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Molecular eDNA-markers, distribution vectors and potential niche shifts of invasive mussels *Dreissena polymorpha* and *Dreissena rostriformis bugensis*

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Molecular eDNA-markers, distribution vectors and potential niche shifts of invasive mussels *Dreissena polymorpha* and *Dreissena rostriformis bugensis*

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[3)] In review in Management of Biological Invasions

[4)] In review in Management of Biological Invasions

Summary

In freshwater ecosystems worldwide, invasive species are a major threat to the biodiversity, and cause high economic costs. Research is strongly needed to find solutions to such environmental and anthropogenic problems, which put ecosystem services at risk. In order to be effective, problem oriented research has to be issue-oriented and act across different disciplines considering the diversity and complexity of the involved processes. The invasion of zebra mussels (*Dreissena polymorpha*) and quagga mussels (*Dreissena rostriformis bugensis*) to North America and Western Europe pose one example of a human induced problem, as both species have strong negative ecological and economic impacts in their invasive range. In Switzerland, the zebra mussel is widely distributed in most rivers and lakes since the 1960ies. On the other hand, the quagga mussel is about to invade, and is expected to colonize an even wider and different range of habitats compared to zebra mussels. In order to plan preventive measures against their further spread we need a profound understanding of the impact, ecology and invasions pathways of these two species. In this thesis, I close some specific knowledge gaps, helping to design preventive measures against the further spread of zebra and quagga mussels in Switzerland. I chose an interdisciplinary research approach involving research tools from various disciplines such as freshwater ecology, evolutionary biology, social sciences and molecular biology.

Knowing the ecological niche with its abiotic and biotic constraints is critical in order to estimate the potential distribution of an invasive species. In chapter 1, I tested whether quagga mussels are more tolerant to low oxygen conditions and low temperatures compared to zebra mussels, as a higher tolerance may allow quagga mussels to colonize the deeper zones of stratified lakes and lakes at higher altitudes. Against my expectations quagga mussels were not more tolerant to oxygen depletion. Instead, I found more pronounced phenotypic variation among populations than among species indicating evolutionary post-invasion responses in both zebra and quagga mussels. My results suggest that adaptive processes need to be considered when setting up predictive models of the future dreissenid distribution and that such processes need to be investigated further.

Overland transport of recreational boats has been shown to be an important distribution vector for zebra and quagga mussels to inland waters. Interviewing boat owners by means of a self-administered questionnaire and assessing biofouling

samples collected from moored boats, I investigated in chapter 2 the vector potential of recreational boating in Switzerland. I demonstrate that mainly seasonally and year-round moored boats pose a high risk of spreading zebra mussels to all navigable lakes. As recreational boating may distribute quagga mussels in a similar way, it is important to take preventive measures tackling this type of vector.

Effectively cleaning the boats and equipment before they are transported overland from one water body to another, may slow down the spread of invasive mussels. The boat cleaning behavior of boat owners have never been studied in detail before the implementation of preventive measures. In chapter 3, I investigated the boat cleaning behavior of boat owners in Switzerland, by studying the boat cleaning rates, cleaning methods, the cleaning motivation and the reasons why boat owners neglect to clean their boat. A high proportion of boat owners reported to clean their boat, either before they transport it overland or after they have detected mussels growing on their boat, but often they did not use effective cleaning methods, such as high pressure washing. Furthermore, I show how the motivation of boat owners influences their cleaning behavior and that well designed information campaigns may increase the cleaning rates and the use of appropriate cleaning methods.

Effective tools for early detection and surveillance are a necessity for the management of invasive species. Therefore, I tested the detection and quantification of zebra and quagga mussels from a simple water sample (eDNA) using PCR (polymerase chain reaction) and qPCR (quantitative PCR) with species specific primers. In this last chapter, I demonstrate that a) the established methods are reliable and inexpensive to detect both mussel species and b) that qPCR has great potential to quantify zebra and quagga mussel biomass from such eDNA samples. After an additional validation process, this method may be used for the surveillance of the population development of the two species.

This thesis emphasizes that interdisciplinary research can effectively tackle environmental problems. By using this approach, I solved some specific questions which are of practical use when planning measures against the further spread of two aquatic invasive species.

Zusammenfassung

In den Süsswasser-Ökosystemen weltweit stellen invasive Arten eine grosse Bedrohung für die biologische Artenvielfalt dar und verursachen hohe Kosten für die Gesellschaft. Weil dadurch die Ökosystemdienstleistungen in Mitleidenschaft gezogen werden, ist es dringend notwendig mittels Forschung Lösungen für solch anthropogene Umweltprobleme zu finden. Um die Probleme effektiv zu lösen, soll sich die Forschung an spezifischen Fragestellungen ausrichten und mithilfe verschiedener Disziplinen arbeiten, um die Vielfalt und Komplexität dieser Probleme zu berücksichtigen. Die Invasion der Zebramuschel (*Dreissena polymorpha*) und der Quaggamuschel (*Dreissena rostriformis bugensis*) in Nordamerika und Westeuropa ist ein Beispiel eines anthropogenen Umweltproblems, welches stark negative Auswirkungen auf die Ökosysteme und hohe ökonomische Kosten in den neu besiedelten Gebieten verursacht. Seit den 1960iger Jahren hat sich die Zebramuschel in vielen Fliessgewässern und Seen der Schweiz angesiedelt. Die Quaggamuschel hingegen hat gerade erst angefangen die Schweizer Gewässer zu besiedeln und kann vermutlich mehr Lebensräume kolonialisieren als die verwandte Zebramuschel. Damit geeignete Vorsorgemass-nahmen geplant werden können, ist es notwendig die Ökologie, die Besiedlungswege und die Auswirkungen dieser gebietsfremden Art zu kennen. In dieser Doktorarbeit schliesse ich konkrete Wissenslücken, deren Antworten helfen werden, geeignete Vorsorgemassnahmen zu entwerfen, um die weitere Verbreitung der Zebra- und Quaggamuschel zu verhindern. Ich habe einen interdisziplinären Ansatz gewählt und bediente mich der Forschungsmethoden aus verschiedensten Disziplinen, wie zum Beispiel aus der Gewässerökologie, der Evolutionsbiologie, den Sozialwissenschaften und der Molekularbiologie.

Die Anforderungen an den Lebensraum und die Randbedingungen der ökologischen Nische einer invasiven Art bestimmen deren potentielles Verbreitungsgebiet. Im ersten Kapitel untersuchte ich, ob Quaggamuscheln mit tiefen Sauerstoffkonzentrationen und tiefen Wassertemperaturen besser umgehen können als Zebramuscheln. Eine höhere Toleranz für diese Messgrössen würde der Quaggamuschel erlauben Seen in grösserer Höhe und tiefere Lebensräume in geschichteten Seen zu besiedeln. Entgegen meinen Erwartungen zeigten die Quaggamuscheln keine höhere Toleranz unter den Sauerstoff zehrenden Bedingungen. Dafür fand ich heraus, dass die phänotypischen Abweichungen zwischen den benutzten Populationen grösser sind als zwischen den Arten, was auf evolutionäre Prozesse nach der erstmaligen Besiedlung hindeutet. Deshalb weisen

meine Resultate darauf hin, dass adaptive Prozesse beim Erstellen von Vorhersagemodellen berücksichtigt werden, und dass solche evolutionären Prozesse bei biologischen Invasionen genauer untersucht werden sollten.

Der Überlandtransport von Freizeitbooten ist bekannt als wichtiger Verbreitungsvektor für Zebra- und Quaggamuscheln in Binnengewässern. Im zweiten Kapitel untersuchte ich das Vektorpotential der Freizeitboote in der Schweiz, in dem Bootsbesitzer mit selbst-auszufüllenden Fragebogen befragt wurden. Zudem wurde der Aufwuchs an im Wasser vertäuten Booten und an Booten beim Auswassern an Bootsrampen untersucht. Meine Daten zeigen, dass hauptsächlich saisonale und ganzjährig im Wasser liegende Boote ein hohes Vektorpotential für die Verbreitung der Zebramuschel in alle schiffbaren Seen darstellt. Weil die Quaggamuschel durch den Freizeitbootsverkehr ähnlich verbreitet werden könnte, ist es dringend notwendig Vorsorgemassnahmen bei diesem Verbreitungsvektor zu treffen.

Die Verbreitung der invasiven Muschelarten könnte auch durch die wirksame Reinigung der Boote und Gerätschaften vor dem Überlandtransport verlangsamt werden. Das Reinigungsverhalten der Bootsbesitzer wurde bisher noch nie vor der Einführung (e.g. Behörden) derartiger Vorsorgemassnahmen eruiert. Im dritten Kapitel prüfte ich das Reinigungsverhalten der Bootsbesitzer in der Schweiz, in dem ich die Bootsreinigungsquote, die Reinigungsmethoden, die Motivation zur Bootsreinigung und die Gründe, warum Bootsbesitzer die Reinigung vernachlässigen untersucht habe. Ein hoher Anteil der Bootsbesitzer gab an ihr Boot vor einem Überlandtransport oder nach Entdeckung von Muschelaufwuchs am Boot zu reinigen. Jedoch wurden häufig Reinigungsmethoden angewandt, welche nicht so wirksam sind wie das Hochdruckreinigungsverfahren. Ich konnte zeigen, dass die Motivation der Bootsbesitzer ihr Reinigungsverhalten beeinflusst, und dass mit diesem Wissen entworfene Informationskampagnen die Reinigungsquote erhöhen und die Anwendung der richtigen Reinigungsmethoden beeinflussen können.

Wirkungsvolle Werkzeuge für die Früherkennung und Überwachung sind eine Notwendigkeit beim Management von invasiven Arten. Mittels artspezifischer Primer testete ich die Erkennung und Quantifizierung von Zebra- und Quaggamuscheln aus Wasserproben (eDNA) unter Anwendung der Polymerase-Kettenreaktion (PCR) und der quantifizierbaren PCR (qPCR). In diesem letzten Kapitel konnte ich demonstrieren, dass a) diese etablierte Methode zuverlässig und kostengünstig die beiden Muschelarten in den eDNA Wasserproben erkennt und b)

qPCR das nötige Potential aufweist, die Biomasse von Zebra- und Quaggamuscheln in dem beprobten Gewässer zu quantifizieren. Nach einem zusätzlichen Validierungsprozess könnte diese Methode für die Überwachung der Populationsdynamik der beiden Arten benutzt werden.

Mit dieser Doktorarbeit möchte ich unterstreichen, dass interdisziplinäre Forschung zur Lösung von Umweltproblemen beitragen kann. Dank dieser Ausrichtung habe ich einige spezifische Fragestellungen zur Verbreitung der beiden invasiven Muschelarten beantwortet, welche von praktischer Bedeutung beim Entwurf der geeigneten Vorsorgemassnahmen sein werden.

General introduction

Problem oriented research on invasive species in freshwaters

Invasive alien species (IAS) are among the most important causes of species extinctions, capable of evoking rapid changes in ecosystems. They are a major threat to biodiversity and human health (Wilcove et al. 1998; Gurevitch and Padilla 2004) and can cause high economic costs, which burden taxpayers and hamper private industry through high re-investment and infrastructure costs (Pimentel et al. 2005). With ongoing globalization of trade and travel, introduction rates of non-native species to new environments have increased dramatically (Meyerson and Mooney 2007). Particularly in freshwater ecosystems, the human mediated transport of these organisms plays a key role in the environmental change of biota (Sala et al. 2000). The negative effects of aquatic invasive alien species can cascade through the whole ecosystem and impair ecosystem functions (Ricciardi and Macisaac 2010). In order to plan and take measures against invasive species we need to estimate their potential future distribution and their potential for negative impacts, for which we need to understand their invasion pathways and their environmental niche.

Environmental research, and particularly invasion biology, is expected to contribute to the solution of such anthropogenic problems, which put ecosystem services at risk (Bocking 2004; Kueffer and Hirsch Hadorn 2008), as it has been repeatedly stated since the UN conference on Environment and Development in 1992 (Funtowicz et al. 1998; Lubchenco 1998). Kueffer and Hirsch Hadorn (2008) present a framework for effective problem oriented research: In order to be effective, problem oriented research has to be issue oriented, has to take the different dynamics, diversity and complexity of the involved processes into account and thus has to act across different disciplines (Hirsch Hadorn et al. 2006). For example, in the case of biological invasions, it is not enough to understand the ecology of the species at hand. Instead, many anthropogenic factors such as the anthropogenic dispersal of a species or the alteration of habitats by human activities have to be taken into account (Estévez et al. 2015). We also need to understand the economic consequences of biological invasions and how legal policies influence the invasion potential. For the mitigation of such environmental problems new policies or behavioral changes in humans are often needed. Further, technology can provide new tools for the surveillance of IAS or for preventive and alleviating measures (Caffrey et al. 2015). Thus integrative ecological research is the key to tackle the complexity of specific real-world problems (Caffrey

et al. 2014). More specifically, a wide range of disciplines (such as social sciences, system analysis, law or technology) need to work together in order to understand the problem and find potential solutions (Kueffer 2006).

My PhD thesis focuses on applied, problem oriented research driven by specific questions, rather than fundamental hypotheses driven science. By its nature, research on biological invasions has a strong orientation towards specific questions, which can be useful for the planning of preventive measures against the spread of nuisance species and for the mitigation of negative impacts (Ruiz 2003). While I was in the process of starting my PhD thesis in 2011 the invasion front of the quagga mussel (*Dreissena rostriformis bugensis*, Andrusov 1897) was moving south towards Switzerland (Molloy et al. 2007; Martens and Schiel 2012; Heiler et al. 2013). Research from North America (Strayer 2009) had already shown a series of negative impacts on ecology and economics caused by the invasive quagga mussel and its congener, the zebra mussel (*Dreissena polymorpha*, Pallas 1771). As the quagga mussel was expected to become a nuisance species in Switzerland, I decided to investigate its invasion potential and answer some of the questions which may help to take preventive or alleviating measures.

My thesis involves research tools from various disciplines, such as freshwater ecology, evolutionary biology, social sciences and molecular biology and is a case study showing how ecologists can make use of various disciplines for solving real world problems. Here, I describe the problem of the imminent quagga mussel invasion to Switzerland and explain which contributions this thesis offers to find appropriate measures. I describe the different disciplines we used to address the problem and explain why we used those different approaches. Subsequently, I present the four chapters, which contain the main results of my PhD thesis. At the end of the thesis, I discuss how the results from the different disciplines and approaches may help to solve the problem, and what we can learn for the planning of measures against the quagga mussel invasion.

The study system and the problem

The zebra mussel, *Dreissena polymorpha*, and the quagga mussel, *Dreissena rostriformis bugensis*, are two closely related species originating from the Ponto-Caspian region (Albrecht et al. 2007). Both are highly invasive in both North America and Europe, exhibit extremely high population densities and strong negative impacts on the ecology (Strayer 2009; Higgins and Vander Zanden 2010) and economy (Pimentel et

al. 2005) in invaded water bodies. These mussels are sessile, byssate bivalves with a planktonic larval stage and extremely high reproductive capacity (Nichols and Black 1994; Ackerman et al. 1994). The two share a similar ecological niche and distribution pathways but experienced very different invasion histories in Western Europe (bij de Vaate et al. 2002; Vanderploeg et al. 2002). Zebra mussels have spread widely in Western Europe since the early 19th century and have reached all larger rivers and lakes in Switzerland since the 1960ies (Kinzelbach 1992; Burla and Ribi 1998; bij de Vaate et al. 2002). In contrast, the quagga mussel has a much shorter invasion history in Western Europe, starting only in 2004 with potentially multiple introductions to the Netherlands and the Main-Rhine-Danube canal (Molloy et al. 2007; Imo et al. 2010; Heiler et al. 2013).

Presently, quagga mussels are expected to spread to lakes and rivers in Switzerland, as they have spread widely in the Rhine River system within a few years and have reached Karlsruhe around 2007 (Martens and Schiel 2012; Heiler et al. 2013; Matthews et al. 2014). We have now demonstrated its presence in the harbor of Basel at the Swiss border by using detection methods based on eDNA with species specific primers (chapter 4), but to our knowledge the quagga mussel has not spread further yet. Compared to the zebra mussel, quagga has a broader reproductive tolerance as it is able to reproduce at lower water temperatures and survive at higher water depths (Mills et al. 1996; Orlova et al. 2005). Additionally, the quagga mussel is able to colonize hard as well as soft substrates (Zhulidov et al. 2010) and may survive better in more oligotrophic water bodies (Baldwin et al. 2002). The introduction of the quagga mussel could therefore lead to a much wider distribution range of dreissenids in Switzerland, and quagga mussels may also establish in those Swiss water bodies and habitats (e.g. deep lake profundal) which are still free of zebra mussels. Adding to the negative impacts of zebra mussels, quagga mussels might significantly increase the issues caused by dreissenid species in the future. Water suppliers and operators of thermal power plants fear that their water intake pipes, which are often located at depths where zebra mussels are only present in very low densities, may be more strongly colonized by quagga mussels and may thus be clogged more often. The consequence of such a scenario would be that pipes need to be cleaned more often with chlorine, drinking water production may be reduced and production costs may increase significantly (Koester 2014). Thus it is urgent to develop strategies for the management of quagga mussels as soon as possible.

Research helping to solve the problem

In order to manage the invasive alien species (IAS) it is of prime importance to recognize the newly arriving IAS, comprehend their distribution pathways and provide predictions for future spread and magnitude of damage in the affected ecosystems (Caraco et al. 2000; Hoy et al. 2010). Furthermore, we need to provide tools to detect and monitor such a species, to reduce its spread and to alleviate its impact. The most effective and most economical way of dealing with IAS, is to prevent an invasion by taking measures to prevent its arrival and spread. After establishment and a lag phase, some alien species are able to spread rapidly in an exponential manner, which is the definition of a species becoming invasive (Lockwood et al. 2007; Miehls et al. 2009). At this stage, it is already very difficult to eliminate IAS from the natural environment. Thus it is important to forecast species invasions and to act ahead of time, before the species is established in a new geographic region of concern (Lockwood et al. 2007). This requires that we would be able to predict or anticipate species invasions. The knowledge from other geographic regions, where a potential invader has already become invasive, is an important source for learning, without forsaking to acknowledge environmental, biological and societal differences between regions.

Concerning the zebra and quagga mussel invasion, a considerable amount of research has examined the impacts of the two species on the environment and economy (Pimentel et al. 2005; Strayer 2009; Higgins and Vander Zanden 2010), their distribution pathways (Johnson and Carlton 1996; Johnson et al. 2001), their environmental niches including both biotic and abiotic interactions (Karatayev et al. 1998; Orlova et al. 2005; Hallstan et al. 2010; Zhulidov et al. 2010) and the competitive interactions between the two species (Baldwin et al. 2002; Karatayev et al. 2011b). Using environmental niche models (Drake and Bossenbroek 2004; Quinn et al. 2014), vector based models (Bossenbroek et al. 2007) or models based on populations dynamics (Mari et al. 2009) researchers have also established predictive models for the distribution potential of either species and in North America authorities and researchers together have implemented a series of measures in order to prevent their further spread (Rothlisberger et al. 2010). Most of the above mentioned research on the invasion of zebra and quagga mussels has been conducted in North America and Eastern Europe. Not all of these findings therefore apply for the situation in Central Europe and Switzerland (as will be shown in chapters 1, 2 and 3). This thesis thus fills some of the knowledge gaps with regard to the particular situation in

Switzerland, focusing on the invasion potential of the quagga mussel to Switzerland. In this case study I can show that problem oriented science can help to provide important knowledge for management.

Chapter 1: The environmental niche

Habitat requirements and the ecological niche constraints (along with distribution pathways) are critical in order to estimate the potential distribution of an invasive alien species. I thus compared some of the main limiting factors for population establishment and growth of zebra and quagga mussels. These limits can then be compared to the current distribution of zebra mussels and form the basis for estimating the potential distribution of quagga mussels in Switzerland. When compared to zebra mussels, quagga mussels have been shown to grow better at lower temperatures, greater depths in lakes (Mills et al. 1993; Roe and MacIsaac 1997; Orlova et al. 2005) and use energy more efficient under low food conditions (Baldwin et al. 2002; Stoeckmann 2003). Therefore, quagga mussels might colonize deep habitats in Swiss lakes and also colder or more oligotrophic lakes at higher altitudes in the future.

Swiss lakes are often deep, cover a large range of trophic states and typically show dimictic cycles, seasonal changes and depth gradients. This means they are different from most European and American lakes. Moreover, they show pronounced oxygen depletion during the summer months (Müller et al. 2012). Hypolimnetic oxygen depletion is also a common phenomenon in some of the large and deep lakes of North America (U.S. EPA GLNOP 2013), but the growth limitation of zebra and quagga mussels by different oxygen levels has never been assessed experimentally (Alexander and McMahon 2004). The main assumption for my first chapter is based on the observation of Stoeckmann (2003) who demonstrated that quagga mussels consumed less oxygen than zebra mussels over a wide range of temperatures. Therefore, I suspected, that oxygen depletion may play an important role for the growth limitation of zebra and quagga mussels in the deep lakes.

In the first chapter, I compared the survival and growth of zebra and quagga mussels in four different oxygen concentrations in two temperatures (De Ventura et al. 2016a). As the quagga mussel has not spread within Switzerland yet, we performed the experiments in the Netherlands, where both species have spread widely. When planning those experiments, I took into account that the mussels may already be adapted to local conditions and that mussel populations may have differences in

their tolerance to low oxygen conditions. It has been repeatedly shown, that evolutionary processes, such as local adaptation, can play an important role along the invasion process and that evolutionary processes may be more important at the invasion front (Lee 2002; Sexton et al. 2009; Lee et al. 2012). Nevertheless, when describing the environmental niche of IAS, invasion biologists often do not consider multiple populations in their experiments. The idea that evolutionary processes and the invasion dynamics act on different time scales is still prevalent in invasion biology, but is not necessarily true. In order to account for population differences and potential adaptation to local conditions I included three populations of each mussel species in a full factorial experiment (chapter 1).

Chapter 2: Distribution vectors

In order to manage and reduce the damage caused by IAS, we need to improve our understanding of the factors that promote invasion. In freshwater ecosystems, human mediated transport of organisms undoubtedly plays a key role (Sala et al. 2000; Kolar and Lodge 2001). The main causes for many introductions of IAS in the Rhine River system have been increased connectedness among watersheds that were historically separate, transoceanic transport in the ballast water of large ships to harbors in the river delta, but also intentional and unintentional transport and release through other human activities (bij de Vaate et al. 2002; Leuven et al. 2009). The primary distribution vectors for zebra and quagga mussels over long distances are shown to be transoceanic transport in ballast water, transport with commercial ships along waterways and passive downstream dispersal of mussel larvae (Ricciardi and Rasmussen 1998; Schneider et al. 1998; Vanderploeg et al. 2002; Leuven et al. 2009). The suspected primary distribution vector for zebra and quagga mussels to more isolated or disconnected water bodies away from the main shipping routes, is the overland transport of recreational boats (Johnson et al. 2001; Minchin et al. 2003; Martens and Schiel 2012).

In Switzerland, the dendritic river networks are strongly fragmented by dams and thus overland transport is required for upstream distribution of mussels. Even if Swiss water bodies are heavily used for recreational boating (with a total 100'000 registered boats), there was no information at hand on the overland transport activities of recreational boaters. Furthermore, only very little was known about whether different types of boats or different boating practices may play different roles for the overland transport of zebra mussels. In chapter 2, boat owners in Switzerland were invited to take a survey where we asked them about the properties

of transported boats, observations on zebra mussel fouling, transport activities and boat cleaning habits (De Ventura et al. 2016b). We interviewed roughly 3'500 registered boat owners using a self-administered questionnaire. We also analyzed biofouling samples taken from boats at launching ramps and from boats moored in harbors, in order to confirm the mussel fouling rates found with the questionnaire. Based on the results from the questionnaire we then estimated the distribution potential of zebra mussels by recreational boating. We further discuss the implications of our findings for the future spread of quagga mussels in Switzerland, as their spread to isolated lakes was also repeatedly linked to the overland transport of recreational boats (Stokstad 2007; Karatayev et al. 2011a; Martens and Schiel 2012).

Chapter 3: Human behavior matters

In the previous chapter, I showed that overland transport of recreational boats is an important distribution vector for zebra mussels in Switzerland. Attached to the exterior of seasonally or year-round moored boats, mussels are transported between water bodies with high enough frequencies to spread both zebra and quagga mussels to all navigable water bodies in Switzerland (De Ventura et al. 2016b). Prevention measures such as instructing boat owners to appropriately clean their boats and equipment to remove organisms and let the boat or equipment dry before transport from one water body to another, may slow down the spread of invasive mussels. Such measures have been implemented and tested widely in North America (Morse 2009; Rothlisberger et al. 2010; Comeau et al. 2011). Nevertheless, the effects of such measures are poorly understood and the boat cleaning behavior of boat owners has never been studied in detail before the implementation of preventive measures. In chapter 3, we thus investigated the boat cleaning behavior of boat owners in Switzerland, where almost no preventive measures have been taken yet. By studying the boat cleaning rates, the cleaning motivation and the reasons why boat owners may neglect to clean their boat, we were able to suggest which behavioral changes are needed in order to increase boat cleaning rates and the use of appropriate boat cleaning methods. We also show how the proposed behavioral changes may be best achieved and what the effects of such behavioral changes on the vector potential of recreational boating may be. Finally, I recommend which measures should be taken urgently in order to prevent the further spread of quagga and zebra mussels in Switzerland.

Chapter 4: New tools for early detection and monitoring

Effective tools for early detection and surveillance are indispensable for the management of IAS (Caffrey et al. 2014). Early detection and quantification of aquatic species by traditional methods, such as kick-net sampling or scuba diving, is often difficult, laborious and potentially inaccurate (Barbour et al. 1999; Stucki 2010), in particular for small or larval stage freshwater invertebrates, which additionally may show patchy distribution patterns (Arscott et al. 2003). Zebra and quagga mussels may be difficult to detect in their early phase of invasion because of low densities and patchy distribution (Burlakova et al. 2006; Lockwood et al. 2007). As we showed in chapter 2, quagga mussels are likely to appear first in the larger lakes in Switzerland (De Ventura et al. 2016b), which are heavily used by recreational boating, and they may colonize the deeper zones first (Mills et al. 1993; Orlova et al. 2005; Zhulidov et al. 2010). Moreover, it has been repeatedly demonstrated that the detection and quantification of species abundances from environmental DNA (eDNA) extracted from water samples has several advantages over traditional surveillance methods (Dejean et al. 2012; Jerde et al. 2013; Goldberg et al. 2013). In the last chapter we assessed the use and utility of eDNA samples with species-specific primers (Bronnenhuber and Wilson 2013). We tested the detection and quantification of zebra and quagga mussels using PCR (polymerase chain reaction) and qPCR (quantitative PCR). We demonstrate that the established methods are inexpensive and reliably detect both zebra and quagga mussels. We also show that eDNA quantification has a great potential to be used for the surveillance of the population development of zebra and quagga mussels.

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Chapter 1

Variability in the phenotypic tolerance to low oxygen in invasive populations of quagga and zebra mussels

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Abstract

Novel biotic or abiotic conditions can cause invasive species to evolve rapidly in their newly invaded habitats and are important factors when predicting species invasions. Zebra mussels (*Dreissena polymorpha*) have a relatively long invasion history in Western Europe, whereas quagga mussels (*Dreissena rostriformis bugensis*) began spreading about a decade ago. In a previous invasion to North America, quagga mussels repeatedly colonized stratified lakes at greater depth than zebra mussels. It would be important to know if the same invasion pattern is expected to repeat in Western Europe, as the quagga are expected to reach deep stratified lakes in the near future. This might require quagga mussels to be more tolerant to the low oxygen conditions at depth than zebra mussels. Therefore, using a fully factorial design, we tested survival of different zebra and quagga mussel populations from Western Europe in four oxygen levels (6%, 33%, 66% and 90%) and two temperature regimes (11°C and 18°C). Surprisingly, survival differences among oxygen and temperature treatments depended more on population origin than on species identity. This finding suggests that populations have undergone rapid and convergent adaptation to local conditions after invasion, in particular to low oxygen. We also found that population-by-environment interactions were more variable among quagga than zebra mussel populations. Our results suggest that rapid evolutionary adaptation to low oxygen conditions needs to be considered when predicting the further spread of zebra and quagga mussels.

Key words

Dreissena polymorpha, *Dreissena rostriformis bugensis*, niche shifts, temperature, oxygen depletion, population-by-environment interactions

Introduction

Recognizing that eco-evolutionary dynamics may be important for natural adaptation has alerted invasive species ecologists to consider ecological and evolutionary processes such as phenotypic plasticity, developmental plasticity and local adaptation when predicting the future range and ecological impact of non-native species (Lee 2002; Lambrinos 2004). Consequently, newly established invasive populations might diverge rapidly in their tolerance to various environmental factors (Pearman et al. 2008; Prentis et al. 2008; Sexton et al. 2009). For example the copepod *Eurytemora affinis,* which is a native salt water species, evolved adaptations to interstitial ion regulation within a few generations of invading freshwater lakes on the east coast of North America (Lee et al. 2012). In another example Huey et al. (2000) found that upon introduction to North America the native European fruit fly, *Drosophila subobscura,* evolved gradual phenotypic adaptation in wing size in response to temperature along the invasion route in less than two decades. These examples emphasize that non-native species might adapt their environmental niche along with the invasion process in response to environment. Such adaptations are known to require sufficient heritable genetic variation, which in non-native species depends on propagule number, source of the introduction and details of the invasion history (Roman and Darling 2007; Ficetola et al. 2008; Brown and Stepien 2010).

The zebra mussel, *Dreissena polymorpha* (Pallas, 1771), and the quagga mussel, *Dreissena rostriformis bugensis* (Andrusov, 1897) are two closely related species originating from the Ponto-Caspian region. Both are invasive in Europe and North America and exhibit extremely high population densities and strong ecological and economic impacts in their invasive range (Pimentel et al. 2005; Strayer 2009; Higgins and Vander Zanden 2010). The Zebra mussel has spread widely across Western Europe since the early 19th century (Kinzelbach 1992) and is now present in most larger rivers and lakes. The quagga mussel has a much shorter invasion history in Western Europe, starting in 2004 with potentially multiple introductions from the Netherlands and the Main - Rhine - Danube canal (Molloy et al. 2007; Imo et al. 2010; Heiler et al. 2013). Presently, quagga mussels are expected to spread further to the deep stratified lakes in vicinity of the alps, which often show pronounced oxygen depletion at depth (Matthews et al. 2014).

Both quagga and zebra mussel populations have high gene flow and express high genetic diversity in their invasive range (Wilson et al. 1999; Muller et al. 2002;

Therriault et al. 2005; Brown and Stepien 2010; Imo et al. 2010). Therefore, populations of either species supposedly have a high potential for genetic adaptation. European quagga mussels show limited genetic differentiation in neutral nuclear markers (Therriault et al. 2005; Imo et al. 2010), whereas zebra mussels express clear divergence among European populations (Muller et al. 2002; Pollux et al. 2003; Rajagopal et al. 2009). These findings are likely to reflect the longer invasion history of the zebra mussel in Western Europe, which has had more time to differentiate and adapt to local conditions. Although quagga mussels started to spread later than zebra mussels in Western Europe, the invasion front is proceeding rapidly (Matthews et al. 2014) and the quagga mussel seems the stronger competitor when both species are present (Mills et al. 1996; Orlova et al. 2005; Karatayev et al. 2011b). The invasion history and population ecology of these species predicts that sufficient genetic variation for evolutionary adaptation should be present, but evidence for evolutionary adaptation in invasive populations has been lacking so far.

In this study, we investigated among-population differences in survival of quagga and zebra mussels in low and high oxygen conditions under two temperature regimes, with the aim of detecting physiological adaptation to low-oxygen conditions. The deep lakes of the alpine region are still free of quagga mussels and often show prolonged phases of oxygen depletion (of variable severity depending on the lake) during summer stratification. The recently invaded shallow lakes in the Netherlands on the other hand show only short phases (several days) of hypoxic conditions during extreme summer heat events. One study showed that zebra mussels are poor oxygen regulators but depending on the temperature are able to survive in low oxygen conditions for a number of days (Johnson and McMahon 1998). Moreover, Stoeckmann and colleagues (2003) showed that quagga mussels of Lake Erie had a lower respiration rate and consumed less oxygen than the sympatric zebra mussels over a range of temperatures. It is not clear if this means that quagga mussels have a higher phenotypic tolerance to low oxygen conditions compared to zebra mussels and whether this might facilitate colonization of the deep hypoxic zones of stratified lakes. As both species colonized water bodies of very different oxygen conditions, with quagga mussels often outcompeting zebra mussels when both species are present, we wanted to know whether differences in colonization patterns arise due to differences in phenotypic tolerance to low oxygen conditions or due to evolutionary adaptation.

We analyzed the population reaction norms for survival in response to oxygen and temperature among Western European populations of quagga and zebra mussels to evaluate the population-by-environment interactions, which can indicate if local adaption has taken place post-invasion. Manipulating oxygen and temperature regimes, we simulated the conditions above and below the summer thermocline in a stratified lake and hypothesized that quagga mussels should show higher survival under these conditions than zebra mussels. In contrast to the very recent (only about one decade) introduction of quagga mussel populations, the time since the introduction of the tested zebra mussel populations was longer, but variable (150 – 40 years). Thus, we hypothesized further that due to their longer invasion history zebra mussel populations would show stronger population-specific adaptive environmental responses.

Methods

Origin of mussels

Samples from six populations of mussels were collected from four locations (Figure 1). Both species were collected from River Main (50.111140N, 8.916910E, Hanau, Germany), and Lake IJsselmeer (52.709983N, 5.493267E, Netherlands). Additionally, zebra mussels were collected from Lake Greifensee (47.349075N, 8.690081E, Switzerland) and quagga mussels from Lake Markermeer (52.531667 N, 5.231083 E, Netherlands). The study lakes have a history of oxygen depletion in the deeper regions. Lake Markermeer and Lake IJsselmeer are large shallow (3.5 m depth and 5 to 6 m depth, respectively) eutrophic lakes in the Netherlands while Lake Greifensee is a smaller and deeper (30 m depth) stratified eutrophic lake in Switzerland. Lake Greifensee shows pronounced oxygen depletion in the hypolimnion during the summer months. For Lake Markermeer and Lake IJsselmeer Noordhuis (2014) reported short periods of stratification and oxygen depletion (a few days) in 2011 and 2012 and mass mortalities of fish and dreissenid mussels were likely due to prolonged stratification and oxygen depletion in the deep zones of the lakes during the extreme summer heat-wave in 2006. Oxygen levels were also reduced in River Main during the summer months but the oxygen concentration never reached levels below 30% oxygenation in the non-stratified River Main (Supplementary Material, Figure S1).

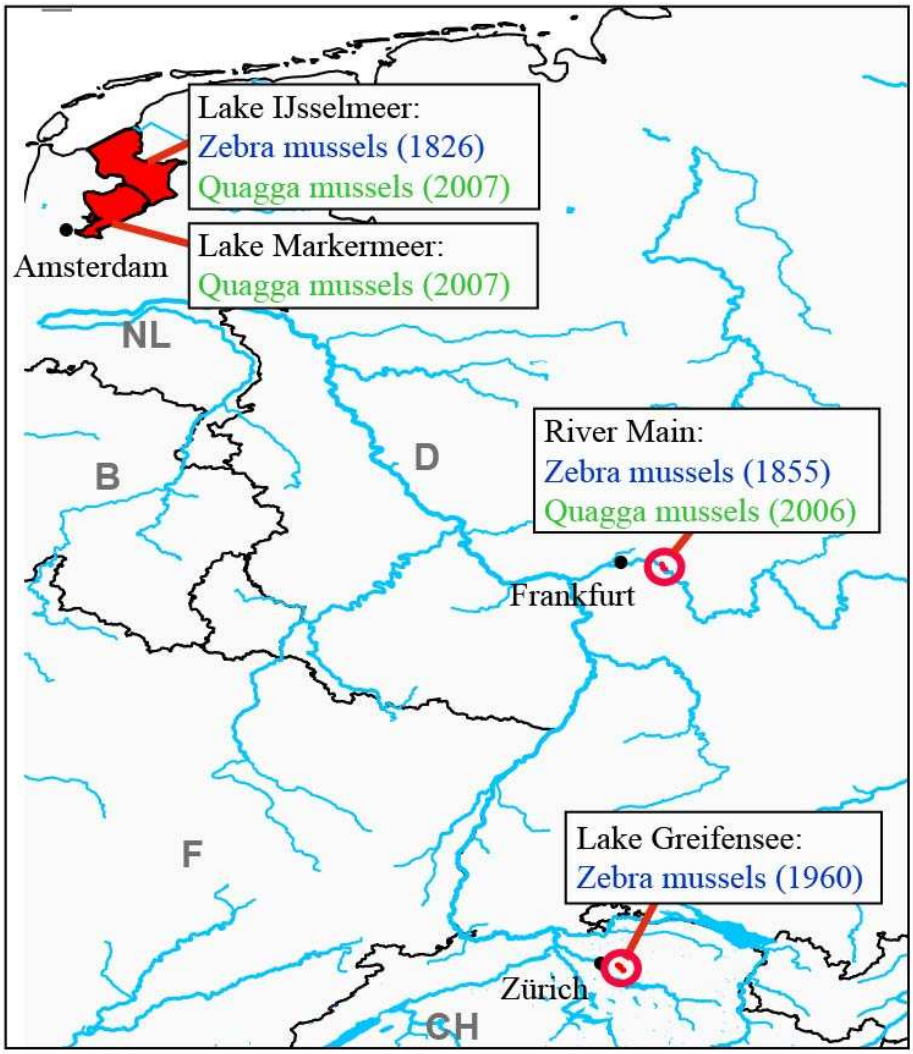

Figure 1 Sampling locations (marked in red) with zebra mussel (*Dreissena polymorpha*) in blue and quagga mussel (*D. rostriformis bugensis*) in green. Lake Markermeer and Lake IJsselmeer are large shallow eutrophic lakes, while Lake Greifensee is a smaller stratified, eutrophic lake. The year of invasion for each population is given in brackets and was retrieved from Kinzelbach (1992) for zebra mussels, and from Heiler et al. (2013) for quagga mussels. Lake IJsselmeer and Lake Markermeer were only constructed in 1932 by cutting the former Zuiderzee (brackish water) from the sea with a dam but zebra mussels had already been present in the surrounding area (Zuiderzee) before that time.

River Main and Lake Greifensee mussels were picked haphazardly from stones near the shore line at roughly 1 m water depth in May 2012 and transported in cooling boxes to the laboratory in Wageningen. In mid-June 2012, we collected mussels from Lake Markermeer (at ca. 3.5 m depth) and Lake IJsselmeer (at ca. 4 m depth) with a bottom dredge (metal frame of 35 x 60 cm, mesh size 5 mm) and sorted them by

species. For each population of mussels, we collected over 2000 individuals of shell length larger than 8 mm. All mussels were acclimated to lab conditions for at least one month before the experiment (started end of July 2012) in aerated ground water at 15°C and fed with 5 ml Shell Fish Diet (Reed Mariculture Inc.) per 1000 mussels per day.

Table 1 Mean shell length (mm) and shell volume (mm^3, estimated as 4/3 x pi x length/2 x height/2 x width/2), the corresponding standard deviation and the total number of experimental mussels (N) for each population, listed as a combination of sampling site (origin) and species.

Origin	Species	N	Variable	Unit	Mean	Standard Dev.
Greifensee	Zebra mussels	384	Shell length	mm	13.40	3.69
IJsselmeer	Quagga	384	Shell length	mm	20.75	4.77
IJsselmeer	Zebra mussels	384	Shell length	mm	13.99	3.14
Main	Quagga	384	Shell length	mm	19.74	5.52
Main	Zebra mussels	384	Shell length	mm	16.06	3.96
Markermeer	Quagga	384	Shell length	mm	13.30	3.27
Greifensee	Zebra mussels	384	Shell	mm^3	421.47	422.52
IJsselmeer	Quagga	384	Shell	mm^3	1252.55	590.89
IJsselmeer	Zebra mussels	384	Shell	mm^3	383.18	239.81
Main	Quagga	384	Shell	mm^3	1645.09	1309.74
Main	Zebra mussels	384	Shell	mm^3	736.06	539.18
Markermeer	Quagga	384	Shell	mm^3	268.88	208.35

Experiment

We assessed survival of mussels of the six study populations under different oxygen and temperature conditions in a mesocosm experiment. We conducted a fully factorial experiment including four replicates of two temperature treatments (11°C and 18°C) crossed with four oxygen levels (6%, 33%, 66% and 90% oxygen saturation) in a total of 32 experimental plastic aquaria (34 x 25 x 16 cm, 9 L, Supplementary Material, Figure S2). Aquaria were held in water baths (glass aquaria of 185 x 50 x 50 cm, equipped with a cooling and heating system, NIOO Institute, Wageningen) to adjust the temperature. Oxygen levels were controlled in a separate tank (20 L plastic bottle) for each oxygen level by bubbling either nitrogen (for the levels of 6%, 33% and 66% oxygen saturation) or oxygen (for 90% oxygen saturation) into the tank. The outflow of each tank was directed to the corresponding aquaria with PVC tubes. Each experimental aquarium was completely filled with water and closed with a lid in order to minimize gas exchange with the surrounding air. The

excess water discharged from the aquaria to the surrounding water bath, while the outflow from the water baths was collected in a common tank and from there pumped back to the four different tanks where the four oxygen levels were controlled. In this way all aquaria were connected to the same closed water circulation system, because we wanted to minimize the nesting effect of the separate oxygen regulating tanks and water baths in the experiment. The flow of nitrogen to each of the oxygen controlling tanks was controlled with a manometer and the resulting oxygen levels were measured with an optical oxygen sensor (LDO101, HACH Company, USA) in the experimental aquaria. The experimental setup is further described in Supplemental Material Figures S2 and S3. The flow speed at the inflow to the experimental aquaria was set between 90 and 110 ml per minute with screw clamps. Flow, temperature and oxygen levels were checked regularly (daily during the first two weeks of the experiment and biweekly later on) in all aquaria and adjusted if deviation from the target value was larger than 1°C for temperature or 5% for oxygen. The experimental setup was filled and run without mussels for three weeks before the start of the experiment in order to adjust experimental conditions. The oxygen concentrations and temperatures monitored in the aquaria throughout the experiment are shown in the Supplementary Material Figure S4 and Table S1 and Figure S5, respectively.

Each aquarium received 12 randomly selected mussels from each population (12 individuals × 6 populations × 32 aquaria = 2304 mussels). Experimental mussels were selected such that they were representative for the size distribution of each mussel population with shell length > 8 mm (Table 1). The experimental size distributions of each population were not different between treatments (Supplementary Material Figure S6). For the quagga mussel populations of River Main and Lake IJsselmeer a stratified random sampling was used in order to ensure that each aquarium contained both small and large mussels. All mussels were marked individually with a color and number using bee tags (Geller GbR) and measured for shell length, width and height with calipers. We tagged and measured one population per day and kept the tagged mussels under acclimatization conditions until the start of the experiment. An additional 60 'baseline' mussels per population were measured and tagged in the same way as the experimental mussels. On the starting day these 'baseline' mussels were stored in -80°C for subsequent analysis of tissue dry weight and all experimental mussels were distributed to their corresponding aquaria of defined temperature and oxygen level. A food mixture consisting of 7.2 ml of Shell Fish Diet and 4 ml of Rotifer Diet (Reed Mariculture Inc.) suspended in aerated groundwater

was equally distributed to the 32 aquaria daily. Every second day we checked all aquaria for dead mussels and removed them. We considered mussels to be dead when they stayed wide open and did not close their shells upon touching. After 65 days the experiment was stopped because almost all mussels had died in some of the tanks.

Statistical analysis

We analyzed the right censored survival of mussels throughout the experiment with a parametric accelerated failure time (AFT) model using Weibull error distribution as recommended in Kleinbaum and Klein (2005). As fixed factors we included oxygen, temperature and either population or species and all two-way interactions. In both models, including either species or population as an explanatory variable, the three-way interactions were not significant and omitted ($p = 0.38$ and $p = 0.72$, respectively). In order to avoid convergence problems resulting from high survival in high oxygen treatments we pooled the data for the three highest oxygen levels (33%, 66% and 90%) as opposed to the low oxygen treatment (6% oxygen). This simplification was justified, because the survival in the three higher oxygen levels did not differ significantly (Weibull model, $p = 0.89$) and the model for the data set with only two oxygen treatment classes gave a slightly reduced AIC (Akaike's Information Criterion). Additionally, we included aquarium as the frailty term (random effect) to account for the variability between experimental units. As mussel volume (or shell length) did not significantly affect mussel survival, it was not included in the final models (Supplementary Material Table S2). We confirmed the assumptions of the AFT Weibull model graphically by plotting the log of the negative log of the survival function (Kleinbaum and Klein 2005). The lines for the different populations were roughly straight, but not parallel, which is in accordance with assumptions of the Weibull model. We calculated the median predicted survival days for each population and all treatment combinations from each of the two survival models: one including populations and one including species. We then used these predictions to compare the reaction norms between the different populations and species.

Table 2 A) Deviance and likelihood statistics and statistical significance of the factors in the Weibull-survival models including oxygen, temperature and population as explanatory factors, **B)** Deviance and likelihood statistics and statistical significance of the factors in the Weibull-survival models including species instead of population as an explanatory variable. Tables show, the explanatory variables and the interactions included, degrees of freedom (D.f.), deviance, residual degrees of freedom, -2 x log-likelihood (-2 x LL) and corresponding p-values (Pr (>Chi)). All significant effects have p-values < 0.001 and are indicated with ***. The AIC gives the value of the Akaike information criterion as a measure of the relative model quality and goodness of fit.

A)	D.f.	Deviance	Resid. D.f.	(-2 x LL)	Pr (>Chi)	Sig.
Intercept	NA	NA	2302	4331	NA	
Oxygen level	1.00	471.4	2301	3859	<0.00001	***
Temperature	1.00	146.3	2300	3713	<0.00001	***
Population	5.00	68.4	2295	3645	<0.00001	***
Frailty term (aquarium)	22.49	83.0	2273	3562	<0.00001	***
Oxygen level x temperature	-0.75	-0.1	2273	3562	0.59	
Oxygen level x population	5.44	30.1	2268	3532	<0.00001	***
Temperature x population	5.17	10.1	2263	3522	0.079	
AIC = 3603						

B)	D.f.	Deviance	Resid. D.f.	(-2 x LL)	Pr (>Chi)	Sig.
Intercept	NA	NA	2302	4331	NA	
Oxygen level	1.00	530.1	2299	3692	<0.00001	***
Temperature	1.00	97.5	2300	4222	<0.00001	***
Species	1.00	11.3	2301	4319	0.0010	***
Frailty term (aquarium)	21.96	78.2	2277	3614	<0.00001	***
Oxygen level x temperature	-0.99	-0.1	2276	3611	0.71	
Oxygen level x species	1.01	0.7	2275	3611	0.42	
Temperature x species	1.05	2.4	2276	3611	0.13	
AIC = 3667						

In order to control for differences in survival due to condition differences of mussels after the acclimatization period, we calculated the condition index (CI, see also Lawrence and Scott 1982) for sixty 'baseline' mussels per population. For all 'baseline' mussels the tissue was retrieved from the shell, freeze dried for three days and the tissue dry mass (mg) was weighed on a high precision scale. The volume (mm^3) was calculated as using the approximation of 4/3 * Π * length/2 * height/2 * width/2 and the condition index (CI) as tissue dry weight (mg) divided by volume (mm^3). The CI was then compared between populations in a linear regression model. The influence of the factors species and population origin was additionally examined in a linear mixed effects model, where location was considered as a nested effect under species.

All analyses were performed in R (R-Core-Team 2014) and the "survival" - package (Therneau 2014) was used for the survival analysis and the "nlme" - package (Pinheiro et al. 2013) for mixed linear models.

Results

Oxygen and temperature had strong and significant effects on survival of mussels in the experiment (Figure 2, Table 2A). The predicted median survival of mussels in the lowest oxygen treatment was 96 days (SE = 16 days) translating to four times lower survival rate than in the high oxygen treatment (389 days, SE = 103 days, $p < 0.0001$, Table 2A). Higher mortality in the low oxygen treatment was observed for all populations in both temperature regimes but there were no significant differences in survival by populations among the three high oxygen treatments 33%, 66% and 90% ($p = 0.88$, data not shown). The predicted mussel survival was 27% lower at 18°C when compared to 11°C (267 days, SE = 65.2 compared to 364.4 days, SE = 97.7, $p < 0.0001$, Table 2A) including all mussels from all populations and treatments. The reduction in survival due to low oxygen was also more pronounced at 18°C in all populations (Figure 2). Against our expectations the survival rate of quagga mussels was significantly lower compared to zebra mussels (272 days, SE = 62.5 days compared to 359 days, SE = 100.4 days, $p < 0.001$, Table 2B).

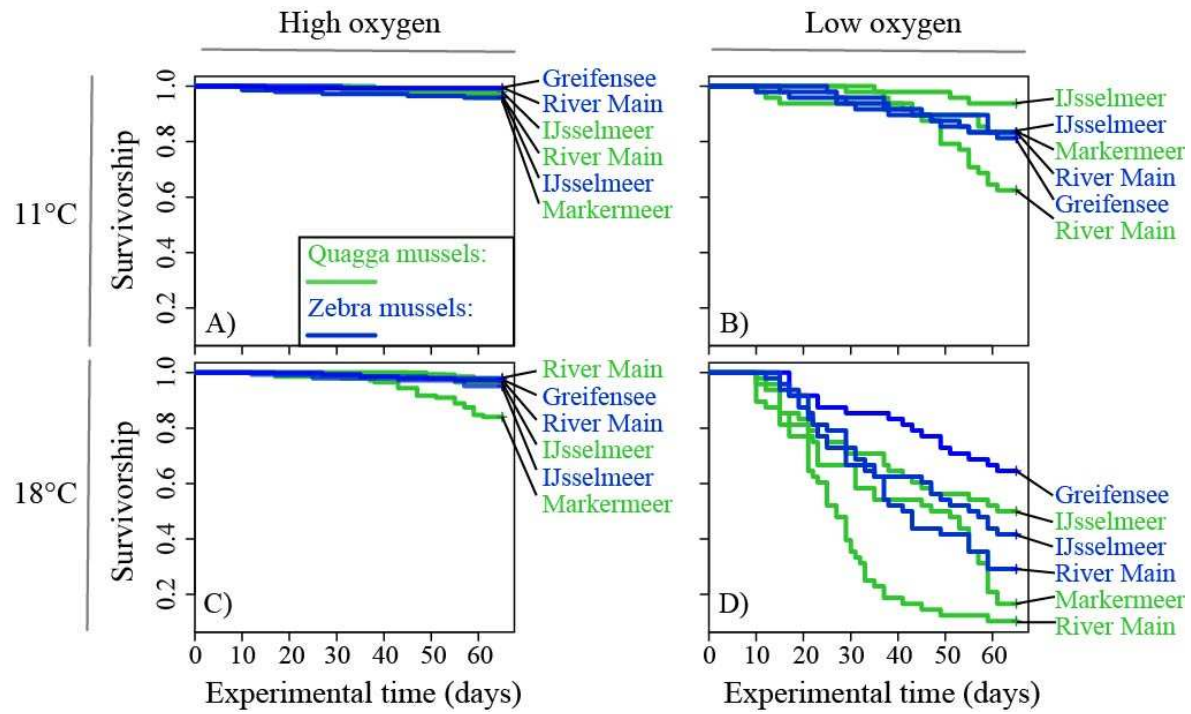

Figure 2 Experimental survivorship curves for the four treatment combinations, after the three highest oxygen levels were pooled: **A)** High oxygen and low temperature, **B)** low oxygen and low temperature, **C)** high oxygen and high temperature, **D)** low oxygen and high temperature. Curves for zebra mussels are depicted in blue and for quagga mussels in green and the populations are indicated on the right of each plot, with populations showing highest survival on top and populations showing lowest survival at the bottom of each list.

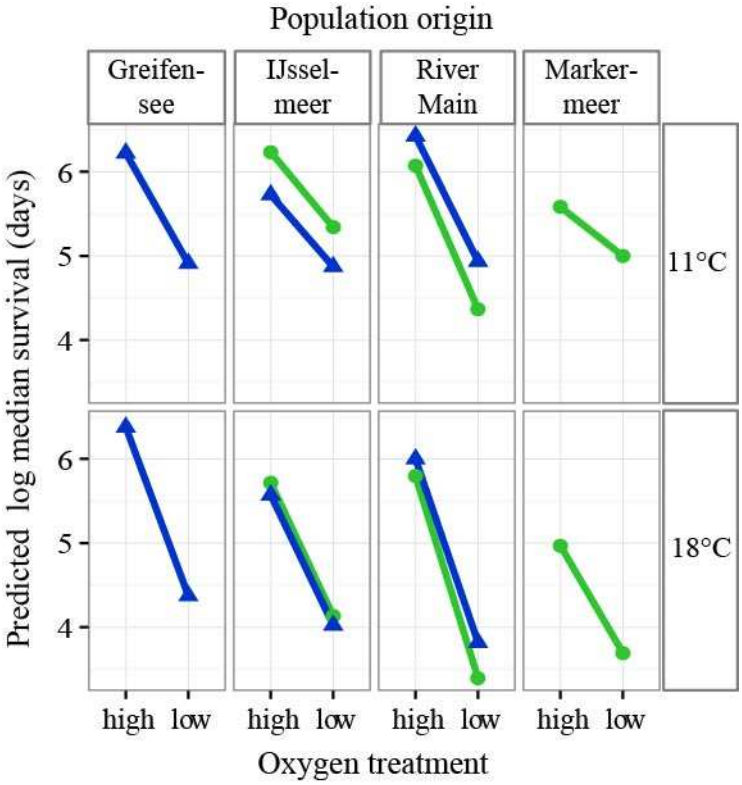

Figure 3 Reaction norms for the natural logarithm of the predicted median survival against high and low oxygen treatment class. Reaction norms for zebra mussels (blue, triangles) and quagga mussels (green, circles) are shown separately for each of the populations and the two temperatures.

We also found strong and significant differences in phenotypic response to oxygen and temperature treatments among populations (Figure 3). Significant interaction between oxygen level and population in survival ($p < 0.0001$, Table 2A) implies origin-specific tolerance differences to oxygen depletion. The model with population as a factor fitted the data better (AIC = 3603) than the model including species (AIC = 3667), suggesting that the differences among species were less pronounced than the differences among study populations. Sensitivity to lowered oxygen varied strikingly between populations showing a strong population-by-environment interaction in both temperatures (Figure 3). The slopes of the reaction norms varied between sites of origin but less so between populations of species coexisting at the same site (Figure 3), showing that responses were more similar within locations than within species. Analyzing survival rates at low oxygen levels separately revealed that in the high temperature treatment both quagga and zebra mussels from River Main survived relatively poorly (Figure 2D) while both Lake IJsselmeer populations had higher tolerance of oxygen and temperature stress. In the low oxygen treatment, the survival profiles differed strongly among both zebra and quagga mussel populations at 18 °C, while differences in survival were less pronounced among zebra mussel populations than among quagga mussel populations at 11°C (Figure 2B).

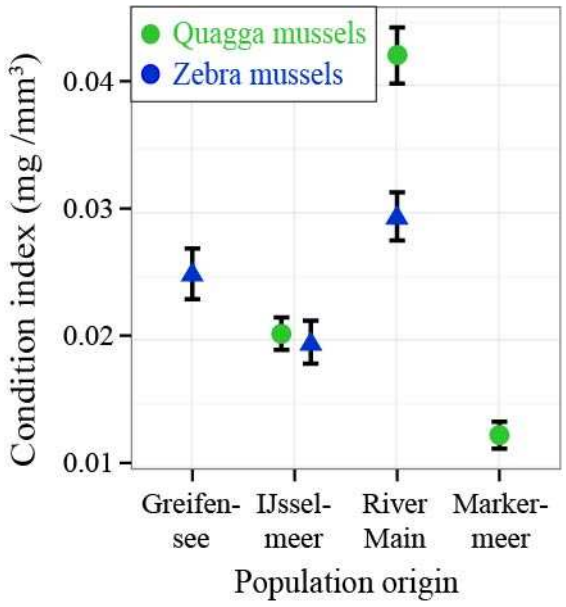

Figure 4 Mean condition index, calculated as soft tissue dry weight (mg) divided by shell volume (mm^3), for zebra mussels (blue triangles) and quagga mussels (green circles) against population origins. Error bars represent standard errors.

The condition indices (CI) for 'baseline' samples were significantly different between populations ($p < 0.0001$, Table S3), but did not predict their ranking in survival at low oxygen in the experiment. Quagga mussels differed more from each other in CI than

zebra mussels and location paired populations better than species (Figure 4, Figure S7). In the mixed effects model population origin explained 70% of the variation in CI while species identity explained only a minor fraction of the variation (1×10^{-5} %, Table S4).

Discussion

We found that the survival of both quagga and zebra mussels was strongly reduced at low oxygen with higher temperature (18°C). Comparable reductions of survivorship due to chronic hypoxia at different temperatures were found experimentally by Johnson and McMahon (1998) for zebra mussels from the Niagara River, Buffalo, New York. Unexpectedly, our results revealed that the survival reaction norms between oxygen treatments depended more on population origin than on species identity. In more detail, we found that the population-by-oxygen interaction differed more among quagga than among zebra mussel populations (Figure 3). Similarly, the origin of the populations explained the variation of recorded condition indices (CIs) better than species identity, but CI did not correlate with the survivorship of the populations in the experiment (Figure 2, Figure 4). In fact, among-population differences in survival under low oxygen reflect the environmental oxygen levels recorded at each sampling location. Under low oxygen conditions both species from River Main showed the lowest survival rate, followed by mussels from the Dutch lakes, while zebra mussels from Lake Greifensee showed the highest survival (Figure 2D). This corresponds to environmental data where Lake Greifensee shows strongest oxygen depletion at the greater depths during summer months, while Lake Markermeer and Lake IJsselmeer experienced more pronounced phases of oxygen depletion at depth than River Main (Supplementary Material S1 and Noordhuis et al. 2014). Hence, the observed pattern might be an indication of local adaptation in the selected populations as the locally experienced environmental conditions are reflected in the reaction norms. Therefore, population origin matters more than species identity in explaining the experimental survival data. As external fertilization in the water column mixes local genotypes in every reproductive event and free-floating larvae have only a very limited ability to select their settling place, it is unlikely that the different sampling depths of the populations influences their genetic background. Of course, we cannot exclude the possibility of developmental phenotypic differences as our experimental mussels were wild-caught.

Zebra and quagga mussel populations from North America and Europe are reported to be genetically diverse outcrossing populations suggesting that genetic bottlenecks should not be limiting adaptation (Wilson et al. 1999; Muller et al. 2002; Therriault et al. 2005; Brown and Stepien 2010; Imo et al. 2010). Western European zebra mussel populations showed clear genetic differentiation (Muller et al. 2002; Pollux et al. 2003; Rajagopal et al. 2009), indicating that the local population genetic structure has emerged through restricted gene-flow, drift and possibly local evolutionary processes in these post-invasion populations. Local adaptation of zebra mussel populations to temperature regimes has already been suggested by Elderkin and Klerks (2001; 2005) who showed a gradient in allele frequency along the Mississippi River, which corresponded to latitudinal distance.

German quagga populations, that were established only one decade ago (Molloy et al. 2007; Heiler et al. 2013), do not show genetic differentiation (Imo et al. 2010). In the face of their recent invasion history it seems remarkable that quagga populations show more variance in response to oxygen levels than zebra mussels and seem to be better adapted to the variable oxygen conditions in the different invaded habitats. The examined populations represent the current invasion front of quagga mussels in Western Europe, which implies that potential adaptive processes can happen in only few generations. This is in accordance with the prediction that rapid local adaptation should be more frequent in populations at the species' range limits (Sexton et al. 2009). A complementary explanation for differences between the species might be that the propagule exchange (gene flow and migration) is lower for quagga mussels than for zebra mussels (Karatayev et al. 2011a) leading to faster genetic divergence among populations at the invasion front. Therefore, we should not exclude the possibility of some degree of rapid local adaptation in quagga mussels, even though the populations are recent. Alternatively, the population differences may also have arisen through pre-invasion local adaptation of different source populations. For example, Heiler et al. (2013) suggested that repeated introductions of quagga mussels from potentially different source populations have occurred in the delta of the River Rhine in the Netherlands and the Rhein-Main-Danube canal in Germany. Distinguishing the two alternatives may not be possible based on the results of this study, but certainly is an important point for future studies and predictions.

Quagga and zebra mussels showed similar sensitivities to low oxygen but on average quagga mussels had a somewhat higher mortality than zebra mussels in the low oxygen treatment, independent of temperature. Zebra mussels were found to have

similarly low survival and low oxygen regulatory capacities under hypoxic conditions (Johnson and McMahon 1998). Garton et al. (2013) suggested similar oxygen regulatory capacities for zebra and quagga mussels based on unpublished results by Johnson and McMahon, who found somewhat lower median lethal times (LT_{50}) compared to our data, with slightly but consistently higher LT_{50} values for zebra mussels. Stoeckmann (2003) showed that quagga mussels of Lake Erie had a lower respiration rate and consumed less oxygen than the sympatric zebra mussels over a range of temperatures. They suggested that in Lake Erie, this difference in respiration rate translated into higher growth and reproductive rates and that this has been one factor promoting the competitive exclusion of zebra mussels by the recently invaded quagga mussel. Assuming that generally lower respiration rates could also be found in the quagga mussel populations tested in our experiment, our results suggest that such differences would not generally translate into better tolerance of low oxygen conditions.

European lakes colonized so far are rather shallow, often well mixed across the water column and experience only short periods of oxygen depletion in summer (Supplementary Material Figure S1). In contrast, the deep lakes in the vicinity of the Alps, where quagga mussels are expected to invade in the near future, show variably strong stratification and oxygen depletion below the thermocline for several months during summer. These hypolimnetic zones are also characterized by lower temperatures and low nutrient conditions. Our results indicate that quagga mussels of Western Europe are more sensitive to lower oxygen levels than sympatric zebra mussels, suggesting that alternative factors should be considered when predicting species-specific depth distribution and the potential displacement of zebra mussels by quagga mussels, which has repeatedly been observed in North American and European lakes. Such alternatives could be better survival and reproduction at lower temperatures (Roe and MacIsaac 1997) and better tolerance of low nutrient conditions. Quagga mussels were found to filter seston, including bacterioplankton, more efficiently than zebra mussels at low seston concentrations (Baldwin et al. 2002; Stoeckmann 2003). A generally higher somatic growth and survivorship in freshwater (Karatayev et al. 2011b) might also give quagga mussels a competitive advantage over zebra mussels. All these factors may allow quagga mussels to better colonize the deeper zones of lakes (as long as they show only moderate levels of hypoxia) and reach higher densities compared to zebra mussels. Yet, the above cited studies did not take the potentially different population background into account.

Assuming that source populations are of Western European origin and adapted to shallow lakes, we predict that invading quagga and zebra mussels should initially have a similar tolerance to low oxygen conditions. In a field experiment by Verhofstad et al. (2013) zebra mussels survived even better than quagga mussels in the deep zones of a Dutch Lake (Lake Cuijk), potentially due to their better tolerance of hypoxic conditions. Nevertheless, quagga mussels might gain an additional competitive advantage over zebra mussels for reaching the deeper zones of these lakes through better post-invasion adaptation to low oxygen conditions given sufficient time.

To conclude, we found more pronounced phenotypic variation among populations than among species which highlights the possibility for post-invasion evolutionary response. Alternatively, these patterns of phenotypic divergence might have arisen pre-invasion through local adaptation of different source populations followed by separate invasions to the investigated locations, or through canalized phenotypic plasticity to environmental conditions at the studied location. The population-by-environment interactions call for further studies where canalized developmental plasticity should be contrasted with adaptive divergence using F1 and F2 lab-reared offspring in a classic common garden design. Different life cycle stages of zebra and quagga mussel may have different ecological requirements and further studies are needed to examine the environmental niche of different life-cycle stages. Despite these constraints, local adaptation in quagga and zebra mussels might promote environmental niche shifts along their invasion fronts in Europe and elsewhere and thus seem to be important for predictive models of future quagga and zebra mussel distributions. Furthermore, our results stress the importance of using multiple populations of a species when the environmental niches of invasive species (or species in general) are investigated in experiments.

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Supplementary material

The following supplementary material is available for this article:

Table S1 The overall mean oxygen saturation for each combination of oxygen level and temperature in the experiment.

Table S2 Deviance and likelihood statistics and statistical significance of the factors in the Weibull-survival models.

Table S3 Linear regression model explaining the condition index (CI) of experimental mussel populations.

Table S4 The mixed effects model explaining the condition index (CI) by species and population origin as nested effects.

Figure S1 Environmental oxygen concentrations and water temperatures in studied water bodies.

Figure S2 Schematic view and pictures of the experimental setup.

Figure S3 Schematic top view of how the oxygen regulating tanks, the water baths and aquaria were arranged.

Figure S4 Experimental oxygen levels measured in the aquaria during the course of the experiment.

Figure S5 Experimental temperatures measured in the aquaria during the course of the experiment.

Figure S6 Mean shell length and estimated mean shell volume for each experimental population (origin, species) and all treatment combinations.

Figure S7 Condition index (CI) as linear regression of tissue dry weight by volume for each population of quagga mussels and zebra mussels.

This material is available as part of online article from:
http://www.aquaticinvasions.net/2016/Supplements/AI_2016_DeVentura_etal_Supplement.pdf

Tables

Table S1 The overall mean oxygen saturation for each combination of oxygen level and temperature setting with number of measurements (N) throughout the course of the experiments, mean, standard deviation (SD) and standard error of the mean (SE).

Oxygen level	**Temperature**	**N**	**Mean**	**SD**	**SE**
6%	11	80	6.12	3.33	0.37
6%	18	84	6.48	1.79	0.20
33%	11	89	32.33	2.74	0.29
33%	18	94	35.53	2.62	0.27
66%	11	90	65.31	7.74	0.82
66%	18	94	70.94	6.47	0.67
90%	11	79	93.95	5.59	0.63
90%	18	83	104.16	4.99	0.55

Table S2 Deviance and likelihood statistics and statistical significance of the factors in the Weibull-survival models including all main effects and interactions which were significant in our model for the different populations (see results, Table 2a) and additionally including volume as a main effect and both two-way interactions of volume by oxygen level and volume by temperature. The frailty term aquarium was also kept in the model. The table shows the explanatory variables and the interactions included, degrees of freedom (D.f.), deviance, residual degrees of freedom, -2 x log-likelihood (-2*LL) and corresponding p-values (Pr (>Chi)). All significant effects have p-values < 0.001 and are indicated with ***. Neither the main effect of volume nor the interactions of oxygen level by volume or temperature by volume showed significant effects on the predicted survival of mussels.

	D.f.	Deviance	Resid. D.f.	(-2*LL)	Pr (>Chi)	Sig.
Intercept	NA	NA	2302	4331	NA	
Oxygen level	1.00	471.4	2301	3859	<0.00001	***
Temperature	1.00	164.4	2295	3645	<0.00001	***
Population	5.00	50.4	2296	3809	<0.00001	***
Volume	1.00	2.3	2294	3642	0.13	
Frailty term (aquarium)	22.25	83.1	2272	3559	<0.00001	***
Oxygen level x population	5.28	31.2	2266	3528	<0.0001	***
Oxygen level x volume	1.09	2.7	2265	3525	0.08	
Temperature x volume	1.02	0.2	2264	3525	0.55	
AIC = 3631						

Table S3 Linear regression model explaining the condition index (CI) as a dependent variable. The populations, the estimated volume of individual mussels and the two-way interaction of these two variables were included as explanatory variables into the model. The table shows the explanatory variables and the interactions included, degrees of freedom (D.f.), sums of squares (Sum Sq), the mean squares (Mean Sq), the F-statistics (F-value), the corresponding p-values (Pr (>F)) and the significance levels (Sig.). Volume and the interaction of population by volume where significant as expected, but the variable population exhibited a much stronger and more significant effect on the CI.

	D.f.	Sum Sq	Mean Sq	F-value	Pr (>F)	Sig.
Population	5	462.55	92.509	1.54E+02	<0.00001	***
Volume	1	3.09	3.09	5.14E+00	0.02	*
Population x volume	5	7.97	1.595	2.65E+00	0.02	*
Residuals	323	194.3	0.602			

Table S4 The mixed effects model explaining the condition index (CI) by species and population origin as nested effects (location nested under species: lme (CI ~ 1, random = ~ 1| species / origin). A) We present the R-output including the standard deviations (Std.Dev.) for the corresponding intercepts and residuals for each of the two the random effects, species and location (representing the population origin) nested under species. There were no fixed effects in the model (CI ~ 1). B) Standard deviations, variances and the explanatory power, showing how much of the variance was explained by either species or the population origin, were calculated from the fixed effects model presented in a).

A) Linear mixed-effects model fit by REML

AIC	BIC	logLik			
884.6397	899.9201	-438.3199			
Random effects:					
Formula: ~1 | species					
	(Intercept)				
Std.Dev.	0.000379				
Formula: ~1 | location %in% species					
	(Intercept)	Residual			
Std.Dev.	1.277758	0.8496686			
Fixed effects: CI ~ 1					
	Value	Std.Error	D.f.	t-value	p-value
(Intercept)	3.092906	0.5237026	332	5.905845	0
Standardized Within-Group Residuals:					
Min	Q1	Med	Q3	Max	
-3.00394184	-0.5324124	-0.05504565	0.48331004	4.93936412	

B) Variance explained by each of the random effects in the linear mixed effects model

Factor	**Std.Dev.**	**Variance**	**Explanatory power (%)**
Species	0.00	1.44E-07	6.10E-06
Origin nested under species	1.28	1.63	69.34
Residuals	0.85	0.72	30.66

Figures

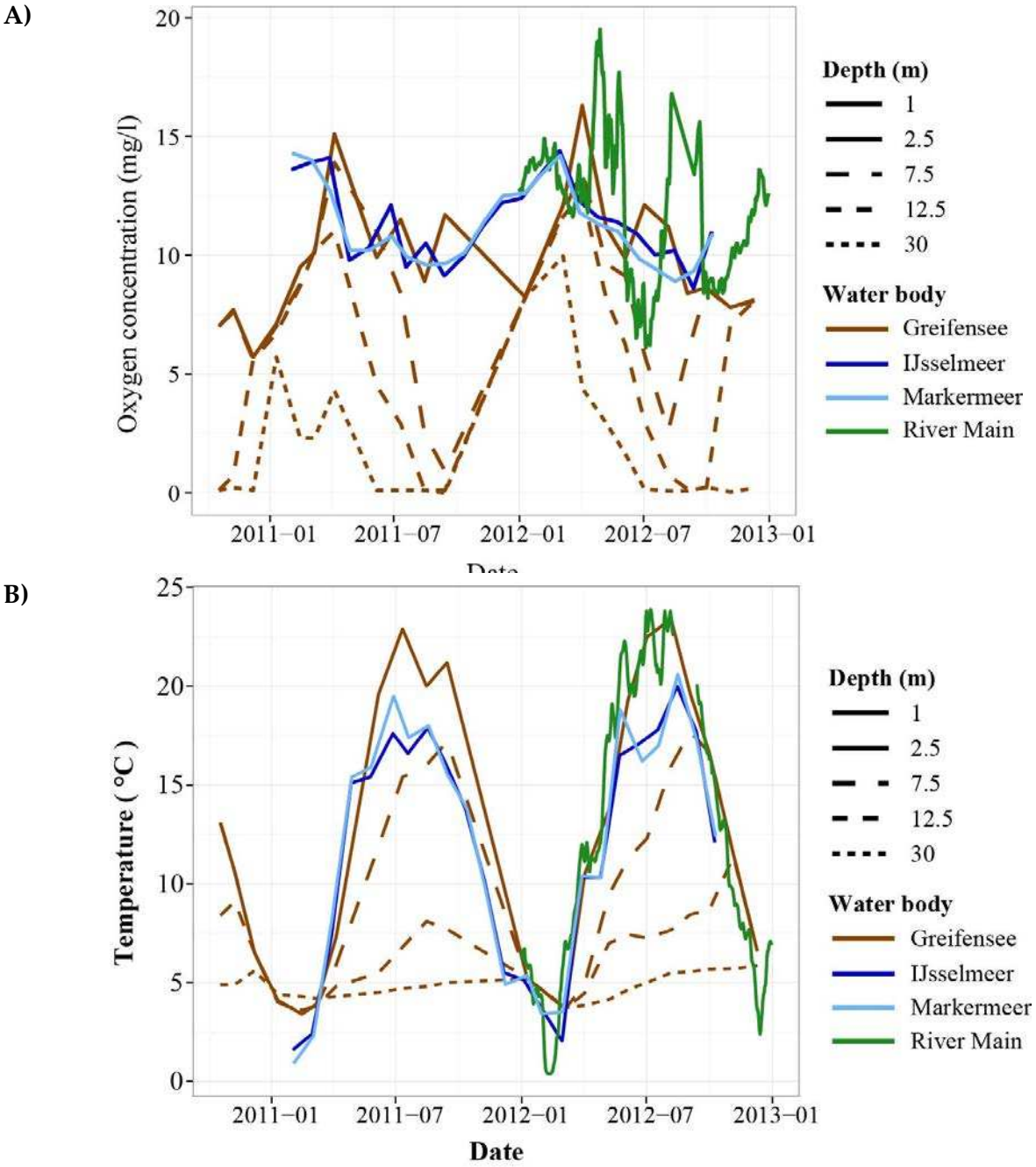


Figure S1 A) Oxygen concentrations and **B)** water temperatures at four different depths in Lake Greifensee (2.5 m, 7.5 m, 12.5 m and 30 m) and at one meter depth in Lake Markermeer and Lake IJsselmeer were measured monthly across the years 2011 and 2012. Oxygen concentrations and water temperatures were measured in the Main River near Kahl in the water column weakly across the year 2012. Measurements for Lake Greifensee were obtained from AWEL (Amt für Wasser Energie und Luft of Canton Zürich, Switzerland), for Lake Markermeer and Lake IJsselmeer from Rijkswaterstad (Dutch national agency of water issues) and for River Main from LfU (Bayerisches Landesamt für Umwelt, Germany).

Additionally, oxygen data at one meter depth in Lake Markermeer and Lake IJsselmeer was only available in fragments but was analyzed in depth by Ruurd Noordhuis for two summers (2011 and 2012) on Lake IJsselmeer data: he found a few two-day events and only two three-day events of pronounced stratification, where oxygen finally was just below 8 mg/l. Nevertheless, mass mortality of Smelt (*Osmerus eperlanus*) and dreissenid mussels occurred during the extreme summer heat-wave in 2006, where the mussel population was reduced down to 10%, likely due to prolonged stratification and oxygen depletion in the deep zones of the lake. The mussel populations recovered during the following years but the population potentially went through a bottleneck with a potential selection pressure on tolerance of low oxygen levels (R. Noordhuis, 2014, personal communication; Deltares, Dutch Technological Institute).

Figure S2 A) Schematic view of the experimental setup with 1) bubbling nitrogen into one of the four oxygen regulating tanks, 2) gas exchange with the surrounding atmosphere, 3) overflow from the oxygen regulating tank (which was directed to eight aquaria, here only one is shown), 4) regulated passive overflow to aquaria (100 ml/min), 5) regulated passive inflow from other oxygen regulating tank, 6) overflow to one common collection tank, 7) pumping water back to the four oxygen regulating tanks. The arrangement of the four oxygen regulating tanks, the eight water baths and the 32 aquaria is shown in Figure S3. **B)** Picture of the experimental setup. Additionally, the oxygen regulating tank (25 l) was connected via a hose to a precedent tank (4l) in order to make the flow distance longer and increase gas exchange efficiency. **C)** Tagged mussels in an experimental aquarium. Colors of tags correspond to the six different populations used in the experiment, while numbers correspond to individual mussels. In the top left corner of the picture the inflow to the aquarium and in the bottom right corner the outflow from the aquarium are shown. **D)** Close up of tagged experimental mussels.

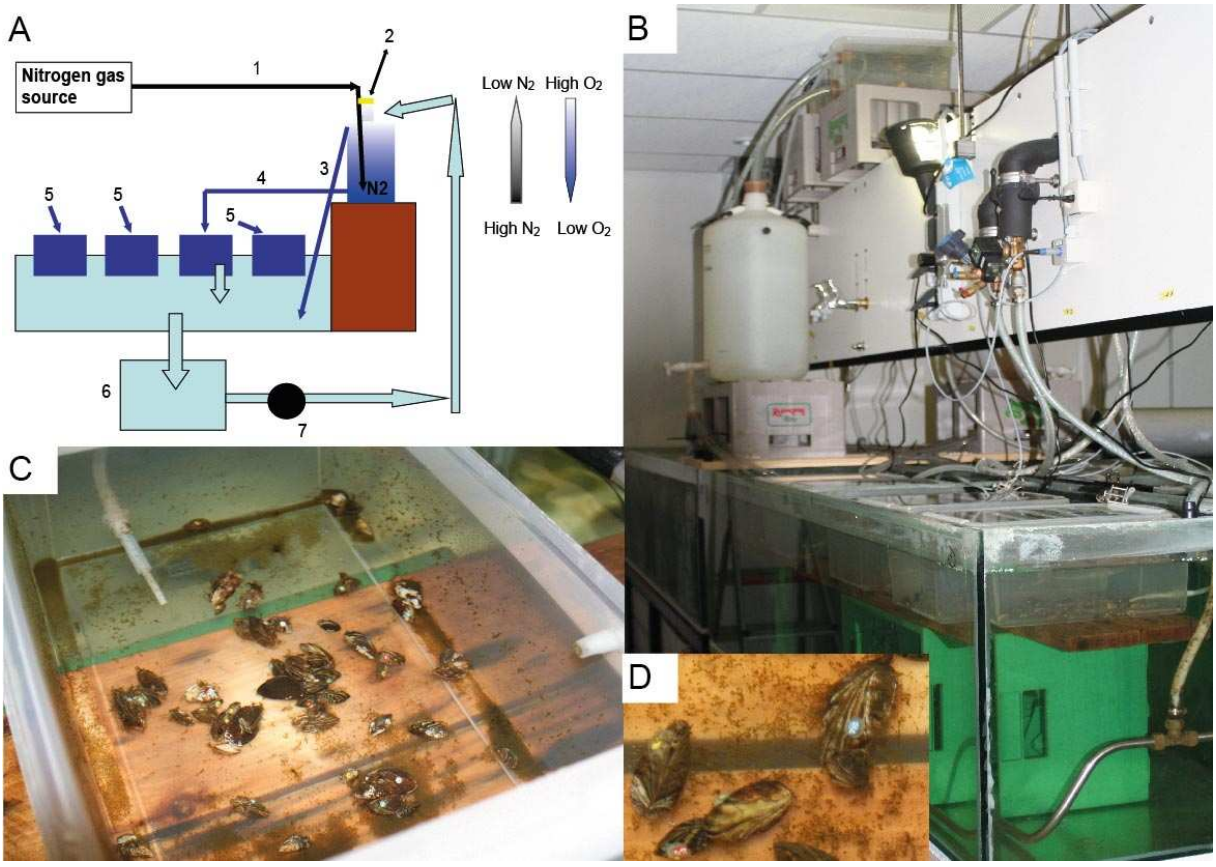


Figure S3 Schematic top view of how the oxygen regulating tanks, the water baths and aquaria were arranged. The four different oxygen levels in the oxygen regulating tanks and the experimental aquaria are represented by the four different shades of red. The oxygen levels were arranged in a random order within each of the water baths. The two temperatures in the water baths are represented with different shades of blue. The black arrows show how the water was pumped from the common water collection tank to the four oxygen regulating tanks which were located higher than the experimental aquaria (using only one pump). The outflow of each of the oxygen regulating tanks was directed to the corresponding aquaria with PVC tubes (passive flow). Each experimental aquarium was completely filled with water and closed with a lid in order to minimize gas exchange with the surrounding air. The excess water discharged from the aquaria to the surrounding water bath, while the outflow from the water baths was collected in a common tank (passive flow). Originally we had intended to set the temperatures in the experiment to 18°C and 4°C, but with the heating and cooling system used, we could not cool the water in the colder tanks below 11°C. The reason for this was that we collected all the water in the overflow from the water baths in the same tank (instead of using separate collection tanks for each of the two temperatures), because we wanted to avoid nesting of the variable temperature in the experiment. Therefore, we had slow but constant inflow of warmer water into the aquaria which needed to be cooled by the aquaria and the cooling system reached its limit. Nevertheless, we found clear differences between the two temperatures, and the mortality at low oxygen mainly seemed to develop more slowly at 11°C compared to 18°C. This effect might simply be more pronounced at 4°C, and we would see a smaller but similar effect of low oxygen on the survival after two months in each of the populations.

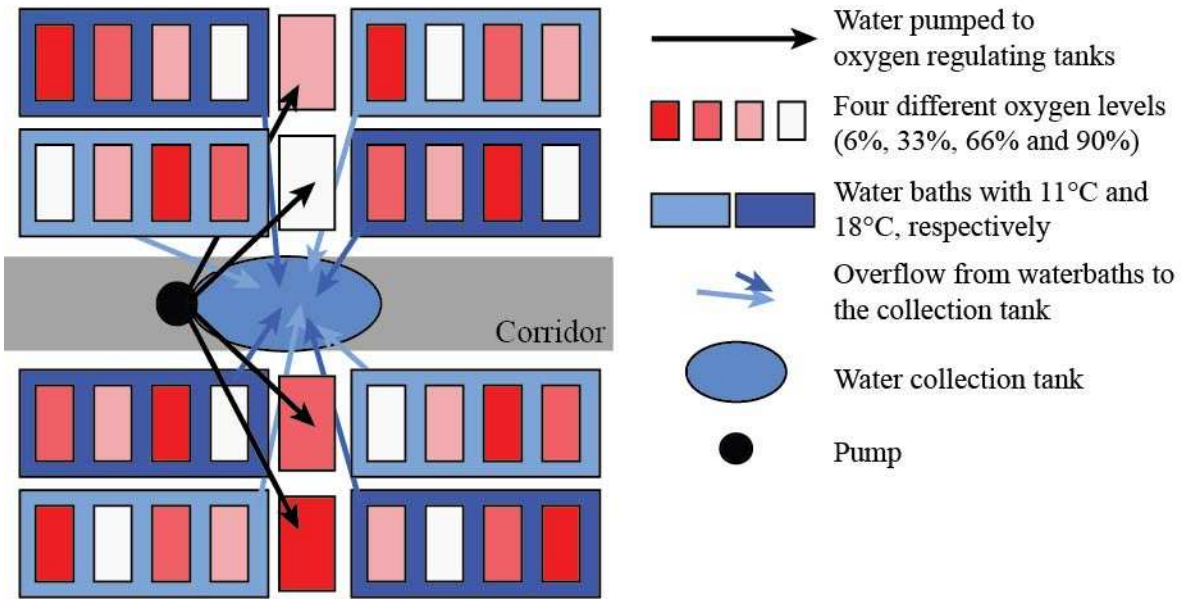
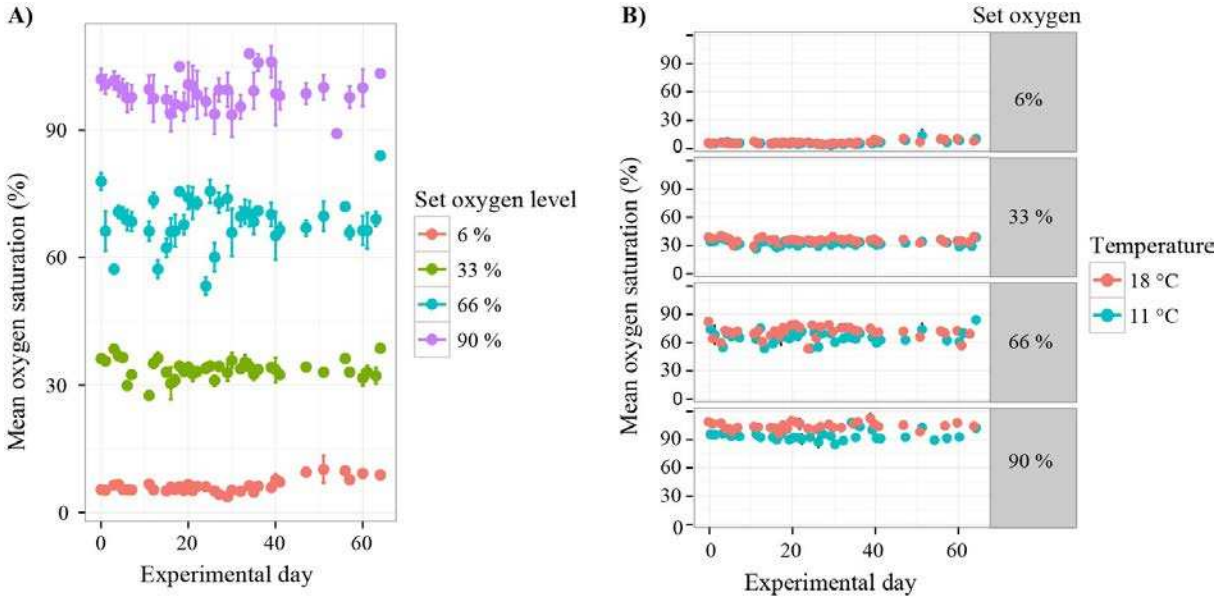


Figure S4 Experimental oxygen levels measured in the aquaria during the course of the experiment. **A)** Mean oxygen saturation (%) for each of the oxygen levels (the mean of the eight aquaria within the same oxygen setting) on each date of measurement. Oxygen concentrations fluctuated more at higher concentrations than at lower concentrations. **B)** Therefore, mean oxygen levels are also shown for each combination of temperature (18°C = red, 11°C = blue) and oxygen level set. Error bars represent standard errors of the mean. At the lowest oxygen level, the oxygen saturation showed relatively small variation and was not different between the two temperatures. (See also Table S1.)



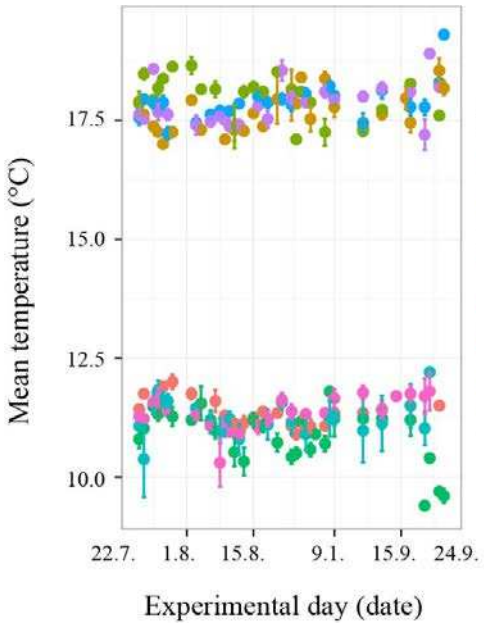


Figure S5 Experimental temperatures measured in the experimental aquaria during the course of the experiment. Temperatures are shown as the mean of the four aquaria within the same water bath on each date of measurement. Error bars represent standard errors of the mean. Water baths A, D, E and H were set to 11 °C (overall mean = 11.4 °C, SE = 0.07) and water baths B, C, F and G were set to 18°C (overall mean = 17.6, SE = 0.074).

Figure S6 A) Mean shell length (mm) and **B)** estimated mean shell volume (mm^3) for each experimental population (origin, species) and all treatment combinations of high and low oxygen and the two temperatures 11°C (blue) and 18°C (red). Error bars show the corresponding confidence intervals.

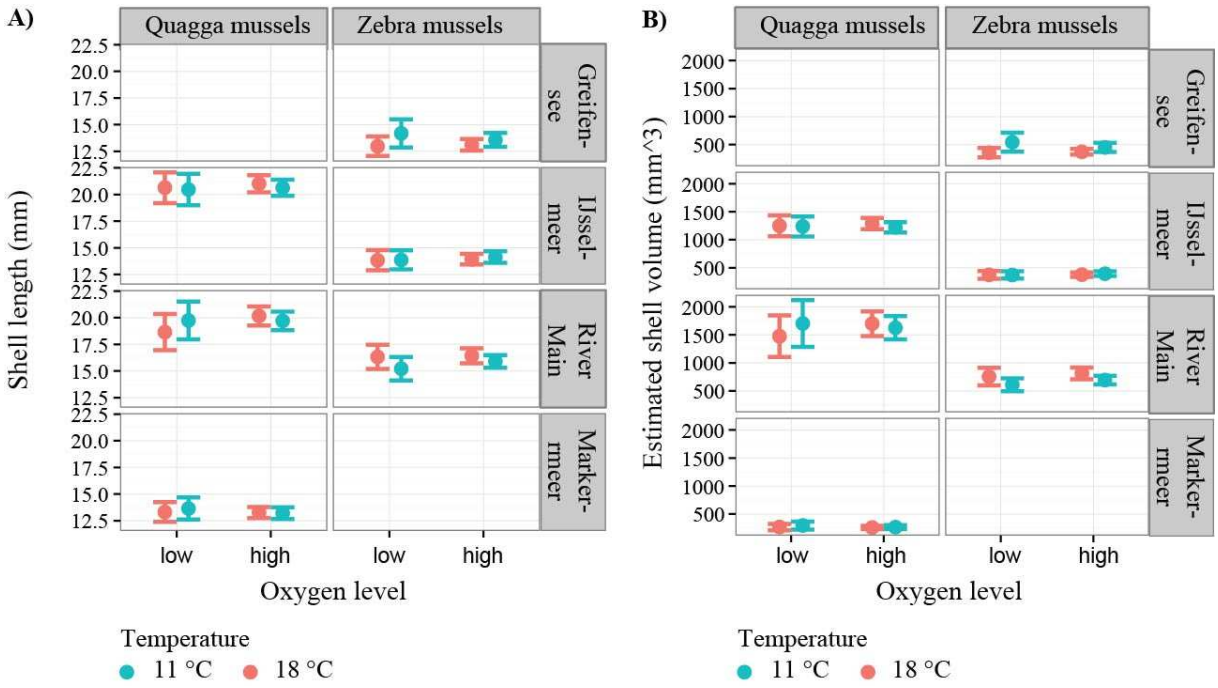
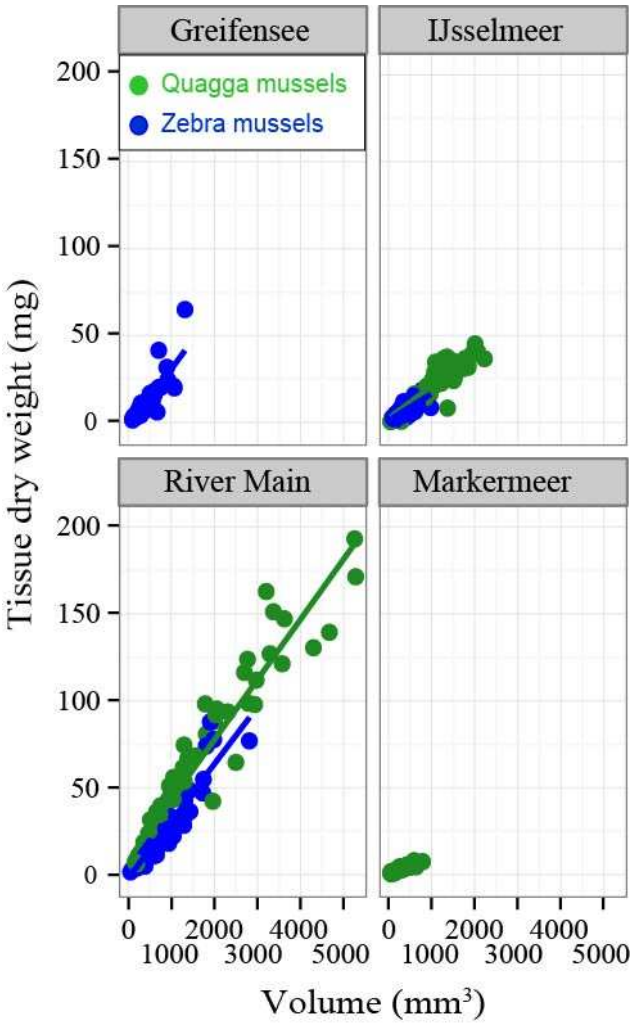


Figure S7 Condition index (CI) as linear regression of tissue dry weight (y-axes, mg) by volume (x-axes, mm^3) for each population of quagga mussels (green) and zebra mussels (blue). The condition index (measured as the slope of the regression) was similar between zebra and quagga mussel populations of the same origin, but was different between sampling sites. Also this interpretation of condition index did not predict the ranking of survival rates of the experimental populations in the experiment. For example, both mussels from Greifensee and River Main show a good condition (steep slope), but mussels from Greifensee survived well at low oxygen conditions while mussels from the River Main showed lower survival rates compared to IJsselmeer mussels at low oxygen.



Chapter 2

Overland transport of recreational boats as a spreading vector of zebra mussel *Dreissena polymorpha*

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Abstract

In aquatic ecosystems invasive species are among the most important threats to biodiversity worldwide. Understanding the dispersal mechanisms of aquatic invaders is very important for protection and management of vulnerable water bodies. Here we ask how recreational boats that are transported overland could contribute to the dispersal of invasive zebra mussels among lakes in Switzerland. Using a questionnaire sent to registered boat owners, we surveyed properties of transported boats and collected information on self-reported mussel fouling and transport activities of boat owners. We also sampled boat hulls at launching ramps and harbors for biofouling invertebrates. Boats that were kept seasonally or year-round in water were found to have high vector potential with mussel fouling rates of more than 40%. However, only about 6% of boats belonging to these groups were transported overland to other water bodies. Considering that approximately 100,000 recreational boats are registered in Switzerland, we estimated that every year around 1,400 boats fouled with mussels are transported overland. Such boats pose a high risk of distributing zebra mussels between water bodies. Our results suggest that there is a considerable risk that recreational boats may spread new fouling species to all navigable water bodies within the study area. We speculate that one such species could be the quagga mussel, which has not yet invaded lakes in Switzerland. On a more positive note, our study has identified the group of high-risk boats so that possible control measures would only affect a relatively small number of boat owners.

Keywords

Recreational boating; invasive species; distribution vectors; *Dreissena polymorpha;* fragmented river networks, small craft boats

Introduction

We need to understand the factors that promote species invasions in order to manage and reduce the damage caused by invasive species. Worldwide, invasive species are among the most important threats to biodiversity in freshwater ecosystems, particularly in lakes (Sala et al. 2000). In freshwater ecosystems, human mediated transport of organisms plays a key role in spread and introduction of invasive species (Reviewed in Havel et al. 2015). For example, in the well-studied Rhine River the number and spread of introduced invasive species has increased dramatically within the last decades (Baur and Schmidlin 2007). Human activities have increased connectedness between watersheds and promoted passive transportation and unintentional release to new areas (Leuven et al. 2009).

Natural dispersal of exclusively aquatic species is often limited by the dendritic structure of waterways (Fagan 2002). Secondary spread of non-native species to more isolated or disconnected water bodies away from the main shipping routes suggests an important role for overland transport by human activities (Johnson et al. 2001; Minchin et al. 2003). For example, overland transport of recreational boats has been shown to function as a vector for surface-fouling zebra mussels (Johnson et al. 2001). Within-lake movement of recreational boats has also been shown to function as a strong vector for the secondary spread of zooplankton and benthic invertebrates (Kelly et al. 2013). However, the headwaters of European river systems are strongly isolated by dams and rarely used for commercial shipping. Nevertheless, many introductions to these freshwater systems have occurred (Kinzelbach 1992; Bacela-Spychalska et al. 2013). It seems then that overland transport is a prerequisite for the spread of non-native species among these headwaters. As commercial boats are rarely transported overland, transport of recreational boats remains the most likely distribution vector (Johnson et al. 2001; Rothlisberger et al. 2010; Kelly et al. 2013; Bacela-Spychalska et al. 2013).

Zebra mussel (*Dreissena polymorpha*, Pallas 1771) represents an invasive species that has benefitted from human activities. The zebra mussel has spread widely across western Europe since the early 19th century (Kinzelbach 1992) and has now colonized most larger rivers and lakes except for highest elevation headwaters. It originates from the Ponto-Caspian region, which is now well connected to Western Europe via three inland channels for commercial shipping (bij de Vaate et al. 2002). Overland dispersal is also likely to have occurred as adult zebra mussels (>10 mm shell length) can survive up to ten days out of water (Ricciardi et al. 1995; Paukstis et

al. 1999). Thus they have the potential to survive overland transport over long distances and colonize new habitats far from invaded source regions.

Indeed, Johnson and Carlton (1996) and Johnson et al. (2001) showed that overland transport of mussels fouling boat equipment and macrophytes attached on the recreational boats enabled the overland transport of zebra mussels to isolated lakes even when the hulls of the boats were not fouled. Both studies focused more on the potential of trailer-kept ("transient") boats to transport zebra mussels over land and less on the boats moored in harbors and marinas over longer periods of time. However, as these authors recognized, mussel fouling directly on the hulls of boats that are kept permanently in water is more likely, and therefore we hypothesize that these boats can also be important as vectors for zebra mussels, even if they might be transported less frequently (Minchin et al. 2006). It is thus important to know the vector potential (the potential of a specific distribution vector to transport non-native species, such as zebra mussels, to new habitats) of different boat types, and how different boat properties and boating practices contribute to the invasion risk associated with overland transport events.

This study addresses this research gap by examining the vector potential of recreational boats that are primarily moored and rarely transported overland for distributing invasive species. The study focuses on transportation of recreational boats in Switzerland and quantifies two key aspects of those that are mostly kept in water: (1) the rate of fouling by zebra mussels and (2) the frequency that they are transported between water bodies in Switzerland. Given that the transport of this type of boat is a rare event, we used a methodology for this study that allows investigating a very large sample efficiently. Specifically, we implemented a large-scale representative survey of boat owners in Switzerland. Due to the risks of biases in self-reported data, we also sampled boats using launch ramps and boats moored in harbors. Finally, because our field sampling showed that mussels on boat hulls were mostly juveniles (1 mm -10 mm in shell length), we experimentally tested how long this size of zebra mussel survives out of water at two temperature regimes. We estimated the vector potential of recreational boats in Switzerland based on which boat categories were fouled with mussels, how frequently they were transported, the classes of water bodies involved, and the time period of the transportation. Moreover, by investigating the transportation routes between different water bodies, we asked specifically if zebra mussels are being transported to small alpine lakes that have not been colonized by zebra mussels so far. Finally, we discuss our results in the context of prevention and management of possible future invasions by fouling

invasive species, for example, the quagga mussel (*Dreissena rostriformis bugensis*, Andrusov 1897), which has not yet spread to the lakes of this study. Our research not only provides new information on the prevalence of mussel fouling and transport of recreational boats in Switzerland, but proposes that it might, at least in some areas, not be the most frequently transported boats that spread invasive species but the least often transported ones.

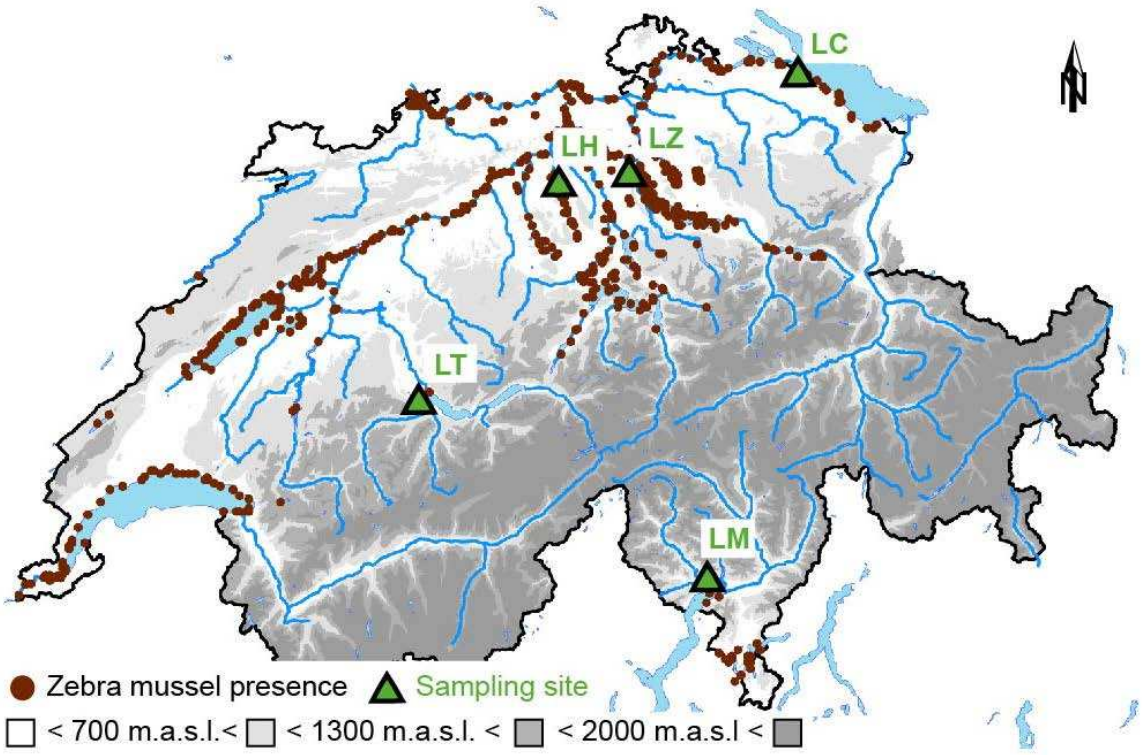

Figure 1 Distribution of zebra mussels in 2011 in Switzerland (brown dots, zebra mussel presence data collected from cantonal offices, the Swiss Centre for the Cartography of Fauna, CSCF, and various environmental offices in Switzerland) and field sampling sites (green triangles, LC: Lake Constance, Bottighofen, 47°64'74.65" N, 9°19'69.72" E, LH: Lake Hallwil, Beinwil am See, 47°26'91.83"N, 8°21'15.72"E, LM: Lake Maggiore, Tenero Campofelice, 46°16'63.53" N, 8°85'42.93" E, LT: Lake Thun, Thun, 46°73'41.84" N, 7°63'01.55" E, LZ: Lake Zürich, Wollishofen, 47°20'25.61"N, 8°32'22.20"E). All field-sampling sites lie below 700 m above sea level. Grey shades show different levels of altitude.

Methods

Study area

We studied boats used on navigable lakes and rivers of Switzerland (Figure 1), representing headwaters of the wider alpine region. Zebra mussels occur in almost all larger lakes and rivers in Switzerland, except those at higher altitudes (Figure 1). To date, the highest lake in Switzerland, where zebra mussels have been found, is on 1250 m.a.s.l.. Commercial shipping between lakes is absent in Swiss waters, but lakes are used intensively for recreational boating with a total of 99,200 private boats registered at cantonal offices in 2013 (Swiss Federal Statistical Office 2014). These boats are distributed over roughly 30 navigable lakes and several rivers and are almost exclusively used for recreational purposes.

Questionnaire

We mailed boat owners self-administered questionnaires (in German and French) that could be filled out on paper or on the internet. The request indicated that participation was voluntary and provided information about the study as well as how the investigators could be contacted in case of questions. The questionnaire consisted of 30 questions about socio-demographic information, the boat characteristics, the presence of fouling organisms on exterior surfaces, the overland transport between water bodies, the cleanliness of the boat and cleaning behavior, and attitudes towards boat cleaning. Filling out the questionnaire took about 15 minutes.

Most questions used in this study were straightforward in design. For example, we simply asked where the boat was normally kept with the possible responses of (1) moored all year around, (2) moored during summer season and (3) kept on land. This third category is referred to as “transient” or “trailered” boats in other studies (e.g. Johnson et al. 2001; Rothlisberger et al. 2010). Table 1 compiles the categories of the questions used in this study. The questions on overland transport between water bodies were more complex, resulting in a separate data set that was used to analyze over land transportation frequencies, routes and durations of transport. For each of their ten most recent overland transport events, they were asked to specify the water body of origin and the destination water body, how long the boat had been in the water body of origin, for how many days the boat was out of water during transport and storage, and how long the boat remained in the destination water body. Boat owners were also asked to inform about the number of transport events within the past five years.

Nine of the 26 cantonal shipping agencies in Switzerland sent the questionnaires to a random sample of 20% of the registered boat owners (Supplementary Material, Figure S1). Overall, about 10 % of all registered boat owners in Switzerland (N = 10'500) received the questionnaire. We received 3561 replies (response rate = 34%). The relative distribution of boat types represented in the replies matched the distribution provided by the cantonal shipping agencies (Swiss Federal Statistical Office 2014), suggesting that the survey responses was representative of the population of boats registered in Switzerland. The distribution of boat types in each canton did not differ significantly between survey data and the Swiss-wide data set (Supplementary Material, Figure S2).

Statistical analysis of the questionnaire

We used logistic regression models to evaluate which of the independent variables assessed in the questionnaire best explained the dependent variables. We used two dependent variables in separate models: mussel fouling (whether boat owners had reported mussel fouling on their boat) and overland transport (whether a boat had been transported overland within the last five years). Only explicit answers were included in the analysis and others, such as 'don't know' or 'other option', were excluded. We performed a similar model selection procedure for both dependent variables. First, we explored the correlation structure between all explanatory variables using a categorical principal component analysis (CatPCA) in SPSS (Statistical Package for the Social Sciences, Version 21). None of the Spearman correlations between pairs of transformed explanatory variables exceeded 0.5, indicating sufficiently low multicollinearity to perform logistic regression analyses. Subsequently, the effects of the explanatory variables were tested for both dependent variables in logistic regression models using the statistical package R, version 3.0.2 (R-Core-Team 2014). The relevant explanatory variables were grouped in sets (Table 1) and variables of each set were first tested separately against the dependent variables. All variables with significant effects and their higher order interactions were then tested again in combined models where we added and removed explanatory variables in a stepwise procedure comparing the explanatory power of the models using AIC (Akaike information criterion) and significance levels of single variables. Since the variable "water body" consisted of many water bodies, and some of them only contained few observations, we summarized water bodies into the following categories: fresh waters abroad, ocean, rivers, large meso-eutrophic lakes (≥ 38 km^2, Ptot > 0.03 mg P / L), medium-sized lakes (≥ 7 km^2), small lakes (< 7 km^2), large oligotrophic lakes (≥ 38 km^2, Ptot < 0.03 mg P / L) and small alpine lakes (< 7

km² above 1300 m.a.s.l.). Lakes were classified by trophic state based on the Hydrological Atlas of Switzerland (Liechti and Jakob 2014). In the logistic regression models for mussel fouling and transport, we excluded fresh waters abroad, oceans and small alpine lakes from the analysis due to small sample sizes (Table 2a).

The data on specific overland transport events, for which origin and destination water bodies had been named in the survey, were analyzed separately. We used Geographical Information System (GIS) to illustrate and quantitatively analyze the frequencies of overland transport between individual water bodies. Subsequently, we investigated how long boats were kept out of water during overland transport and quantified transport frequencies between water body categories.

Field sampling

To verify the mussel fouling rates reported in the questionnaire, we investigated frequency and intensity of mussel fouling on recreational boats by inspecting boats and sampling hulls in five lakes (Figure 1, Lake Constance and Lake Zürich, two large mesotrophic lakes, Lake Thun, a large oligotrophic lake, Lake Hallwil, a medium eutrophic lake and Lake Maggiore, a large mesotrophic lake south of the alps) over the months of June, July and August 2013. In order to include both, boats kept on land and boats year-round or seasonally moored, we used two different assessment methods.

For boats kept on land, we visited public boat ramps during sunny weekends, and inspected all boats being launched or taken out of the water during the time we were present at the ramp (total N = 119 boats). We also asked the owners of the inspected boats where they normally kept their boat, where they had transported their boat from and how long their boat had been kept in water before our inspection. We estimated the amount of zebra mussels and other organisms visible to the naked eye by inspecting all surfaces of the boat including the engine and niche areas (crevices, seams and rivets) on the outside of the boat. Additionally, we checked for macrophytes attached to the boat (Johnson et al. 2001). To check for very small mussels and other small organisms, we scraped an area of 20x20 cm² of any boat that had visible fouling (14 out of 119 boats). These samples were kept in 70% ethanol and subsequently analyzed using a dissecting microscope (magnification: 5x – 40x).

For boats moored year-round or seasonally, we directly inspected 30 boats per lake underwater in August 2013 (Figure 1). In a first step, we snorkeled around the boat and estimated the amount of mussels visible by eye or by touch at the boat hull, around the motor, or at the keel/sword. In a second step, we pooled samples from

different surfaces of the boat (one each from the hull, the motor, and the keel/sword area) taken while snorkeling. Surfaces were scraped and the removed material caught with a zooplankton net (250 μm mesh size). We aimed at scraping off about the same area per boat as for the sampling at boat ramps (an area of roughly 20 x 20 cm^2). Samples were transported to the lab in a cooler and analyzed using a dissecting microscope within 24 hours. The samples were checked for zebra mussels and other animals that could originate from the boat hull. The shell length of five individual zebra mussels per life cycle stage (plantigrade larva, juveniles and adult mussels) was measured for five boat samples per site.

We examined the effect of boat type (sailing boat, motor boat), and water body (Lake Zürich, Lake Hallwil, Lake Thun, Lake Constance and Lake Maggiore) on mussel fouling (presence/absence) in a logistic regression model in R (R-Core-Team 2014).

Survival experiment

As we found mainly small mussels on the inspected boats, we experimentally tested survival of juvenile mussels (shell length: 1 – 10 mm) to aerial exposure. We installed 48 plastic disks in Lake Greifensee (coordinates: 47° 20′ 58″ N, 8° 40′ 49″ E) on which zebra mussel larvae settled and grew between July and September 2013. Disks were then retrieved from the lake and transported to the lab in a cooler. Within 6 hours, the number of mussels was counted for each disk using a dissecting microscope (magnification 5x - 40x) and the shell length of 20 haphazardly chosen individuals per disk was measured to evaluate the size range of the mussels. Mussels shell length ranged between 1 – 13 mm with a median of 3.7 mm and the size distribution on the settlement plates did not differ between treatments and time points. Subsequently the mussel-fouled disks were kept in air in two different environmental chambers at 12°C and 25°C with a similar range of humidity (70% – 80%). The average day temperature in Zürich, Switzerland, in July is 25°C, while 12°C represents the average day temperature in early spring or late autumn (http://www.climatedata.eu). After 18, 42, 66 and 90 h, four disks per temperature treatment were randomly chosen and the number of alive and dead mussels was identified under the dissecting microscope. A mussel was scored as dead when its shell was open and no movement was detected upon physical stimulation (Paukstis et al. 1999). As a positive control, three disks were kept in aerated lake water in each environmental chamber and assessed for mussel survival at each time point.

Table 1 Survey summary showing all variables assessed in the survey and discussed in this paper. The relevant variables were grouped in sets (such as mussel fouling, overland transport, boat location, boat properties, boat usage and boat cleaning). In the model selection process, independent variables of each set were first tested separately against the dependent variables (see methods, statistical analysis). For each variable, the response categories and the corresponding proportions are shown. Explanations on selected variables: * Dry stored boats are mostly kept on a trailer, at home or in a storage facility. They are only launched when used, mostly for rather short periods of time (hours - several days). ** Boats without motor are the boats belonging to neither of the two categories rowing boats and motorboats, but were mostly wooden boats, rowing boats or flat bottomed or punt-like boats

Variable sets	**Variables**	**Variable categories**						
Location	Water body	Water body and water body category (see text and Figure 2 and Figure 5)						
Location	Boat storage type	Year-round in water		Season in water		Dry*		Other
		22.9%		49.6%		25.2%		1.4%
Properties	Boat type	Motorboat		Sailing boat		Boat without motor**		Other option
		50.7%		39.9%		8.2%		1.3%
Properties	Motor type	Z-drive		Shaft driven		Outboarder		Other
		29.6%		12%		58.4%		4.7%
Properties	Sailing boat type	Yole		Keel boat		Catamaran		Other type
		22.7%		66%		6%		5.3%
Properties	Boat material	Wood		Glass fiber		Aluminium		Other material
		14.2		75.4%		4.2%		4.5%
Properties	Boat length	0 - 2.5 m		2.6 - 6.5 m		6.6 – 10 m		>10 m
		1.8%		57.2%		34.8%		4.6%
Usage	Boat usage	Pleasure		Competition		Fishing		Water sport
		82.5%		10.3%		17.8%		13.5%
Usage	Launching infrastructure	Crane		Ramp		Nothing		Other
		38.2%		41%		15.8%		4.9%
Prevention	Antifouling paint	Yes		No		Don't know		
		67.4%		23.3%		7.5%		
Cleaning	Cleaning location	Shipyard/public station		In the water		At land		At home
		38.5%		8.2%		26%		23.2%
Cleaning	Reaction to fouling	No reaction		Removed all		Tried removing		Other reaction
		8.3%		74.1%		14.6%		2.9%
Fouling	Mussel fouling	Yes		No				
		35%		65%				
Fouling	Fouling area	Boat hull		Motor		Keel/Sword		Water in boat
		62.2%		38.1%		25.5%		0.9%
Transport	Overland transport (events / 5 years)	0	1-4	5-8	9-12	13-16	17-20	>20
		88.8%	7.4%	1.6%	0.6%	0.2%	0.2%	1.2%

Results

Descriptive statistics of the questionnaire

Table 1 summarizes the results of the questionnaire for the relevant variables discussed in this paper. The "home" water bodies, where the boats were normally kept, are not shown in the table. In total, 42 water bodies of variable sizes were listed. The number of boats varied largely between water bodies between 1 (Lake Davos) and 670 (Lake Geneva). As 47 different water bodies were reported in the questionnaire and it was difficult to analyze and interpret the results for each location separately, we grouped the water bodies into categories as described in the method section. After grouping we included five out of eight categories of locations for the statistical analysis: rivers (275 boats), large meso-eutrophic lakes (2160 boats), medium lakes (425 boats), small lakes (24 boats), and large oligotrophic lakes (436 boats). Small alpine lakes (3 boats) were not included in the analysis but are still shown in the figures as a separate category (Figure 2 and 5).

Mussel fouling

In total 35% of the boat owners stated that they had found zebra mussels on their boat. Within this group, two thirds had found mussels at the boat hull while many also reported that mussels were found in the engine area (38%) and niche areas around the keel or sword (26%). Boat owners also mentioned the cooling system, fish bucket and rudder as frequently fouled areas.

The best-fitting logistic regression model for mussel fouling included boat storage, water body category, boat type, water sports, fishing and the two-way interaction between boat storage and boat type as explanatory variables (AIC = 3143.6, residual deviance = 3111.6 on 3096 d.f.). Boats that stayed throughout the year in the water were more likely to be fouled by mussels (60%) than boats seasonally kept in water (40%), whereas boats that were kept on land were rarely fouled by zebra mussels (2.9%, $P < 0.0001$, Table 2a). The probability of mussel fouling was higher in the large and medium size eutrophic lakes and varied significantly between water body categories ($P < 0.0001$, Table 2a, Figure 2a). Although not analyzed in detail, single water bodies varied considerably in the mussel fouling rates (between 0% in Lake Brienz and 63% in Lake Pfäffikon). Two weaker but significant predictors of mussel fouling were boat type and the interaction between boat type and boat storage (for details see Supplementary Material, Figure S3). Boats used for water sports had less mussel fouling while the boats used for fishing were fouled more frequently (Table 2a). Reported mussel fouling was also consistently higher in boats which were

painted with antifouling (Supplementary Material Figure S4). This counterintuitive result may be due to the fact, that boat owners who often have problems with mussel fouling are more likely to use antifouling paint. As the use of antifouling may be strongly dependent on the likelihood of mussel fouling (rather than the other way around) we excluded antifouling as an explanatory variable from the model.

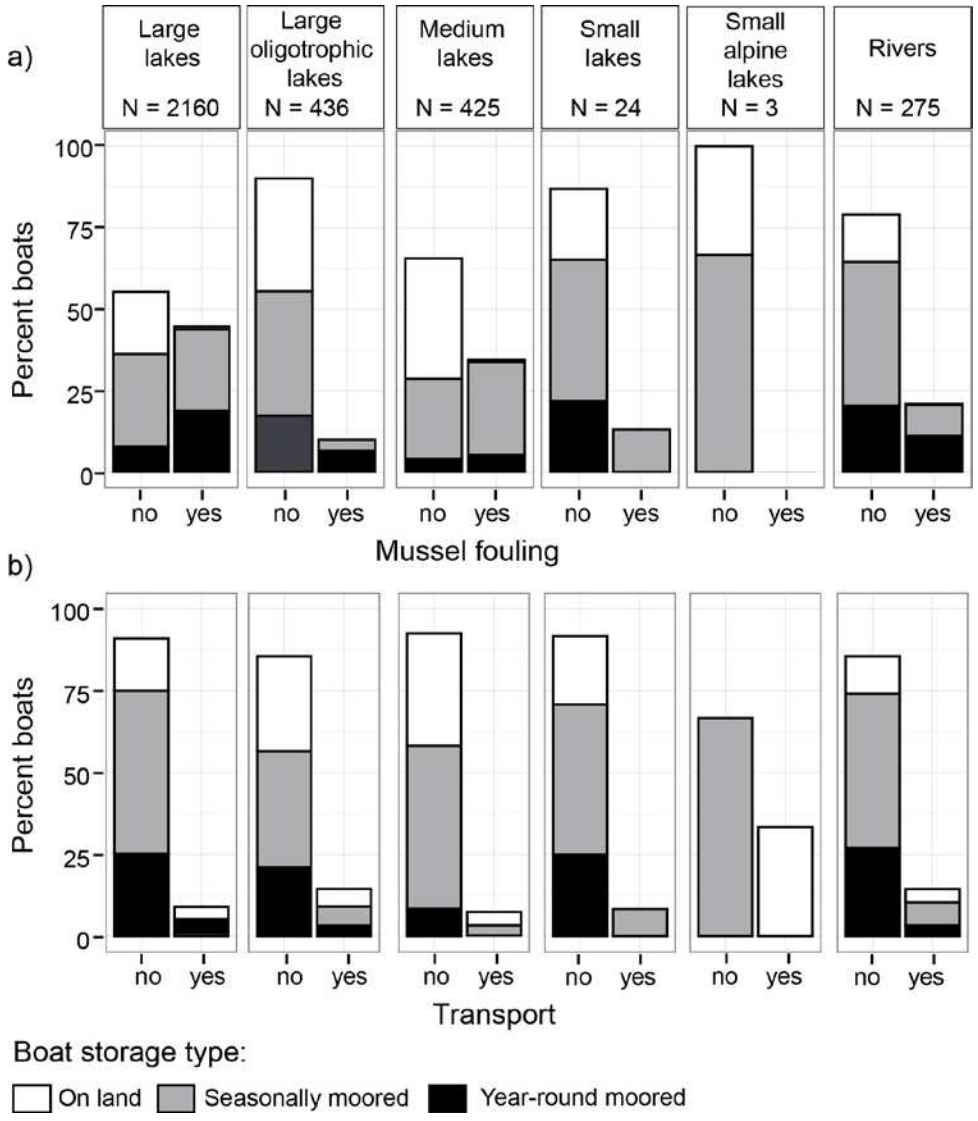

Figure 2 Percentage of boats for which boat owners reported: a) mussels growing on their boat (mussel fouling = yes) and no mussels growing on their boat (mussel fouling = no) and b) whether they had transported (transport = yes) or not transported (transport = no) their boat at least once overland within the last five years. Within each bar the different shades of grey represent boat storage types: boats moored year-round (black), moored seasonally (grey) and boats kept on land (dry, white). Percentages are shown separately for each of the water body categories rivers, large meso-eutrophic lakes (≥ 38km2, Ptot > 0.03 mg P / L), medium sized lakes (≥ 7 km2), small lakes (< 7 km2), large oligotrophic lakes (≥ 38km2, Ptot < 0.03 mg P / L) and small alpine lakes (< 7 km2, above 1300 m.a.s.l.).

Table 2 Results of the final logistic regression models with **a)** mussel fouling on boats (whether boat owners reported mussel fouling in the survey) and **b)** overland transport of boats (whether boat owners reported an overland transport within the last five years) as dependent variables. The columns show the included independent variables, the Likelihood Ratio chi-square statistics for each effect (LR Chisq), the degrees of freedom (D.f.), corresponding P-values, significance levels (Sig.) variable categories, and for each category the number of boats fouled (N fouled) in a) or transported (N transported) in b), respectively, and percent of boats fouled in a) or transported in b), respectively. The odds ratios and the corresponding confidence intervals are presented in the supplementary material (Supplementary Material, Table S1).

a) Dependent variable: mussel fouling yes/no							
Independent effects	**LR Chisq**	**D.f.**	**P-value**	**Sig.**	**Categories**	**N fouled**	**% fouled**
Water body categ.	241.46	4	< 0.0001	***	Figure 2 a)		
Boat storage	653.9	2	< 0.0001	***	year round in water	503	60.3
					seasonal in water	729	40.5
					dry location	26	2.9
Boat type	10.73	2	< 0.005	**	motor boat	581	32.1
					sailing boat	574	40.1
					without motor	76	26.9
Water sports	8.78	1	< 0.005	**	yes	112	22.7
					no	1154	37.0
Fishing	8.45	1	< 0.005	**	yes	268	42.1
					no	997	33.5
Boat storage * boat type	10.29	4	< 0.05	*			

b) Dependent variable: boat transport yes/no							
Independent effects	**LR Chisq**	**D.f.**	**P-value**	**Sig.**	**Categories**	**N transported**	**% transported**
Water body category	26.16	4	< 0.0001	***	Figure 2 b)		
Boat storage	56.48	2	< 0.0001	***	year round in water	52	6.1
					seasonal in water	163	8.8
					dry location	198	21.2
Boat type	8.91	2	< 0.05	*	motor boat	192	10.3
					sailing boat	197	13.4
					without motor	18	6.1
Water sports	6.14	1	< 0.05	*	yes	77	15.2
					no	340	10.6
Competitions	73.23	1	< 0.0001	***	yes	108	28.0
					no	309	9.3
Boat storage * boat type	15.36	4	< 0.01	*			

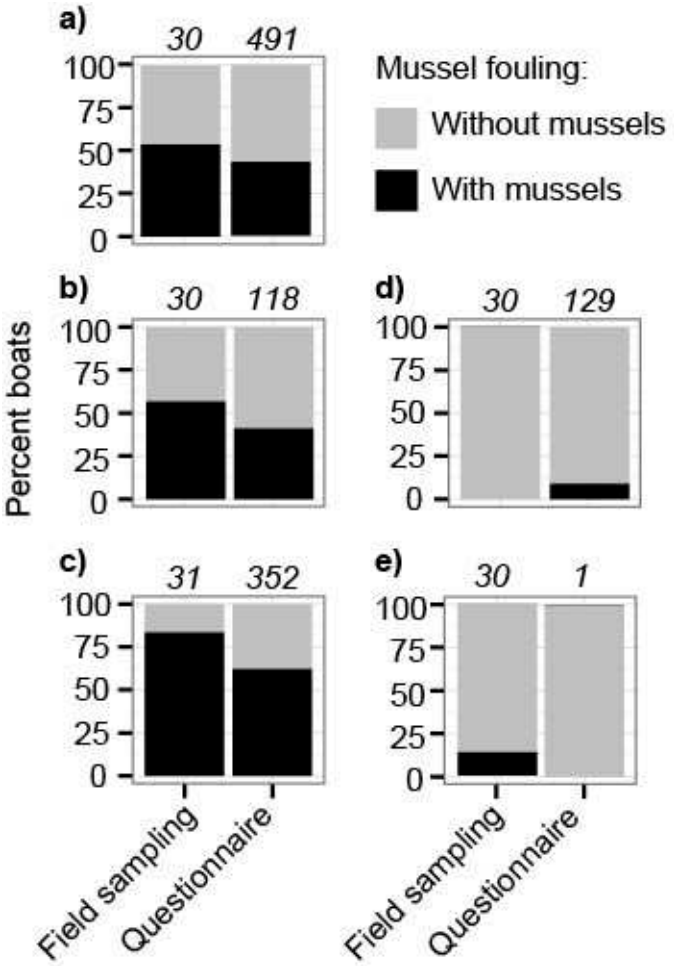

Figure 3 Percentages of boats on which zebra mussels were found (black bars) or not found (grey bars) for our under-water sampling (one harbor per lake) and the survey data on boats which were kept in water seasonally or year-round. Percentages are given for each of the five Lakes **a)** Lake Constance, **b)** Lake Hallwil, **c)** Lake Zürich, **d)** Lake Thun and **e)** Lake Maggiore. For each lake and each assessment method, the number of boats (N) represents 100% of boats assessed (for the field sampling) or 100% of boats for which either presence or absence of mussels was reported (for the questionnaire data).

The biological sampling confirmed that the type of boat storage and the type of water body are important for mussel fouling. None of the 119 boats inspected at boat ramps were fouled with mussels and no zebra mussels or other aquatic animals of interest were found in the 14 biofilm samples we collected at the boat ramps. Most of the inspected boats had been in the water only for a short period of time (1 – 3 days) before the examination and were normally kept on a trailer (85%). We also did not find any macrophytes entangled on any of these inspected boats and trailers. In contrast, the 150 underwater samples scraped from boat hulls revealed a mussel fouling rate that was comparable to the questionnaire for boats kept in water either seasonally or all year around (41% in underwater samples vs. 44% in the questionnaire, Figure 3). The presence of mussels in the biological samples was also highly dependent on the sampled water body (Chi-square = 64.7, d.f. = 4, $P < 0.0001$), but it was independent of boat type (Chi-square = 0.46, d.f. = 2, $P = 0.79$). On average, boats of all lakes carried rather small mussels (between 0.5 – 4 mm long), and we only found larger mussels (up to 2 cm) on boats in Lake Zürich. For most fouled boats, mussels were found on all exterior surfaces in contact with water such as the

boat hull, the engine area and the keel, independent of boat type, boat material or motor type. Numbers of mussels estimated from our underwater samples ranged from less than 100 (for 42.9% of fouled boats) to several 1000 (for 23.3% of fouled boats) per m^2. Besides mussels, we found also other organisms such as bryozoan resting stages (statoblasts) or egg clutches of benthic invertebrates, but hardly any other benthic invertebrates such as the amphipod *Dikerogammarus villosus.*

Overland transport frequencies of boats

In our questionnaire, 11.4% of boat owners stated that they have transported their boat between water bodies at least once within the last five years and we calculated an average of 1.2 transport events per year within this group. The best logistic regression model explaining overland transport probability included boat storage type, water body category, boat type, water sports, competitions, and the two-way interaction between boat storage and boat type (AIC = 1922.2, residual deviance = 1892.2 on 3206 d.f.). As for mussel fouling, the probability of transport depended again the most on the boat storage type ($P < 0.0001$, Table 2b), but in this case, boats that were kept on land had a higher probability of being transported (21.2%) than seasonally or year round moored boats (8.8% and 6.1% respectively). Furthermore, the transport probability was significantly different among the water body categories ($P < 0.0001$, Table 2b) being higher for boats from large oligotrophic lakes and rivers compared to the other categories (Figure 4 and Figure 5).

Weaker significant effects on transport probability were found for boat type ($P < 0.02$, Table 2b) and for the interaction between storage type and boat type ($P < 0.05$, Table 2b). Among year-round and seasonally moored boats, the transport probabilities were elevated for sailing boats and motor boats and significantly lower for boats without motors (with probabilities of 8.1%, 8.3% and 3.7%, respectively). Sailing boats were only significantly more often transported over land among boats kept on land (with transport probabilities of 29.5% for sailing boats, compared to 17.4% for motor boats and 9.8% for boats without motor). Furthermore, boats used for water sports and competition were more likely to be transported (Table 2b), but only 13.5% and 10.3% of boats belonged to these categories, respectively.

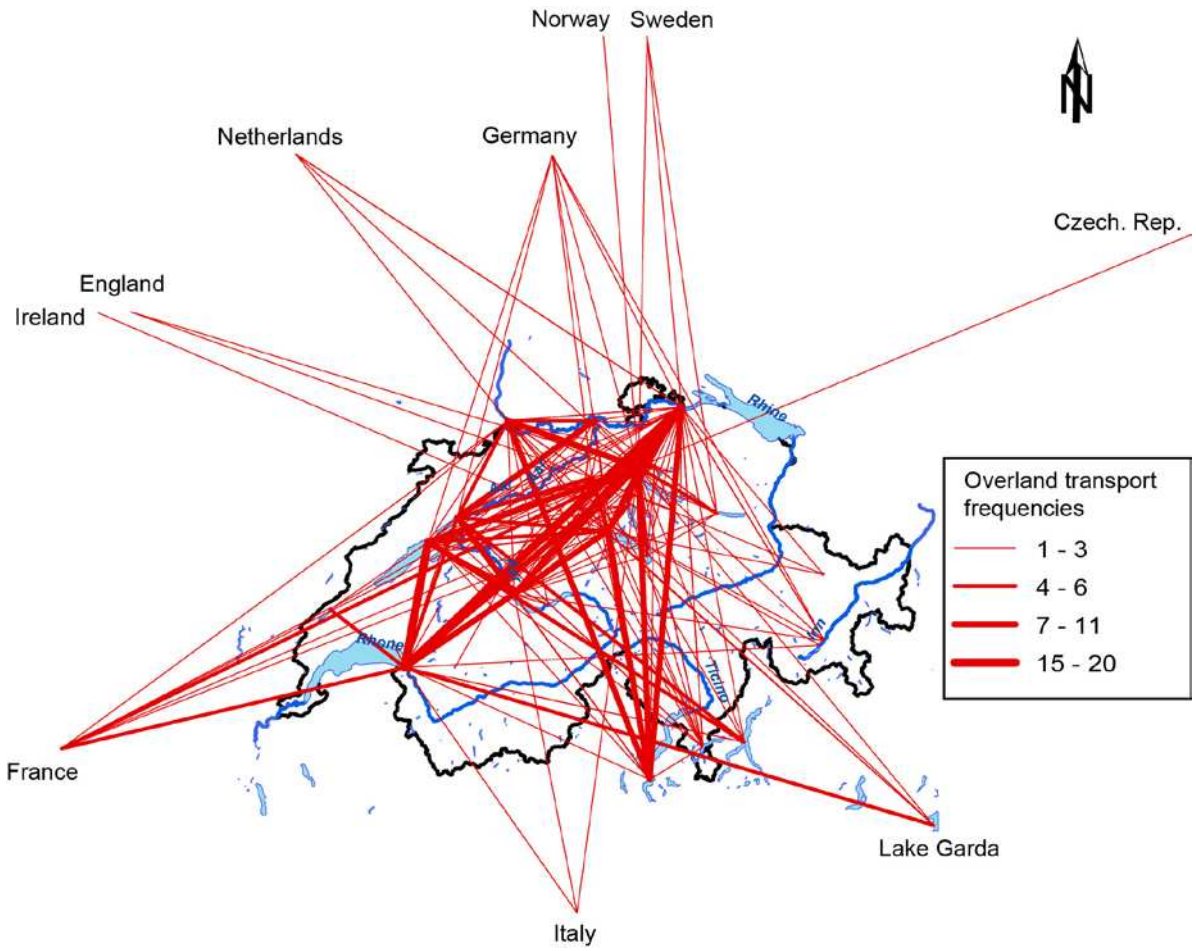

Figure 4 Overland transport network based on survey data including all named overland routes with given start and end water bodies. The network contains only transport events between different fresh water bodies and frequencies (numbers of events in past five years as reported in surveys) of transport routes are symbolized by the thickness of the lines. Connections to water bodies abroad were summarized to the ten categories named in the figure.

Transportation routes and time spent in transport

In the questionnaire, a total of 745 overland transport events between water bodies within the past five years were reported. The routes and frequencies of the reported transport events among freshwaters are shown in the transport network including a total of 40 different water bodies (Figure 4). The main transport routes are between Lake Zürich, Lake Geneva, Lake Constance, Lake Neuchatel and Lake Maggiore. Almost all navigable water bodies were named at least once in the transport network. Results of the questionnaire also showed that transport events to and from abroad took place frequently. Among all transported boats, 71% of overland transport events lasted less than ten days and 50% lasted less than two days. When we analyzed the number of transport events between different water body categories that lasted less than two days, most occurred between different large meso-eutrophic lakes and from

large meso-eutrophic to medium-sized lakes (for seasonally moored boats, Figure 5b) and freshwaters abroad (for boats kept on land, Figure 5c). Among moored boats, a relatively high proportion of boats was transported from large or medium-sized lakes and rivers, which indicates a higher probability of mussel transport to target lakes (Figure 5a and b, see discussion). The few mussel-free, small alpine lakes were also a destination for such transport events, even if only rarely (Figure 5b, seven events from medium sized and large meso-eutrophic lakes had been reported for seasonally moored boats). On the other hand, the large oligotrophic lakes, of which only one is still free of zebra mussels, are often a target for transport events of moored boats.

Survival experiment

Survival of juvenile mussels subjected to desiccation stress was between one and two days at 25°C and between two and four days at 12°C (Supplementary Material, Figure S5). At 25°C most mussels were still alive after one day but almost all of them had died after two days while at 12°C mussel survival declined more slowly and some mussels survived up to three days.

Frequency of high-risk boat transport

We defined high-risk boat transfers as boats that are fouled by mussels, transported over land, and launched into the water within a time period when mussel survival is likely. We found mussel fouling to be largely determined by the boat being kept in the water (Table 2a). Based on our analysis the probability for a boat to be moored, transported and simultaneously fouled with mussels is 2.2% (Figure 6). Furthermore, we found that 67% of all moored boats, when transported overland, are launched within less than two days out of water. We used two days as a threshold because we found small mussels on most moored boats and larger mussels (>10mm) only on relatively few boats and because most small mussels died between day one and day two of our experiment while almost none survived more than two days. Based on this assumption we estimated that 1.4% of all boats in Switzerland are high-risk boats (note that using a much more conservative level of the period on land of one day or less would reduce our estimate by only 39%, i.e., 0.85% of all boats). On average, boats belonging to this high-risk group were reported to be transported 0.55 times per year. Considering that there are approximately 100,000 recreational boats in Switzerland (Swiss Federal Statistical Office 2014), we estimate that around 800 overland transport events between Swiss water bodies take place every year that present a high risk of new mussel introductions (Figure 6).

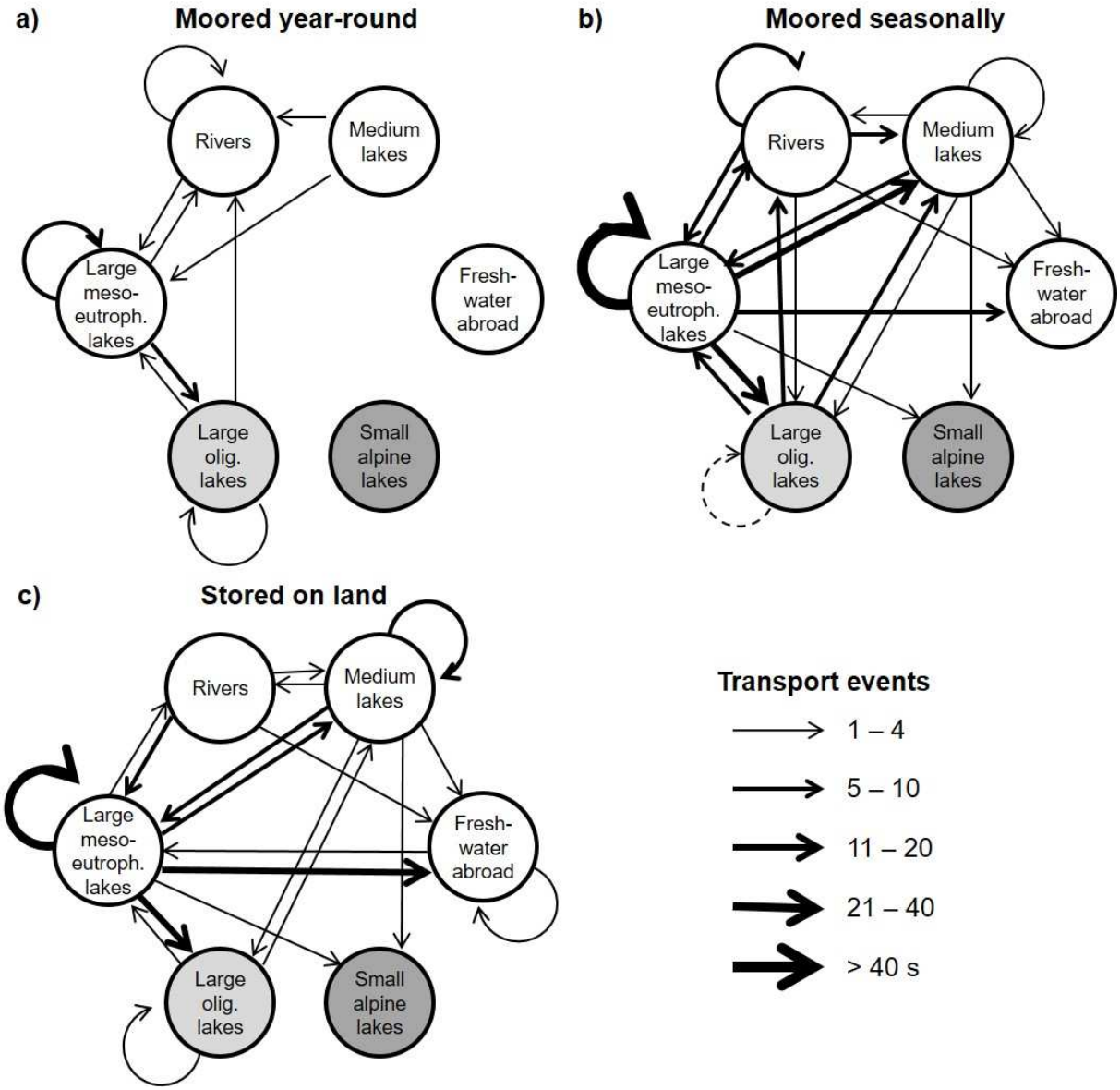

Figure 5 Number of transport events from one water body to another grouped as transport events between different categories of water bodies such as rivers, large meso-eutrophic lakes (≥ 38km2, Ptot > 0.03 mg P / L), medium sized lakes (≥ 7 km2), small lakes (< 7 km2), large oligotrophic lakes (≥ 38 km2, Ptot < 0.03 mg P / L), small alpine lakes (< 7km2, above 1300 m.a.s.l.) and freshwater abroad. The number of transport events, as they were reported in the questionnaire for the past five years, is shown as thickness of the arrows between circles (0-4 events: 1 pt., 5-10: 2 pt., 11-20: 3 pt., 21-40: 4 pt, 40: 5 pt). Only boats which were kept two days or less out of water during the transport for boats moored a) year-round, b) seasonally and c) stored on land were included. The dark grey circle represents the small alpine lakes, which are still free of zebra mussels, and the light grey circle represents the large oligotrophic lakes, of which only one is still free of zebra mussels.

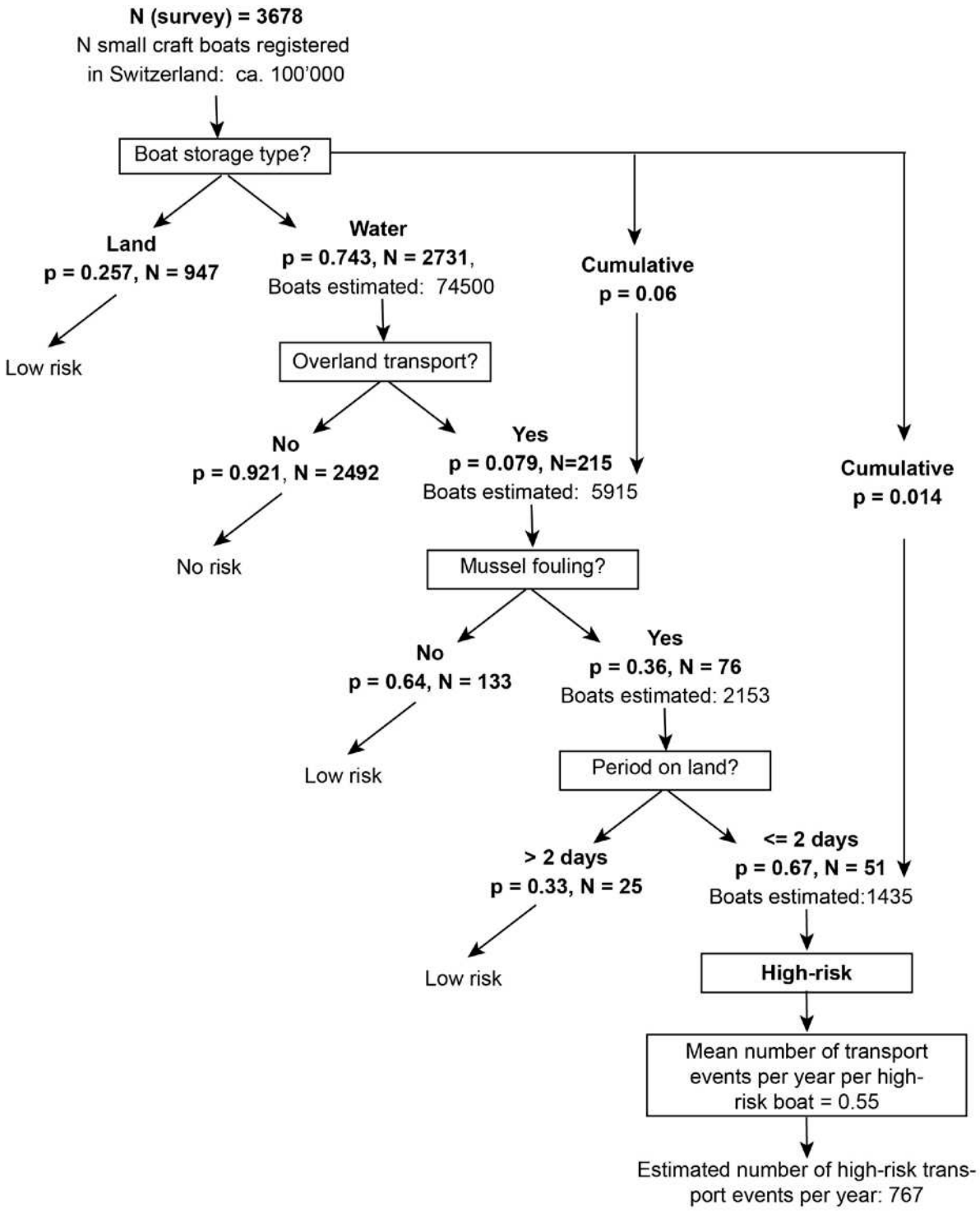

Figure 6 Decision tree showing how we estimated the number of high-risk overland transport events between water bodies within Switzerland. We used four criteria (in boxes) to filter the data to estimate the proportion of high-risk boats for overland spread of zebra mussels. The resulting categories (bold), with proportions, the resulting numbers of boats from the survey (bold), the estimated number of boats in Switzerland (regular) after selection with cumulated criteria are presented after each step. At each step the few boats for which we did not have an answer to the corresponding question (NA's) were ignored to calculate the percentages and they are also not shown in the figure.

Discussion

The focus of previous research has been on boats transported with high frequency and kept on land (often on a trailer) for most of the time. However, our results demonstrate that this class of boats is not an important vector for the spread of the zebra mussel in Switzerland. We found that the main responsible factor for the spread of zebra mussels is likely the rarely transported boats that are moored year-round or seasonally and are thus most often fouled with mussels. This relatively small group of recreational boats is then a high-risk vector for zebra mussel in Switzerland. We estimated that up to 800 high-risk overland transport events take place each year (Figure 6). Considering that we found transport events among almost all navigable water bodies in Switzerland, this number may be high enough for creating a significant propagule pressure for the spread of this and other fouling species (Lockwood et al. 2005). As we also found bryozoan statoblasts and egg clutches of benthic invertebrates in our fouling samples, we suggest that various fouling organisms are continuously transported by recreational boating between water bodies in Switzerland.

Both our survey and our field sampling campaign revealed that between 40% and 50% year-round or seasonally moored recreational boats carried mussels. Although the use of these boats is usually limited to a single water body, they are still occasionally transported over land and thus are an important distribution vector for zebra mussels. In contrast, boats kept on trailers are more often moved between different water bodies, but are rarely fouled with mussels, undoubtedly because they are used only for short periods in water (e.g. few days), which is certainly too short a period for successful mussel settlement (Wainman et al. 1996; Kavouras and Maki 2003). Johnson et al. (2001) also did not find mussel fouling on trailered boats at boat ramps. Instead, they found that zebra mussels were mainly transported overland attached to aquatic macrophytes, which were frequently entangled to such boats, equipment or trailers. In contrast, we did not find any macrophytes or other organisms on boats or boat trailers, because dense macrophyte beds are not common in Swiss navigable waters and because navigation near shore, where macrophytes are more common, is strictly regulated or even prohibited.

The most effective vector for the spread of zebra mussels may thus be boats that are kept for more than one season in water and harbor larger mussels (10 mm – 20 mm) attached to the hull. Such larger zebra mussels may be reproductive at the age of about one year (Ram et al. 1996). After overland transport, these mussels might not only fall off in the target lake, but also reproduce, releasing millions of gametes

forming larvae and introducing a cohort of juvenile mussels into the destination water body. Our survey data showed that almost all overland transport events took place between May and September, overlapping widely with the spawning season of zebra and quagga mussels (Supplementary Material, Figure S6). Thus we speculate that even a few transport events of fouled boats to an uninvaded lake can lead to establishment of a new mussel population, assuming that the environmental conditions are suitable for survival and reproduction.

Our data on distribution and frequency of transport events suggests that the resulting propagule pressure in the water bodies varies considerably and may not be correlated to geographic distance among them but depend more on the size and popularity of the water bodies serving as recreational targets. The main boat transportation routes are between the large lakes such as Lake Constance, Lake Geneva, Lake Zürich and Lake Lucerne (Figure 4). In general, the larger productive lakes located in rather densely populated areas (Liechti and Jakob 2014) also have the highest mussel densities and highest fouling rates on boats. These same lakes also harbor highest numbers of recreational boats and serve as the starting point for many boat transport events of both on land-stored and moored boats (Figure 5). Similarly, Bossenbroek et al. (2007) showed in a gravity model, that the transportation rate of zebra mussels through recreational boating was high among already invaded lakes in the U.S., but the probability of a transport to uninvaded lakes was low. Their model was in accordance with the observation, that the range expansion of zebra mussels had slowed down in recent years (Johnson et al. 2006). We observed a similar effect on the range expansion of zebra mussels in Switzerland. Unfortunately, the data on zebra mussel invasion to Switzerland are not detailed enough to analyze how, with hindsight, the pattern of present spread of zebra mussels matches the boat transportation pattern we discovered. But at least we can predict that these lakes are likely to serve as important hubs for the further spread of zebra mussels and other fouling species. In more recent invasions of lakes and rivers in Switzerland, for example by *Dikerogammarus villosus* (Hesselschwerdt et al. 2008; Bacela-Spychalska et al. 2013), one surprising pattern has been that the invasive range extended rapidly including considerable jumps over longer overland distances between water bodies. Such a pattern has been related to over land transport of recreational boats (Bacela-Spychalska et al. 2013) and is compatible with the transportation frequencies and routes of recreational boats shown by this study.

An interesting point emerging from our questionnaire is that some alpine lakes at higher altitude serve as a target for boat transport (Figure 5), but seem to have remained uninvaded by zebra mussels (To date, zebra mussels have not been found above 1300 m.a.s.l., Figure 1). As the frequency of transport events is low, from these data it is not clear whether the propagule pressure in these lakes was too low for the establishment of zebra mussel populations or whether the environmental niche did not allow the establishment of a new population (Rouget and Richardson 2003; Von Holle and Simberloff 2005; Simberloff 2009). We suspect the latter. Duration of the warm water period (>12°C) in the summer is hypothesized to be the limiting boundary for the spread of zebra mussels (Borcherding 1991). In higher elevation warm water period in the summer is short and may constrain the reproductive cycle of the mussels.

In the end the question of how to manage the propagule pressure of fouling species in vulnerable water bodies remains. We consider that in the lower elevation lakes where transport events occur at higher frequency the propagule pressure can be taken as continuous in the sense that the introductions are frequent enough that the location will eventually be colonized. Regulation of boat movements is relatively lax in Switzerland. We suggest that well-targeted measures against unintended transport of mussels by recreational boats, which have already been tested intensively in the U.S. (see e.g. ANS-Task-Force 2015), could significantly reduce the propagule pressure of fouling species. Our data shows that it might be sufficient to focus prevention measures to the relatively small group of boats that are moored seasonally or all year-round and transported. We estimated that this group comprises roughly 6% of all registered boats in Switzerland (about 6000 boats). With measures focusing on this group, the roughly 800 transport events with a high risk of transporting mussels between water bodies could be prevented (Figure 6). Such measures should include detailed information to the boat owners who moor their boats permanently or seasonally, opportunities for easy and effective cleaning of the boats at boat ramps and enforcement of regulations for safe boat transfer. We estimate that focusing measures on the transport of boats that are kept year-round or seasonally in water would cover about 90% of all high risk transport events. Our data illustrates that it is of prime importance to adapt management measures to the behavior of potential human-mediated vectors specific to a region or country.

We suggest that our vector data could be combined with an environmental niche model (Elith and Leathwick 2009; Kearney and Porter 2009) to evaluate the probability of establishment both as a function of transport frequency (propagule

pressure) and ecological niche availability (resistance of native ecosystems, Leung and Mandrak 2007). This approach would be particularly relevant for modelling future spread of zebra mussels to higher elevation lakes, which may become vulnerable to invasion as climate change proceeds. Such models could also be used to predict spread of potential new invasions like the quagga mussel, *Dreissena rostriformis bugensis*. Quagga mussels have already been reported in the Rhine River and many of Dutch lakes (also densely colonizing shallow areas and harbors), and there is a clear risk that it will spread to lakes in Switzerland (Martens and Schiel 2012; Matthews et al. 2014). Quagga mussels were found to have lower rates of spread than zebra mussels on large spatial scales potentially due to differences in populations dynamics and habitat preferences (Karatayev et al. 2011). Another reason may be that quagga mussels are less resistant to desiccation than zebra mussels and thus the proportion of surviving quagga mussels during over land transport may be lower (Ricciardi et al. 1995). Nevertheless, quagga mussels can attach to hard surfaces such as boat hulls, and their spread to isolated lakes has been repeatedly linked to the overland transport of recreational boats (Stokstad 2007; Karatayev et al. 2011; Martens and Schiel 2012). Because quagga mussels were found to survive and reproduce better at lower temperatures (Roe and MacIsaac 1997) and under low food conditions (Baldwin et al. 2002) than zebra mussels they may also colonize the colder, oligotrophic lakes in higher elevations, which have not been colonized by zebra mussels so far. Future research should focus on collecting environmental data on the possible target lakes to parameterize environmental niche models.

Concluding remarks

Although the zebra mussel is now widely established in Swiss waters, our study provides quantitative information on a key vector of secondary spread that can be used to prevent or slow down the further spread of this species as well as of other fouling species that pose a significant invasion risk, such as bryozoa and amphipods. The quagga mussel is of particular concern as it is already found in the Rhine River and has a great potential to spread similarly throughout Swiss navigable waters (but see Bossenbroek et al. 2007). We know now, however, that a particular group of boats could play a major role as a likely vector for spreading quagga, and this knowledge permits more efficient prevention measures if swift action is taken. Specifically, any recreational boats that have been moored in mussel-invaded waters outside of the country and are then transported to Switzerland are a real risk for quagga mussel introduction. It should be possible to stop these boats at the border, quarantine and

thoroughly clean them to prevent quagga mussel introduction. Moreover, from our data we can predict which lakes in the observed area are most likely to be invaded next by quagga mussels, and we may use this knowledge for future monitoring plans and measures reducing the spread of quagga mussels among Swiss lakes should they become established.

Acknowledgments

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Supplementary Material

Figures

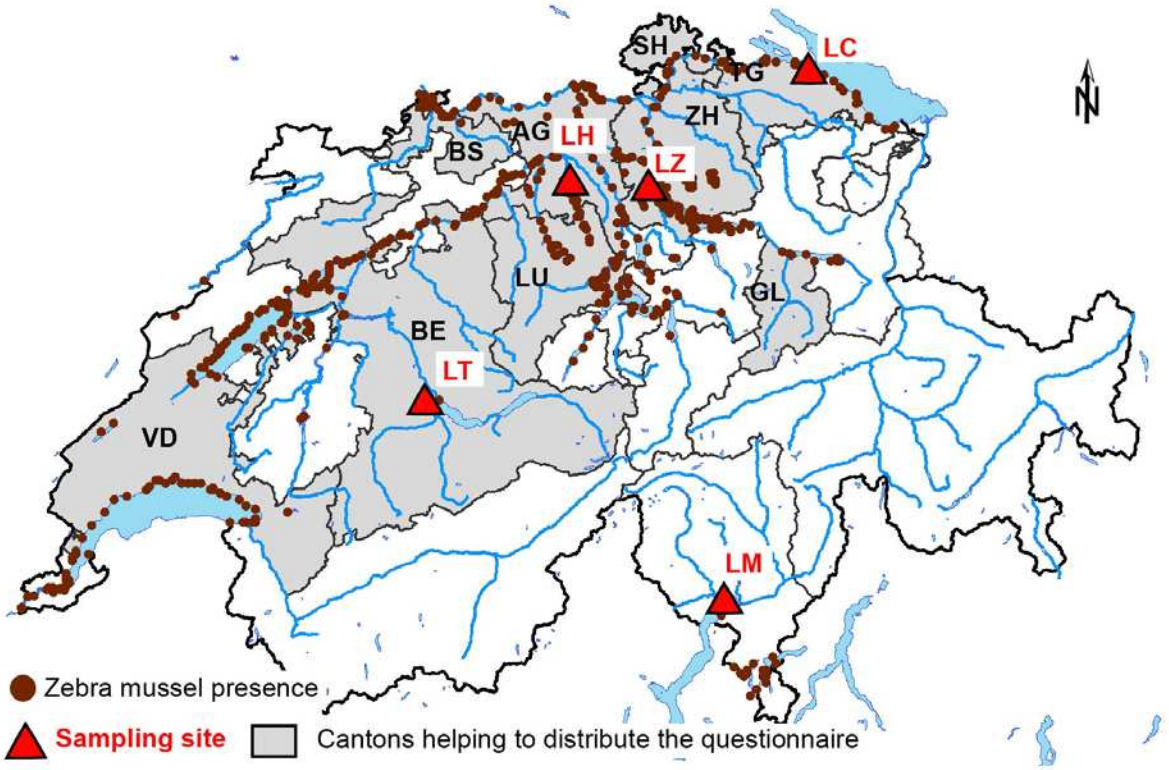

Figure S1 Map of Switzerland showing the nine out of 26 Cantons, who's shipping agencies supported the distribution of the questionnaire shaded in grey. German speaking cantons Aargau (AG), Basel (BS), Glarus (GL), Luzern (LU), Schaffhausen (SH), Thurgau (TG) and Zürich (ZH), the French speaking canton Vaud (VD) and the bilingual canton Bern (BE). The questionnaire was available in German and French. Additionally the distribution of zebra mussels from 2011 (brown dots, data collected from cantonal offices, CSCF and environmental offices) and the field sampling sites (red triangles, LC: Lake Constance, LH: Lake Hallwil, LM: Lake Maggiore, LT: Lake Thun, LZ: Lake Zürich) are shown.

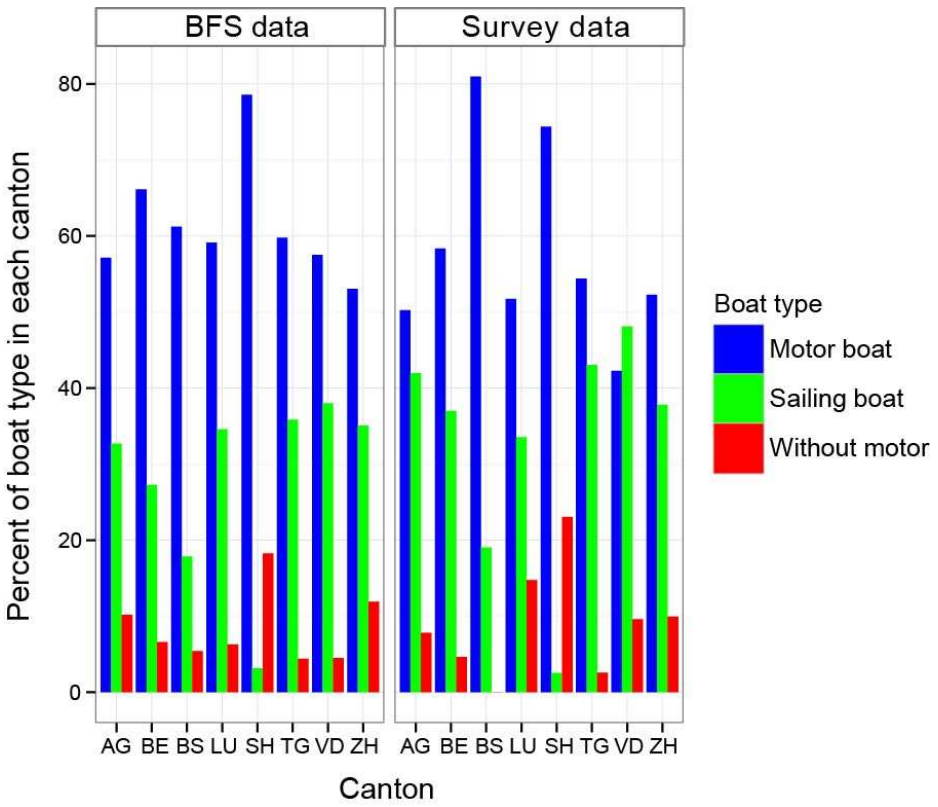

Figure S2 Percentage of boat type is shown for the cantons of Aargau (AG), Bern (BE), Basel (BS), Luzern (LU), Schaffhausen (SH), Thurgau (TG), Vaud (VD) and Zürich (ZH). Motorboats are shown in blue, sailing boats in green, and boats without motor (e.g. rowing boats or flat bottom wooden boats) are shown in red. Percentages are shown for data retrieved from the cantonal offices from the Swiss Federal Statistical Office (BFS) and from our survey data.

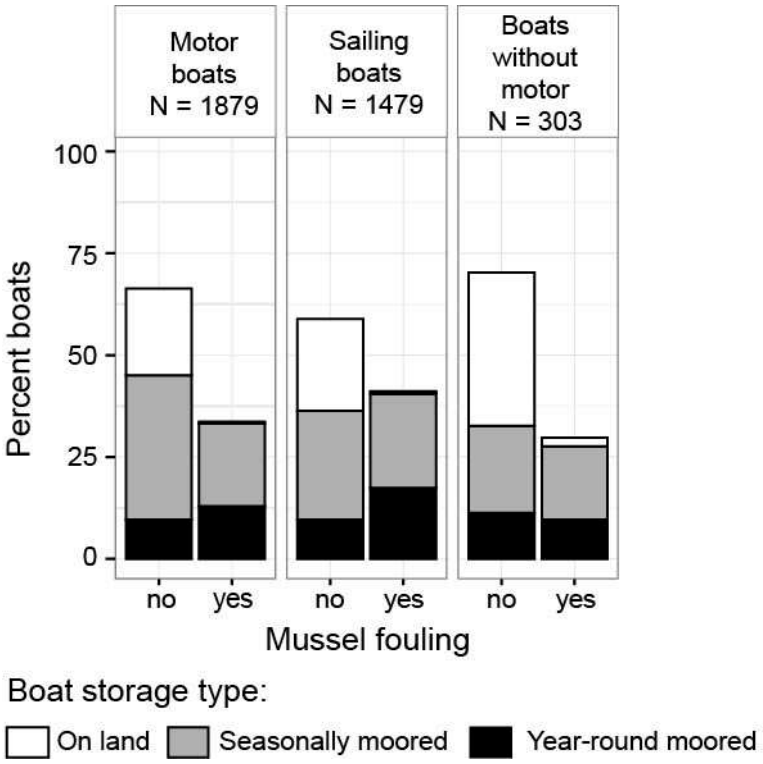

Figure S3 Percentage of boats for which boat owners reported mussel fouling in the questionnaire. Percentage of fouled (Mussel fouling = yes) and not fouled (Mussel fouling = no) boats, for each of the boat storage types: kept on land (white bars), seasonally moored (grey) and year-round moored (black). Percentages are given for each of the three boat types: motor boats, sailing boats and boats without motor. For each boat type, the bars add up to 100%, representing the number of boats (N) assessed in each category. For example, within boats kept in water, sailing boats had higher proportion of fouling compared to motor boats.

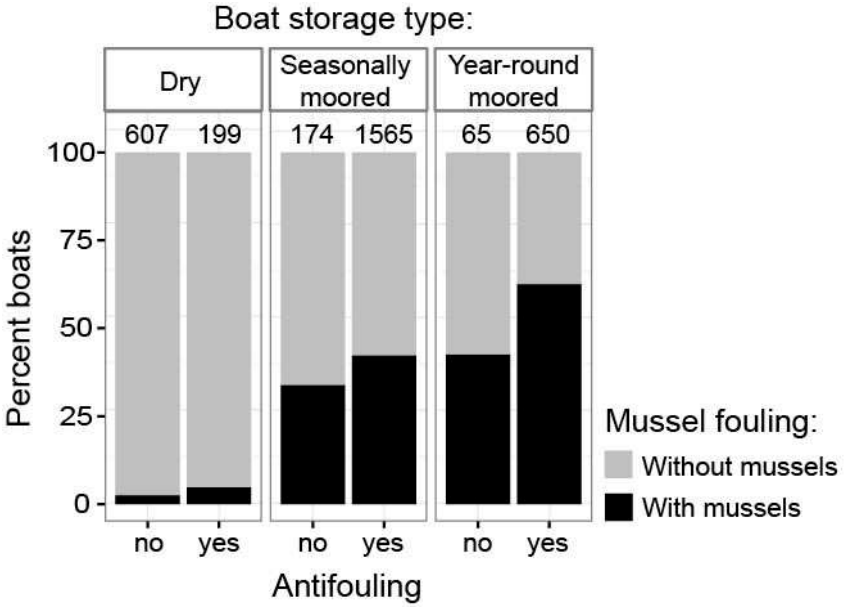

Figure S4 Percentage of boats for which boat owners reported mussel fouling in the questionnaire. Percentage of fouled (black bars) and not fouled (grey bars) boats for boats with (Antifouling = yes) or without antifouling paint (Antifouling = no), for each of the boat storage types (in different panels from left to right: kept on land, seasonally moored, all year-round moored). For each boat storage type and antifouling treatment group, the bars add up to 100%, and the numbers of boats assessed (N) is given for each of these combinations. For example, within seasonally moored boats, the 1565 boats treated with antifouling had higher proportion of fouling compared to the 174 boats without antifouling.

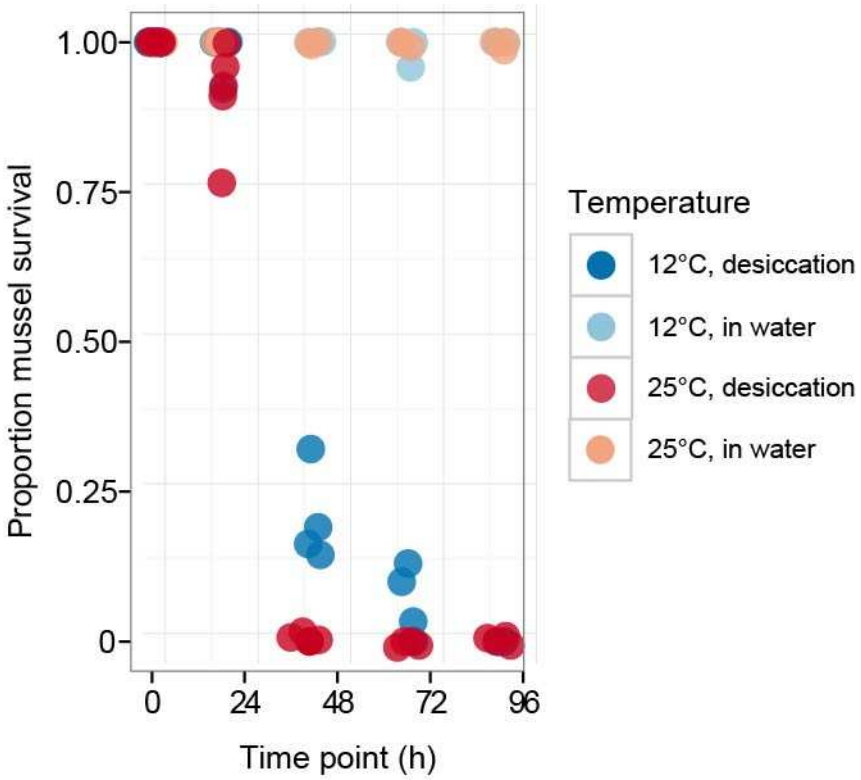

Figure S5 Proportion of survival of juvenile mussels (shell length: 0.5 – 10 mm, median 3.7 mm) under aerial exposure at the two temperatures 12°C (blue) and 25°C (red). The proportion of survival was measured at five different time points (0: start of the experiment, 1: 18 h, 2: 42 h, 3: 66 h and 4: 90 h). The light shaded dots show the positive controls where the plates with mussels were kept in aerated water at 12°C (light blue) and 25°C (light red), respectively.

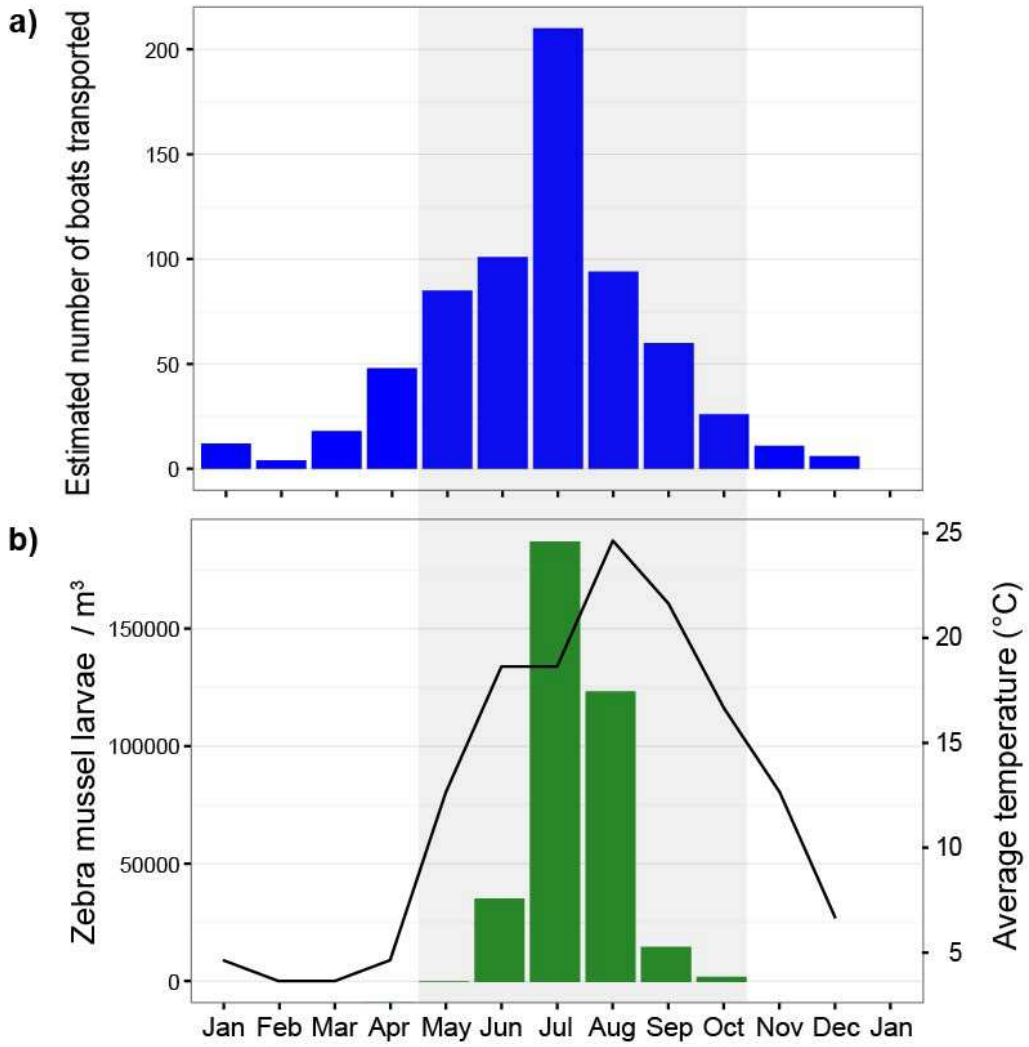

Figure S6 a) Estimated number of over land transports of recreational boats per months during the years 2008 - 2013 (blue bars, survey data). **b)** Number of zebra mussel larvae in Lake Zürich measured routinely at the beginning of the months May - October by the water supply station in Zürich, as average numbers per m^3 of monthly measurements in 2008 - 2013 (green bars). Average monthly water temperatures in Lake Zürich in °C (black line, y axes on the right hand side). The shaded area indicates the overlap of the spawning season of zebra mussels (May - October) with the over land boat transport frequencies.

Tables

Table S1 Odds ratios and corresponding confidence intervals of the final logistic regression models with **a)** mussel fouling on boats (whether boat owners reported mussel fouling in the survey) and **b)** overland transport of boats (whether boat owners reported an overland transport within the last five years) as dependent variables. The columns show the included independent effects, the reference level for each of the independent effects, the corresponding factor categories, the odds ratios (OR) and the 2.5% and 95% confidence intervals.

a) Dependent variable: mussel fouling yes/no					
Independent effects	**Reference category**	**Factor categories**	**OR**	**2.5%**	**97.5%**
		(Intercept)	0.03	0.01	0.06
Water body category	large meso-eutr. lakes	large oligotrophic lakes	0.13	0.09	0.18
		medium lakes	0.94	0.71	1.24
		rivers	0.25	0.17	0.34
		small lakes	0.12	0.03	0.38
Boat storage	dry	seasonal in water	25.36	13.19	56.68
		year round in water	58.91	29.78	134.26
Boat type	motor boats	sailing boat	1.12	0.41	3.10
		without motor	2.01	0.59	6.23
Water sports	no	yes	0.66	0.50	0.87
Fishing	no	yes	1.44	1.13	1.84
Boat storage * boat type	dry:motor boat	seasonal in water:sailing boat	1.21	0.43	3.41
		year-round in water:sailing boat	1.30	0.45	3.79
		seasonal in water:without motor	0.96	0.28	3.56
		year-round in water:without motor	0.30	0.08	1.20

b) Dependent variable: boat transport yes/no					
Independent effects	**Reference category**	**Factor categories**	**OR**	**2.5%**	**97.5%**
		(Intercept)	0.11	0.07	0.15
Water body category	large meso-eutr. lakes	large oligotrophic lakes	1.60	1.15	2.21
		medium lakes	0.69	0.44	1.05
		rivers	2.28	1.48	3.44
		small lakes	2.46	0.36	10.14
Boat storage	dry	seasonal in water	0.63	0.42	0.94
		year round in water	0.54	0.32	0.89
Boat type	motor boats	sailing boat	1.90	1.24	2.94
		without motor	0.43	0.14	1.06
Water sports	no	yes	1.53	1.09	2.11
Competitions	no	yes	4.61	3.27	6.52
Boat storage * boat type	dry:motor boat	seasonal in water:sailing boat	0.37	0.21	0.65
		year-round in water:sailing boat	0.35	0.17	0.73
		seasonal in water:without motor	1.26	0.31	5.08
		year-round in water:without motor	0.68	0.03	4.80

Chapter 3

Motivation and awareness of boat owners for boat cleaning to prevent spread of invasive species

In review

Authors

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Abstract

Overland transport of recreational boats is among the most important distribution vectors for aquatic invasive zebra and quagga mussels to inland waters. Simple measures such as instructing boat owners how to prepare their boat for transport without carrying invasive species are considered to be important prevention measures. Nevertheless, the net effects of such measures are poorly understood and the boat cleaning behavior of boat owners has never been studied in detail before the implementation of such preventive measures. We investigated the boat cleaning behavior of boat owners in Switzerland using a self-report questionnaire, where almost no preventive measures have been taken yet. We found that the self-reported boat cleaning rates are high, with 92% of boaters cleaning their boat upon finding mussels attached to their boat and 84% of them cleaning their boat before a transport. Nevertheless, only half of the boat owners report using high pressure washing to clean their boat before an overland transport and many still use inappropriate cleaning methods. Our model shows that the boat cleaning behavior could be significantly improved by changing how boat owners value the perceived costs and the perceived benefits of cleaning as well as their awareness for the potential negative impacts on aquatic ecosystems caused by non-native species. With regard to a possible spread of zebra mussels to uninfested alpine lakes and the imminent spread of quagga mussels in Switzerland, we conclude that Swiss public would be open to accept implementation of prevention measures, similar to those applied in the US.

Keywords

Recreational boating; distribution vectors; boat cleaning, *Dreissena polymorpha;* behavioral change, preventive measures

Introduction

Invasive species are among the most important threats to biodiversity in aquatic ecosystems (Sala et al. 2000). Most aquatic invasive species cause high economic costs, and once they are established and reach high population densities, it is nearly impossible to eliminate them from their invasive range (Pimentel et al. 2005). Thus, it is of high importance to prevent the spread of such species to not yet invaded habitats. The natural spread of exclusively aquatic species is generally limited by the dendritic structure of river-lake systems (Fagan 2002). Overland transport of recreational boats has been shown to be among the most important distribution vectors for the ongoing secondary spread of aquatic invasive species in such systems (Johnson et al. 2001; Leung et al. 2004; MacIsaac et al. 2004). Overland transport has been demonstrated for species such as the spiny water flea (MacIsaac et al. 2004), the Eurasian water mill foil (Buchan and Padilla 2000), the killer shrimp (Bacela-Spychalska et al. 2013), and zebra mussels (Johnson et al. 2001; De Ventura et al. 2016). The transport of aquatic invasive species was shown to be mostly unintentional (Johnson and Carlton 1996). Organisms were found to be transported in bilge wells, live wells, bait buckets, attached to the boat exterior or entangled in macrophytes, which were attached to the boat or the boat trailer (Johnson et al. 2001).

Research on boats as vectors has focused on the two closely related invasive species, the quagga mussel (*Dreissena rostriformis bugensis* Andrusov, 1897) and the zebra mussel (*Dreissena polymorpha* Pallas, 1771), because they have a series of negative impacts on the ecology of invaded rivers and lakes (Ricciardi et al. 1995b; Vanderploeg et al. 2002; Strayer 2009) and impose high socioeconomic costs (Pimentel et al. 2005). Overland transport of small boats was shown to be mainly responsible for the distribution of these two species to inland waters (Padilla et al. 1996; Bossenbroek et al. 2001). In North America, zebra mussels were mostly transported overland entangled in macrophytes, which were unintentionally transported by trailered boats (Johnson et al. 2001). In Switzerland, macrophytes are rarely entangled with boats or boat trailers, but zebra mussels are transported directly attached to the boat hull, propeller, keel, engine area or other irregularities on the boat exterior (De Ventura et al. 2016). In our previous study we found that roughly 40% of year-round or seasonally moored boats carried zebra mussels and 5% of those were transported between water bodies and without being kept out of water longer than two days, allowing the survival of mussels during the overland transport. We estimated that roughly 700 of such boats, imposing a high risk of

distributing zebra mussels, were transported between water bodies every year, and transports took place between all navigable water bodies in Switzerland. To date, zebra mussels occur in almost all larger lakes and rivers in Switzerland, except those at higher altitudes (Figure 1) while the quagga mussel, a congener of the zebra mussel, has only recently been detected in the Swiss Rhine (De Ventura 2015, unpublished data) and is expected to spread widely in Switzerland via recreational boating (Martens and Schiel 2012; De Ventura et al. 2016). Thus it is important to prevent the further distribution of zebra and quagga mussels through the overland transport of small craft boats. A reduction in the strength of this vector would lead to reduced propagule pressure on uninfested lakes and rivers (Lockwood et al. 2005; Simberloff 2009) and may greatly reduce the establishment risk of zebra and quagga mussels in these water bodies (Bossenbroek et al. 2001; Leung and Mandrak 2007).

In Switzerland (and in most other European countries), only very little action has been taken by local authorities to reduce the spread of invasive species through recreational boats. To our knowledge there are no regulations addressing this problem and only few tentative campaigns have informed boat users about invasive species and boat cleaning measures. However, in North America a series of measures have been implemented to prevent the spread of zebra and quagga mussels (Rothlisberger et al. 2010). In regional and national information campaigns, boat owners were instructed to clean their boats and equipment, remove organisms and let the boat and equipment dry before an overland transport (ANS-Task-Force 2015). Recommended methods for cleaning were mostly hot water spraying or high pressure washing. In some states (e.g. Wisconsin and Minnesota) such regulations have been imposed by law and a fine has to be paid if the demanded action is omitted (http://dnr.wi.gov/topic/invasives/boat.html). In a study from U.K., Anderson et al. (2014) have shown that also 64% of anglers and 79% of canoeists use their boat in different river catchments within a fortnight and likely distribute invasive species. In the same study only 12% of anglers and 50% of canoeists cleaned their equipment before transporting it to a new catchment, but if canoeists had heard of the “Check, Clean, Dry” campaign, they cleaned their boat and equipment 40% more often (http://www.nonnativespecies.org/checkcleandry/).

Table 1 Summary of measures asked from boat users and other people dealing with aquatic organisms on US lakes and rivers. This is an example of rules implemented by the "Stop Aquatic Hitchhikers" campaign of the U.S national Aquatic Nuisance Species task force (ANS-Task-Force 2015).

	Action to take	**When**	**Detailed action**
A)	Remove all visible mud, plants and animals from boats trailers and boat equipment	Before leaving any water body	• Remove any visible plants, plant fragments, fish or animals. • Remove mud and dirt since it too may contain a hitchhiker. • Do not transport any potential hitchhiker, even back to your home. Leave them at the site you visited.
B)	Eliminate all water from the boat and boat equipment	Before transporting the boat to another water body	• Eliminate all water from every conceivable. • Remove water from motors, jet drives, live wells, boat hulls, scuba tanks and regulators, boots, waders, bait buckets, seaplane floats, swimming floats.
C)	Clean and dry anything that came into contact with the water	Before transporting the boat to another water body	• Use hot (< 40° C or 104° F) or salt water to clean your equipment. • Wash your dog with water as warm as possible and brush its coat. • If hot water is not available, use high pressure washing. • If possible, allow for 5 days of drying time before entering new waters
D)	Do not release any plants or animals into a water body which do not originate from the same water body	In general	• Do not release anything from your aquarium into or near a body of water. • Do not release unused bait into the waters you are fishing.

However, only little research has been done on the efficacy of such regulations, information campaigns and boat cleaning methods. The most effective method to completely remove fouling mussels from a boat was shown to be hot water sprays (Morse 2009; Comeau et al. 2011) or high pressure washing (Rothlisberger et al. 2010). Rothlisberger et al. (2010) showed that in the area of Wisconsin roughly 60% of boat owners did not always clean their boat before a transport and they confirmed that small-craft boats are still an important distribution vector, even after prevention measures have been taken (since the 1990ies). To our knowledge the cleaning behavior of boat owners has never been studied before major prevention measures have been taken. Furthermore, no research has been done to investigate the motivation of the boat owners for boat cleaning and the reasons why they would not clean their boat. Consequently, there is no secured knowledge about which

arguments may best convince boat owners to help preventing the spread of invasive species. In order to assess the net effect of regulations and information campaigns it would be necessary to assess the situation before the measures are taken. Specifically studying cleaning behavior, cleaning motivation and overland transportation habits might help to better design measures, target the right audience and help to find the right arguments to convince boat owners to appropriately clean their boats before an overland transport.

We therefore investigated the boat cleaning habits of boat owners in Switzerland by using a questionnaire. We evaluated the data of the questionnaire considering the results from our earlier study on mussel fouling and boat transportation frequencies (De Ventura et al. 2016). In this earlier study we found that year-round and seasonally in water kept boats had the highest potential for overland transport of zebra mussels between different water bodies, while boats kept on land (also referred to trailered boats or transient boats elsewhere) almost never harbored mussels, other aquatic invertebrates or macrophytes. Consequently, we focused our analysis of boat cleaning on year-round and seasonally in water kept boats and excluded boats normally kept on land from our analysis. We analyzed the results of the questionnaire to investigate the following questions:

1. How often do boat owners clean their boats and how do they value the boat cleaning?
2. Which criteria determine whether boat owners clean their boats after they have detected mussels?
3. Which boat owners use high pressure washing before transporting their boat to another water body?

Based on the results for these three questions, we built a model estimating to which extent a) increasing the awareness for the damage on ecosystems caused by aquatic non-native species b) decreasing the perceived costs for boat cleaning or c) increasing the perceived benefits of boat cleaning, may improve the boat cleaning rate and the probability to choose high pressure washing as a method. We then estimated how much boat cleaning, together with the implementation of these measures, could reduce the strength of overland boat transport as a distribution vector. Finally, we discuss the importance of prevention measures with regard to a possible spread of zebra mussels to uninfested alpine lakes and the imminent spread of quagga mussels in Switzerland.

Methods

Survey

We investigated the cleaning behavior of boat owners in Switzerland using a large scale questionnaire. Data from boat owners were gathered by self-administered questionnaires (in German and French), which were distributed by mail and could be filled out on paper or in the internet. The questionnaire consisted of 30 items asking about a) socio-demographic data, b) characteristics of the boats, c) presence of fouling organisms at the boat exterior, d) overland transport events between water bodies, e) cleaning habits, f) cleaning motivation and g) the awareness towards problems of aquatic invasive species. In more detail, we asked boat owners how they evaluated different types of costs and benefits of boat cleaning, how much money they spend for boat cleaning per year, whether they think that recreational boats are an important distribution vector for aquatic animals and whether they think that non-native species may have negative impacts on aquatic ecosystems. Table 2 compiles the categories of the items used in this study. The invitation letter informed boat owners that participation was voluntary, and how the investigators could be contacted in case of questions. Filling out the questionnaire took about 15 minutes. Most items used in the analyses presented here are straightforward in design (e.g., simply asking where the boat was normally kept with the answering options: moored all year-round, moored during summer season, kept on land).

Nine of the total 26 cantonal shipping agencies sent the questionnaires to a random sample of 20% of the registered boat owners (Figure 1). Roughly 10 500 boat owners received the survey and 3561 of them filled in and returned the questionnaire (response rate = 34%). The boats of responders were distributed over roughly 30 navigable lakes and several rivers, which are almost exclusively and intensively used for recreational boating. Cases of overland transport by boat owners were reported for all navigable Swiss lakes and the areal coverage of the sampling is shown in Figure 1. With a total of 99 200 private boats registered at cantonal offices in 2013 (Swiss Federal Statistical Office 2014) the returned questionnaires covered roughly 3.7% of all registered boats in Switzerland. Among the 3561 boat owners who returned the questionnaire 89% were men (average age 57.3 ± 13.1 years). The distribution of boat types reported in the survey is similar to the distribution provided by the cantonal shipping agencies (Swiss Federal Statistical Office 2014), indicating that the survey is representative for the registered boats in Switzerland (Supplementary Material, Figure S1).

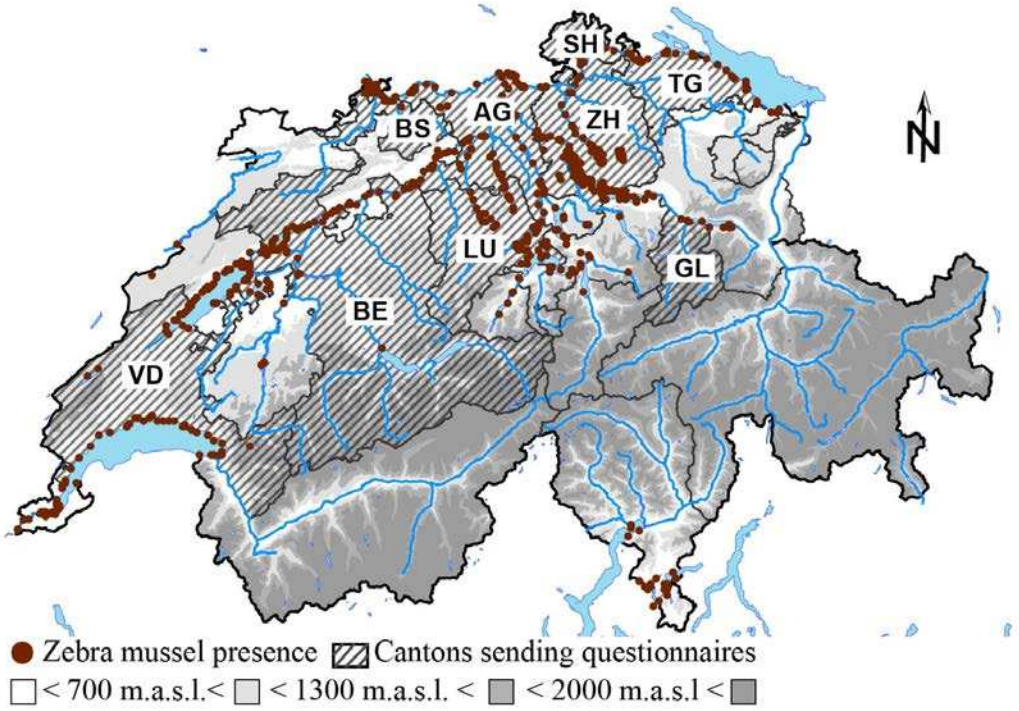

Figure 1 Zebra mussel distribution in Switzerland (brown dots) and the cantons involved in this study (striped area). German speaking cantons Aargau (AG), Basel (BS), Glarus (GL), Luzern (LU), Schaffhausen (SH), Thurgau (TG) and Zürich (ZH), the French speaking canton Vaud (VD) and the bilingual canton Bern (BE). The questionnaire was available in German and French. Data on the distribution of zebra mussels was collected in 2011 from cantonal offices, the CSCF (Swiss Centre of Faunistic Cartography) and environmental offices. Grey shades show different levels of altitude. To date, zebra mussels have not been found above 1300 m.a.s.l. (darker grey and darkest grey shades).

Table 2 A summary of the survey results showing all variables discussed in this paper. For each variable, the response categories, the corresponding proportions and the total number of answers (N) included in the analysis are shown. The dependent variables used in our models are highlighted in grey. For the analysis of boat cleaning we used a subset of survey data including only owners of year-round or seasonally moored boats (panel B). For the analysis of high pressure washing rate (panel C), we included only year-round and seasonally moored boats, for which at least one overland transport between different water bodies was reported. Boats without motor are the boats belonging to neither of the two categories sailing boats and motor boats, but were mostly wooden boats, rowing boats or flat bottomed or punt-like boats.

A) Question	**Results for all boats**					**N**
Where did you normally keep (store) your boat?	Year-round in water	Seasonally in water	On land (dry)	Other		3765
	22.9%	49.6%	25.2%	1.4%		
B) Questions	**Results for year-round and seasonally moored boats**					**N**
How did you react after you had detected mussels or other aquatic animals attached to your boat?	No reaction	Removed all	Tried removing	Other reaction		1256
	8.3%	74.2%	16.8%	2.6%		
Of which type is your boat?	Motor boat	Sailing boat	Boat without motor**			2731
	52.1%	41.6%	6.3%			
Which material does the hull of your boat consist of?	Wood	Glass fiber	Aluminum	Other material		2731
	15.0%	79.1%	3.3%	2.6%		
To which length category does your boat belong to?	0 - 2.5 m	2.6 - 6.5 m	6.6 – 10 m	>10 m		2731
	0.9%	47.4%	45.3%	6.3%		
What do you use your boat for?	Pleasure	Competition	Fishing	Water sport		2731
	85.5%	10.6%	18.4%	12.9%		
Did you find mussels or other organisms, attached to your boat?	Yes	No	Other organisms			2731
	53.0%	46.8%	6.8%			
Where did you find mussels on your boat?	Boat hull	Motor	Keel/Sword	Water in boat		2731
	61.8%	38.2%	25.7%	0.8%		
Did you use antifouling paint to prevent fouling on your boat?	Yes	No				2554
	90.6%	9.4%				
Where did you normally clean your boat?	Shipyard/public station	In the water	At land	At home		2577
	49.9%	9.2%	21.7%	19.2%		
How do you estimate probability that recreational boats distribute organisms between water bodies?	very low	low	medium	high	very high	2731
	4.9.%	9.0%	16.3%	24.8%	44.1%	
How do you estimate the benefit for aquatic ecosystems, if recreational boats helped to distribute organisms among them?	very low	low	medium	high	very high	2731
	36.0%	15.6%	31.3%	9.7%	7.3%	
How do you estimate the damage on aquatic ecosystems, if recreational boats helped to distribute organisms among them?	very low	low	medium	high	very high	2731
	3.7%	8.3%	23.1%	33.4%	31.5%	
Have you transported your boat from one lake to another within the past 5 years?	Yes	No				2577
	8.0%	92.0%				

Continued on the following page

C) Questions	Results for moored and transported boats					N
How often did you use the following cleaning method before transporting your boat to another water body?	Never	Rarely	Sometimes (50%)	Mostly	Always	N
Scratching	77.5%	3.7%	3.2%	5.4%	10.2%	187
Brushing	59.4%	7.0%	6.4%	7.0%	20.3%	187
Low pressure water spraying	53.5%	3.2%	5.3%	9.1%	28.9%	187
High pressure washing	31.6%	8.0%	5.3%	12.8%	42.2%	187
In case you did not clean your boat before a transport, what are the reasons?	yes	no				N
Lack of time	29.0%	71.0%				100
Lack of motivation	8.0%	92.0%				100
The boat was believed to be clean	55.0%	45.0%				
Indifference	8.0%	92.0%				100
High costs	1.0%	99.0%				100
Forgetting	1.0%	99.0%				100

Statistical analysis of the survey data

Generally, we included only explicit answers in the analysis and answer categories, such as 'don't know' or 'other option' were excluded. We used logistic regression models to evaluate which of the independent variables assessed in the survey best explained a) whether boat owners cleaned their boat or not after they had found mussels on their boat and b) whether boat owners used high pressure washing to clean their boat before an overland transport. The variable 'reaction to fouling' asked boat owners how they reacted after they had detected mussels on their boat (Table 2). Possible answers were 'no reaction', 'all removed', 'unsuccessful attempt to remove all mussels', 'boat cleaned by dockyard'. From this questionnaire item 'reaction to fouling', we created the binary variable 'cleaning' containing the two categories 'yes' or 'no' by recoding the answers of 'no reaction' into the category 'no' and the answers for 'all removed', 'unsuccessful attempt to remove all mussels' and 'boat cleaned by dockyard' into the category 'yes'. To allow for a robust statistical test on the usage of high pressure washing, we created a new binary variable 'high pressure washing' by recoding the answers from the question about high pressure washing frequencies 'never', into the category 'often'. 'rarely' and 'sometimes' into the new category 'rare' and the answers 'mostly' and 'always'

We performed a similar model selection procedure for both dependent variables. In all the analysis we included only seasonally or year-round moored boats and excluded land kept boats from the data set. First, we explored the correlation structure between all continuous and ordinal explanatory variables, which we

intended to test in the two models for a) boat cleaning rate and b) whether high pressure washing was used rarely or often before a transport in R (R-Core-Team 2014), using the function 'rcorr' of the 'Hmisc' package (Harrell 2013). Second we tested the Spearman correlation structure of all explanatory variables, including categorical variables for each of the two models a) and b) in separate categorical principal component analyses (CatPCA) in SPSS (IBM-Corporation 2012). Some of the Spearman correlations between pairs of explanatory variables exceeded 0.5, indicating high multi-collinearity and precaution for multivariate regression analyses (Supplementary Material Table S2).

Among variables for which boat owners estimated the different types of costs and benefits of removing mussels from their boats, the variables describing different types of costs such as difficulty, time expenditure, perceived monetary costs, and strenuousness were highly intercorrelated; while the variables describing the benefit and importance of cleaning were highly correlated as well (Supplementary Material Table S1, Tables S2). We thus further explored the correlation structure among cost and benefit variables in a CatPCA in SPSS. In the resulting two-dimensional model, dimension 1 represented the four cost variables and dimension 2 represented the two benefit variables (Figure 2). We thus retrieved the object scores from the CatPCA-model for each of the two dimensions and used the resulting two summarizing variables (one for cost and one for benefit) in the subsequent logistic regressions. For plotting and analyzing the data we normalized the object scores for the benefit dimension and the cost dimension by dividing the scores with the standard deviation of the mean. Subsequently, we shifted the object scores by subtracting the minimum negative value in order to get rid of negative values and then scaled them with a factor such that all values lay between 0 and 4. We did this because this was the scale we used in the questionnaire and it would be an intuitive scale to interpret the results. The results for the logistic regression model were the same with transformed and untransformed data.

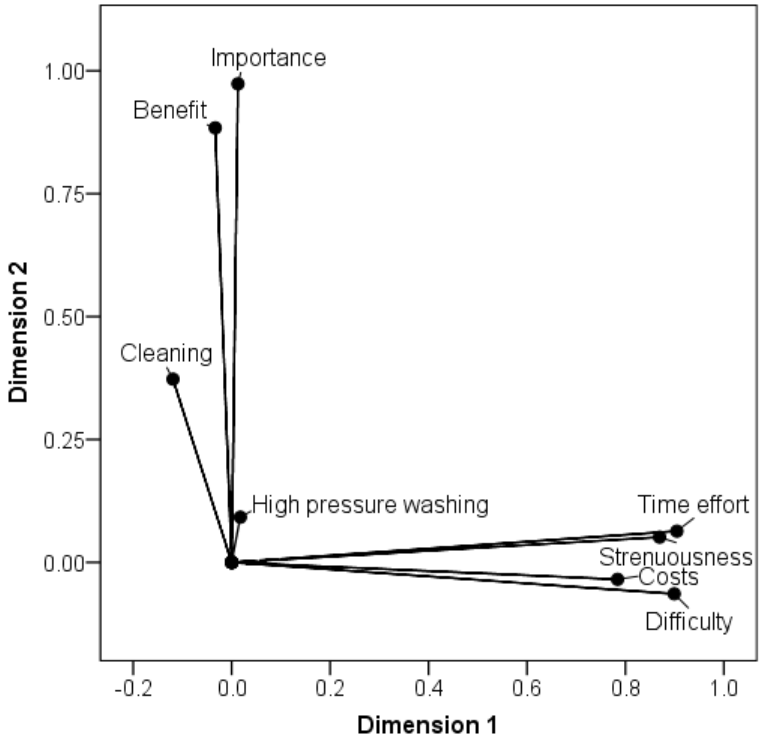

Figure 2 Two dimensional plot of the CatPCA (categorical principal component analysis) for cost-benefit variables such as difficulty, time expenditure, monetary costs and strenuousness, benefit and importance. Cleaning (as binary variable with the options 'yes' and 'no') and high pressure washing (as binary variable with the options 'rare' and 'often') were added to the CatPCA as supplementary variables and thus did not influence the calculated correlation structure but are shown as component loadings in relation to the dimension for cost (dimension 1) and the dimension for benefit (dimension 2).

The effects of the explanatory variables were tested for the two dependent variables in logistic regression models using the statistical package R, version 3.0.2 (R-Core-Team 2014). We tested the effects of variables for which we expected significant effects in stepwise forward model selection procedure based on p-values and also taking into account Akaike Information Criterion (Table 3b and 4b). We performed the model selection with inserting the independent variables in different orders and also tested the models in a backward model selection procedure in the same way. The different procedures always resulted in the same final model including the same significant effects.

From the resulting models we calculated the predicted probabilities for a) uncleaned boats and b) use of high pressure washing, as function of explanatory variables (Table 3a and 4a). We calculated the predicted probabilities for uncleaned boats by evaluating the final model excluding the boat type as an explanatory factor. (Because the influence on the predictions through the variable boat type were relatively weak

and not important for the overall predictions.) We subsequently calculated the predicted probabilities for each category of the independent variables perceived costs, perceived benefits or the awareness for the damage on ecosystems evoked by aquatic non-native species while holding the other two independent variables at their mean values (Figure 5A, 5B and 5C). We calculated the predicted probabilities for high pressure washing from the final model including the explanatory variables boat storage type and awareness for the damage on ecosystems (Figure 5D). We further assumed that it would be difficult to change the perception of boat owners which already perceived costs low and benefits and the damage on ecosystems high. On the other hand, the values of perceived costs above the population mean (= 1.3) and the values of perceived benefits or the awareness for the damage on ecosystems below the population means (3.35 and 3.8, respectively) might be changed more easily. Thus we present in three scenarios how the cleaning rate will change if the perceived costs, benefits and the estimated damage on ecosystems are shifted below or above certain values for all boat owners, which lay close to the population means (perceived costs below 2, perceived benefit above 3 and awareness for the damage on ecosystems above 4, Figure 5). Similarly, we present a scenario how the rate of high pressure washing will change if the estimated damage on ecosystems is shifted above a value of 4 for all boat owners.

Table 3 A) Results of the final logistic regression model with boat cleaning (whether owners of seasonally or year-round moored boats reported NOT to clean their boat after detecting mussels on the boat exterior) as dependent variable. We included all the explanatory variables for which we found significant effects. (AIC = 555.6, residual deviance = 543.6 on 1112 degrees of freedom, Loglikelihood = -271.8). The columns show the included independent effects, the Likelihood Ratio chi-square statistics for each effect (LR Chisq), the degrees of freedom (Df), corresponding p-values, variable categories, odds ratios (OR), with confidence intervals (OR CI 2.5% and OR CI 97.5%), number of uncleaned boats (N), and percent of boats which were not cleaned. **B)** We show one example of the stepwise forward model selection procedure based on p-values and AIC. The columns show the variables at each of the model selection steps, the p-value of the corresponding variable in this step, the resulting AIC value and whether the variable was kept in the model or discarded for the next step.

A) Dependent variable: cleaning = no

Independent variable	LR Chisq	Df	p-value	Sig.	Factor category	OR	OR CI 2.5%	OR CI 97.5%	N not cleaning	% not cleaning
Boat type	8.7	2	< 0.05	*	Sailing boat	ref. for boat type			33	6.9
					Motor boat	1.95	1.20	3.24	66	11.7
					Boat without motor	0.80	0.21	2.38	5	6.7
Cost dimension	9.2	1	< 0.005	**	Continuous	1.64	1.25	2.76		
Benefit dimension	51.3	1	< 0.001	***	Continuous	0.16	0.05	0.17		
Damage ecosystem	10.6	1	< 0.005	**	Ordinal	0.72	0.59	0.88		

B) Forward Stepwise selection based on p-values and AIC

Variable added	p-value of added variable in the model	AIC of the resulting model	Keep or remove variable from the model
Null Model: Cleaning (yes or no) ~ 1		715.32	
Estimated benefits for cleaning	< 2.2e-16	640.27	keep
Estimated costs for cleaning	< 0.001	629.86	keep
Awareness for "damage on ecosystems"	< 0.005	565.38	keep
Boat type	< 0.05	555.59	keep
Boat use for competitions	0.30	556.43	remove
Awareness for "distribution of invasive species through recreational boating"	0.44	545.43	remove
Monetary cleaning costs	0.51	504.01	remove
Antifouling usage	0.18	515.01	remove
Boat material	0.96	561.23	remove
Boat use for fishing	0.97	557.54	remove
Boat storage type	0.70	557.44	remove
Overland transport (yes or no)	0.57	555.45	remove

Table 4 A) Results of the final logistic regression models with use of high pressure washing (whether boat owners reported to clean their boat rarely or often before a transport event) as dependent variable. We analyzed the data for owners of year-round and seasonally moored boats, who had reported at least one over land transport event from one lake to another within the past five years. We included all the explanatory variables with significant effects (AIC = 209.46, residual deviance = 203.46 on 158 degrees of freedom (Df), Loglikelihood = -101.7 with Df =3). The columns show the included independent effects, the corresponding Likelihood Ratio chi-square statistics (LR Chisq), degrees of freedom (Df), p-values, variable categories, odds ratios (OR), with confidence intervals (OR CI 2.5% and OR CI 97.5%), number of boats often high pressure washed (N), and percent of boats which were often high pressure washed before a transport. **B)** We show one example of the stepwise forward model selection procedure based on p-values and AIC. The columns show the variables at each of the model selection steps, the p-value of the corresponding variable in this step, the resulting AIC value and whether the variable was kept in the model or discarded for the next step.

A) Dependent variable: cleaning with high pressure washing

Independent variable	LR Chisq	Df	p-value	Sig.	Factor category	OR	OR CI 2.5%	OR CI 97.5%	N often steamed	% often steamed
Boat storage type	8.8	1	< 0.005	**	Perennially in water	ref. for storage type			15	35.7
					Seasonally in water	3.18	1.58	7.06	85	63.4
Damage ecosystem	6.9	1	< 0.01	**	Ordinal	1.49	1.11	2.04		

B) Forward Stepwise selection based on p values and AIC

Variable added	p-value of added variable in the model	AIC of the resulting model	Keep or remove variable from the model
Null Model: High pressure washing ~ 1		242.7	
Boat storage type	< 0.005	234.72	keep
Mussel fouling	0.26	232.55	remove
Awareness for "damage on ecosystems"	< 0.01	209.46	keep
Awareness for "distribution of invasive species through recreational boating"	0.75	206.33	remove
Boat use for competitions	0.98	211.46	remove
Boat type	0.71	211.33	remove
Boat material	0.26	210.89	remove
Antifouling usage	0.20	200.32	remove
Estimated benefits for cleaning	0.26	210.22	remove
Estimated costs for cleaning	0.94	211.46	remove
Monetary cleaning costs	0.06	196.57	remove

Results

How often do boat owners clean their boats and how do they value the boat cleaning?

When owners of seasonally or year-round moored boats were asked how they reacted after they had detected mussels growing on their boats, roughly 25% did not clean or only unsuccessfully clean their boats. While 8.4% of boat owners admitted, that they did not clean their boats at all, 16.8% of boat owners reported that they had unsuccessfully tried to remove mussels from their boat (Table 2). On average, the estimated monetary cleaning costs per year were moderate with CHF 346 and CHF 363 for seasonally or year-round moored boats, respectively, but these costs varied considerably between individuals (between 0 CHF and 10000 CHF). When asked how they valued the costs and benefits of cleaning on a scale between 1 (low) and 5 (high), most boat owners estimated the different types of costs moderate (mean scores for difficulty, time expenditure, monetary costs and strenuousness were 2.4, 2.5, 2.1 and 2.6, respectively) while they estimated the benefit and the importance of cleaning higher (mean scores for benefit and importance were 4 and 3.7, respectively, Figure 3). When we explored the correlation structure between cost-benefit variables in categorical principal component analysis (CatPCA), a two-dimensional model explained 79% of the total variance in the data. Dimension 1 explained 75% of the variance of the four cost variables while dimension 2 explained 87% of the variance of the two benefit variables (Figure 2). These two principal components were then used in the statistical analysis for boat cleaning and the use of high pressure washing. The scores for the benefit of cleaning were relatively high (mean = 3.35 on a scale between 0 and 4, SE = 0.005) while the scores for the costs were rather moderate (mean = 1.31 on a scale between 0 and 4, SE = 0.009).

Among all boat owners who kept their boats year-round or seasonally in water, 83% reported to clean their boats always or mostly before they transport it overland, with one of the methods: scraping, brushing, low pressure spraying or high pressure washing (Table 2). However, only 55% of these boat owners always or most of the time used high pressure washing before a transport (Table 2). The most often named reason why boat owners did not clean their boat before a transport was that they thought their boat was already clean (55% named that reason). Another often named reason was the lack of time (29%), while other reasons such as lack of motivation, indifference, high costs or simply forgetting to clean were named rarely with 8%, 8%, 1% and 1%, respectively (Table 2).

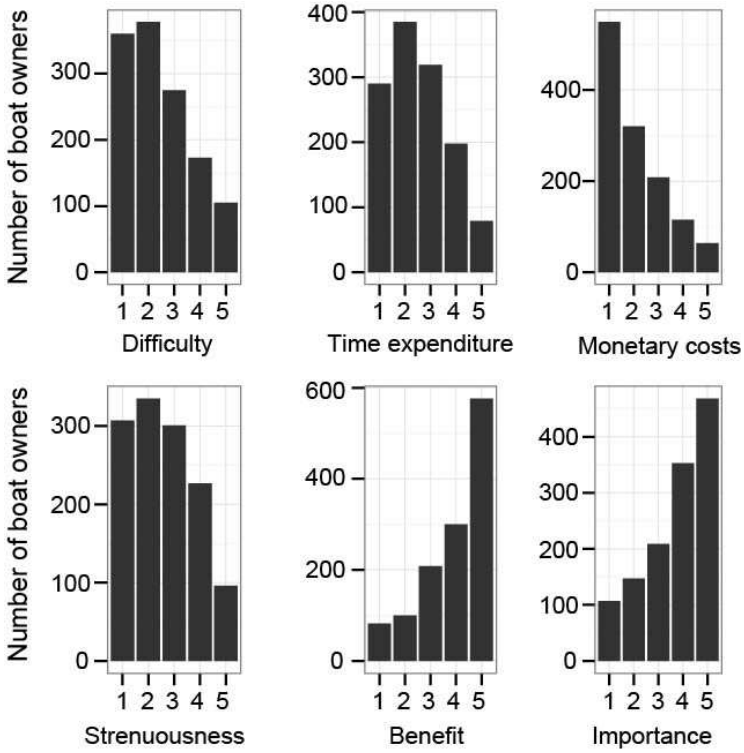

Figure 3 The histograms show how many owners of seasonally or year-round moored boats estimated difficulty, time expenditure, (monetary) costs, strenuousness, benefit and importance of boat cleaning to be very low (1), low (2), medium (3), high (4) or very high (5).

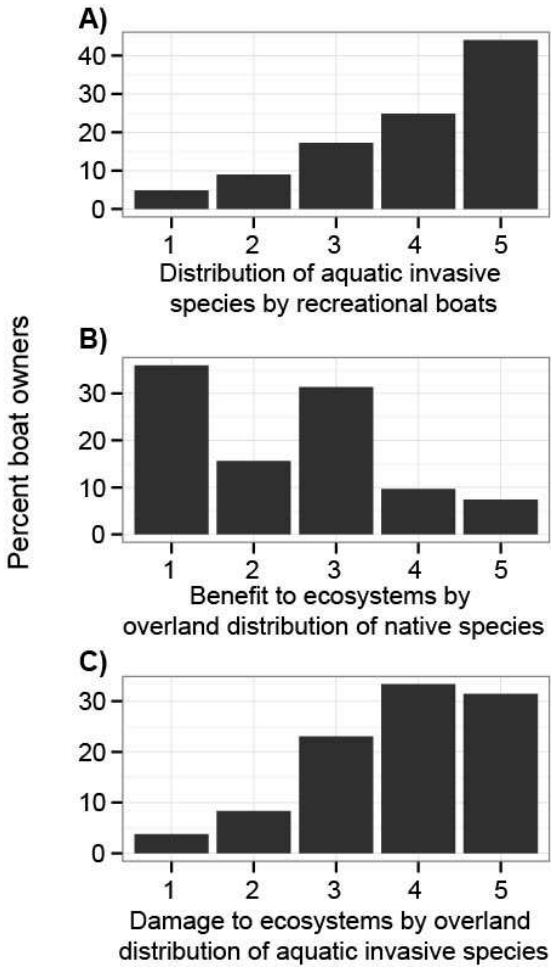

Figure 4 Percentage of boat owners who valued A) the probability that aquatic invasive species are distributed by overland transport of recreational boats, B) the probability that aquatic ecosystems benefit from the distribution of native species by recreational boating and C) the probability of damage evoked through the spread of non-native species on aquatic ecosystems either very low (1), low (2), medium (3), high (4) or very high (5).

On average boat owners consider it likely that recreational boats transport aquatic animals between water bodies (3.9 on a range between 1 and 5, Figure 4) and that there are negative effects on ecosystems if boats spread non-native aquatic animals between water bodies (3.8 on a range between 1 and 5). They were rather undecided whether the distribution of aquatic species through recreational boating might also have beneficial effects for the aquatic ecosystems (2.3 on a range between 1 and 5).

Which factors determine whether boat owners clean their boat after they have detected mussels?

We examined what reasons were behind not cleaning the boat after mussels had been found attached to the boat. As expected, boat owners who were concerned of the costs of cleaning (as perceived difficulty, time expenditure, estimated monetary costs and strenuousness of cleaning) cleaned their boat significantly less often ($p < 0.005$, odds ratio = 1.64, Table 3a, Figure 5A). Also the boat owners who valued the benefit of cleaning lower were significantly more reluctant to clean ($p < 0.001$, odds ratio = 0.16, Table 3a, Figure 5B). How boat owners rated the negative effects of the spread of non-native species on ecosystems was strongly and positively correlated with the declared frequency of cleaning ($p < 0.001$, Table 3a, Figure 5C). Against our expectations, the cleaning rate did not depend on whether boat owners were aware of the fact that recreational boats might spread aquatic species between water bodies (Table 3b). Also the estimated monetary cleaning costs did not significantly (and negatively) influence whether boat owners cleaned their boat or not. On a side note, owners of motor boats cleaned significantly less often (11.7% did not clean), compared to owners of sailing boats (5.9% did not clean) or boats without motor (6.7% did not clean, Table 2a).

Our model predicts that reducing the perceived costs for all boat owners below a value of 2 would reduce the average probability of a boat not to be cleaned at all (after mussels have been detected) to 5.4%, compared to 8.1% of boat owners who reported not to clean in our questionnaire (Figure 5A and 6A). To accomplish such a shift, perceived costs would only need to be reduced in16% of boat owners ($N_{>2}$ = 182). If the perceived benefit of cleaning would be increased above a value of 3 for boat owners with lower values (14% of boat owners, $N_{<3}$ = 158) the average probability of a boat owner not to clean his boat would be reduced to 5.4% (Figure 5B). Increasing the awareness for the damage on ecosystems above a level of 4 for boat owners with lower values (31% of boat owners, $N_{<4}$ = 368) would have a similar effect and decrease the average probability of not cleaning to 5.1% (Figure 5C). If, for

all boat owners, perceived costs were reduced to a level of 2 and, at the same time, perceived benefits and the awareness for the damage on ecosystems were increased to a level of 3 and 4, respectively, the average probability for a boat owner not to clean his boat could be decreased to 1.5%.

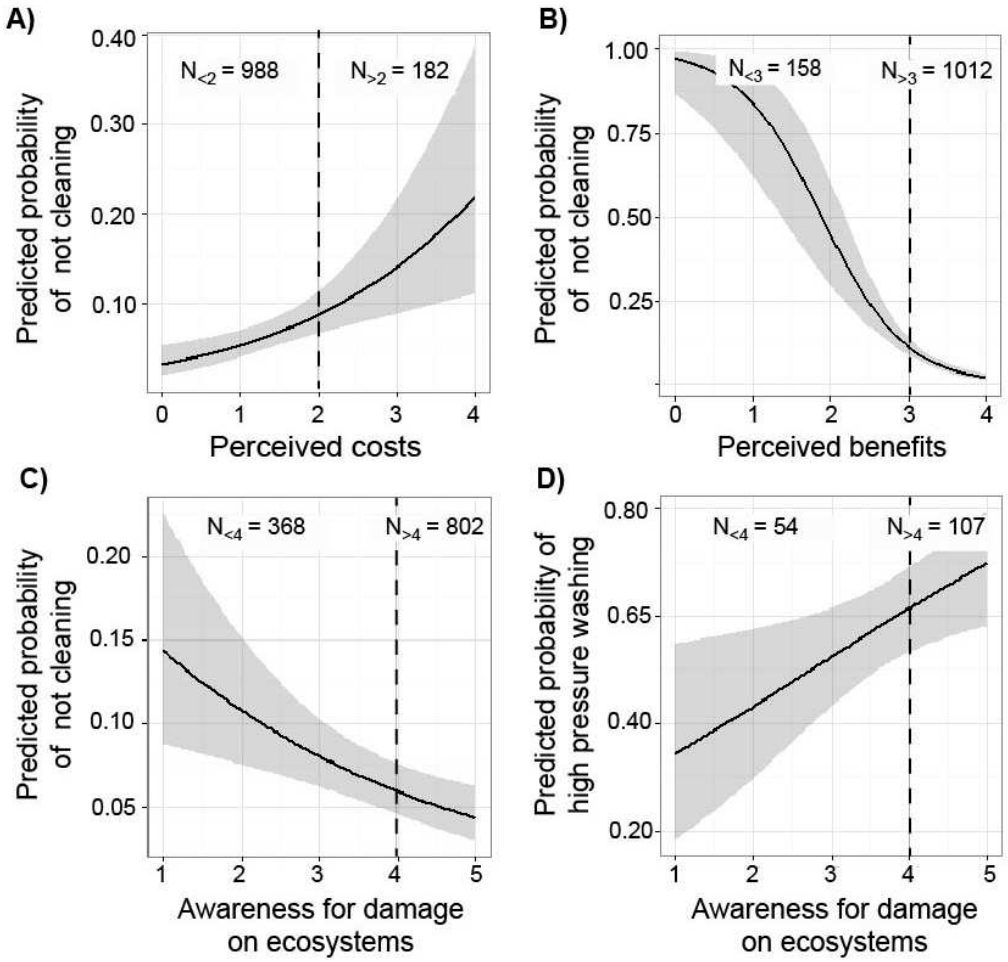

Figure 5 The predicted probabilities for boat owners not to clean their boat after finding mussels on the boat exterior, for the different levels of A) perceived costs (values between 0 = very low and 4 = very high), B) perceived benefits (values between 0 = very low and 4 = very high) and C) the awareness for the damage on ecosystems evoked by aquatic invasive species (values between 1 = very low and 5 = very high). The grey area depicts the standard errors of the predicted probabilities. D) The predicted probabilities for boat owners to clean their boat with high pressure washing before transporting a boat, for the different levels of how boat owners valued the damage on aquatic ecosystems evoked through the spread of non-native species. In the four predictive scenarios, we assume that measures may shift the values of perceived costs below 2, of perceived benefits above 3, and of the awareness for the negative impacts above 4, indicated by the dashed lines. We also show how many boat owners belonged to the group below or above the dashed lines (N).

Which boat owners use high pressure washing before transporting their boat to another water body?

Surprisingly, boats which were kept seasonally in water were significantly more often cleaned by high pressure washing before a transport (63% often high pressure washed $p < 0.0005$, Table 4) than boats which were kept year-round in water (36% often high pressure washed). The higher boat owners estimated the ecological damage evoked by non-native species on aquatic ecosystems, the more often they cleaned their boat by high pressure washing ($p < 0.01$, odds ratio = 1.49, Figure 5D). Our model predicts that increasing this type of awareness above a level of 4 for all boat owners, would increase the average proportion of boat owners using high pressure washing from 55% to 66% (Figure 6B). Against our expectations none of the two composite variables 'cleaning costs' or 'cleaning benefit' nor the variables 'awareness for the distribution of aquatic species by recreational boating', 'boat usage types', 'boat material' or 'mussel fouling' showed a significant effect on the rate of using high pressure washing. Also motorboats and sailing boats were not different from each other in their rates of high pressure washing. Boats of the category "without motor" were never cleaned with high pressure washing before a transport but as there were only six boats in this category (within the data set used to test for effects on rate of high pressure washing) they could not be included in the logistic regression model.

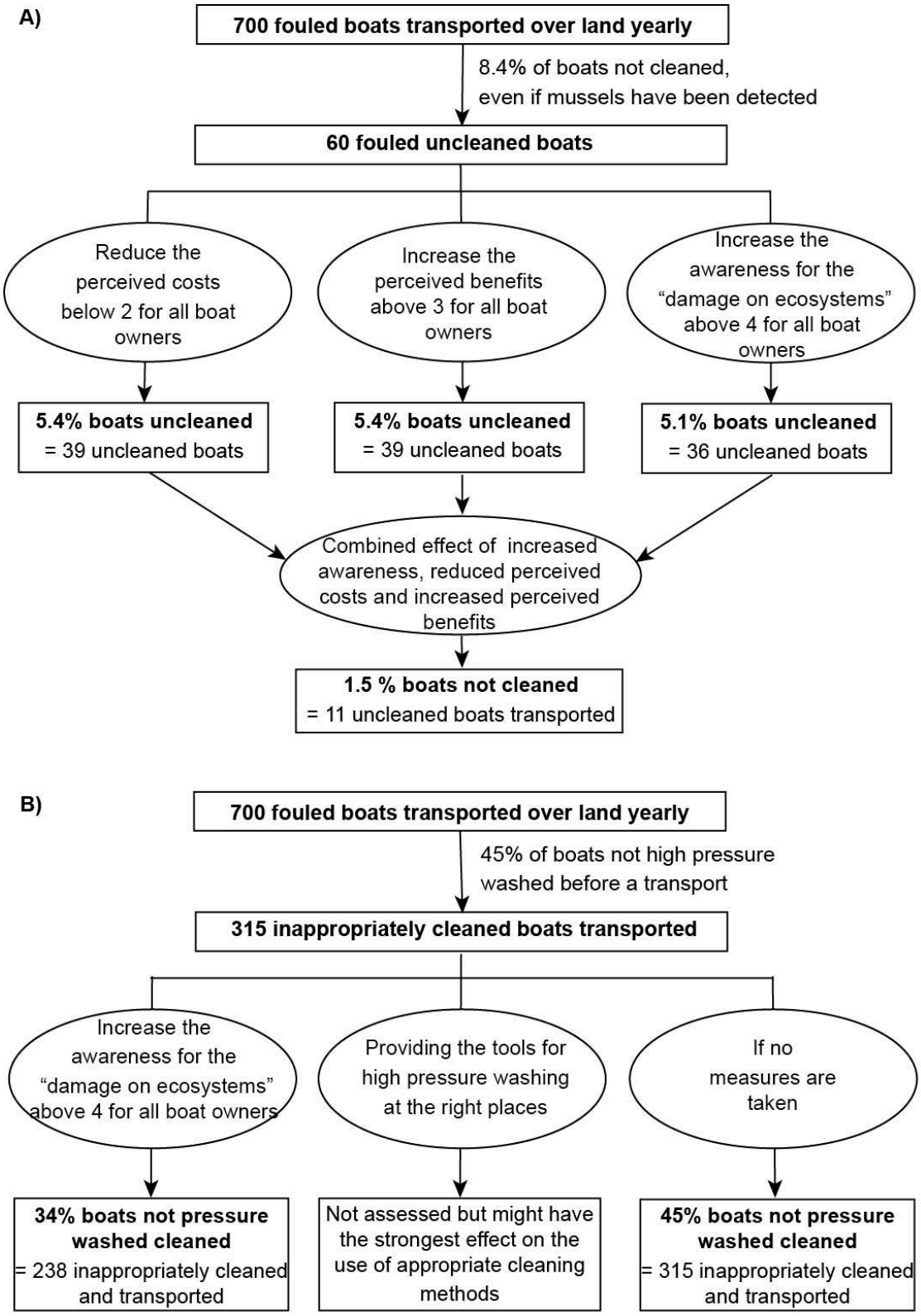

Figure 6 Flow chart showing the effects on boat cleaning behavior and the potential of recreational boating as a distribution vector for zebra mussels. A) Effects on whether a boat is cleaned after mussels have been detected, B) effects of whether boats are cleaned with high pressure washing before a transport.

Discussion

Our results show that only 8.5% of boat owners, who kept their boat year-round or seasonally in water, were not willing to clean their boat when they detected fouling organisms such as zebra mussels on their boats. Moreover, 83% of boat owners reported to clean their boats always or mostly before they transport it overland, using either scraping, brushing, low pressure spraying or high pressure washing. These can be considered high rates, as for now there are no regulations in Switzerland and only very little measures such as information campaigns have been undertaken to encourage boat owners to clean their boats and prevent transport of invasive species overland. Yet, self-reported boat cleaning rates are higher than the results of the North American study by Rothlisberger (2010), who found that two thirds of boat owners did not always clean their boat before a transport. Thus the underlying willingness to clean the boat seems to be high in Switzerland and the behavior of boat owners might be different in Switzerland from those in the U.S. Unfortunately, we have not found any information about boat cleaning rates in the Great Lakes region before authorities started to take measures. Thus it is difficult to say what the impact of those measures and information campaigns were.

For Switzerland, we previously estimated roughly 700 transport events of recreational boats carrying zebra mussels between waterbodies per year (De Ventura et al. 2016). Transport events between all navigable water bodies were recorded, with the lowest propagule pressure to the uninfested oligotrophic alpine lakes. Continued or increased propagule pressure or the arrival of another fouling species, which might have an environmental niche that is more suitable to the habitats in the alpine lakes, might put those lakes at risk for an invasion. For example the quagga mussel, a congener of the zebra mussel, has recently arrived in the Rhine river in Switzerland and was shown to colonize water bodies with lower temperatures (Roe and MacIsaac 1997) and lower nutrient loads (Baldwin et al. 2002) compared to zebra mussels. Moreover, quagga mussels can also attach to hard surfaces such as boat hulls and their spread to isolated lakes was repeatedly linked to the overland transport of recreational boats (Stokstad 2007; Karatayev et al. 2011a; Martens and Schiel 2012). Therefore, the quagga mussel might well spread via recreational boating to most water bodies in Switzerland including the mussel free alpine lakes. It might not be possible to completely prevent the spread of invasive fouling species, even if strong prevention measures are taken. Nevertheless, as the successful establishment of an invasive species to new habitats was shown to depend on the propagule pressure (Lambrinos 2004; Sexton et al. 2009), a reduction in vector strength might largely

reduce the speed and also the probability of population establishment in uninfested water bodies.

The predictions retrieved from our model on boat cleaning show, that measures changing the perceived costs and benefits and the awareness for the damage on ecosystems evoked by non-native species, could have a strong effect on boat cleaning rates. A reduction in the perceived costs such as time expenditure, strenuousness or difficulty may be achieved by providing good cleaning facilities and cleaning instructions to boat owners at harbors and boat ramps. Moreover, providing information on the problems caused by species invasions may increase the awareness for the damage caused by non-native species. Our dataset comprised of relatively few boat owners who transported a water kept boat overland and also filled in the table for cleaning methods (N = 187). Therefore, we drew first conclusions about the willingness of boat owners to clean their boat from our larger data set on whether boat owners were willing to clean upon detecting mussels on their boat (N=2731). We assumed that above mentioned measures may have the strongest effect on boat owners which declared perceived costs above the population mean (= 1.3) and perceived benefits or the awareness for the damage on ecosystems below the population means (3.35 and 3.8, respectively). Thus we present three scenarios where the three independent variables are shifted below or above certain values for all boat owners, which lay close to the population means (Figure 5). All three scenarios would considerably reduce the proportion of uncleaned boats to 5.4%, 5.4% and 5.1% respectively, compared to 8.1% if no measures are taken (Figure 6). Our model further predicts that, if measures acted on all the three aspects, shifting them simultaneously to values as described above, the reduction in uncleaned boats would be significantly higher leaving only roughly 1.5% boats uncleaned (Figure 6).

Even if cleaning rates are high and might be even considerably increased by the measures just described, only half of the investigated boat owners always or most of the time use high pressure washing to clean their boat before a transport. High pressure washing and hot water sprays (Morse 2009; Comeau et al. 2011) were shown to be the most effective methods to completely remove fouling mussels. Since boat owners in our study area rarely have the equipment for washing with hot water available (neither at harbor, boat ramps, or at home), we did not distinguish between high pressure washing with hot or cold water in the questionnaire. Nevertheless, our results suggest that the number of transports of infested boats is reduced by roughly 50% through boat cleaning, in a best case scenario (assuming that boat owners who

declared to high pressure wash their boat also have used this method effectively). As the other cleaning methods such as scraping, brushing or low pressure rinsing are less effective, increasing the rate of high pressure washing may be crucial to considerably reduce the proportion of boats transporting zebra mussels and potentially other invasive species (e.g. quagga mussels or resting stages of invasive bryozoans). In an ideal situation authorities and boating clubs would provide washing stations with high pressure and hot water at each harbor, boat ramp or other places where boats are frequently taken out of the water along with information panels on how to clean a boat appropriately and effectively. The effects of providing high pressure washing stations on the rate of appropriate boat cleaning were not tested in this study. However, such measures would certainly help to convince more boat owners to use this method when washing their boat. Moreover, informing boat owners about the fact that recreational boats might spread non-native species and that those species may have negative impacts on ecosystems and socioeconomics, could increase the rate of high pressure washing considerably to 64%, which is, in our opinion, not sufficient (Table 4a, Figure 6B).

A relatively high proportion of boat owners (16.5%) realized that it is difficult to clean the boat completely from mussels and reported that they had not succeeded to do so. From our discussions with workers at shipyards, we learned that, even with good knowhow and the appropriate equipment, it can be difficult to remove all mussels from the engine area and other irregularities on the boat exterior. Furthermore, considering that a large proportion of moored boats were infested with very small mussels (> 5mm), which are hardly detected by the unexperienced eye (De Ventura et al. 2016), a significant proportion of boat owners might also not have realized, that their boat was infested. For example, seasonally moored boats were 0.31 times less often high pressure washed (Table 3a) than year-round moored boats. Such boats, in particular early in the season, do not have an extensive biofilm yet and harbor only few small mussels, while boats which are kept year-round in water are more likely to be seriously fouled with mussels and thus were expected to be more often washed with high pressure. For those reasons, the proportion of boat owners not removing (all) mussels from their boats might be underestimated in our data. As the results on whether boat owners clean their boat a) upon finding mussels or b) before a transport showed similar cleaning rates, the cleaning rate itself might not be so much overestimated as the cleaning success.

This finding again shows the importance of clear instructions on boat cleaning skills and providing appropriate boat cleaning facilities to boat owners, or having boats cleaned in shipyards were appropriate cleaning tools and know-how are present. In order to do that, we need to scrutinize what the best practices are and test which cleaning methods work best and how they are best instructed to boat owners. Additionally, a quarantine time where boats are dried and kept at least two weeks out of water before they can be transported to a new water body might prevent the overland transport of invasive mussels. Zebra mussels larger than 10 mm can survive up to 10 days out of water(Ricciardi et al. 1995a) while smaller zebra mussels were found to survive for up to two days at 25°C on air (De Ventura et al. 2016). For seasonally or year-round moored boats, half of the boats were kept only two days or less on land, likely allowing the survival of zebra mussels during the transport. The problem with a quarantine time is that mussels might survive in places in or at the boat which stay moist during the quarantine time and which may be the same places, which are also difficult to clean. Nevertheless, we think that an increase in appropriate boat cleaning potentially coupled with a quarantine time may greatly reduce the overland transportation rate of zebra mussels and other aquatic invasive species.

Conclusions

Most boat owners cleaned their boat when they detected mussels and also before transporting their boat overland. Most of them were also aware that recreational boats can distribute aquatic invasive species and that those may have negative impacts on ecosystems. This might indicate that boat owners are generally amenable to information campaigns providing information on invasive species problematics and advice on how to prevent the spread of invasive species through appropriate boat cleaning. Firstly, it seems to be necessary to inform boat owners about appropriate cleaning methods to eliminate invasive species from their boat effectively. Secondly, the cleaning rate might be increased by changing how people perceive the costs and benefits of cleaning and how they value the damage on aquatic ecosystems caused by the distribution of non-native species. We thus suggest that a) information campaigns on appropriate boat cleaning methods and potential negative impacts of aquatic invasive species on ecosystems and socioeconomics are carried out at all potentially infested water bodies, b) high pressure washing facilities are provided by authorities in these places and c) a quarantine time is recommended for seasonally and year-round moored boats. Cleaning is hard to control even if it is

regulated and also a quarantine time might not always be followed. Nevertheless, if underlying willingness for cleaning a boat is as high as in our study, the above mentioned measures may be effective enough to reduce the overland transport of mussels significantly and thus effectively slow down or even prevent the further spread of zebra and quagga mussels.

We want to highlight here that, for Switzerland, measures should be taken as soon as possible, since the quagga mussel has already arrived in a harbor in Basel (De Ventura 2015, see chapter 4) and might spread further from there. Quagga mussels were often found to spread slower than zebra mussels but in many cases eventually invaded the same habitat as the zebra mussel and displaced its congener (Karatayev et al. 2011b). We thus urgently recommend to apply the same measures to prevent the distribution with recreational boats as described for the zebra mussel above.

Acknowledgements

We thank the cantonal waterways and shipping offices for helping us with sending our survey to a random sample of boat owners. Special thanks also go to all boat owners who filled in the questionnaire and the boatyards providing us with information on boat cleaning methods used in Switzerland. This work was financed by the Swiss Federal Office for the Environment (FOEN) and the Swiss Federal Institute for Environmental Sciences and Technology (Eawag). Data on the registered boats in Switzerland were provided by the Swiss Federal Statistical Office. This study was conducted in strict compliance with the ethical principles of the American Psychological Association (APA) and the Declaration of Helsinki. It underlies the ethics review board of the ETH, Swiss Federal Institute of Technology Zurich. This review board exempts the survey studies that do not comprise an intervention from obtaining an ethical approval.

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Supplementary Material

Figures

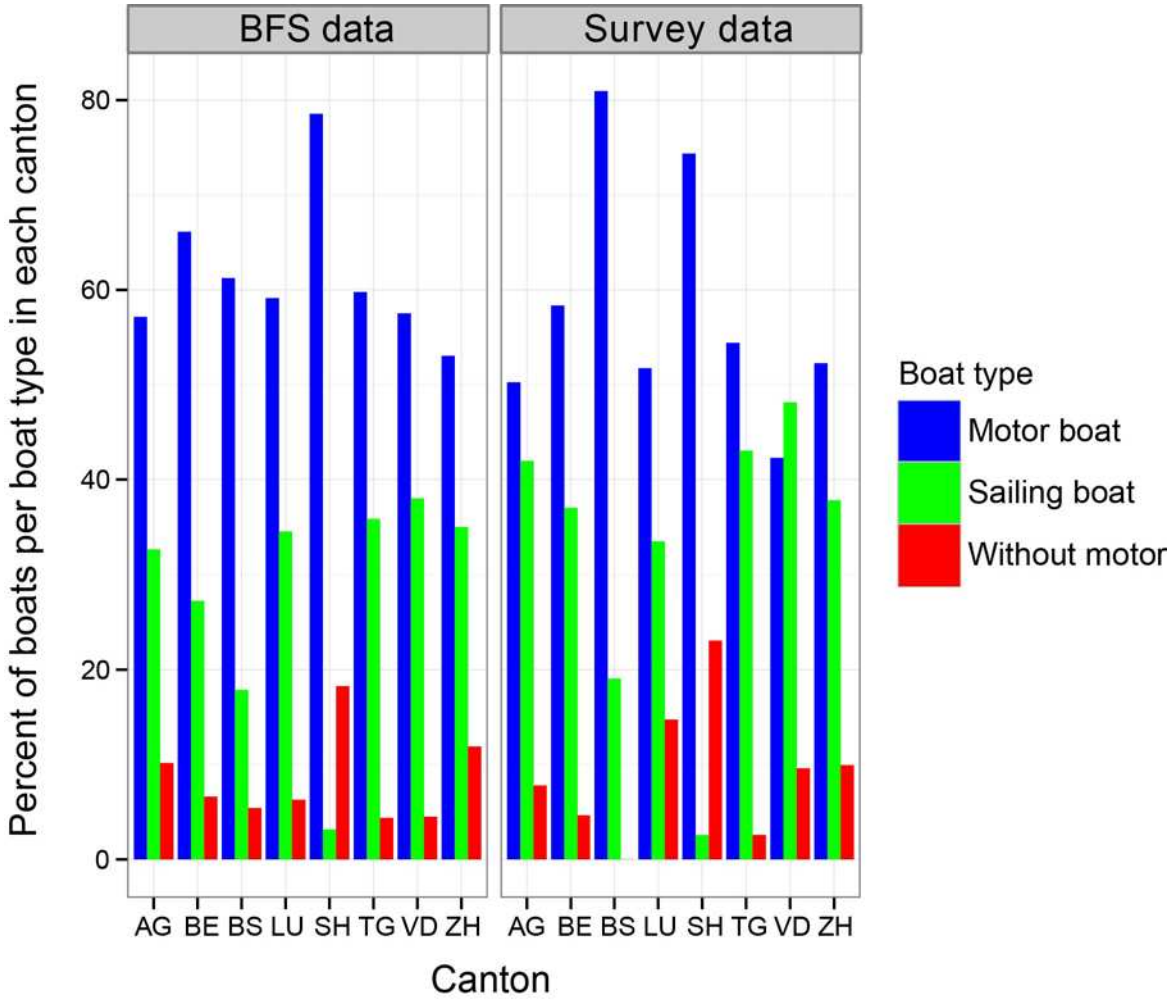

Figure S1 Percent of boats per boat type are shown for each of the cantons Aargau (AG), Bern (BE), Basel (BS), Luzern (LU), Schaffhausen (SH), Thurgau (TG), Vaud (VD) and Zürich (ZH). Motorboats are shown in blue, sailing boats in green, and boats without motor (e.g. wooden boats, rowing boats or flat bottomed or punt-like boats) are shown in red. Percentages are shown for data retrieved from the data bases of cantonal offices from the Swiss Federal Statistical Office (BFS) and for our survey data.

Tables

Table S1 Overview on the spearman correlation structure of continuous and ordinal explanatory variables tested in the models for boat cleaning rate and the rate of high pressure washing. Only seasonally or year-round moored boats were included and the correlations were calculated with the rcorr function of the Hmisc package in R. Dark grey fields have R^2-values > 0.5 and light grey fields R^2-values higher > 0.3. Difficulty, time expenditure, monetary costs and strenuousness correlate strongly with each other (R^2 > 0.55), while benefit correlates strongly with importance (R^2 > 0.6).

		Percieved costs					Percieved benefit					
	Monetary cleaning costs	Difficulty	Time expenditure	Percieved monetary costs	Strenuousness	Dimension cost	Benefit	Importance	Dimension benefit	Awareness for "distribution AIS"	Awareness for benefit on ecosystems	Awareness for "damage on ecosystems
Monetary cleaning costs	1	0.05	0.03	0.25	0.07	0.09	0.15	0.15	0.15	0.02	-0.01	0.05
Difficulty	0.05	1	0.71	0.49	0.67	0.85	-0.08	-0.09	-0.06	0.01	0.01	0.01
time expenditure	0.03	0.71	1	0.58	0.77	0.9	-0.05	-0.02	0.08	0.06	0.02	0.02
Percieved monetary costs	0.25	0.49	0.58	1	0.56	0.73	-0.07	-0.05	-0.04	0.03	0.07	-0.05
Strenuousness	0.07	0.67	0.77	0.56	1	0.89	-0.05	-0.03	0.07	0.04	0.01	-0.02
Dimension cost	0.09	0.85	0.9	0.73	0.89	1	-0.12	-0.08	-0.02	0.04	0.03	-0.02
Benefit	0.15	-0.08	-0.05	-0.07	-0.05	-0.12	1	0.59	0.84	0.12	-0.16	0.17
Importance	0.15	-0.09	-0.02	-0.05	-0.03	-0.08	0.59	1	0.86	0.13	-0.14	0.19
Dimension benefit	0.15	-0.06	0.08	-0.04	0.07	-0.02	0.84	0.86	1	0.14	-0.15	0.2
Awareness for "distribution AIS"	0.02	0.01	0.06	0.03	0.04	0.04	0.12	0.13	0.14	1	-0.15	0.34
Awareness for benefit on ecosystems	-0.01	0.01	0.02	0.07	0.01	0.03	-0.16	-0.14	-0.15	-0.15	1	-0.18
Awareness for "damage on ecosystems	0.05	0.01	0.02	-0.05	-0.02	-0.02	0.17	0.19	0.2	0.34	-0.18	1

Table S2 Spearman correlations of explanatory variables, including categorical variables, tested in the models for **a)** boat cleaning rate and **b)** the rate of high pressure washing. Only seasonally or year-round moored boats were included and the correlation structure was analyzed with CatPCA (categorical principal component analysis) using SPSS. Dark grey fields have R^2-values > 0.5 and light grey fields R^2-values higher > 0.3.

a)

Correlation structure of transformed variables																	
							Perceived costs and benefits										
	Boat type	Hull material	Antifouling	Competition	Fishing	Boat storage	Estimated costs	Difficulty	Time expenditure	Monetary costs	Benefit	Tediousness	Importance	Distribution potential	Damage to ecosystems by AIS	Overland transport
Boat type	1.00	0.18	0.26	0.33	-0.32	0.04	0.17	-0.01	-0.06	0.03	0.02	-0.03	-0.01	-0.01	-0.01	0.02
Hull material	0.18	1.00	0.26	0.07	-0.07	-0.01	0.07	-0.05	-0.04	-0.01	0.01	-0.04	0.02	0.00	-0.02	-0.02
Antifouling	0.26	0.26	1.00	0.09	-0.12	0.04	0.18	0.00	-0.02	0.02	-0.03	0.00	-0.04	-0.02	0.02	0.02
Competition	0.33	0.07	0.09	1.00	-0.15	-0.07	0.05	-0.06	-0.06	-0.02	0.04	-0.05	0.04	0.00	-0.03	0.11
Fishing	-0.32	-0.07	-0.12	-0.15	1.00	0.17	-0.10	0.03	0.04	0.03	-0.03	0.04	-0.05	0.03	0.05	-0.02
Boat storage	0.04	-0.01	0.04	-0.07	0.17	1.00	0.03	0.08	0.11	0.15	-0.06	0.10	-0.14	0.05	0.00	-0.05
Estimated costs	0.17	0.07	0.18	0.05	-0.10	0.03	1.00	0.01	0.02	0.11	0.06	0.04	0.05	-0.02	-0.02	-0.01
Difficulty	-0.01	-0.05	0.00	-0.06	0.03	0.08	0.01	1.00	0.72	0.53	-0.22	0.67	-0.24	0.03	0.01	-0.01
Time expenditure	-0.06	-0.04	-0.02	-0.06	0.04	0.11	0.02	0.72	1.00	0.62	-0.26	0.77	-0.26	0.06	0.02	-0.01
Monetary costs	0.03	-0.01	0.02	-0.02	0.03	0.15	0.11	0.53	0.62	1.00	-0.38	0.62	-0.39	0.05	0.01	0.00
Benefit	0.02	0.01	-0.03	0.04	-0.03	-0.06	0.06	-0.22	-0.26	-0.38	1.00	-0.26	0.73	0.01	0.01	0.04
Tediousness	-0.03	-0.04	0.00	-0.05	0.04	0.10	0.04	0.67	0.77	0.62	-0.26	1.00	-0.27	0.05	0.00	0.01
Importance	-0.01	0.02	-0.04	0.04	-0.05	-0.14	0.05	-0.24	-0.26	-0.39	0.73	-0.27	1.00	0.00	0.01	0.05
Distribution potential	-0.01	0.00	-0.02	0.00	0.03	0.05	-0.02	0.03	0.06	0.05	0.01	0.05	0.00	1.00	0.28	-0.02
Damage to ecosystems by AIS	-0.01	-0.02	0.02	-0.03	0.05	0.00	-0.02	0.01	0.02	0.01	0.01	0.00	0.01	0.28	1.00	-0.06
Overland transport	0.02	-0.02	0.02	0.11	-0.02	-0.05	-0.01	-0.01	-0.01	0.00	0.04	0.01	0.05	-0.02	-0.06	1.00
Dimension	1.00	2.00	3.00	4.00	5.00	6.00	7.00	8.00	9.00	10.00	11.00	12.00	13.00	14.00	15.00	16.00
Eigenvalue	3.41	1.86	1.42	1.30	1.15	1.02	0.97	0.90	0.79	0.74	0.67	0.54	0.42	0.32	0.27	0.22

b)

Correlation structure of transformed variables

							Perceived costs and benefits								
	Boat type	Hull material	Antifouling	Competition	Boat storage	Estimated costs	Difficulty	Time expenditure	Monetary costs	Benefit	Tediousness	Importance	Distribution potential	Damage to ecosystems by AIS	Mussel fouling
Boat type	1.00	0.12	0.21	0.37	0.05	0.13	-0.02	-0.07	0.03	0.01	-0.03	-0.02	0.00	0.04	0.10
Hull material	0.12	1.00	0.26	0.06	0.01	0.08	-0.05	-0.04	0.00	0.00	-0.03	0.01	0.01	0.01	0.00
Antifouling	0.21	0.26	1.00	0.09	0.04	0.18	0.00	-0.02	0.02	-0.01	0.01	-0.03	-0.01	0.02	0.10
Competition	0.37	0.06	0.09	1.00	-0.07	0.05	-0.06	-0.06	-0.02	0.04	-0.05	0.05	0.00	0.00	0.03
Boat storage	0.05	0.01	0.04	-0.07	1.00	0.03	0.09	0.11	0.15	-0.06	0.11	-0.15	0.07	0.07	0.18
Estimated costs	0.13	0.08	0.18	0.05	0.03	1.00	0.01	0.02	0.11	0.07	0.04	0.06	-0.04	0.00	-0.01
Difficulty	-0.02	-0.05	0.00	-0.06	0.09	0.01	1.00	0.72	0.55	-0.23	0.68	-0.25	0.02	0.03	0.27
Time expenditure	-0.07	-0.04	-0.02	-0.06	0.11	0.02	0.72	1.00	0.63	-0.25	0.77	-0.25	0.05	0.04	0.31
Monetary costs	0.03	0.00	0.02	-0.02	0.15	0.11	0.55	0.63	1.00	-0.37	0.63	-0.37	0.04	0.02	0.49
Benefit	0.01	0.00	-0.01	0.04	-0.06	0.07	-0.23	-0.25	-0.37	1.00	-0.25	0.73	0.05	0.04	-0.55
Tediousness	-0.03	-0.03	0.01	-0.05	0.11	0.04	0.68	0.77	0.63	-0.25	1.00	-0.26	0.04	0.03	0.32
Importance	-0.02	0.01	-0.03	0.05	-0.15	0.06	-0.25	-0.25	-0.37	0.73	-0.26	1.00	0.04	0.04	-0.60
Distribution potential	0.00	0.01	-0.01	0.00	0.07	-0.04	0.02	0.05	0.04	0.05	0.04	0.04	1.00	0.33	0.06
Damage to ecosystems by AIS	0.04	0.01	0.02	0.00	0.07	0.00	0.03	0.04	0.02	0.04	0.03	0.04	0.33	1.00	0.08
Mussel fouling	0.10	0.00	0.10	0.03	0.18	-0.01	0.27	0.31	0.49	-0.55	0.32	-0.60	0.06	0.08	1.00
Dimension	1.00	2.00	3.00	4.00	5.00	6.00	7.00	8.00	9.00	10.00	11.00	12.00	13.00	14.00	15.00
Eigenvalue	3.78	1.71	1.59	1.35	1.11	0.97	0.89	0.73	0.67	0.59	0.48	0.36	0.31	0.26	0.22

Chapter 4

Molecular eDNA markers for early detection and surveillance of invasive zebra and quagga mussels

In review

Authors

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Abstract

Early detection and monitoring of invasive species is important for the development of effective mitigation measures directed at minimising the negative effects of invaders. Aquatic invasive species are typically rare in the early stages of invasion and detection using costly and time-consuming field surveys is often challenging. Environmental DNA (eDNA) methods are increasingly applied in freshwater systems to detect and quantify target species: eDNA methods can be applied with relative ease to detect invasive aquatic species over large geographic scales and across the invasion fronts. In this study we develop and test eDNA detection and quantification methods for invasive zebra and quagga mussels. Both mussel species have invaded widely in North America and Europe and show strong negative ecosystem-wide impacts. We extracted DNA from filtered water samples which we collected along the Rhine catchment, including the known invasion area of the zebra mussel and the expected invasion front of the quagga mussel. Standard PCR (end-point PCR) and quantitative PCR (qPCR) method were compared for detection and qPCR was used to quantify the eDNA signal for each species. Our results show that the invasion front of the quagga mussel has moved southwards, including areas where this species had not been detected previously with traditional benthic invertebrate sampling methods. Standard PCR and qPCR performed similarly in detection of both of the mussel species. Moreover, the eDNA quantification of both species showed high precision within sampling site and matched with expected densities of zebra and quagga mussels based on previous field survey studies. Nevertheless, we recommend further validation of eDNA quantification as a proxy for zebra and quagga mussel density or biomass. The tested eDNA methods are cost effective and have the potential to be widely applied for the surveillance of zebra and quagga mussels in the future.

Keywords

eDNA, Targeted species detection, Freshwater, *Dreissena polymorpha, Dreissena rostriformis bugensis*, Invasive species, Cytochrome c oxidase I, qPCR

Introduction

Invasive species have strong negative impacts on biodiversity and cause high economic costs in fresh water systems worldwide (Sala et al. 2000). Early detection and surveillance of invasive species is important in order to plan measures to slow down their spread and to mitigate their effects. Nevertheless, early detection and quantification of aquatic invasive species, e.g. by kick-net sampling or scuba diving is often difficult, laborious and potentially inaccurate (Barbour et al. 1999; Stucki 2010), in particular for small freshwater invertebrates, which often have patchy distribution patterns (Arscott et al. 2003). The detection of non-native species with the potential to become invasive can be particularly difficult where densities are low. This is often the case during the early lag-phase of establishment (Lockwood et al. 2007), or when primary habitats are particularly inaccessible for surveys, e.g. in deeper lakes. In such cases, the detection and quantification of species from environmental DNA (eDNA) extracted from water samples may have several advantages over traditional surveillance methods, as has been demonstrated for the American bullfrog (Dejean et al. 2012), the Asian carp (Jerde et al. 2013) or the invasive New Zealand mudsnail (Goldberg et al. 2013).

The major advantage of the eDNA method is that the target organisms do not need to be found and determined as specimens. Instead it only requires collection of water samples, concentrating the organic material and extracting the eDNA, e.g. from filter papers. eDNA comprises extracellular and cell-bound DNA which organisms release as a by-product of excretion or the shedding of cells (e.g. in hair or skin) into the water column (Thomsen and Willerslev 2015). The occurrence of target organisms in the eDNA samples can then be detected by end-point Polymerase Chain Reaction (standard PCR) with species specific primers (Goldberg et al. 2013; Mächler et al. 2014). Particularly when applied to one or a few species of interest, eDNA approaches coupled with species or lineage-specific PCR may allow assessment of species occurrence in high temporal and spatial resolution. Further, application of standardized sampling and molecular protocols may allow comparisons across studies and surveillance programs (Thomsen and Willerslev 2015).

Quantitative PCR (qPCR) with species specific primers allows the quantification of target DNA in eDNA samples and was shown to be more sensitive to lower copy numbers than standard PCR (Wilcox et al. 2013). Such quantitative eDNA estimates may be used as a proxy for population densities at a specific location. Several

researchers have successfully correlated eDNA concentrations with densities or biomass of target freshwater organisms in captivity, e.g. for fish (Takahara et al. 2012), amphibians (Thomsen et al. 2012) or New Zealand mudsnails (Goldberg et al. 2013). For two amphibian species, density estimates from field survey data have shown good positive correlation with eDNA estimates of population size (Pilliod et al. 2013). However, only very few studies have investigated the potential of standard PCR and qPCR for early detection and quantification of invasive species over wide geographic scales and few have assessed the potential of eDNA quantification for more than one species within the same freshwater system and compared the results to field survey data.

The zebra mussel (*Dreissena polymorpha*, Pallas 1771) and the quagga mussel (*Dreissena rostriformis bugensis*, Andrusov 1897) are two closely related species originating from the Ponto-Caspian region. Both species are invasive in North America and Europe (Mills et al. 1996; Therriault et al. 2005; Zhulidov et al. 2010), with strong negative impacts on the ecology of the invaded water bodies (Vanderploeg et al. 2002; Strayer 2009; Higgins and Vander Zanden 2010) and economics (Pimentel et al. 2005). The two species share a similar life cycle, both produce pseudofaeces and exhibit similarly high filtration rates (Ackerman et al. 1994; Diggins 2000), and may thus also show comparably high eDNA shedding rates. Since the 19th century the zebra mussel has colonized most larger rivers and navigable lakes in Western Europe and started colonizing Swiss water bodies in the 1960s. The quagga mussel only arrived around 2004 in the Netherlands and in the Rhine-Main-Danube channel (Imo et al. 2010; Heiler et al. 2013) and is currently spreading southwards along the Rhine system (Matthews et al. 2014). In Switzerland, the quagga mussel had not been detected before this study. The quagga mussel was found to cope better with low temperatures (Roe and MacIsaac 1997) and lower nutrient levels (Baldwin et al. 2002) than the zebra mussel and may thus colonize colder or more oligotrophic water bodies, for example in higher altitudes. They may also colonize lentic systems to greater depths, potentially attaching to surfaces and clogging water intake pipes of drinking water plants.

In order to plan measures against the further spread of quagga mussels and for the mitigation of expected negative impacts, it is important to monitor the spread of these species. We therefore examined if eDNA methods using standard PCR and qPCR with species specific primers provide an efficient and cost effective method for the surveillance of zebra and quagga mussels. We collected water samples along the

River Rhine system, from Lake Constance to the Lower Rhine in the Netherlands (Figure 1). While quagga mussels had previously not been detected upstream of Kehl (Kinzelbach 1992; bij de Vaate et al. 2002; Heiler et al. 2013), zebra mussels were known to be present at all sampling sites, thus serving as a positive control for the species specific eDNA detection. As eDNA is washed downstream, the samples may represent the upstream community up to several kilometers upstream of the sampling site (Deiner and Altermatt 2014). We chose to use filtration and eDNA extraction methods previously used by Deiner et al. (Deiner et al. 2015) who filtered and extracted DNA from water samples in a dedicated DNA-free facility in the laboratory. In addition, we also filtered water samples directly in the field, in order to find out whether this simpler approach was free of cross-contamination between sampling sites. Applying standard PCR and qPCR using the species specific primers published by (Bronnenhuber and Wilson 2013) we addressed the following points:

1. We tested the detection of zebra and quagga mussels in field filtered and lab filtered eDNA samples using standard PCR. In particular, we were interested in whether the quagga mussel can be detected upstream of Kehl, where it has not been reported so far.
2. We estimated the lowest concentration of target DNA of zebra and quagga mussels that can still be quantified in eDNA samples with EvaGreen qPCR method.
3. We quantified and compared the concentrations of target eDNA of zebra and quagga mussel with qPCR.
4. Finally, we discuss the potential application of eDNA detection and quantification with standard PCR and qPCR for the surveillance of invasive zebra and quagga mussels.

Methods

Field sampling

Environmental DNA (eDNA) samples were collected at twelve sites in the River Rhine catchment in July and August 2014 (Figure 1, Table 1). At each site, three water samples of 1 L volume were collected from the shore (water depth approx. 1 m) with a clean 10 L bucket, which was rinsed five times few meters downstream of the actual sampling site before the sample was taken. Each sample was filtered directly in the field on a glass fibre filter (GF/F Glass fibre filters, 25 mm diameter, 0.7 µm average pore size) using a clean filter holder (GE Healthcare, Whatman) and a

disposable 50 mL syringe. In order to prevent contamination of filters with non-site specific eDNA, filters were only touched with clean forceps and filter holder and syringes only with new disposable gloves. Filter holders, and forceps were bleached (10 % bleach solution) and treated with UV-light for 20 minutes, while filters were also treated with UV-light before use. For the filtration of each sample we needed between one and four filters, depending on the amount of organic and inorganic material present in the water. Filters were placed into fresh 1.5 mL Eppendorf tubes, frozen immediately in a liquid nitrogen dewar and kept at -80°C until DNA was extracted. As negative field controls we brought 1 L of UV-treated DNA-free water to each site and filtered it according to the above procedure. For nine sampling sites we also collected water samples by submerging a 1 L octagonal PET bottle (VWR International, Radnor, PA, USA) with a gloved hand just below the surface near the shore. The water samples were transported in an ice filled cooling box and filtered within 36 hours in a laminar flow hood in a DNA-clean facility the same way as described above for the field filtered samples. All bottles were previously rinsed with 10 % bleach, rinsed well with water and pre-decontaminated by a 20 minute UV-light treatment and sealed before use. As negative lab controls, we transported 1 L of UV treated DNA-free water to each of the field sites, where it was filled into an octagonal PET bottle and subsequently treated it like the lab filtered samples.

Table 1 eDNA sampling sites with geographic information, sampling date, filtration method, expected quagga mussel presence and sample ID. The filtration method indicates where the samples were filtered. We also show if we expected to find quagga mussels. The names in bold letter indicate how the samples are named throughout the paper.

Locality	Water body	Longitude	Latitude	Sampling date	Filtration method	Quagga expected	ID
Altnau	Lake Constance	47.622719	9.269722	01.07.2014	field & lab	No	LC
Diessenhofen	Rhine River	47.691267	8.749828	01.07.2014	field & lab	No	DH
Möhlin	Rhine River	47.585061	7.833133	03.07.2014	field & lab	No	Mö
Basel (harbor)	Rhine River	47.588978	7.592456	03.07.2014	field & lab	Unsure	Ba
Kehl (harbor)	Rhine River	48.606792	7.821083	11.07.2014	field & lab	Yes	Ke
Karlsruhe (harbor)	Rhine River	49.016597	8.303797	10.07.2014	field & lab	Yes	Ka
Dettenheim	Lake Giesen	49.157083	8.392094	10.07.2014	field & lab	Yes	LG
Worms	Rhine River	49.622911	8.383000	09.07.2014	field & lab	Yes	Wo
Hanau	Main River	50.111783	8.917033	09.07.2014	field & lab	Yes	Ha
Wageningen	Rhine River	51.957622	5.673594	12.08.2014	field only	Yes	Wa
Lelystad	Lake IJsselmeer	52.546583	5.454114	11.08.2014	field only	Yes	IJ
Almere	Lake Markemeer	52.490903	5.386439	11.08.2014	field only	Yes	Ma

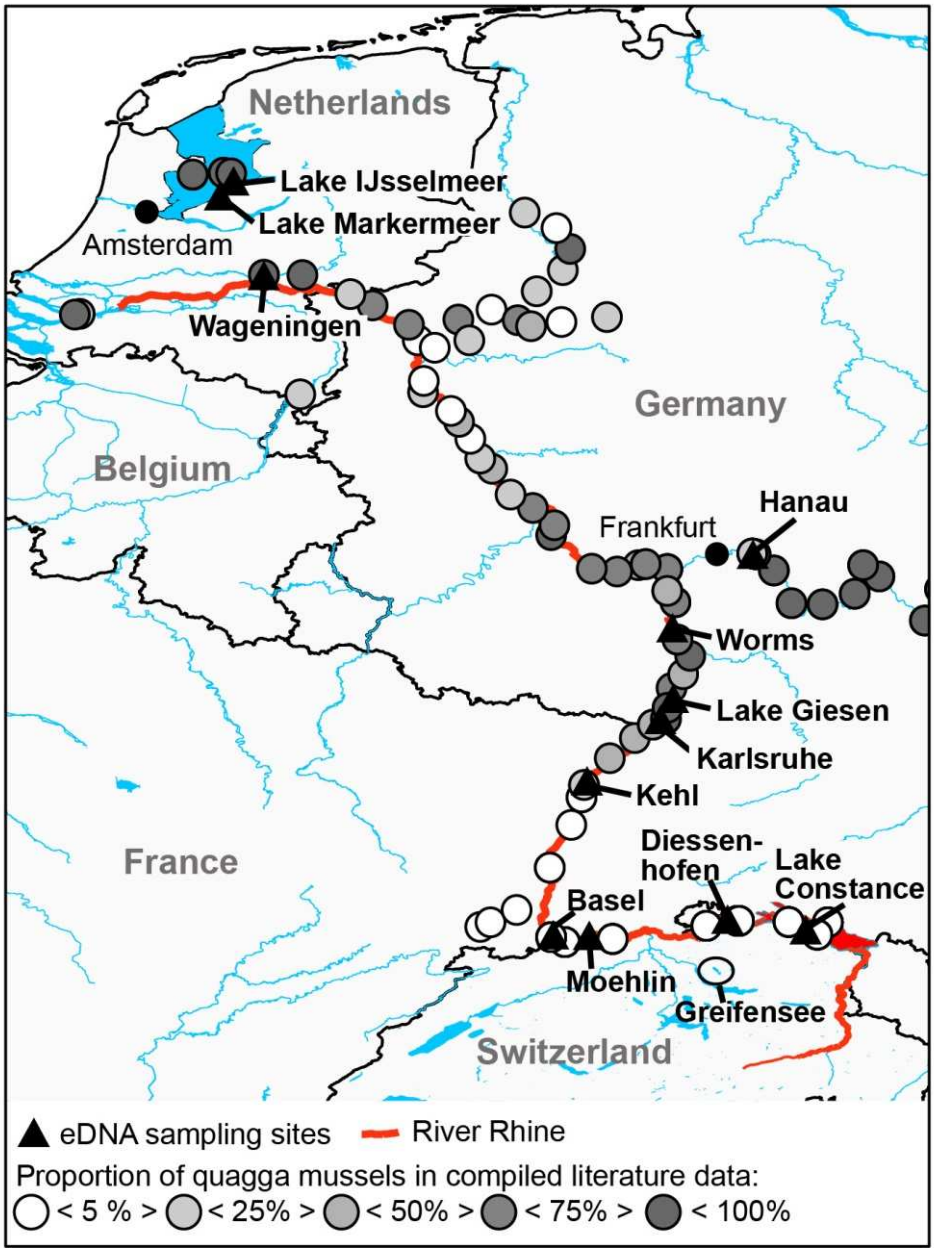

Figure 1 Sampling sites (black triangles), where eDNA samples were collected along the River Rhine catchment. Mussels were collected from Lake IJsselmeer, Lake Markermeer Hanau and Greifensee for tissue extracted DNA. Circles with different shades of grey indicate the proportion of quagga mussel density (individuals per m2) in relation to the total dreissenid density (zebra plus quagga mussels). These density estimates originate from field survey data, which we compiled from the literature. Most field density estimates for the Rivers Rhine and Main were collected in 2009 by Heiler et al.(2013), those for the Swiss River Rhine and Lake Constance by John Hesselschwerdt and Jutta Mürle in 2014 (Hesselschwerdt et al. 2014), those for Lake Markermeer and Lake IJsselmeer in 2011 by Matthews et al. (2014) and in 2012 by Heiler et. al (2013), and those from the Lower Rhine in Wageningen where collected in 2011 by Matthews. et al (2015) and Leuven et al. (2014).

eDNA extraction protocols

For targeted detection of zebra and quagga mussels, we extracted eDNA from the filters using the DNeasy Blood & Tissue Kit (Qiagen GmbH, Hilden, Germany) with some modifications as described in Deiner et al. (2015), except for the following steps: After lysis we shook the content in the closed upside down Eppendorf tube towards the lid, and with a sharp pointed needle punched a hole into the bottom of the tube. Subsequently each tube was inserted into a second 1.5 ml Eppendorf tube and centrifuged for 3 min at 6000 g. The extraction was continued with the flow-through, while the upper tube containing the dry filter was discarded. The extractions were performed in a laminar flow hood in a dedicated DNA-clean facility as described by Fulton (2012) and Deiner (2015) and all equipment including pipettes and needles were treated with UV-light for 20 minutes before use. For the twelve negative extraction controls we used clean, UV-treated filters. For those samples for which we used more than one filter (per 1 L sample), we pooled equal amounts of extract from each filter. For the eDNA quantification with qPCR, we corrected the eDNA concentration estimates for higher total elution volumes of pooled samples. In 14 test extractions we measured eDNA concentrations between 0.9 and 6.4 ng/µl with Qubit 2.0 dsDNA HS Assay Kit (Invitrogen, Carlsbad, CA, USA). All DNA extracts were kept at 4°C until further use.

Species specific primers

We used the species specific primers published by Bronnenhuber and Wilson (2013) for PCR amplification, targeting mitochondrial DNA (Table 2). For species detection we used the DbuCOI3 primer pair, amplifying a fragment of 164 bp of the quagga mussel COI-sequence, and DpoCOI3 primer pair amplifying a 254 bp fragment of the zebra mussel COI-sequence. We tested both primer pairs for species specificity in PCR's using DNA from the tissue of four zebra and four quagga mussels (zebra mussel tissue originating from Lake Greifensee (2x), Lake IJsselmeer and Lake Markermeer, and quagga mussel tissue originating from River Main (2x), Lake IJsselmeer and Lake Markermeer). DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen GmbH, Hilden, Germany), following the manufacturer's protocol and PCRs performed and products visualized as described below. We also blasted each of the primer sequences against the nucleotide nr/nt data base on Genbank (http://www.ncbi.nlm.nih.gov/genbank/) testing for potential amplification of non-target sequences of other aquatic species.

Table 2 Primers used for detection of zebra and quagga mussels (Bronnenhuber and Wilson 2013). We show the primer sequences and the mismatches with the primer sequence at the same locus of the congener species (either zebra or quagga mussel) are shown in bold font. Further we present the number of mismatches in the primer sequence (MMs), the length of the amplified fragment, the annealing temperature used in the PCR protocols (TA), the estimated melting temperature (TM) and GC-content of the primer sequences (% GC). For either of the two primer pairs, no cross amplification (cross amp.) with the non-target species (either zebra or quagga mussels, respectively) was detected by Bronnenhuber and Wilson (2013) or by us.

Target species	Primer	Primer sequence	MMs	Fragment length	TA	TM	% GC
Quagga mussel	DbuCOI3F	G**G**GGTTGAAC**A**TTATAYCC**A**CC**G**TT	4	164	66	57	48
	DbuCOI3R	AAA**C**TGATGACA**C**CC**G**GCACG	3			57.7	57
Zebra mussel	DpoCOI3F	GCT**A**AG**G**GC**A**CCTGGAAG**C**GT	4	254	66	59	61
	DpoCOI3R	C**A**CC**C**CC**G**AATCC**T**CC**T**TC**C**CT	6			59.3	63

Detection of target species by standard PCR

In order to detect the presence of zebra and quagga mussel DNA, we amplified target sequences with PCR, multiplexing DpoCOI3 and DbuCOI3 primers and visualized the products on an agarose gel. We tested all samples filtered in the field and in the laboratory and included four types of negative controls (Supplementary Material Table 1): field filtered negative controls (N=10), lab filtered negative controls (N=7), extraction negative controls (N=14) and PCR controls (N=12) containing only UV-light treated nuclease-free water (Sigma). PCR's of each sample were run in triplicate. If not all of the three triplicates were unambiguously, either only positive or only negative for the detection of the target species, the PCR was again repeated in triplicate for the ambiguous samples in order to exclude false positive or false negative results. For PCRs on eDNA and tissue extracted DNA we used Multiplex PCR Master Mix (Qiagen GmbH, Hilden, Germany) following the manufacturer's instructions. The final concentrations of forward and reverse primers were 0.2 μM and we used 2 μL of extracted eDNA per 15 μL reaction volume. The thermal cycling regime was 95°C for 15 min, followed by 35 cycles of 95°C for 30 s, 66°C for 90 s and 72°C for 90 s. A final extension step of 72°C for 10 min was carried out and the PCR product was stored at 4°C until further analysis. We confirmed the resulting PCR products on a 1.4% agarose gel stained with PeqGreen (Peqlab, Erlanden, Germany) and compared them to a 100bp ladder (Promega, Madison, WI, USA).

Quantifying target species by quantitative PCR

Quantitative PCR (qPCR) reactions were run in triplicates on a LightCycler 480 Real-Time PCR System (Hoffmann-La Roche Ltd) in 15 μL reaction volumes using the same protocol and reagent concentrations as described for the PCR above, except that we added 0.75 μL Evagreen to each reaction and run separate singleplex tests for zebra and quagga mussel primers. For each of the pooled samples, the eDNA concentration was diluted two times and 3 μL of the dilute was used in the qPCR reaction (for exceptions see Supplementary Material Table 1) in order to have enough volume for all qPCR replicates. We tested only lab filtered samples except for the sites Wageningen, Lake IJsselmeer and Lake Markermeer where only field filtered samples were available. We also included seven filtration negative controls, five extraction controls and one PCR control containing only UV-light treated nuclease-free water.

As qPCR standards we amplified PCR product from tissue extracted DNA for each primer pair (DbuCOI3 and DpoCOI3). Each PCR product was then purified using the centrifugation protocol of the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA), eluted with 120 μL nuclease-free water and the concentration of the elute measured with Qubit. qPCR standards were subsequently prepared from the purified PCR products in a 10 x dilution series for each target species (Figure 3) in DNA-low binding tubes (Eppendorf AG, Hamburg, Germany), kept at 4°C and used in qPCR within 48 hours. The DNA concentrations were measured for the two highest concentrated standards of each dilution series with Qubit and calculated for all other standard dilutions. All samples, negative controls and standards were run on the same qPCR plate. Zebra mussel standards containing between 1.1×10^{10} and 1.1 sequences per μL and quagga mussel standards containing between 8×10^{9} and 0.8 sequences per μL were included in six replicates. Amplification curves, Cq values, melting temperatures and melting curves were analysed in the Light Cycler 480 Software, Version 1.5 (Roche Diagnostics). We calculated the number of target sequence copies per μL eDNA sample as the concentration extrapolated from the standard curve (ng/μL) divided by the dilution factor (Supplementary Material Table 1) and converted the result to g/μL, which was subsequently divided by the molar weight of the target sequence and multiplied with 6.022×10^{23} (Avogadro constant). For each sample, we also calculated the quagga mussel ratio as the mean concentration of quagga mussel copies divided by the sum of quagga and zebra

mussel copies. All calculations and visualizations of the data were done with the statistical program R (R-Core-Team 2014).

We excluded all replicates of samples and negative controls from the qPCR data set for which the height and shape of melting curves did not match with those of the corresponding standards. Three controls for the DpoCOI3 amplification, could not be excluded by this procedure, but had a higher Cq value than the lowest amplifying standard (Supplementary Material Figure S1). These three negative samples and eDNA samples which showed higher Cq values than the lowest amplifying standards were repeated in standard (end-point) PCR to confirm false positive and false negative results. None of the negative controls amplified and were thus excluded (Supplementary Material, Figure S2). For the quantitative analysis of eDNA concentrations we did not exclude any samples for which the presence of target sequences was confirmed by standard PCR.

Confirmation of target sequences by sequencing of PCR products

To confirm the species specificity of the primers we sequenced the PCR products of one lab filtered sample per site and a field filtered sample from Wageningen, Lake IJsselmeer and Lake Markermeer. Singleplex PCR's were run for each primer pair with 2 µL eDNA per reaction. A second, nested PCR with 30 µL reaction volume was performed for each sample and primer pair with 4 µL of 1:100 diluted PCR product from the first PCR, in order to get enough product for sequencing. Each product from the second PCR was checked on an agarose gel, a subsample was purified using the centrifugation protocol of the Wizard SV Gel and PCR Clean-Up System and eluted with 40 µL of nuclease-free water. We sent 15 µL of each purified product to Microsynth (Microsynth, Switzerland) for sequencing. The resulting sequences were aligned in Mega version 6 (Tamura et al. 2013) and compared to COI sequences of zebra mussels and quagga mussels downloaded from Genbank and blasted against the nucleotide nr/nt data base on Genbank.

Results

Primer specificity testing

The DpoCOI3 and DbuCOI3 primer pairs detected and quantified eDNA of zebra and quagga mussels in a species specific manner. Neither of the two primer pairs amplified non-target DNA from the mussel tissue samples nor from any of the eDNA sampling sites. Among the 37 zebra mussel COI sequences, which we found on Genbank (Table S2) and which contained both primer binding sites of DpoCOI3, we found one mismatch with the DpoCOI3 forward primer in one single zebra mussel sequence (Accession number: JQ435817, origin: Romania). We also found only one mismatch with the DbuCOI3 reverse primer in one single quagga mussel sequence (Accession number: JQ435816.1, origin: Romania), among the 15 sequences containing both primer binding sites of DbuCOI3. Each primer had three, four or six mismatches with the non-target dreissenid binding site (Table 2).When blasted against the nucleotide collection (nr/nt) in Genbank, both primer pairs showed a similarity of less than 80% with any non-target sequence.

Species detection by standard PCR

Zebra mussels were detected in eDNA samples from all sites, in almost all pooled field filtered and lab filtered samples (Figure 2). Only one field filtered sample from Lake Markermeer (N = 2) and one from Lake IJsselmeer (N = 3) did not amplify the zebra mussel target, potentially reflecting very low zebra mussel abundances. The quagga mussel target was detected in all pooled field filtered and lab filtered samples, except the ones collected upstream of Basel. The quagga mussel primers also amplified in the eDNA samples from Basel, where the quagga mussel had not been detected so far. None of our PCR controls, extraction controls or lab filtered or field filtered controls amplified any zebra or quagga mussel eDNA, except for three field filtered controls from Möhlin, Worms and Hanau, for which we found amplification of zebra mussel DNA (data not shown). As we could not exclude the possibility of contamination during filtration in these cases, only lab filtered samples were used for the quantitative PCR, where possible.

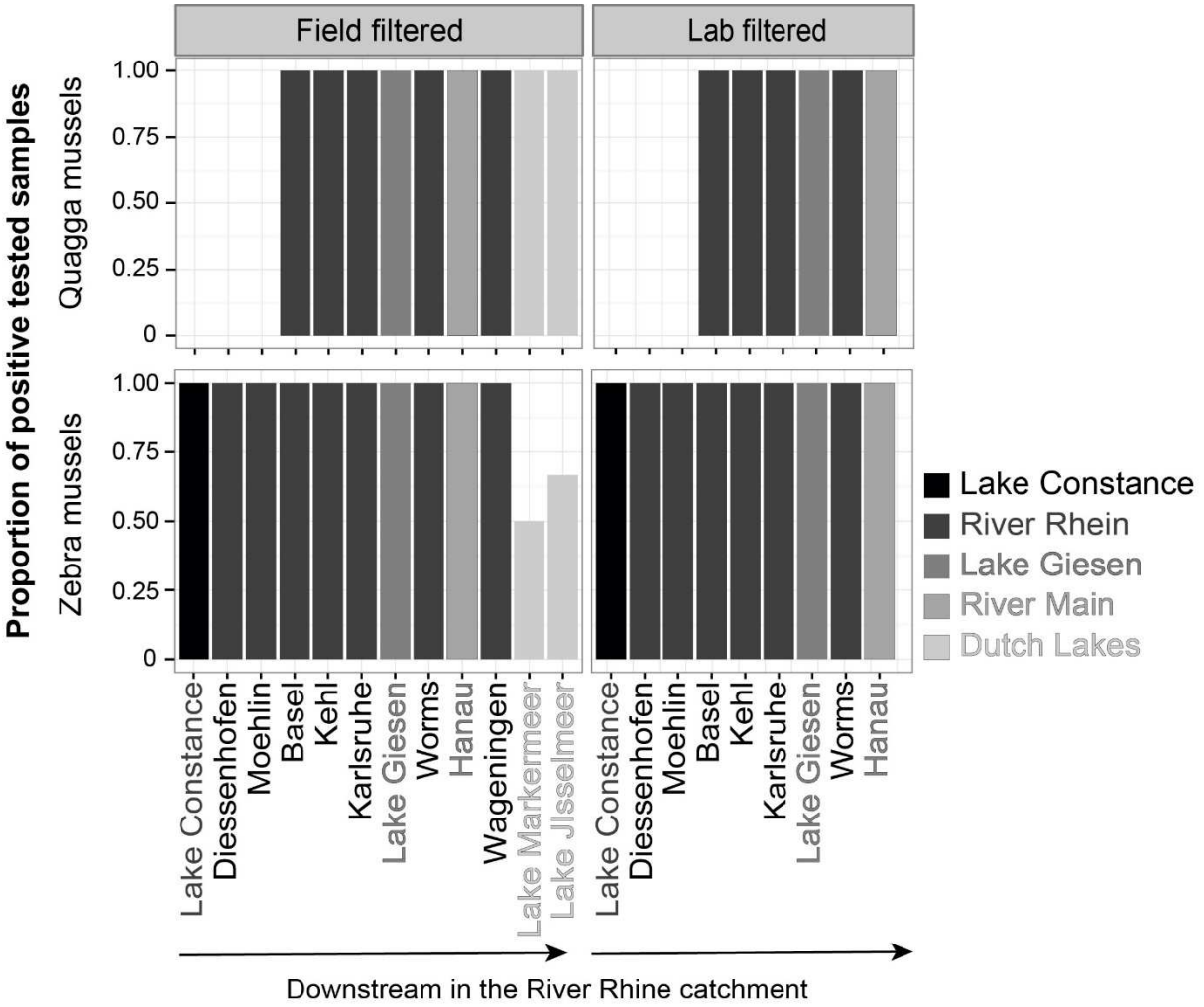

Figure 2 Testing the presence of quagga mussels (upper panel) and zebra mussels (lower panel) on the three eDNA replicates per site (N = 3). Except for Lake Markermeer (N = 2) where one sample had to be discarded. Extracted eDNA samples were analyzed with species specific primers using standard (end-point) PCR. The Y-axis indicates the proportion of positive samples per sampling site. Grey shades indicate to which water body the sampling sites belong to (see also Figure 1). The downstream direction of the River Rhine along which samples haven been collected, is indicated by an arrow.

Quantification of zebra and quagga mussel target sequences by qPCR

For both targets the lowest standard dilution (lowest quagga mussel standard: 6.6 x 10^{-11} ng/µL, lowest zebra mussel standard: 1.5 x 10^{-10} ng/µL) did not amplify in all of the replicates and the limit of quantification LOQ was designated as the second lowest standard dilution for both standard curves, which amplified in all replicates (quagga mussel standard: 6.6 x 10^{-10} ng/µL, Cq value of 31.7 ± SE = 0.37, zebra mussel standard: 1.5 x 10^{-9} ng/µL, Cq value of 30.8 ± SE = 0.28, see Figure 3). The average

LOQ for the quagga mussel target was 8.1 copies per µL (SE = 2.3, equivalent to a mean Cq value of 31.7, SE ± 1.9) while the average LOQ for the zebra mussel target was 12 copies per µL (SE ± 2.6, equivalent to a mean Cq value of 30.7, SE ± 1.2). The quagga mussel standard curve had an amplification efficiency of 98.6% and a slope of -3.39, while the amplification efficiency was 99.0% and the slope -3.37 for the zebra mussel standard curve. We were able to identify all the 24 PCR products which we had amplified with the DpoCOI3 or the DbuCOI3 primer pair as either zebra mussel or quagga mussel DNA, respectively by sequencing and subsequent blasting.

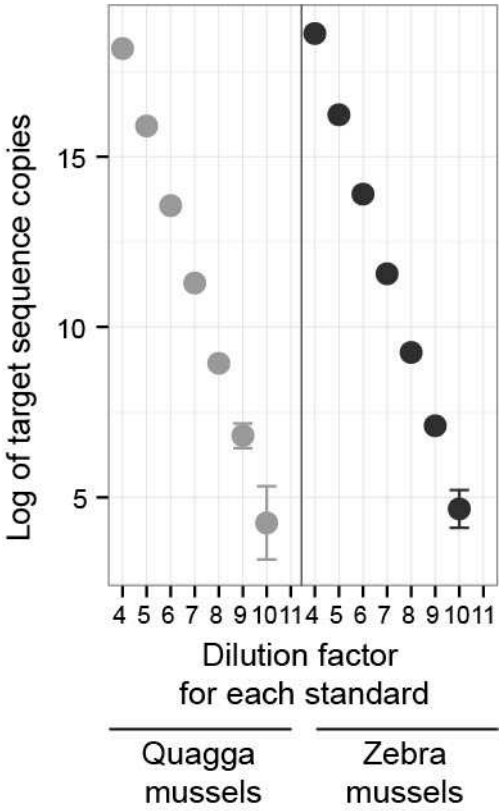

Figure 3 Means of log concentrations (log number of target sequence copies / µL) for each standard dilution of quagga mussel (grey circles) and zebra mussel (black circles) target sequences. The error bars show standard errors of the mean. A dilution factor of 4 represents the highest standard concentration (8×10^9 sequence copies / µL for quagga mussels and 1.1×10^{10} sequence copies / µL for zebra mussels). A dilution factor of 11 represents the lowest standard concentration (0.8 sequence copies / µL for quagga mussels and 1.1 sequence copies / µL for zebra mussels). The lowest concentrated standards of both standard curves with a dilution factor of 11 did not amplify.

We found a comparable pattern of zebra and quagga mussel presence with qPCR as with PCR presented above. Zebra mussel DNA was detected at all sites, while quagga mussel DNA was present at all sites except those upstream of Basel (Figure 4a). Mean numbers of detected eDNA copies per µL per site and species and corresponding standard errors are shown in Figure 4. In the Rhine in Basel the number of detected eDNA copies per µL was clearly lower for quagga (9.5×10^3 seq / µL, SE ± 1.1×10^3) than for zebra mussels (4.5×10^5 seq/ µL, SE ± 8.6×10^4), while in all other locations downstream of Basel the concentration of quagga eDNA copies was higher. The highest concentration of zebra mussel DNA was found in Diessenhofen

(2.8 x 10^6 seq / µL, SE ± 3.9 x 10^5), where also field samplings of zebra mussels few kilometers upstream showed extremely high densities (Hesselschwerdt et al. 2014). The lowest zebra mussel signals we found in Lake Markermeer and Lake IJsselmeer, agreeing with a survey conducted in 2012 (handpicking on shore, data not shown). As a previous study by Heiler and colleagues (2013) mainly focused on the ratio of quagga mussel abundances to total dreissenid abundances (zebra plus quagga mussels) from field survey data, we also present those ratios calculated from our qPCR data (Figure 4b). The ratio was still very low for Basel (0.02, SE ± 0.0017) but high for all other sites downstream of Basel, except for Hanau (0.45, SE ± 0.061). Interestingly, for Lake IJsselmeer and in the River Rhine near Worms the ratio was almost 100%, with ratios of 0.991 (SE ± 0.004) and 0.996 (SE ± 0.0036), respectively (Figure 4b).

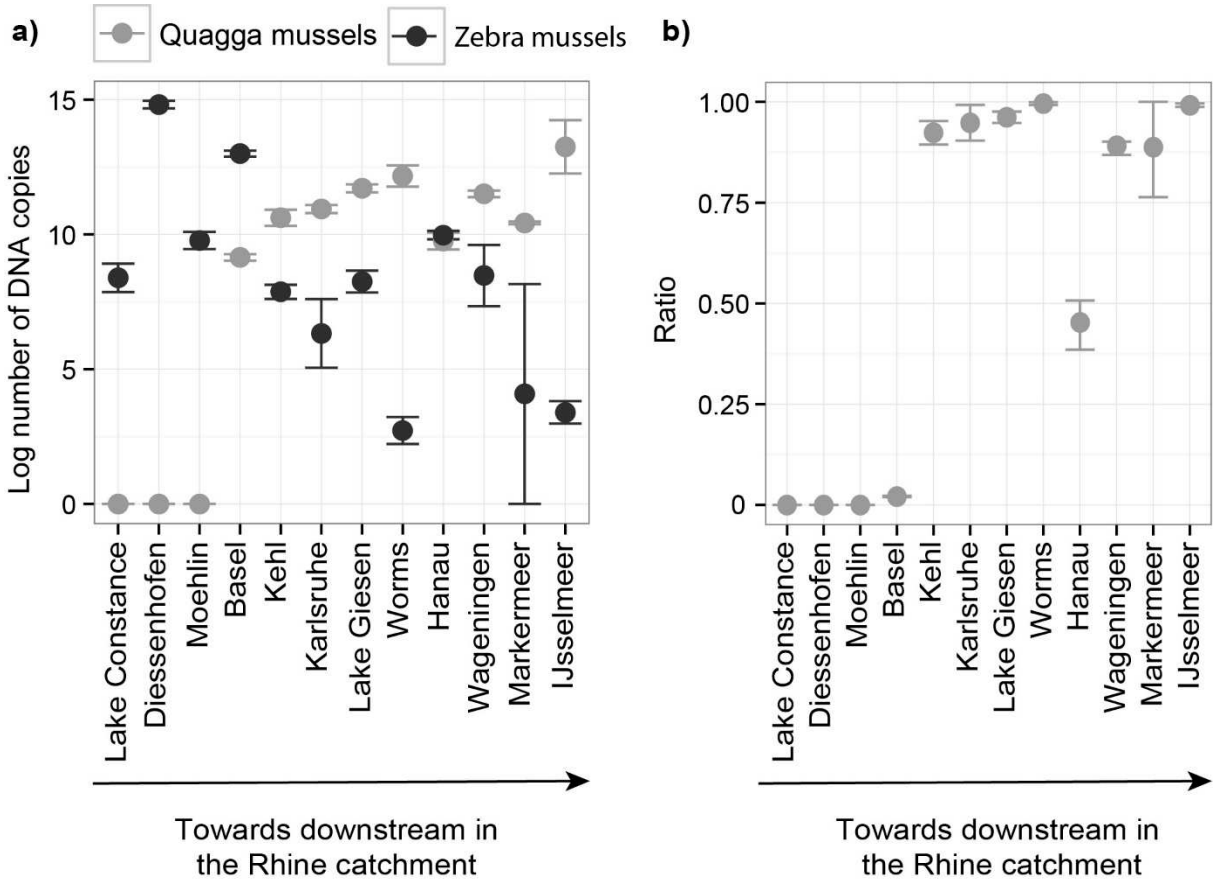

Figure 4 a) Means$_{\text{sample replicates}}$ of the mean$_{\text{technical replicates}}$ logarithmic concentrations (log number of target sequence copies/µL) for each sampling site as a proxy for zebra and quagga mussel biomass. The error bars show standard errors of the mean. **b)** Ratio of quagga mussels eDNA concentrations to the total dreissenid eDNA concentration (zebra plus quagga mussels) measured as number of target sequence copies/µL for each sampling site. The error bars show the standard errors of the mean ratio.

Discussion

Using an eDNA approach we were able to detect quagga mussels in all sampling sites downstream of Basel and for the first time also in the harbour in Basel (Figure 2). Since 2006 the quagga mussel has rapidly expanded southwards in the Rhine system and has been detected as far south as Karlsruhe in 2007 (Martens et al. 2009). Therefore, the appearance of the quagga mussel in Basel has been expected for several years. However, quagga mussels had not been detected in monitoring programs using traditional benthic invertebrate sampling methods including kicknet sampling, surber-sampling and scuba diving in the upper Rhine around Basel run by environmental offices and authorities (Figure 1).

Our study shows that zebra and quagga mussels can be detected reliably in eDNA samples using standard PCR with the species specific primers used in this study. All of the samples collected upstream of Basel, where the quagga mussel had not been found by traditional sampling, failed to amplify the target sequence based on the eDNA sample (Figure 2). The only false positives occurred for the zebra mussel, in three field filtered controls, likely due to eDNA cross-contamination during handling of the filters in the field. Nevertheless, cross-contamination between sites is unlikely as we used new or cleaned equipment for each sampling site. The advantage of this filtration method is that filters can be frozen directly in the field and water samples do not need to be transported to the lab, during which the eDNA might degrade. Despite the need for careful controls, the detection of mussels with species specific PCR is reliable, inexpensive and less time consuming compared to traditional sampling (Thomsen and Willerslev 2015).

For both species, we estimated a qPCR quantification limit of roughly 10 eDNA copies per μL (Figure 3). This translates to minimum lower quantification limit of $10x10^6$ eDNA copies per L collected water sample, assuming that no eDNA got lost during the sampling, transport, filtration and extraction processes and that there were no PCR inhibiting substances retained in the eDNA extracts. PCR inhibiting substances may be co-extracted with the eDNA and may lead to inaccurate detection or quantification of eDNA (McKee et al. 2015; Sigsgaard et al. 2015). Using the Qiagen PCR Multiplex Master Mix we hoped to mitigate PCR inhibition. Nevertheless, filters clogged easily and retained high amounts of organic and inorganic material in some sites (Worms, Hanau, Wageningen, Lake Markermeer and Lake IJsselmeer) and thus we cannot exclude PCR inhibition completely.

Therefore, we recommend spiking of eDNA samples with synthetic DNA oligonucleotides of a known length and concentration to be quantified along with the target eDNA in order to control for potential PCR inhibition. Furthermore, the amplification efficiencies in our qPCR experiment were similarly high for both species and thus the estimated eDNA concentrations may directly be compared between the species and may be used as a proxy for zebra and quagga mussel biomass in the field.

Comparing eDNA concentrations of zebra and quagga mussels with qPCR revealed similar concentration patterns as we had expected based on previous knowledge of zebra and quagga mussel abundances in the field sites. In mesocosm experiments with fish or amphibians, eDNA quantification has been shown to correlate well with known densities or biomass of the target organisms (Takahara et al. 2012; Thomsen et al. 2012). Pilliod et al. (2013) also found strong correlation of eDNA quantification with density estimates from field survey data in two amphibian species. Most field survey data for dreissenids in the River Rhine catchment stem from older sampling campaigns in 2008 and 2009. Knowing that the quagga mussel invasion front has shifted southwards since then, with quagga mussels displacing zebra mussels, this data cannot be directly compared with our eDNA quantifications in a statistical model. Nevertheless, zebra mussel eDNA concentrations were high in the upper Rhine and decreased towards the lower Rhine (Figure 4a), where they were shown to be increasingly displaced by quagga mussels (Heiler et al. 2013). In contrast, quagga mussel eDNA concentrations were lowest in Basel and increased northwards with increasingly long invasion history, except for Hanau and Lake Markermeer (Figure 4a). Also the quagga mussel ratio was still low in Basel where the quagga has invaded most recently, but was close to 100% at sites with longer invasions history. Our results thus confirm the displacement of zebra by quagga mussels (Figure 4b).

Our qPCR approach revealed relatively small differences between samples within site leading to small standard errors and indicating high precision within site (Figure 4a). The variation was clearly larger for samples with low eDNA concentrations close to the detection limit. Nevertheless, many other factors may confound eDNA quantifications by influencing the production and decay rates of target eDNA in freshwater systems. For example eDNA shedding depends on the species identity (Mächler et al. 2014), temperature and diet of the studied organism, while decay rates, depend on environmental factors such as temperature or light exposure (Klymus et al. 2015). Furthermore, the predominance of different life history stages

and seasonality of target organisms may strongly influence the eDNA quantity. For example, we cannot completely exclude that we caught zebra or quagga mussel larvae in our water samples, which may have led to a strong signal in the eDNA quantification. Despite all these confounding factors qPCR may allow to follow the population development over different geographic and temporal scales. We recommend that the use of eDNA quantification as a proxy for zebra and quagga mussel densities need to be validated in mesocosm experiments with known mussel densities or in comparison with more recent field data specifically assessed for such a study.

Conclusions

Our study shows that eDNA detection with standard PCR is a reliable method for the targeted early detection and surveillance of zebra and quagga mussels. This method is inexpensive, fast if applied for a series of samples from different sampling sites and does not need very complicated equipment, except of a simple PCR-cycler and a gel casting system. In our case, eDNA detection with standard PCR was not only cheaper and simpler but also a more robust method than qPCR. It was less prone to false positives as it has lower sensitivity and also less prone to false negatives as qPCR signals at very low eDNA target concentrations were sometimes ambiguous and had to be confirmed with standard PCR.

We show that eDNA quantification as a proxy for measures of zebra and quagga mussel biomass is a promising technique for the future. Particularly in lotic environments, target eDNA will be washed in and out with certain rates (Jane et al. 2015) and the eDNA signal will possibly only be lost several kilometres downstream of a point source (Deiner and Altermatt 2014). Thus eDNA quantification as a proxy for organism densities may not be appropriate in small scale studies but may apply for the quantification of invasive species on larger geographic scales as presented in our study. In combination, traditional PCR and qPCR are powerful tools for the early detection and surveillance of specific species. The two techniques may be applied to various (potentially) invasive species or other organisms of high interest, such as diseases of aquatic organisms, for example cray fish plague (Strand 2013) or parasitic fish diseases such as the proliferative kidney disease or bryozoans as its intermediate hosts (Anderson et al. 1999; Okamura et al. 2011).

In order to manage invasive species, it is important to detect new invaders early on and follow their population development in the early phase of invasion. For the early detection of quagga mussels in Switzerland, we recommend that water samples are

taken repeatedly across the year at neuralgic water bodies and assessed with traditional PCR. Sampling sites could be in the upper Rhine or in lakes such as Constance, Geneva or Zürich, which are used intensively for recreational boating and are thus at high risk for the quagga mussel invasion (De Ventura et al. 2016). In the case of invasion, eDNA quantification will then help to follow the population development of zebra and quagga mussels over time and reveal the potential displacement of the zebra mussel populations by the quagga mussel.

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Supplementary Material

Figures

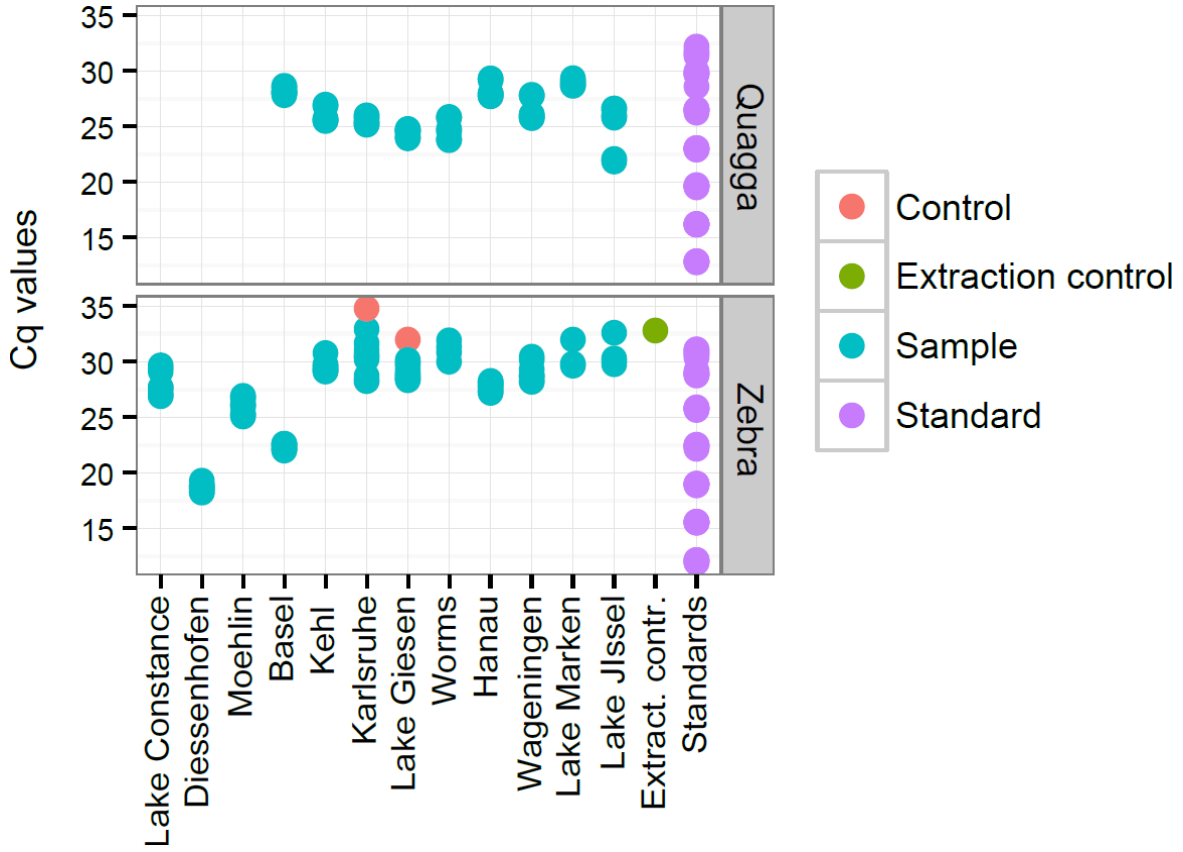

Figure S1 Cq values for all pooled samples, controls, extraction controls and standards, which amplified in the qPCR and were not excluded by the melting temperature or the shape of the melting curve. None of the PCR controls amplified or could not excluded by melting curve analysis. Three controls for the zebra mussel quantification, could not be excluded by this procedure, but had a higher Cq value than the lowest amplifying standard.

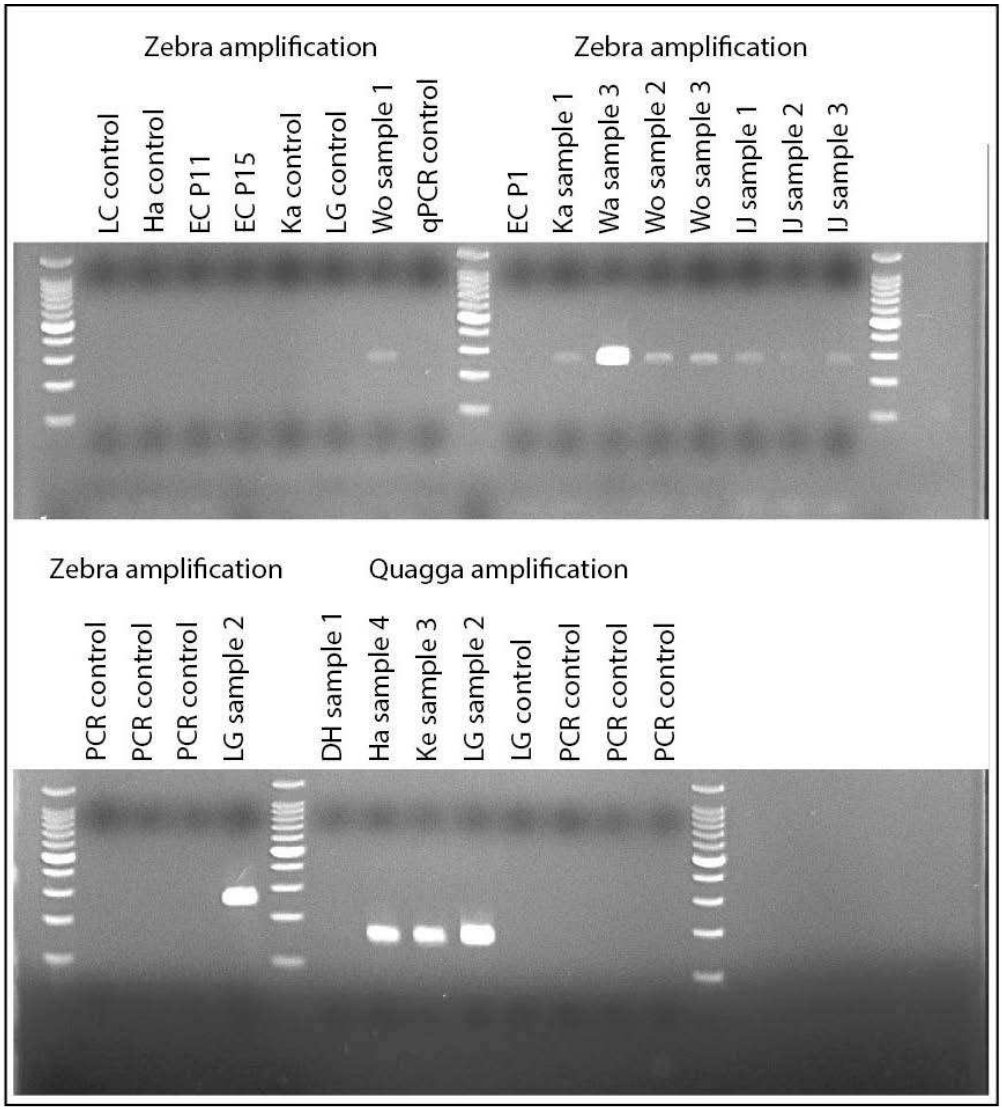

Figure S2 Results from the traditional PCR, where we re-amplified the DpoCOI3 target (zebra mussel) and the DbuCOI3 target (quagga mussel) from negative controls, which revealed suspiciously low Cq values in the qPCR, and samples with higher Cq values than the lowest amplifying standards. All of the eDNA samples amplified and the positive amplification in the qPCR could be confirmed. None of the negative controls amplified and could thus be excluded. Labels: control: field or lab filtered control samples, EC: extraction control, qPCR control: same nuclease free water as used in the qPCR run, PCR control: nuclease free water, sample: some of the eDNA samples also tested in qPCR before, LC: Lake Constance, Ha: Hanau, Ka: Karlsruhe, LG: Lake Giessen, Wo: Worms, Wa: Wageningen, IJ: Lake IJsselmeer, DH: Diessenhofen, Ke: Kehl.

Tables

Table S1 List of pooled samples showing sampling sites, sample types, zebra and quagga mussel detections, use in qPCR for eDNA quantification, dilution factors, numbers of filters used and resulting filter dilution factors.

Sites	Type	Pool ID	Zebra mussel detected	Quagga mussel detected	Volume tested in PCR	Tested in qPCR	Dilution of eDNA for qPCR	Number of filters used	Filter dilution factor
None	Clean filter extracted	EC_P1	0	0	2 µl	Yes	0.5	1	1
None	Clean filter extracted	EC_P11	0	0	2 µl	Yes	0.5	1	1
None	Clean filter extracted	EC_P12	0	0	2 µl	Yes	0.5	1	1
None	Clean filter extracted	EC_P13	0	0	2 µl	Yes	0.5	1	1
None	Clean filter extracted	EC_P15	0	0	2 µl	Yes	0.5	1	1
None	Clean filter extracted	EC_P16	0	0	2 µl	No	NA	1	1
None	Clean filter extracted	EC_P2	0	0	2 µl	No	NA	1	1
None	Clean filter extracted	EC_P3	0	0	2 µl	No	NA	1	1
None	Clean filter extracted	EC_P4	0	0	2 µl	No	NA	1	1
None	Clean filter extracted	EC_P5	0	0	2 µl	No	NA	1	1
None	Clean filter extracted	EC_P6	0	0	2 µl	No	NA	1	1
None	Clean filter extracted	EC_P7	0	0	2 µl	No	NA	1	1
None	Clean filter extracted	EC_P8	0	0	2 µl	No	NA	1	1
None	Clean filter extracted	EC_P9	0	0	2 µl	No	NA	1	1
Basel	field filtered sample	Ba_P4	1	1	2 µl	No	NA	2	0.5
Basel	field filtered sample	Ba_P5	1	1	2 µl	No	NA	2	0.5
Basel	field filtered sample	Ba_P6	1	1	2 µl	No	NA	2	0.5
Lake Constance	field filtered sample	LC_P4	1	0	2 µl	No	NA	1	1
Lake Constance	field filtered sample	LC_P5	1	0	2 µl	No	NA	1	1
Lake Constance	field filtered sample	LC_P6	1	0	2 µl	No	NA	1	1
Lake Giesen	field filtered sample	LG_P4	1	1	2 µl	No	NA	2	0.5
Lake Giesen	field filtered sample	LG_P5	1	1	2 µl	No	NA	2	0.5

Lake Giesen	field filtered sample	LG_P6	1	1	2 µl	No	NA	2	0.5
Diessenhofen	field filtered sample	DH_P4	1	0	2 µl	No	NA	1	1
Diessenhofen	field filtered sample	DH_P5	1	0	2 µl	No	NA	1	1
Diessenhofen	field filtered sample	DH_P6	1	0	2 µl	No	NA	1	1
Hanau	field filtered sample	Ha_P1	1	1	2 µl	No	NA	4	0.25
Hanau	field filtered sample	Ha_P2	1	1	2 µl	No	NA	4	0.25
Hanau	field filtered sample	Ha_P3	1	1	2 µl	No	NA	4	0.25
Lake IJsselmeer	field filtered sample	IJ_P1	0	1	2 µl	Yes	0.25	4	0.25
Lake IJsselmeer	field filtered sample	IJ_P2	1	1	2 µl	Yes	0.1	4	0.25
Lake IJsselmeer	field filtered sample	IJ_P3	1	1	2 µl	Yes	0.25	4	0.25
Karlsruhe	field filtered sample	Ka_P4	1	1	2 µl	No	NA	2	0.5
Karlsruhe	field filtered sample	Ka_P5	1	1	2 µl	No	NA	2	0.5
Karlsruhe	field filtered sample	Ka_P6	1	1	2 µl	No	NA	2	0.5
Kehl	field filtered sample	Ke_P4	1	1	2 µl	No	NA	2	0.5
Kehl	field filtered sample	Ke_P5	1	1	2 µl	No	NA	2	0.5
Kehl	field filtered sample	Ke_P6	1	1	2 µl	No	NA	2	0.5
Lake Markermeer	field filtered sample	Ma_P2	0	1	2 µl	Yes	0.333	4	0.25
Lake Markermeer	field filtered sample	Ma_P3	1	1	2 µl	Yes	0.333	4	0.25
Moehlin	field filtered sample	Moe_P4	1	0	2 µl	No	NA	1	1
Moehlin	field filtered sample	Moe_P5	1	0	2 µl	No	NA	1	1
Moehlin	field filtered sample	Moe_P6	1	0	2 µl	No	NA	1	1
Wageningen	field filtered sample	Wa_P1	1	1	2 µl	Yes	0.5	4	0.25
Wageningen	field filtered sample	Wa_P2	1	1	2 µl	Yes	0.1	4	0.25
Wageningen	field filtered sample	Wa_P3	1	1	2 µl	Yes	0.5	4	0.25
Worms	field filtered sample	Wo_P4	1	1	2 µl	No	NA	4	0.25
Worms	field filtered sample	Wo_P5	1	1	2 µl	No	NA	4	0.25
Worms	field filtered sample	Wo_P6	1	1	2 µl	No	NA	4	0.25
Basel	field negative controlcontrol	Ba_PC	0	0	2 µl	No	NA	1	1

Lake Constance	field negative controlcontrol	LC_PC	0	0	2 µl	No	NA	1	1
Lake Giesen	field negative controlcontrol	LG_PC	1	1	2 µl	No	NA	1	1
Hanau	field negative controlcontrol	Ha_PC	1	0	2 µl	No	NA	1	1
Lake IJsselmeer	field negative controlcontrol	IJ_PMQ	0	0	2 µl	No	NA	1	1
Karlsruhe	field negative controlcontrol	Ka_PC	0	0	2 µl	No	NA	1	1
Kehl	field negative controlcontrol	Ke_PC	0	0	2 µl	No	NA	1	1
Moehlin	field negative controlcontrol	Moe_PC	1	0	2 µl	No	NA	1	1
Wageningen	field negative controlcontrol	Wa_PMQ	0	0	2 µl	No	NA	1	1
Worms	field negative controlcontrol	Wo_PC	1	0	2 µl	No	NA	1	1
Basel	lab filtered sample	Ba_P1	1	1	2 µl	No	0.5	2	0.5
Basel	lab filtered sample	Ba_P2	1	1	2 µl	No	0.5	2	0.5
Basel	lab filtered sample	Ba_P3	1	1	2 µl	No	0.5	2	0.5
Lake Constance	lab filtered sample	LC_P1	1	0	2 µl	Yes	0.5	1	1
Lake Constance	lab filtered sample	LC_P2	1	0	2 µl	Yes	0.5	1	1
Lake Constance	lab filtered sample	LC_P3	1	0	2 µl	Yes	0.5	1	1
Lake Giesen	lab filtered sample	LG_P1	1	1	2 µl	Yes	0.5	2	0.5
Lake Giesen	lab filtered sample	LG_P2	1	1	2 µl	Yes	0.5	2	0.5
Lake Giesen	lab filtered sample	LG_P3	1	1	2 µl	Yes	0.5	2	0.5
Diessenhofen	lab filtered sample	DH_P1	1	0	2 µl	Yes	0.5	1	1
Diessenhofen	lab filtered sample	DH_P2	1	0	2 µl	Yes	0.5	1	1
Diessenhofen	lab filtered sample	DH_P3	1	0	2 µl	Yes	0.5	1	1
Hanau	lab filtered sample	Ha_P4	1	1	2 µl	Yes	0.5	4	0.25
Hanau	lab filtered sample	Ha_P5	1	1	2 µl	Yes	0.5	4	0.25
Hanau	lab filtered sample	Ha_P6	1	1	2 µl	Yes	0.5	4	0.25
Karlsruhe	lab filtered sample	Ka_P1	1	1	2 µl	Yes	0.5	2	0.5
Karlsruhe	lab filtered sample	Ka_P2	1	1	2 µl	Yes	0.5	2	0.5
Karlsruhe	lab filtered sample	Ka_P3	1	1	2 µl	Yes	0.5	2	0.5
Kehl	lab filtered sample	Ke_P1	1	1	2 µl	Yes	0.5	2	0.5
Kehl	lab filtered sample	Ke_P2	1	1	2 µl	Yes	0.5	2	0.5
Kehl	lab filtered sample	Ke_P3	1	1	2 µl	Yes	0.5	2	0.5
Moehlin	lab filtered sample	Moe_P1	1	0	2 µl	Yes	0.5	1	1
Moehlin	lab filtered sample	Moe_P2	1	0	2 µl	Yes	0.5	1	1
Moehlin	lab filtered sample	Moe_P3	1	0	2 µl	Yes	0.5	1	1
Worms	lab filtered sample	Wo_P1	1	1	2 µl	Yes	0.5	4	0.25
Worms	lab filtered sample	Wo_P2	1	1	2 µl	Yes	0.5	4	0.25
Worms	lab filtered sample	Wo_P3	1	1	2 µl	Yes	0.5	4	0.25
Basel	lab negative control	Ba_PMQ	0	0	2 µl	Yes	0.5	1	1
Lake Constance	lab negative control	LC_PMQ	0	0	2 µl	Yes	0.5	1	1
Lake Giesen	lab negative control	LG_PMQ	0	0	2 µl	Yes	0.5	1	1
Hanau	lab negative control	Ha_PMQ	0	0	2 µl	Yes	0.5	1	1
Karlsruhe	lab negative control	Ka_PMQ	0	0	2 µl	Yes	0.5	1	1

Kehl	lab negative control	Ke_PMQ	0	0	2 µl	Yes	0.5	1	1
Worms	lab negative control	Wo_PMQ	0	0	2 µl	Yes	0.5	1	1
None	PCR control	nuclease free water	0	0	2 µl	Yes	NA	1	1
None	PCR control		0	0	2 µl	No	NA	1	1
None	PCR control		0	0	2 µl	No	NA	1	1
None	PCR control		0	0	2 µl	No	NA	1	1
None	PCR control		0	0	2 µl	No	NA	1	1
None	PCR control		0	0	2 µl	No	NA	1	1
None	PCR control		0	0	2 µl	No	NA	1	1
None	PCR control		0	0	2 µl	No	NA	1	1
None	PCR control		0	0	2 µl	No	NA	1	1
None	PCR control		0	0	2 µl	No	NA	1	1
None	PCR control		0	0	2 µl	No	NA	1	1

Table S2 List of quagga and zebra mussel sequences, which we downloaded from Genbank. We show the geographic origin for those samples for which it was indicated on Genbank, the accession number, the species identity and the corresponding forward and reverse primer binding sites used in PCR and qPCR extracted from each sequence.

Origin	Accession number	Species	Forward primer binding site	Reverse primer binding site
gb	DQ840132.1	D. rostriformis bugensis	GGGGTTGAACATTATATCCACCGTT	CGTGCCGGGTGTCATCAGTTT
gb	DQ840133.1	D. rostriformis bugensis	GGGGTTGAACATTATATCCACCGTT	CGTGCCGGGTGTCATCAGTTT
gb	AF479637.1	D. rostriformis bugensis	GGGGTTGAACATTATATCCACCGTT	CGTGCCGGGTGTCATCAGTTT
gb	AF495877.1	D. rostriformis bugensis	GGGGTTGAACATTATATCCACCGTT	CGTGCCGGGTGTCATCAGTTT
Lake Balaton	JQ435816.1	D. rostriformis bugensis	GGGGTTGAACATTATATCCACCGTT	CGTCCCGGGTGTCATCAGTTT
Lake Balaton	JQ771943.1	D. rostriformis bugensis	GGGGTTGAACATTATATCCACCGTT	CGTGCCGGGTGTCATCAGTTT
Lake Balaton	JQ771944.1	D. rostriformis bugensis	GGGGTTGAACATTATATCCACCGTT	CGTGCCGGGTGTCATCAGTTT
Lake Balaton	JQ771945.1	D. rostriformis bugensis	GGGGTTGAACATTATATCCACCGTT	CGTGCCGGGTGTCATCAGTTT
Lake Balaton	JQ771946.1	D. rostriformis bugensis	GGGGTTGAACATTATATCCACCGTT	CGTGCCGGGTGTCATCAGTTT
Lake Balaton	JQ771947.1	D. rostriformis bugensis	GGGGTTGAACATTATATCCACCGTT	CGTGCCGGGTGTCATCAGTTT
Lake Balaton	JQ771948.1	D. rostriformis bugensis	GGGGTTGAACATTATATCCACCGTT	CGTGCCGGGTGTCATCAGTTT
Lake Balaton	JQ771949.1	D. rostriformis bugensis	GGGGTTGAACATTATATCCACCGTT	CGTGCCGGGTGTCATCAGTTT
Lake Balaton	JQ771950.1	D. rostriformis bugensis	GGGGTTGAACATTATATCCACCGTT	CGTGCCGGGTGTCATCAGTTT

Lake Balaton	JX099436.1	D. rostriformis bugensis	GGGGTTGAACATTATATCCACCGTT	CGTGCCGGGTGTCATCAGTTT
Netherlands	EF080861.1	D. rostriformis bugensis	GGGGTTGAACATTATATCCACCGTT	CGTGCCGGGTGTCATCAGTTT
emb	AM749000.1	D. polymorpha	GCTAAGGGCACCTGGAAGCGT	AGGGAAGGAGGATTCGGGGGTG
emb	AM748996.1	D. polymorpha	GCTAAGGGCACCTGGAAGCGT	AGGGAAGGAGGATTCGGGGGTG
emb	AM748992.1	D. polymorpha	GCTAAGGGCACCTGGAAGCGT	AGGGAAGGAGGATTCGGGGGTG
emb	AM748991.1	D. polymorpha	GCTAAGGGCACCTGGAAGCGT	AGGGAAGGAGGATTCGGGGGTG
emb	AM748987.1	D. polymorpha	GCTAAGGGCACCTGGAAGCGT	AGGGAAGGAGGATTCGGGGGTG
emb	AM748984.1	D. polymorpha	GCTAAGGGCACCTGGAAGCGT	AGGGAAGGAGGATTCGGGGGTG
emb	AM748983.1	D. polymorpha	GCTAAGGGCACCTGGAAGCGT	AGGGAAGGAGGATTCGGGGGTG
emb	AM748982.1	D. polymorpha	GCTAAGGGCACCTGGAAGCGT	AGGGAAGGAGGATTCGGGGGTG
emb	AM748981.1	D. polymorpha	GCTAAGGGCACCTGGAAGCGT	AGGGAAGGAGGATTCGGGGGTG
emb	AM748980.1	D. polymorpha	GCTAAGGGCACCTGGAAGCGT	AGGGAAGGAGGATTCGGGGGTG
emb	AM748979.1	D. polymorpha	GCTAAGGGCACCTGGAAGCGT	AGGGAAGGAGGATTCGGGGGTG
emb	AM748978.1	D. polymorpha	GCTAAGGGCACCTGGAAGCGT	AGGGAAGGAGGATTCGGGGGTG
emb	AM746677.1	D. polymorpha	GCTAAGGGCACCTGGAAGCGT	AGGGAAGGAGGATTCGGGGGTG
emb	AM749001.1	D. polymorpha	GCTAAGGGCACCTGGAAGCGT	AGGGAAGGAGGATTCGGGGGTG
emb	AM748999.1	D. polymorpha	GCTAAGGGCACCTGGAAGCGT	AGGGAAGGAGGATTCGGGGGTG
emb	AM748990.1	D. polymorpha	GCTAAGGGCACCTGGAAGCGT	AGGGAAGGAGGATTCGGGGGTG
emb	AM748989.1	D. polymorpha	GCTAAGGGCACCTGGAAGCGT	AGGGAAGGAGGATTCGGGGGTG
emb	AM748988.1	D. polymorpha	GCTAAGGGCACCTGGAAGCGT	AGGGAAGGAGGATTCGGGGGTG
emb	AM748985.1	D. polymorpha	GCTAAGGGCACCTGGAAGCGT	AGGGAAGGAGGATTCGGGGGTG
emb	AM748976.1	D. polymorpha	GCTAAGGGCACCTGGAAGCGT	AGGGAAGGAGGATTCGGGGGTG
emb	AM748975.1	D. polymorpha	GCTAAGGGCACCTGGAAGCGT	AGGGAAGGAGGATTCGGGGGTG
Germany	AM748986.1	D. polymorpha	GCTAAGGGCACCTGGAAGCGT	AGGGAAGGAGGATTCGGGGGTG
Germany	AM748999.1	D. polymorpha	GCTAAGGGCACCTGGAAGCGT	AGGGAAGGAGGATTCGGGGGTG
gb	DQ840124.1	D. polymorpha	GCTAAGGGCACCTGGAAGCGT	AGGGAAGGAGGATTCGGGGGTG
gb	JX099437.1	D. polymorpha	GCTAAGGGCACCTGGAAGCGT	AGGGAAGGAGGATTCGGGGGTG
gb	JQ771953.1	D. polymorpha	GCTAAGGGCACCTGGAAGCGT	AGGGAAGGAGGATTCGGGGGTG
gb	JQ771952.1	D. polymorpha	GCTAAGGGCACCTGGAAGCGT	AGGGAAGGAGGATTCGGGGGTG
gb	JQ771951.1	D. polymorpha	GCTAAGGGCACCTGGAAGCGT	AGGGAAGGAGGATTCGGGGGTG

gb	AF479636.1	D. polymorpha	GCTAAGGGCACCTGGAAGCGT	AGGGAAGGAGGATTCGGGGGTG
gb	JQ435817.1	D. polymorpha	GCCAAGGGCACCTGGAAGCGT	AGGGAAGGAGGATTCGGGGGTG
gb	AF120663.1	D. polymorpha	GCTAAGGGCACCTGGAAGCGT	AGGGAAGGAGGATTCGGGGGTG
Italy	AM748997.1	D. polymorpha	GCTAAGGGCACCTGGAAGCGT	AGGGAAGGAGGATTCGGGGGTG
Italy	AM748977.1	D. polymorpha	GCTAAGGGCACCTGGAAGCGT	AGGGAAGGAGGATTCGGGGGTG
Ponto Caspian	DQ840125.1	D. polymorpha	GCTAAGGGCACCTGGAAGCGT	AGGGAAGGAGGATTCGGGGGTG
Ponto Caspian	DQ840123.1	D. polymorpha	GCTAAGGGCACCTGGAAGCGT	AGGGAAGGAGGATTCGGGGGTG
Ponto Caspian	DQ840122.1	D. polymorpha	GCTAAGGGCACCTGGAAGCGT	AGGGAAGGAGGATTCGGGGGTG
Ponto Caspian	DQ840121.1	D. polymorpha	GCTAAGGGCACCTGGAAGCGT	AGGGAAGGAGGATTCGGGGGTG
Turkey	EF414493.1	D. polymorpha	GCTAAGGGCACCTGGAAGCGT	AGGGAAGGAGGATTCGGGGGTG

General discussion

The four research chapters of my thesis examine four different aspects of the zebra and quagga mussel invasion. The results of all four chapters can be used to draw practical consequences on the management of these species, and they also open up new research questions. In the following discussion, I explain for each of the chapters the management consequences, the open research questions and some of the aspects I worked on during my thesis, which were not included in the thesis chapters.

Chapter 1: The environmental niche may shift during the invasions process

When investigating the environmental niche of a species it is necessary to examine several populations, in order to reveal local evolutionary processes. These might differ among populations and lead to phenotypes which reflect the invasion history and local adaptation instead of fixed species-level traits. There are two main findings from chapter 1, which have to be considered for the future management of invasive zebra and quagga mussels:

First, we found more pronounced phenotypic variation among populations than among species indicating evolutionary post-invasion responses in zebra and quagga mussels. Elderkin and Klerks (2001; 2005) have already suggested local adaptation of zebra mussel populations to different temperature regimes. The results of chapter 1 indicate that local adaptation in quagga and zebra mussels might promote environmental niche shifts along their invasion front and that it is necessary to consider adaptive processes when setting up predictive models of the future dreissenid distribution. Researchers have repeatedly shown that rapid evolutionary changes in invasive alien species (IAS) may happen at the same time scale than the invasion process (Huey 2000; Gilchrist et al. 2001; Prentis et al. 2008). In several empirical studies these processes have been shown to be important for invasion potential, for example enabling a species to invade new habitats (Lee et al. 2012; Buckley and Bridle 2014), accelerating the invasion (Phillips et al. 2006; Brown et al. 2014) or profoundly changing the invasion dynamics (Fronhofer and Altermatt 2015). Nevertheless, only very few modelling studies have taken these processes into account (Perkins et al. 2013; Kubisch et al. 2014). However, evolutionary processes may play an important role for the invasion potential of a species and can differ among populations, especially if these differ in their invasion history.

The second main finding of this chapter was that there are no clear differences between the two species in their tolerance to moderate periods of low oxygen. In our experiments we applied low oxygen conditions and only after several weeks the mussels of both species started to show increased mortality. Also in the shallow Dutch Lakes Markermeer and IJsselmeer, both species have been observed to survive occasional short periods of oxygen depletion of several days (Noordhuis et al. 2014). However, Noordhuis (2014, personal communication) observed that the dreissenid mussel population was reduced down to 10% in Lake Ijssel during the extreme summer heat-wave in 2006, likely due to prolonged (several weeks) stratification and oxygen depletion in the deeper zones of the lake.

Moreover, quagga mussels have often been found to displace zebra mussels in various types of water bodies (Mills et al. 1996; Therriault et al. 2005; Zhulidov et al. 2010), with quagga mussels starting to outcompete the zebra mussel in the deeper habitats first. This competitive advantage of quagga mussels was repeatedly attributed to their superior tolerance (over zebra mussels) to low oxygen levels (Karatayev et al. 1998). Our results in chapter 1 indicate that low oxygen tolerance is not an important factor explaining this competitive advantage. The species-specific depth distribution could also be explained by increased survival and reproduction at lower temperatures (Roe and MacIsaac 1997), increased tolerance to low nutrient conditions (Baldwin et al. 2002; Stoeckmann 2003) or generally higher somatic growth and survivorship (Karatayev et al. 2011) of quagga mussels compared to zebra mussels. One causal explanation for the better growth at low nutrient conditions may be that quagga mussels have a freshwater origin, while zebra mussels originate from brackish water conditions and subsequently evolved towards freshwater conditions (Karatayev et al. 1998; Orlova et al. 2005). Consequently, zebra mussels may have higher energy demands to maintain the internal ion concentrations in freshwater, as Lee et al. (2011) showed for recent copepod invasions from salt water to freshwater. This hypothesis could be tested by comparing the enzyme activity of transmembrane ion transporters of zebra and quagga mussels under different salinities. For all above mentioned reasons, quagga mussels may still colonize deeper zones of the deep alpine lakes in Switzerland compared to zebra mussels. Therefore, water suppliers and water managers should stay alarmed, despite our results.

Chapter 2: Recreational boats as a potent vector for zebra mussels

In chapter 2, I show that by means of overland transport seasonally and year-round moored boats have a high potential of distributing zebra mussels to all navigable lakes in Switzerland. Recreational boats have also been shown to be a strong vector for the secondary spread of zooplankton and benthic invertebrates via in-water transport between interconnected waterways (Kelly et al. 2013). A study by Johnson et al. (2001) assigned the strongest vector potential to the overland transport of trailered boats. In contrast, I found, that trailered boats, which are mostly kept on land when not used, play only a minor role for the overland spread of zebra mussels in Switzerland. Differences in the outcome of the above mentioned studies may be based on different environments (translocation of macrophytes and entangled mussels plays only a minor role in Switzerland as opposed to the North American studies) or different boating practices, or may be due to different research methods. For example, Johnson et al. (2001) focused mainly on trailered boats while I covered a wider range of boat types and boating practices. I also excluded some types of boats from the study: for example, inflatable boats, kayaks and canoes. As most of these boats are kept dry when not used, I expected the excluded boat types to be unimportant as vector for zebra mussels.

I suggest that research projects on distribution vectors of invasive species have to be tailored to local circumstances in order to reveal the critical vectors. Thus, the results from non-local studies should not be taken at face value and conclusions should be scrutinized carefully for similarities before adopting them. For Switzerland, I suggest that preventive measures against the further spread of zebra and quagga mussels are directed to seasonally and year-round moored boats (see also chapter 3). As quagga mussels are likely to be transported by recreational boating with similar frequencies than zebra mussels, measures need to be taken soon if the spread of quagga is to be restrained in Switzerland.

The overland transport of zebra mussel larvae by recreational boats may be a second important vector with some spreading potential (Kelly et al. 2013; Dalton and Cottrell 2013), which I did not investigate in detail. Choi et al. (2013) demonstrated experimentally that veliger larvae of the quagga mussel can survive for several days in the bilge water of boats, depending on the water temperature. Johnson and colleagues (2001) found veliger larvae of the zebra mussel in all kind of water filled enclosures of overland transported boats. In the same study veliger larvae were also found in the water cooling system of two boats. From personal communication with

boat owners and employees of boat yards in Switzerland, I understood that zebra mussel larvae often surpass the filters and enter the cooling systems of motor boats. Consequently, zebra mussels grow up within the cooling system of boats and damage the engine. In 2013 I visited a boat yard and inspected various types of boat engines and cooling systems. Based on this insight, I recommend that this potential vector should be examined more closely, taking different types of cooling systems and boating practices into account. However, as dreissenid larvae were found to have very low survival rates in their natural environment (Wacker 2010), it may be rather unlikely that the translocation of larvae by recreational boats leads to the establishment of a new mussel population in an unifested lake.

Chapter 3: Behavior matters

In this chapter I show how a social science study, combined with ecological expertise can greatly help to design preventive measures. In Switzerland, a high proportion of boat owners clean their boat, either before they transport it overland or after they have detected mussels growing on their boat. Boat cleaning should reduce the potential of recreational boats to spread zebra and quagga mussels overland. Nevertheless, boat owners often do not use the appropriate cleaning methods, such as high pressure washing, in order to remove all mussels from the boat exterior. In chapter 3, I show that the motivation of boat owners influences their cleaning behavior. It is not only the technical effectivity of a cleaning method (Morse 2009; Rothlisberger et al. 2010; Comeau et al. 2011), but also the psychology of boat owners that has to be taken into account. Consequently, I recommend that information campaigns are carried out to change the perception of boat owners, in order to increase boat cleaning rates and the use of appropriate cleaning methods. Such an information campaign should convey that a) boat cleaning is beneficial, b) boat cleaning is inexpensive and c) appropriate boat cleaning helps to keep water bodies free from harmful invasive species. Besides supplying boat owners with information on the benefits of boat cleaning, high pressure washing facilities and the necessary instructions need to be provided by authorities at all water bodies potentially infested with quagga mussels. I communicated our findings from the chapters 2 and 3 to authorities and the public, which is an important step in the process of environmental problem solving (Kueffer et al. 2012). Based on these inputs, authorities are now discussing first steps in order to prevent the further spread of the quagga mussel in Switzerland. It would be a highly interesting follow-up study, and

most likely the first time, to re-assess boat cleaning rates, boat cleaning motivation and practices after recommended measures have been applied for some time.

Chapter 4: New tools for early detection and monitoring

The eDNA application presented in chapter 4, using traditional PCR, is able to detect zebra and quagga mussels reliably, when studied on a regional geographic scale. The presented method is inexpensive and only a minimum of laboratory equipment is needed, like a simple PCR-cycler and a gel casting system. Therefore, it can be easily applied as a monitoring tool to detect the presence of zebra and quagga mussels. In Switzerland such a tool may be highly useful to monitor the future spread of quagga mussels. Samples should be taken periodically in those water bodies, where an invasion of quagga is expected. Once established, the relatively simple method allows to process many samples in a short time and at low cost (Jerde et al. 2013; Sigsgaard et al. 2015). Therefore, it may also be applied by environmental offices or governmental (in Switzerland Cantonal) laboratories. The eDNA quantification of zebra and quagga mussels, as a proxy for biomass on a regional geographic scale, needs further validation but is clearly an option for practical application in the future (Lodge et al. 2012). In general, species specific surveillance and monitoring using eDNA could be easily applied for many species of interest. It may not only be used for the early detection of harmful species but also for other cryptic organisms, like endangered species or the assessment of various kinds of biosecurity risks (Thomsen et al. 2012b; Lodge et al. 2012).

The potential utility of eDNA reaches much further. The use of universal primers in combination with next-generation sequencing allows the detection and possibly quantification of multiple species from eDNA sample and is generally referred to as metabarcoding (Taberlet et al. 2012; Thomsen et al. 2012b). With this method the detection and surveillance of whole communities can be done without the need for taxonomic expertise. However, metabarcoding has two major drawbacks compared to targeted eDNA detection: variability in primer efficiencies for different species (Deagle et al. 2014) and dependence on the barcoding databases which may be incomplete (Kvist 2013). Nevertheless, metabarcoding has already been used for various applications, such as the detection of rare fish species (Thomsen et al. 2012a), or the assessment of the impact of salmon farming on the benthic foraminiferal community (Pochon et al. 2015). Other researchers use metabarcoding for the development of eDNA-based biomonitoring tools (Elbrecht and Leese 2015), with the long-term objective to use these methods for the assessment of freshwater

biodiversity in the context of the European Water Framework Directive. However, in the short to mid-term time frame species targeted methods as we have demonstrated them in chapter 4, may be more readily applied for the purposes of freshwater management.

Concluding remarks

In the thesis at hand, I used an interdisciplinary approach to answer some of the a priori suspected knowledge gaps, and I show how we filled some of these gaps. The interdisciplinary approach helped to tackle important issues, which need to be solved in order to ensure good practice for the management of invasive zebra and quagga mussels in Switzerland.

When comparing the environmental tolerance of zebra and quagga mussels I did not find support for our initial hypothesis that quagga mussels have an inherently higher tolerance towards oxygen depletion. Although our experimental results did not directly explain the environmental niche differences of zebra and quagga mussels observed in field studies, they raised the important question whether rapid local adaptation may play an important role for the zebra and quagga mussel invasion. I recommend that this line of research should be followed up so that the role of evolutionary processes can be considered more precisely when predicting the future zebra and quagga mussel invasion. Taken together with observations from other scientists, I conclude that quagga mussels may also colonize habitats which zebra mussels were not able to colonize (see discussion of chapter 1). They may not only replace zebra mussels but also trigger additional negative ecological and economic impact. Consequently, water managers and authorities should stay alarmed.

I also described an early warning system for zebra and quagga mussels using an eDNA approach, which may be put into practice in a joint effort of authorities, cantonal offices, environmental offices and the Swiss Federal Institute of Aquatic Science and Technology (Eawag). The early detection of potential invaders is one of the key issues for the management of invasive species and is indispensable for the application of preventive measures (Caffrey et al. 2014). Similarly to our example, eDNA detection and surveillance methods may also be established for other species expected to become invasive. The method might be used to scan periodically taken water samples for a whole series of potential invaders.

As we have detected the eDNA of quagga mussels in the harbor of Basel, the implementation of preventive measures against the further spread of quagga mussels

is urgent. Also the European Inland Fisheries and Aquaculture Advisory Commission highlighted the importance of research on effective IAS control strategies and of rapid responses once a potentially harmful invasive species is detected in a region of interest (Caffrey et al. 2015). Combining a social science approach with invasion ecology, I was able to show that recreational boating is an important distribution vector of zebra and quagga mussels. My results also suggest how the spread of these species could be reduced and how such preventive measures could be ensured. I have informed cantonal and federal authorities and the public about the results of this study and the recommended measures. Kueffer et al. (2012) shows that this type of outreach to the public and authorities is a crucial step in problem oriented research (in their paper referred to as the salience challenge). It is now mainly up to the authorities to take up the issue and implement the recommended preventive measures which we would support.

To conclude, I hope that my thesis gives a good example of how problem oriented research approaches may have direct implications for management practices in the field of aquatic invasive species. The transdisciplinary approach I used in my thesis including different research methods from various disciplines was challenging, but also highly rewarding and gave me a good insight how research can help to solve specific environmental problems.

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2008	Post-graduate studies in biology didactics, ETH Zürich
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Research experience:

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2008 - 2010	Scientific assistant for teaching and research at the department of aquatic ecology at EAWAG, Dübendorf, Switzerland
2004	M.Sc. thesis: "Dynamics of cell cycle proteins in Schwann cells" at the Institute of Cell Biology, ETH Zürich, Supervisor Prof. Ulrich Suter.

Further working experience:

2008 - 2010	Executive board of the young green party, Zürich, Switzerland
2007	Project leader for the environmental organisation Veraldarvinir in Reykjavik, Iceland
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1998 - 2000	Internship at Biotechnology laboratory of Cilag AG, Schaffhausen

List of publications:

De Ventura L, Weissert N, Tobias R, Kopp K, Jokela J (2016b) Overland transport of recreational boats as a spreading vector of zebra mussel *Dreissena polymorpha*. *Biological Invasions* 18: 1451–1466, doi: 10.1007/s10530-016-1094-5

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De Ventura L., Weissert N., Tobias R., Kopp K. and Jokela J., Analysis of boat cleaning behaviour in recreational boating and the potential effect on the spread of non – native species.

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Sarpe D., Mandemakers J., De Ventura L., van Donk E., Dionisio Pires L. M., de Senerpont Domis L., The modest and the demanding: Quagga and zebra mussel stoichiometry.

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2011 De Ventura L., Kopp K. and Jokela J. “Aquatic neozoa: Future distribution of the invasive Quagga mussel in Switzerland", 3 Years PhD project, discretionary fund, Swiss Federal Institute of Aquatic Science and Technology, EAWAG, Switzerland

Selected conference contributions

2015 De Ventura L., Sarpe D., Kopp K. and Jokela J. 2014, Rapid physiological adaptation to low oxygen in invasive populations of both quagga and zebra mussels, 9 Symposium for European Freshwater Sciences (SEFS), Geneva, July 5-10, 2015

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2014 De Ventura L., Weissert N., Tobias R., Kopp K. and Jokela J. 2014, Recreational boating: an overland distribution vector for the invasive freshwater mussel *Dreissena polymorpha*, talk at the Annual Meeting of the German Society for Limnology (DGL), Magdeburg, 29. 9 – 2. 10 2014

2013 Eawag symposium

Invited speaker:

2015 The Quagga mussel invasion to Switzerland, Lunch Seminar, Rotary Club, Wetzikon, Switzerland

2015 The Quagga mussel invasion to Switzerland, Group Seminar, Institute of Limnology, Konstanz, Switzerland

2014 Predicting the Quagga mussel invasion to Switzerland, Department Seminar, Swiss Federal Institute for Forest, Snow and Landscape Research, WSL, Birmensdorf, Switzerland

Stakeholder outreach activities during my PhD-project

2015 Publication of a factsheet on reacreational boating as a distribution vector for zebra mussels: L. De Ventura, K. Kopp, A. Bryner, Freizeitboot-Transporte verbreiten gebietsfremde Arten in Gewässern, Eawag, Switzerland, 2015

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