26-01-03.88; TUM	A	-	-	-	47.687064°	11.16427°	x
1	5	*N. major*	Germany, Bavaria, Staffelsee, 4.6; 2015-10-05; Rüegg S.; 26-01-03.89; TUM	A	-	-	-	47.687064°	11.16427°	x
1	5	*N. major*	Germany, Bavaria, Staffelsee, 4.7; 2015-10-05; Rüegg S.; 26-01-03.90; TUM	A	-	-	-	47.687064°	11.16427°	x
1	5	*N. major*	Germany, Bavaria, Staffelsee, 6.1; 2015-10-05; Rüegg S.; 26-01-03.91; TUM	A	-	-	-	47.697183°	11.140881°	x
1	5	*N. marina*	Germany, Bavaria, Staffelsee, 6.2; 2015-10-05; Rüegg S.; 26-01-02.93; TUM	B	-	-	-	47.697183°	11.140881°	x
1	5	*N. major*	Germany, Bavaria, Staffelsee, 6.3; 2015-10-05; Rüegg S.; 26-01-03.92; TUM	A	-	-	-	47.697183°	11.140881°	x
1	5	*N. marina*	Germany, Bavaria, Staffelsee, 6.4; 2015-10-05; Rüegg S.; 26-01-02.94; TUM	B	-	-	-	47.697183°	11.140881°	x
1	5	*N. major*	Germany, Bavaria, Staffelsee, 6.5; 2015-10-05; Rüegg S.; 26-01-03.93; TUM	A	-	-	-	47.697183°	11.140881°	x
1	5	*N. marina s.l.*	Germany, Bavaria, Staffelsee, 6.6; 2015-10-05; Rüegg S.; 26-01-05.33; TUM	hybrid	-	-	-	47.697183°	11.140881°	x
1	5	*N. major*	Germany, Bavaria, Staffelsee, 8.25; 2015-09-17; Rüegg S., A. Schmitz, C. Böhme; 26-01-03.94; TUM	A	-	-	-	47.699197°	11.161717°	x
1	5	*N. marina*	Germany, Bavaria, Staffelsee, 8.26; 2015-09-17; Rüegg S., A. Schmitz, C. Böhme; 26-01-02.95; TUM	B	-	-	-	47.699197°	11.161717°	x
1	5	*N. marina*	Germany, Bavaria, Staffelsee, 8.27; 2015-09-17; Rüegg S., A. Schmitz, C. Böhme; 26-01-02.96; TUM	B	-	-	-	47.699197°	11.161717°	x
1	5	*N. marina s.l.*	Germany, Bavaria, Staffelsee, 8.28; 2015-09-17; Rüegg S., A. Schmitz, C. Böhme; 26-01-05.34; TUM	hybrid	-	-	-	47.699197°	11.161717°	x
1	5	*N. major*	Germany, Bavaria, Staffelsee, 9.21; 2015-09-17; Rüegg S., A. Schmitz, C. Böhme; 26-01-03.95; TUM	A	-	-	-	47.710441°	11.169561°	x
1	5	*N. marina s.l.*	Germany, Bavaria, Staffelsee, 9.22; 2015-09-17; Rüegg S., A. Schmitz, C. Böhme; 26-01-05.35; TUM	hybrid	-	-	-	47.710441°	11.169561°	x
1	5	*N. marina s.l.*	Germany, Bavaria, Staffelsee, 9.23; 2015-09-17; Rüegg S., A. Schmitz, C. Böhme; 26-01-05.36; TUM	hybrid	-	-	-	47.710441°	11.169561°	x
1	5	*N. marina s.l.*	Germany, Bavaria, Staffelsee, 9.24; 2015-09-17; Rüegg S., A. Schmitz, C. Böhme; 26-01-05.37; TUM	hybrid	-	-	-	47.710441°	11.169561°	x
1	5	*N. major*	Germany, Bavaria, Staffelsee, 10.17; 2015-09-17; Rüegg S., A. Schmitz, C. Böhme; 26-01-03.96; TUM	A	-	-	-	47.704025°	11.174819°	x
1	5	*N. marina*	Germany, Bavaria, Staffelsee, 10.18; 2015-09-17; Rüegg S., A. Schmitz, C. Böhme; 26-01-02.97; TUM	B	-	-	-	47.704025°	11.174819°	x
1	5	*N. marina s.l.*	Germany, Bavaria, Staffelsee, 10.19; 2015-09-17; Rüegg S., A. Schmitz, C. Böhme; 26-01-05.38; TUM	hybrid	-	-	-	47.704025°	11.174819°	x
1	5	*N. marina*	Germany, Bavaria, Staffelsee, 10.20; 2015-09-17; Rüegg S., A. Schmitz, C. Böhme; 26-01-02.98; TUM	B	-	-	-	47.704025°	11.174819°	x
1	4	*N. major*	Germany, Bavaria, Staffelsee, 12.13; 2015-09-17; Rüegg S., A. Schmitz, C. Böhme; 26-01-03.97; TUM	A	-	-	-	47.68975°	11.178956°	x
1	5	*N. marina s.l.*	Germany, Bavaria, Staffelsee, 12.14; 2015-09-17; Rüegg S., A. Schmitz, C. Böhme; 26-01-05.39; TUM	hybrid	-	-	-	47.68975°	11.178956°	x

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1	5	*N. marina s.l.*	Germany, Bavaria, Staffelsee, 12.15; 2015-09-17; Rüegg S., A. Schmitz, C. Böhme; 26-01-05.40; TUM	hybrid	-	-	-	47.68975°	11.178956°	x
1	5	*N. marina*	Germany, Bavaria, Staffelsee, 12.16; 2015-09-17; Rüegg S., A. Schmitz, C. Böhme; 26-01-02.99; TUM	B	-	-	-	47.68975°	11.178956°	x
1	5	*N. major*	Germany, Bavaria, Abtsdorfer See; A1; 2015-09-16; Rüegg S., Heinrich, L.; 26-01-03.64; TUM	A	-	-	-	47.914986°	12.904111°	x
1	5	*N. major*	Germany, Bavaria, Abtsdorfer See; A2; 2015-09-16; Rüegg S., Heinrich, L.; 26-01-03.65; TUM	A	-	-	-	47.914986°	12.904111°	x
1	5	*N. major*	Germany, Bavaria, Abtsdorfer See; A3; 2015-09-16; Rüegg S., Heinrich, L.; 26-01-03.66; TUM	A	-	-	-	47.914986°	12.904111°	x
1	5	*N. major*	Germany, Bavaria, Abtsdorfer See; B1; 2015-09-16; Rüegg S., Heinrich, L.; 26-01-03.67; TUM	A	-	-	-	47.914986°	12.904111°	x
1	4	*N. major*	Germany, Bavaria, Abtsdorfer See; B2; 2015-09-16; Rüegg S., Heinrich, L.; 26-01-03.68; TUM	A	-	-	-	47.914986°	12.904111°	x
1	5	*N. major*	Germany, Bavaria, Abtsdorfer See; B3; 2015-09-16; Rüegg S., Heinrich, L.; 26-01-03.69; TUM	A	-	-	-	47.914986°	12.904111°	x
1	5	*N. major*	Germany, Bavaria, Abtsdorfer See; C1; 2015-09-16; Rüegg S., Heinrich, L.; 26-01-03.70; TUM	A	-	-	-	47.914986°	12.904111°	x
1	5	*N. major*	Germany, Bavaria, Abtsdorfer See; C2; 2015-09-16; Rüegg S., Heinrich, L.; 26-01-03.71; TUM	A	-	-	-	47.914986°	12.904111°	x
1	5	*N. major*	Germany, Bavaria, Abtsdorfer See; D1; 2015-09-16; Rüegg S., Heinrich, L.; 26-01-03.72; TUM	A	-	-	-	47.914986°	12.904111°	x
1	5	*N. major*	Germany, Bavaria, Abtsdorfer See; D2; 2015-09-16; Rüegg S., Heinrich, L.; 26-01-03.73; TUM	A	-	-	-	47.914986°	12.904111°	x
1	5	*N. major*	Germany, Bavaria, Abtsdorfer See; D3; 2015-09-16; Rüegg S., Heinrich, L.; 26-01-03.74; TUM	A	-	-	-	47.914986°	12.904111°	x
1	5	*N. major*	Germany, Bavaria, Abtsdorfer See; E1; 2015-09-16; Rüegg S., Heinrich, L.; 26-01-03.75; TUM	A	-	-	-	47.914986°	12.904111°	x
1	5	*N. major*	Germany, Bavaria, Abtsdorfer See; E2; 2015-09-16; Rüegg S., Heinrich, L.; 26-01-03.76; TUM	A	-	-	-	47.914986°	12.904111°	x
1	5	*N. major*	Germany, Bavaria, Abtsdorfer See; E3; 2015-09-16; Rüegg S., Heinrich, L.; 26-01-03.77; TUM	A	-	-	-	47.914986°	12.904111°	x
1	5	*N. major*	Germany, Bavaria, Abtsdorfer See; F1; 2015-09-16; Rüegg S., Heinrich, L.; 26-01-03.78; TUM	A	-	-	-	47.914986°	12.904111°	x
1	5	*N. major*	Germany, Bavaria, Abtsdorfer See; F2; 2015-09-16; Rüegg S., Heinrich, L.; 26-01-03.79; TUM	A	-	-	-	47.914986°	12.904111°	x
1	5	*N. major*	Germany, Bavaria, Abtsdorfer See; F3; 2015-09-16; Rüegg S., Heinrich, L.; 26-01-03.80; TUM	A	-	-	-	47.914986°	12.904111°	x
-	-	*N. major*	Germany, Bavaria, Soyensee; 2011-07-25; Schümann M.; 26-01-03.21; TUM	A	A	KT596489	KT596641	48.106139°	12.206744°	x
-	-	*N. major*	Germany, Bavaria, Donaustauf ; 2011-08-16; Schümann M. ; 26-01-03.14; TUM	A	A	KT596464	KT596628	49.025919°	12.200375°	x
-	-	*N. major*	Austria, Salzburg Land, Mattsee; 2012-08-06; Rüegg S.; 26-01-03.61; TUM	A	A	KT596445	KT596620	47.968289°	13.080669°	x
-	-	*N. marina*	Germany, Bavaria, Starnberger See, Allmannshausen; 2011-08-08; Schümann M.; TUM	B	B	KT596546	KT596647	47.921239°	11.293892°	x
-	-	*N. marina*	Germany, Brandenburg, Pinnower See; 2011-08-06; Kronseder K.; 26-01-02.38; TUM	B	B	KT596561	KT596658	51.962697°	14.518939°	x

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10	30	*N. marina*	Germany, Bavaria, Abtsdorfer See; 2010-09-08; Wutz K.; 26-01-03.1; TUM	A	-	KT596457	-	47.916798°	12.902125°	-
10	30	*N. marina*	Germany, Bavaria, Ammersee T1; 2010-08-26; Wutz K.; 26-01-02.3; TUM** *	B	-	KT596459	-	48.001752°	11.099192°	-
10	30	*N. marina*	Germany, Bavaria, Ammersee T2; 2010-08-26; Wutz K.; TUM*	B	-	-	-	48.069474°	11.110245°	-
10	30	*N. marina*	Germany, Bavaria, Ammersee T3; 2010-08-26; Wutz K.; 26-01-02.4; TUM*	B	-	-	-	48.002300°	11.166625°	-
10	30	*N. marina*	Germany, Baden-Württemberg, Bodensee; 2010-07-23; Wutz K. ; 26-01-02.5; TUM*	B	-	KT596450	-	47.696606°	9.073502°	-
10	30	*N. marina*	Germany, Bavaria, Chiemsee, Aiterbacher Winkel; 2010-08-28; Wutz K. ; 26-01-02.6; TUM*	B	-	KT596461	-	47.875782°	12.354595°	-
10	30	*N. major*	Germany, Baden-Württemberg / Bavaria, Degersee; 2010-08-31; Wutz K.; 26-01-03.2; TUM*	A	-	KT596452	-	47.610045°	9.656357°	-
10	30	*N. major*	Germany, Bavaria, Donaustauf T1; 2010-09-28; Wutz K.; 26-01-03.3; TUM*	A	-	KT596463	-	48.939661°	12.518887°	-
10	30	*N. major*	Germany, Bavaria, Donaustauf T2; 2010-09-28; Wutz K.; 26-01-03.4; TUM*	A	-	-	-	48.938225°	12.513682°	-
10	30	*N. major*	Germany, Bavaria, Großer Brombachsee; 2010-09-28; Wutz K.; 26-01-03.5; TUM*	A	-	KT596474	-	49.133584°	10.961295°	-
10	30	*N. major*	Germany, Bavaria, Großwelzheimer Badesee; 2010-08-24; Wutz K.; TUM*	A	-	KT596476	-	50.058403°	9.014778°	-
10	30	*N. marina*	Germany, Bavaria, Eggstätt-Hemhofer Seenplatte, Langbürgner See; 2010-09-01; Wutz K. ; 26-01-03.6; TUM*	B	-	KT596466	-	47.910346°	12.347376°	-
10	30	*N. major*	Germany, Baden-Württemberg, Mindelsee; 2010-08-23; Wutz K.; 26-01-03.6; TUM*	A	-	KT596453	-	47.753548°	9.016367°	-
9	26	*N. major*	Germany, Baden-Württemberg, Muttelsee; 2010-08-31; Wutz K.; TUM*	A	-	KT596454	-	47.618584°	9.668209°	-
10	30	*N. marina*	Germany, Bavaria, Eggstätt-Hemhofer Seenplatte, Pelhamersee; 2011-07-05; Wutz K.; 60-01-05-3; TUM*	B	-	-	-	47.933228°	12.343783°	-
10	30	*N. marina*	Germany, Bavaria, Eggstätt-Hemhofer Seenplatte, Pilsensee T1; 2010-08-16; Wutz K.; 26-01-02.8; TUM*	B	-	KT596471	-	47.929431°	12.345086°	-
10	30	*N. marina*	Germany, Bavaria, Eggstätt-Hemhofer Seenplatte, Pilsensee T2; 2010-08-16; Wutz K.; 26-01-02.9; TUM*	B	-	-	-	48.032301°	11.196908°	-
10	30	*N. marina*	Germany, Bavaria, Eggstätt-Hemhofer Seenplatte, Pilsensee T3; 2010-08-16; Wutz K.; 26-01-02.10; TUM*	B	-	-	-	48.019839°	11.190176°	-
7	21	*N. major*	Germany, Bavaria / Hesse, See Emma Nord; 2010-08-24; Wutz K.; TUM*	A	-	KT596455	-	50.075660°	8.998755°	-
10	30	*N. marina*	Germany, Bavaria, See Freigericht-Ost; 2010-08-24; Wutz K.; 26-01-03.7; TUM*	A	-	KT596485	-	50.082776°	9.008973°	-
10	30	*N. marina*	Germany, Bavaria, Simssee; 2010-09-01; Wutz K.; 26-01-02.11; TUM*	B	-	KT596486	-	47.887072°	12.245457°	-
10	29	*N. major*	Germany, Bavaria, Soyensee; 2010-09-01; Wutz K.; 26-01-03.8; TUM*	A	-	KT596488	-	48.106138°	12.206744°	-
10	30	*N. marina*	Germany, Bavaria, Starnberger See T1; 2010-08-18; Wutz K.; T 4; 26-01-02.13; TUM*	B	-	KT596544	-	47.858883°	11.302090°	-
10	30	*N. marina*	Germany, Bavaria, Starnberger See T2; 2010-08-18; Wutz K.; T 4; 26-01-02.14; TUM*	B	-	-	-	47.935402°	11.299637°	-
10	30	*N. marina*	Germany, Bavaria, Starnberger See T3; 2010-08-18; Wutz K.; T 4; 26-01-02.15; TUM*	B	-	-	-	47.930441°	11.332686°	-
10	30	*N. marina*	Germany, Bavaria, Starnberger See T4; 2010-08-18; Wutz K.; T 4; 26-01-02.16; TUM*	B	-	-	-	47.820708°	11.320249°	-
10	30	*N. marina*	Germany, Bavaria, Tachinger See, 2010-09-08, Wutz K., 26-01-02.18, T 2; TUM*	B	-	KT596547	-	47.987217°	12.748589°	-
10	30	*N. major*	Germany, Bavaria, Waginger See; 2010-09-08; Wutz K.; 26-01-03.12; TUM*	A	-	KT596549	-	47.929279°	12.800611°	-
9	26	*N. major*	Germany, Brandenburg, Werlsee; 2010-08-01; Wutz K.; TUM*	A	-	KT596565	-	52.421186°	13.813587°	-
10	30	*N. marina*	Germany, Bavaria, Wörthsee T1, 2010-08-16, Wutz K., 26-01-02.19, T 1; TUM*	B	B	KT596551	KT596650	48.053692°	11.160887°	-
10	30	*N. marina*	Germany, Bavaria, Wörthsee T2, 2010-08-16, Wutz K., 26-01-02.20, T 1; TUM*	B	-	-	-	48.044505°	11.169708°	-
10	29	*N. marina*	Germany, Bavaria, Wörthsee T3, 2010-08-16, Wutz K., 26-01-02.21, T 1; TUM*	B	-	-	-	48.068693°	11.184704°	-

Appendix A3

Mean values of relative growth rates (RGR; [d^{-1}]) and other plant growth parameters: shoot and root dry weight SR DR in the different macrophytes species at the end of the study (n = 3 - 6). Brackets indicate standard deviation (SDs). Where no SD is given n = 1. A: Algae, H: CDOM (colored organic matter), R: reference condition, S: SPM (suspended particulate matter). Different superscript letters indicate statistical differences at the 0.05 significance level (*post hoc* Tukey's test or Dunn's test).

		Means and standard deviations (SDs)		
Species	**Tank**	**RGR**	**SR DW**	**R/S**
		relative growth rate	shoot + root dry weight	root to shoot biomass ratio (dry weight)
		[g g^{-1} d^{-1}]	**[g]**	**[g g^{-1}]**
Elodea nuttallii	**R**	0.03 (0.01)	0.56 (0.85)	0.1 (0.04)
	A	0.04 (0.01)	1.61 (1.46)	0.06 (0.02)
	H	0.03 (0.01)	0.04 (0.02)	0.1 (0.03)
	S	0.03 (0.02)	1.30 (2.2)	0.05 (0.02)
Hydrilla verticillata	**R**	0.007 (0.01) [a]	0.03 (0.02) [a]	0.06 (0.01)
	H	-0.003 (0.01) [ab]	0.01 (0.02) [ab]	0.05 (0.03)
	A	-0.02 (0.01) [b]	0 (0.01) [b]	0.06 (0.02)
	S	-0.002 (0.02) [ab]	0.03 (0.05) [ab]	0.06 (0.02)
Lagarosiphon major	**R**	0.014 (0.01)	0.07 (0.08)	0.13 (0.08)
	H	0.002 (0.01)	0.02 (0.02)	0.1 (0.03)
	A	0.003 (0.01)	0.02 (0.02)	0.06 (0.02)
	S	0.007 (0.01)	0.03 (0.02)	0.09 (0.03)
Myriophyllum verticillatum	**R**	0.006 (0.005)	0.03 (0.01)	0.12 (0.06)
	H	0.006 (0.01)	0.02 (0.02)	0.14 (0.07)
	A	-0.002 (0.002)	0.03 (0.03)	0.13 (0.15)
	S	0.001 (0.01)	0.05 (0.02)	0.06 (0.02)
Najas major	**R**	0.04 (0.01)	2.12 (1.66)	0.01 (-)
	H	0.03 (0.02)	3.21 (4.99)	0.06 (-)
	A	0.03 (0.01)	2.23 (2.21)	0.03 (-)
	S	0.05 (0.01)	4.12 (2.77)	0.01 (-)
Najas marina	**R**	0.03 (0.01)	1.78 (1.49)	0.02 (-)
	H	0.03 (0.002)	0.3 (0.05)	0.05 (-)
	A	0.01 (0.02)	0.6 (0.53)	0.03 (-)
	S	0.05 (0.01)	2.4 (2.24)	0.01 (-)