DOCTORAL THESIS

Management of Freshwater Aquatic Invasive Species (AIS)

A thesis submitted in fulfilment of the requirements
for the degree of
Doctor of Philosophy (Ph. D.)
in
Evolutionary Biology and Ecology
at the
Department of Biology, University of Ferrara

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November, 2018

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DOTTORATO DI RICERCA
IN BIOLOGIA EVOLUZIONISTICA ED ECOLOGIA
(XXXI CICLO)

Gestione delle specie aliene invasive acquatiche

Settore Scientifico Disciplinare BIO/05

Anni 2015/2018

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DECLARATION AND STATEMENTS

The candidate confirms that the work submitted is her own, except where work which has formed part of jointly-authored publications has been included. The contribution of the candidate and the other authors to this work has been explicitly indicated in each concerning chapter. The candidate confirms that appropriate credit has been given within the thesis where reference has been made to the work of others.

Iva Johović developed the idea, conducted the experiments, analyzed the data and wrote the manuscript. Elena Tricarico, Alberto Inghilesi and Felicita Scapini formulated the idea, supervised the research and contributed to writing the manuscript. Camilla Verrucchi provided assistance in conducting the experiments. Lorenzo Lazzaro provided advice with statistical analysis.

ETHICAL STATEMENT OF COMPLIANCE WITH STANDARDS OF RESEARCH INVOLVING ANIMALS AND WORK IN PROTECTED AREAS

The study involves animals as the focus of the research subjects and all applicable international, national and institutional guidelines for the care and use of animals were followed. Study also included work in protected area, in particular Sites of Comunity Importance SCIs: “Lago di Sibolla” and “Bosco di Chiusi – Paduletta di Ramone”, of heterogeneous wetland mosaics with habitats of EC interest (3150, 3160, 7150, 7210) and all necesary authorisations were obtained for the duration of the whole research.

ACKNOWLEDGMENTS

Sincere thanks go to my supervisors, Elena Tricarico and Felicita Scapini. You have offered guidance and support when needed, but also allowed me freedom to make my own mistakes. I also thank other researchers at the University of Florence and University of Évora for continued general support and feedback, especially Alberto Inghilesi, Pedro Anastácio, Mafalda Gama, Filipe Banha and Annamaria Nocita.

I am extremely grateful for a Marie Skłodowska-Curie actions (MSCA) scholarship which fully funded this research.

Thanks to my fellow PhD students at the University of Florence, University of Oviedo and University of Swansea: Roberta Skukan, Philip Haubrock, Teja Muha, Matteo Rolla, Marta

Rodriguez-Rey, Anaïs Rey and Sabine Rech for always being available for thoughtful discussion and reassuring conversation.

Lastly, I am indefinitely grateful for the support of my family and friends. Mum, Andreja, Tomislav, Gordana and Tim, your persistent support has given me a solid foundation and the confidence to complete this PhD. Pasquale, Steffano, Elisa, Paula and Sarah thanks for making me feel at home in Florence.

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ABSTRACT

English version

Across the globe, invasive alien species (IAS) are the second major threat to biodiversity. Freshwater ecosystems are among the richest ecosystems on earth and today their biodiversity is declining at greater rates than the most affected terrestrial ecosystems. The negative ecological and economic effects of invasive species on the world's ecosystems have been so profound that some biologists have suggested that we are entering a new era, the Homogocene. Homogocene represents a time period in which all of the continents are connected into a "New Pangaea" through human activities that allow species to move between land masses in a way that they were not able to do before. This has caused a striking increase in the spread of invasive species and further rise is expected in the future. Preventing the introduction of IAS is considered the most environmentally preferable and cost-effective form for their management following the hierarchical approach proposed by the Convention on Biological Diversity and adopted in the recent EU Regulation 1143/2014 on invasive alien species which came into force in January 2015. Prevention of the introduction is proposed as the first step in CBD's hierarchical approach, followed by prevention of the establishment of new introduced species, where an immediate eradication is considered the preferable solution and, if that is not feasible, as a third step, containment and long-term control measures should be implemented together with mitigation actions.

This PhD thesis focuses on aquatic invasive species (AIS) – species that have evolved to live primarily in aquatic habitats and novel tools and measures for their prevention and control. The thesis is organized in four parts. It assessed current state of management of selected AIS (Chapter 1), showed how to create risk maps for invasive species and provided the example how risk maps can assist in prevention of invasive species spread and in conservation decision making (Chapter 2). Then it examined the effectiveness of existing control measure (Chapter 3) and conducted research to develop novel control method (Chapter 4 & Chapter 5). This thesis highlights the complexity of the management of two AIS, *P. clarkii* and *L. catesbeianus* and, although the studied species differ, offers recommendations to improve prevention, new control method for crayfish control, and suggestions to improve the efficacy of traditional control methods. Discussed tools, measures and recommendations comply with the vision of IAS management by both EU and CBD. Risk maps allow effective management plan to be developed in advance of invasion, success of trapping –main

traditional technique to manage invasive crayfish species– can be improved in line with new findings and novel method – releasing manually sterilized males– offers species-specific technique that causes no environmental contamination and non-target impacts. Set of carefully planned management responses (covering both prevention and control/eradication) that can be safely applied against invasive species in different environments, would offer a solid strategy to fight the worst invasive species and restore native biodiversity.

Italian version

In tutto il mondo le specie aliene invasive (IAS) sono la seconda principale minaccia alla biodiversità. Gli ecosistemi di acqua dolce sono tra i più ricchi ecosistemi sulla terra e oggi la loro biodiversità è in declino con un tasso di gran lunga maggiore rispetto agli altri ecosistemi terrestri interessati dal fenomeno. Gli effetti negativi, sia in termini ecologici che economici, sugli ecosistemi a livello mondiale dovuti alle IAS sono stati così intensi che alcuni biologi hanno suggerito che stiamo entrando in una nuova era, detta "Homogocene". L' Homogocene rappresenta un periodo in cui tutti i continenti sono connessi tra loro in una "Nuova Pangea", attraverso le attività umane che permettono alle specie di muoversi tra i continenti come non erano in grado di fare prima. Questo ha causato un forte incremento della diffusione di specie invasive e un ulteriore aumento è previsto in futuro. Prevenire l'introduzione di IAS è considerata la forma preferibile di gestione delle IAS in termini di impatto ambientale e di costi secondo l'approccio gerarchico proposto dalla Convenzione sulla Diversità Biologica adottata anche nel nuovo Regolamento Europeo 1143/2014 sulle specie aliene invasive, entrato in vigore nel gennaio 2015. Dopo la prevenzione, una pronta individuazione e una eradicazione rapida è considerata la migliore soluzione e, in caso tale misura non sia fattibile, come terzo punto è previsto che il contenimento e il controllo a lungo termine siano messi in pratica assieme ad azioni di mitigazione degli impatti.

Questa tesi di dottorato si è occupata di specie invasive acquatiche (AIS) e metodi e misure per la loro prevenzione e controllo. La tesi è organizzata in quattro parti. E' stato valutatol'attuale stato dell'arte nella gestione di AIS selezionate (Capitolo 1) ed è stato esaminato come creare mappe di rischio per le specie invasive, fornendo esempi su come queste possano essere di aiuto nella prevenzione della diffusione di specie invasive e nelle politiche di conservazione (Capitolo 2), In seguito sono stati esaminati l'efficacia delle misure di controllo esistenti (Capitolo 3) e sono state condotte ricerche per sviluppare nuovi metodi di controllo (Capitoli 4 e 5). Questa tesi sottolinea la complessità della gestione di due AIS, *P. clarkii* and *L. catesbeianus*, e offre raccomandazioni per il miglioramento della prevenzione e nuovi metodi di controllo per il gambero rosso, e suggerimenti per migliorare l'efficacia dei metodi di controllo tradizionali. I metodi, le misure e le raccomandazioni discusse soddisfano la visione della gestione delle IAS sia della UE che della CBD. Le mappe di rischio permettono un efficiente piano di gestione da sviluppare prima dell'invasione, il successo del trappolaggio – la principale tecnica tradizionale per la gestione di specie invasive di gambero – può essere migliorato alla luce dei nuovi risultati, e un nuovo metodo – il rilascio di maschi sterilizzati manualmente – offre una tecnica specie-specifica che non determina contaminazioni ambientali o impatti indesiderati. Una serie di risposte di gestione accuratamente pianificate (comprendenti sia la prevenzione che il controllo/eradicazione) che possono essere applicate con sicurezza contro le specie invasive in diversi ambienti, offrono una solida strategia per combattere le peggiori specie invasive e ristabilire la biodiversità nativa.

CHAPTER 1. GENERAL INTRODUCTION

Invasive alien species (IAS) are, based on definition by the United Nations Convention on Biological Diversity (1992), –species introduced from elsewhere whose introduction and/or spread outside their natural past or present distribution threatens biological diversity. IAS are found in all taxonomic groups. They include viruses, fungi, algae, mosses, ferns, higher plants, invertebrates, fish, amphibians, reptiles, birds and mammals (Kaur et al., 2014) that are among the greatest threats to global biodiversity. Their numbers are increasing rapidly (Sala et al., 2000; Seebens et al. 2017) causing global biodiversity loss and environmental change in habitats, communities and ecosystems (Mack et al., 2000), and are economically expensive (Leung et al., 2002). IAS have invaded and affected native biota and habitats on all continents and caused hundreds of extinctions, especially under "island" conditions, either in real islands or in ecological islands, such as aquatic ecosystems (McNeely, 2001). As such, they are considered priority issue under United Nations Convention on Biological Diversity (2010), with Aichi Biodiversity Target 9 declaring that "By 2020, invasive alien species and pathways are identified and prioritized, priority species are controlled or eradicated, and measures are in place to manage pathways to prevent their introduction and establishment". EU recently developed a framework to address the threats posed by IAS and Regulation No. 1143/2014 on IAS came into force in January 2015 (European Commission, 2014). The rate of biological invasions is generally increasing, and it may exacerbate even more with climate change (Caffrey et al., 2014) with greater needs than ever to assess the risk posed by IAS, predict and prevent future invasions and stop the spread of already established populations.

In this introduction, I provide a background to IAS, strategies and recommendations given by international policies on how to address them, and present available control methods in management of alien crayfish species that are considered one of the most successful IAS in freshwater environments.

1.1. A BACKGROUND TO INVASIVE ALIEN SPECIES

Humans have transported and traded animal and plant species for centuries. It is possible to track human mediated transport as far as back to the end of Middle Ages and period associated with European rediscovery of Americas and global exploration that have led to radical movements of species between the Old World and the New world (Hulme, 2009). A second increase coincides with the Industrial Revolution, when trade improved by the construction of canals, highways and railways; the highest rates of introductions occurred in the last 25 years and globalization (Hulme, 2009). The rapid growth of international trade, transport and tourism has caused a striking increase in the translocation of species (Hulme, 2006; Hulme, 2009). Further raise of translocations is expected in the future, with the raise of economic and ecological consequences. Most important factors limiting the deliberate distribution of species through history and today showed to be whether a species possessed traits valuable for domestication, or, for a species that is not introduced deliberately, whether its traits allow it to be transported by human vectors (Ricciardi, 2007).

This time period we live in, in which geographic barriers are being removed and all of the continents are connected into a ‘‘New Pangaea’’ through human activities that are allowing species to move between land masses causing loss of global biodiversity and regional homogenization, is metaphorically called the Homogocene (Strayer, 2010).

IAS, or species introduced from elsewhere who threaten biological diversity, have attracted the attention of scientists since 18th century, yet invasion biology came to be a field only after the book ‘‘The Ecology of Invasions by Animals and Plants’’, published in 1958 by Charles Elton (Thomaz et al., 2015). In the past two decades, there has been an explosion of research interest in human-mediated invasions, that helped us in our understanding of the invasion processes (Blackburn et al., 2011) and prepared the information for creation of international policies to combat IAS. Elton’s book lacked the precise definition of “invasive species” (Pereyra, 2016) and several definitions have been in use since then, proposed by several authors; Richardson et al. (2000) suggested that authors should provide a definition of invasive species in their articles to avoid controversies. In this thesis earlier-mentioned definition of IAS from United Nations Convention on Biological Diversity (CBD) will be used in which IAS is a species whose introduction and/or spread outside their natural past or present distribution threatens biological diversity.

In order for an introduced species to become invasive, it must progress through a series of stages of the invasion process, yet there is little agreement as to how these stages should be distinguished and named. Two main frameworks by Richardson (2000) and Williamson (1996) are adopted by the majority of animal and plant ecologists; Blackburn et al. (2011) merged these two and suggested a united framework that better clarifies invasion process. That framework recognizes:

transport, introduction, establishment and spread as main stages of invasion process (Blackburn et al., 2011; Figure 1.1.).

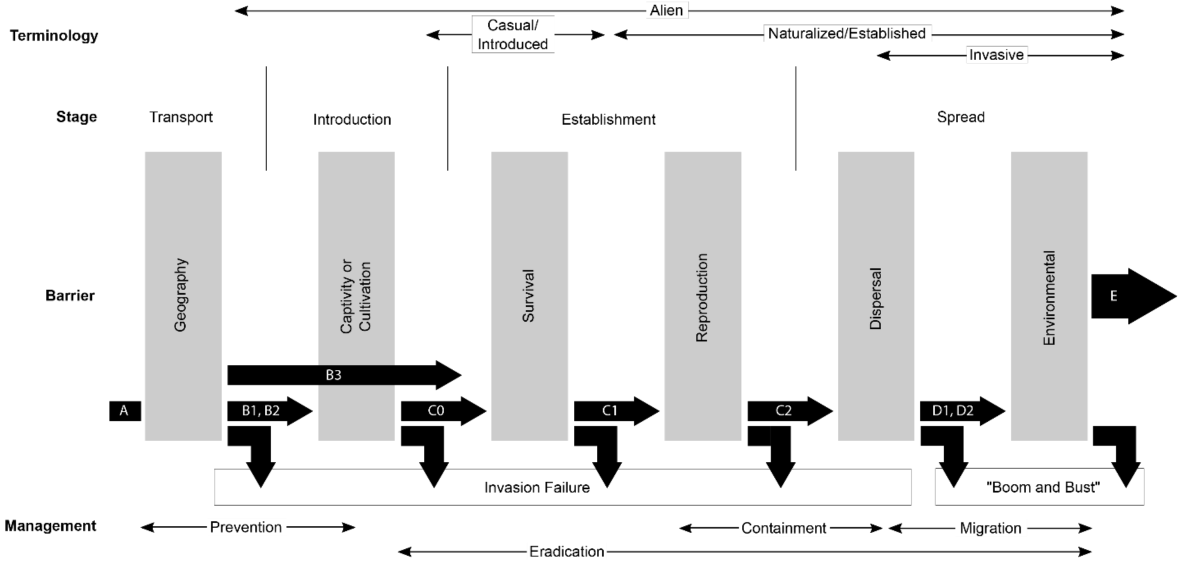

Figure 1.1. Stages of invasion process adapted from Blackburn et al. (2011). The diagram shows stages of the invasion process that species need to pass through in order to become invasive in a recipient ecosystem. Letters A to E represent populations: A - not transported beyond limit of native range; B - transported beyond limit, but in captivity (B1), cultivation (B2) or directly released (B3); C - individuals released into the wild, but incapable of surviving (C0), reproducing (C1), not self-sustaining (C2) or self-sustaining (C3); D – self-sustaining in the wild and surviving significant distance from the original point of introduction (D1) and reproducing (D2); E - fully invasive species with individuals dispersing, surviving and reproducing

Each stage is characterized by a series of environmental, reproductive and dispersal related barriers; it was estimated, as the “rule of tens”, that only as few as ten percent of transported species will appear in wild, ten percent of those will become established and again ten percent of those will become invasive (Williamson & Fitter, 1996). In short, from the initial number of species moved beyond their native range, the majority pose no threat for the recipient ecosystem. The organisms that overcome those barriers generally evolve from a period of low abundance followed by a rapid increase of abundance and then a period of stabilization (Zaiko et al., 2014).

Propagule pressure, also termed “introduction effort” is one of the key elements in understanding why some species fail to establish, while others succeed (Lockwood et al., 2005). Introduction effort represents the number and frequency of individuals arriving in a recipient region (Colautti et al., 2006; Simberloff, 2009a) and it is emerging as a consistent predictor of establishment success. High propagule pressure enhances establishment success by increasing genetic variability (Lockwood et al., 2005) and the likelihood that the introduced individuals will overcome the environmental stochasticity and survive (Simberloff, 2009a).

Pathway is another important element in understanding of biological invasion phenomenon. Pathway is any means (e.g. vector, route) that allows the entry or spread of IAS to some region. Their contribution is changing in the importance over time (Essl et al., 2015). Understanding pathways, how they underlay and mediate biological invasions and their shift in importance also has important role has important role in effective management of IAS.

1.1.1. Invasions in freshwater environments

The richest ecosystems on earth are freshwater ecosystems (Abramovitz, 1996). Despite the fact that rivers, lakes, and wetlands occupy less than 1% of the Earth's surface and only 0.01% of the World's water (Dudgeon et al., 2005), freshwater ecosystems support ~10% of all known species (Strayer and Dudgeon, 2010).

Inland waters are now also experiencing declines in biodiversity far greater than those in the most affected terrestrial ecosystems (Sala et al., 2000; Dudgeon et al., 2005; Vaughn, 2010), and at least 20% of all freshwater species are either extinct or at risk of extinction (Abramovitz, 1996). As a natural resource, they contribute about 20% (about US$6.6 trillion) to the estimated annual global value of the entire biosphere (Gherardi, 2010), which makes them also an enormous economical resource. Preserving their remaining biodiversity is challenging in part due to the high susceptibility of freshwater ecosystems to biological invasions (Wilcove et al., 1998; Havel et al., 2015).

As a result of thousand years of human exploitation of these waterways for food, commerce, and recreation, hundreds of freshwater species, including fish, crayfish and amphibians have deliberately been moved outside their native ranges (Tricarico et al., 2016), mainly because of their economic importance (Fenoglio et al., 2016). Even the distribution of species that are today regarded as native to a given region appears to owe much to human intervention (Gherardi, 2010). The earliest recording of a freshwater fish introduction in most European countries is that of the common carp *Cyprinus carpio* (Copp et al., 2005) which was introduced into western Europe approximately 2,000 years ago, most probably facilitated by the Romans (Balon, 1995). Historical records and genetic studies suggest also that the white-clawed crayfish *Austropotamobius pallipes,* which is now protected under the European Union (EU) Nature Directives, seems to have been moved to Ireland from France by monastic orders in the 12th century (Gherardi, 2010). The introduction of aquatic invasive species (AIS) –invasive alien species (IAS) that have evolved to live primarily in aquatic habitats– intensified in Europe during the mid- to late 19th century under the promotion of the 'acclimatisation' societies (such as the Société Impériale Zoologique d'Acclimatation in France and Society for Acclimatisation of Animals, Birds, Fishes, Insects and Vegetables in the United

Kingdom). Those societies which did not understand dangers of animal introductions introduced species with the purpose of ‘enriching’ environments that were considered “deficient”.

Transportation of animals outside their natural ranges accelerated greatly as methods of transportation have improved and trade barriers relaxed (Copp et al., 2005). Today, the country with the highest number of unintentional alien introductions in Europe is Italy, due to ineffective or absent controls on legal and illegal introductions and translocations, while Germany, which is considered the country with the longest history of importing alien freshwater fishes, has the lowest number of intentional introductions (Copp et al., 2005).

The Millennium Ecosystem Assessment (2005) revealed that freshwater ecosystems had experienced a high impact from invasive alien species over the past century and that these impacts were continuing to rapidly rise relatively to other ecosystems. IAS have been reported as one of the major contributors to freshwater biodiversity loss today (Collen et al., 2014).

From 481 freshwater invasive alien species registered in DAISIE (Delivering Alien Invasive Species Inventories for Europe) database, one third is known to cause ecological and economic impacts, compared to only ~16% of 1071 marine invasive alien species, which raises particular concern for native freshwater organisms (Vilà et al., 2010). The World Wildlife Fund (WWF)’s Living Planet Report of 2014 showed that populations of freshwater species (76%) declined more rapidly between 1970 and 2010 than marine (39%) and terrestrial (39%) populations.

Freshwater ecosystems appear more vulnerable to introduced predators than are marine and terrestrial ecosystems because of fewer defence mechanisms and greater naïveté of native organisms toward novel predators (Cox & Lima, 2006; Vilà et al., 2010). Inland waters are also particularly vulnerable to the introduction of IAS due to their exposure to human activities related to multiple transmission vectors and pathways along which new species can be either accidentally or intentionally introduced, such as extensive aquaculture, fishing and leisure (those activities resulted collectively responsible for almost 40% of aquatic species introductions into Europe; Gallardo & Aldridge, 2013). Besides, the ecological resilience and recovery potential of freshwater systems have been already reduced by human caused degradation (e.g. pollution, habitat destruction), increasing the likelihood that alien species will successfully invade these systems (Dudgeon et al., 2006).

DAISIE reflects the foresight of the European Commission in identifying the need for an inventory of alien species (Vilà et al., 2010). From 12,122 alien species present in the list, experts identified "100 of the worst" European invasive species list, of which 15 are freshwater organisms (Table 1.1.) and only the subphylum Crustacea (4), superclass Osteichthyes (3), phyla Mollusca (2) and Magnoliophyta (2) are represented with multiple species.

Table 1.1. Worst European freshwater invasive species by taxa (DAISIE, 2016)

Taxa	Species in the DAISIE list (n)	Species
Crustacea	4	Fishhook waterflea, *Cercopagis pengoi* Killer shrimp, *Dikerogammarus villosus* Chinese mitten crab, *Eriocheir sinensis* Red swamp crayfish, *Procambarus clarkii*
Osteichthyes	3	Round goby, *Neogobius melanostomus* Topmouth gudgeon, *Pseudorasbora parva* Brook trout, *Salvelinus fontinalis*
Mollusca	2	Asian clam, *Corbicula fluminea* Zebra mussel, *Dreissena polymorpha*
Magnoliophyta	2	New Zealand pigmyweed, *Crassula helmsii* American waterweed, *Elodea canadensis*
Cnidaria	1	Freshwater hydroid, *Cordylophora caspia*
Nematoda	1	Swim-bladder nematode, *Anguillicola crassus*
Chromista	1	Crayfish plague, *Aphanomyces astaci*
Platyhelminthes	1	Salmon fluke, *Gyrodactylus salaris*

Assessment of impact -on ecosystem services- between alien plants, vertebrates and invertebrates in terrestrial, freshwater and marine environments showed that freshwater invaders exhibit a large number of different impact types per species, with nine each reported for zebra mussel (*Dreissena polymorpha*), brook trout (*Salvelinus fontinalis*) and red swamp crayfish (*Procambarus clarkii*) (Vilà et al., 2010).All three representatives of different freshwater taxons showed to mainly affect supporting, regulating, and, ultimately, provisioning services of aquatic ecosystems (e.g., zebra mussels through the alteration of water quality and bioaccumulation, brook trouts through altering native populations of salmonids through hybridization and reduction in numbers of other freshwater taxa (Vilà et al., 2010), red swamp crayfish through reductions in valued edible native species, induction of wide changes in ecological communities and increased costs to agriculture and water management (Souty-Grosset et al., 2016).

Alien species can also interact with one another and facilitate each other's establishment and/or continued existence, leading to accelerated impacts on native ecosystems (Simberloff & Von Holle, 1999). Presence of co-evolved alien fish, such as centrarchid sunfish, can facilitate the invasion of American bullfrog, *Lithobates catesbeanus*, contributing to the decline of native amphibians, especially because adult and juvenile bullfrogs are unpalatable to them, giving them competitive advantage (Adams et al., 2003). Some freshwater invaders, such as North American crayfish and American bullfrog, act as vectors of other freshwater invaders facilitating their spread. North American crayfish species are the native hosts of *Aphanomices astaci*, the causative agent of crayfish plague (Martín-Torrijos et al., 2017), while American bullfrog has been linked to the spread of the causal agent of amphibian fungal skin disease chytridiomycosis by *Batrachochytrium dendrobatidis*

(Bd) (Yap et al., 2018). Crayfish plague has devastated native European crayfish populations and enhanced the survival of their vectors, which gave them competitive advantage, since they are resistant to infection. Epidemiology of Bd infection and its impact on wild amphibians at the population level have not yet been elucidated. Mortality and declines due to chytridiomycosis depend on environmental context and in some species (e.g. common midwife toad *Alytes obstetricans,* Laurenti, 1768) they were evident in high altitude regions while at lower altitudes animals were infected in the absence of declines (Walker et al., 2010). More recently, mortality has been detected in the endangered Sardinian newt *Euproctus platycephalus* (Gravenhorst, 1829) which has been in decline since the early 1980s and Tyrrhenian painted frog *Discoglossus sardus* (Tschudi, 1837) (Duffus & Cunningham, 2010).

1.1.2. Invasive alien crayfish

Freshwater crayfishes belong to the decapod infraorder Astacidea (Lodge et al., 2012). Presently there is 644 known species of crayfishes (Gherardi, 2010) with an average of five–ten species still being described each year (Crandall & Buhay, 2007). Crayfishes are classified into two major groups that are geographically and morphologically distinct: Astacoidea (families Astacidae and Cambaridae) and Parastacoidea (single family Parastacidae) (Lodge et al., 2012). The Astacidae are distributed in Europe and the Pacific drainage of North America, and the Cambaridae are in both East Asia (Asian Cambaridae) and the Atlantic drainage of North America (American Cambaridae) (Kawai & Saito, 2001).

The freshwater crayfishes are distributed across all the continents but the African and Antarctic ones (Taylor, 2001), in a wide range of habitats including rivers, streams, ponds, lakes, wetlands and caves (Crandall & Buhay, 2007). Crayfish typically acts as a keystone species in freshwater communities (Ilhéu et al., 2007), interacting with organisms at multiple trophic levels, acting as both predator and prey, and affecting ecosystem processes through their omnivorous feeding behaviour (Geiger et al., 2005). They also act as ecosystem engineers modifying the structure of the habitat for other organisms by burrowing into the sediment (Johnson et al., 2011) and affecting population and community structure of benthos or egg survival of gravel-breeding fish (Statzner et al., 2000).

Crayfish have been of interest to humans throughout history namely as a food source for commercial harvest (Gherardi, 2011) and bait for predatory fish (Reynolds, 2011); crayfish fragments and gastroliths have been found in 28,000 years old aboriginal cooking hearths in Australia (Gherardi, 2011). This might also explain so many human-mediated translocations of alien crayfish that have occurred intentionally all over the world. Crayfish species have been introduced to various parts of the world for aquacultural purposes (Ackefors, 2000; Holdich et al., 2014), for stocking in lakes and rivers (Ackefors, 2000) and also as an attempt to alleviate poverty in under developed areas (Gherardi, 2006). Frequently, their introductions later result in escapes, or deliberate releases, from cultivated populations or aquaria into the wild, where they often become established in their new territories (Holdich et al., 2014).

Some of the most harmful and widely distributed freshwater IAS are crayfish (Stebbing et al., 2014). Compared to other AIS, the spread of crayfish species is favoured by several characteristics: their uniquely large individual adult size, frequent high abundance and population biomass, and an unusual trophic position as they are extremely omnivorous — many species are capable of predation on other large organisms, including fish and amphibians, herbivory, and detritivory (Lodge et al., 2012). Red swamp crayfish *Procambarus clarkii* (Girard, 1852) can also survive for relatively long periods out of water and to travel long distances (Gherardi, 2007). Although, the number of introduced species that become invasive is on average small, today 46% of all freshwater decapod species in Europe are invasive aliens (Williamson, 1996; Vilá et al., 2010; Hänfling et al., 2011;). Of the 644 currently known and described crayfish species, 28 have established viable populations outside of their native range (Gherardi, 2010). The success of invasive crayfish species is likely to depend on the life history traits of these species, in comparison to the native species, with which they are competing. To understand which biological and ecological traits make some crayfish species prone to invasion, Larson and Olden (2010) performed trait analysis for 77 (involving 13 invasive) crayfish species native to the south-eastern United States and discovered that all species that had previously invaded other regions had more general habitat requirements, larger adult size and higher fecundity compared to assessed non-invasive crayfish species.

Today, some of the most successful aquatic invasive species worldwide belong to the subphylum Crustacea (Devin et al., 2005; Weis, 2010; DAISIE, 2016) (Table 1.1.): more than half of all invasive freshwater species within Europe (40) and more than a third within North America (29) are crustaceans (Karatayev et al., 2009). In the Upper Rhine River, Germany, crustaceans made up about 50% of alien species (Bernauer & Jansen, 2006), which accounts for 13 species of decapods, in both North America and Europe.

Compared to others alien species in Europe, non-indigenous crayfish species or NICS (species of crayfish that don’t occur naturally in some region) showed to be much more successful in their establishment in Europe, resulting with the establishment of nine of the ten species introduced in Europe (Holdich et al., 2009). While globally invasive crayfish species, which came into view as causing biggest concerns, are: the red swamp crayfish, signal crayfish (*Pacifastacus leniusculus*), spiny-cheek crayfish (*Orconectes limosus*), rusty crayfish (*Orconectes rusticus*), Turkish crayfish (*Astacus leptodactylus*) and common yabby (*Cherax destructor*) (Souty-Grosset et al., 2006), the five main invasive crayfish species in Europe are: the marbled crayfish (*Procambarus virginalis*), red swamp crayfish , signal crayfish, spiny-cheek crayfish and virile crayfish (*Orconectes virilis*), now all included in the list of the 49 "invasive alien species of Union concern" (European Union, 2017; Table 1.2.).

Table 1.2. "Invasive alien crayfish species of Union concern" and their native and invasive range (Souty-Grosset et al. 2006; Taylor et al., 2007; Kouba et al., 2014)

Species	Native range	Invasive range
Procambarus virginalis	Florida and Georgia	2 European teritories
Procambarus clarkii	South-central USA and Nothern Mexico	15 European territories
Pacifastacus leniusculus	North-western USA and South-western Canada	29 European territories
Orconectes limosus	North America	22 European territories
Orconectes virilis	North-central USA	2 European teritories

With 29 invaded territories, *P. leniusculus* is the most widespread NICS in Europe, followed by *O. limosus* (22) and *P. clarkii* (15) (Kouba et al., 2014; Table 1.2.). *P. leniusculus* was firstly introduced to Sweden in 1960, *O. limosus* in Germany in 1890 and *P. clarkii* in Spain in 1973 (Ackefors, 2000). These three North American crayfish species were intentionally introduced into European countries by well-intentioned authorities and entrepreneurs, who sought to restore freshwater crayfish numbers in Europe, which had already declined before the arrival of the American species, due to outbreak of crayfish plague and habitat destruction (Gherardi & Holdich, 1999). What they did not know was that all the three species may act as carriers of the plague, when exposed to it. Introduction of NICS in Europe has resulted with detrimental consequences, bringing population decline in native crayfish species to up to 80% (Richman et al., 2015). Studies on ecological impacts have shown that invasive crayfish often enhance the effects of other stressors, e.g. habitat loss and degradation, pollution and overexploitation on decline of crayfish populations, due to their competitive superiority over native species and coupled with reproductive interference (Gherardi, 2007). In invaded ecosystems, introduced North American crayfish species represent the largest invertebrates and directly impact prey or competitor species, which lack efficient defence adaptations to them, and alter their abundance and distribution (Vilà et al., 2010). Community effects on indigenous species can also be caused by indirect mechanisms, for instance, through transmission of pathogens or through their omnivory by changing interactions within communities and causing cascading effects on food webs (Gherardi, 2007). NICS are also known to display a wide plasticity in feeding behavior, switching from detritivore/scavenger to herbivore/carnivore in response to availability of food. As an example, in the invasive range of signal crayfish (*P. leniusculus*) it altered community structure by removing eggs, juveniles and adults of several aquatic species (Peay et al., 2009), outcompeted native crayfish populations for food and habitat space (Vorburger & Ribi, 1999), introduced the crayfish plague, which they are resistant to and is lethal to European crayfish species, and caused habitat degradation and flooding by burrowing (Alderman,1993). The species is also linked to rapid decline of native white claw crayfish, *A. pallipes,* in Great Britain (Bubb et al., 2004), which is now listed as “endangered” on the IUCN red list (2018). Crayfish predation in wild populations has been linked also to amphibian declines (Cruz & Rebelo, 2005).

1.2. MANAGEMENT OF BIOLOGICAL INVASIONS

In management of biological invasions an ounce of prevention is worth a pound of a cure (Leung et al. 2002; Mooney, 2005). This has been especially true for aquatic ecosystems where IAS are often not even noticed until their impacts become apparent (Zaiko at al., 2014). Prevention has been emphasized as the most cost-effective way for managing IAS by international policies such as CBD (2010) and “The IAS Regulation” of EU (2014). The CBD (2010) endorses a three-stage approach for IAS management, with prevention of introduction as the first step followed by prevention of the establishment of new introduced species, where an immediate eradication is considered the preferable solution and, if that is not feasible, as a third step, containment and long-term control measures should be implemented together with mitigation actions.

Introductions can be prevented, but intentional and unintentional introductions may require quite different responses, with the intentional introductions demanding effective quarantine and impact assessment, while the unintentional invasions require effective monitoring (McNeely, 2000). Unsurprisingly, many countries have established means of preventing the import of human pathogens and pest species of agriculture and forestry, as the essential minimum of any invasive species management programme (McNeely, 2000). Post introduction management options for IAS populations are eradication, control and containment. They are mainly restricted to early stages of invasion (Genovesi & Shine, 2004; Essl et al., 2011), when populations are at low density or still confined to a small area. While eradication involves removal of the whole population from a specific area, containment involves restricting the range of the alien species population and should limit the spread from an invaded area, while control aims to lead to long-term reduction in IAS population size towards an acceptable level. If none of these options prove possible to apply against the invader, managers must learn to at least mitigate any harmful impacts caused by invasion.

With newly arrived species, early intervention maximizes the probability of effective management (Simberloff, 2001). Considering the CBD principles, in the early stages of invasion, when populations of IAS are small and localized, priority over other measures should be given to eradication. Costs of management later accelerates exponentially, the longer the species is in the recipient ecosystem (Williams et al.,2010). Management and mitigation costs are estimated to be at least 12.5 billions of euros/year in Europe, but probably passing 20 billions of euros/year (Shine et al., 2009). In USA the same costs have been estimated on 120 billions of American dollars/year (Pimentel et al., 2005). Every dollar spent in prevention and control in USA returns on average 17 dollars in prevented expenditure (Caplat & Coutts, 2011).

Eradication of IAS when they get abundant is difficult. In European countries eradication appears to be only occasionally considered, with many limits preventing its wider application as a management option in the region. Genovesi (2005) reported the list of 37 eradication programs recorded in total for Europe, with almost 90% eradications carried out on islands and rat (*Rattus* spp.) and common rabbit (*Oryctolagus cuniculus* Linneaus, 1758) as the most targeted species. There are only rare recent attempts of eradications being performed for plants, alien invertebrates or marine organisms. Legal inadequacy (several IAS are protected by nation laws in some EU countries), scarce resources, lack of concern and public support or even public opposition (Genovesi, 2005) are just some of the reasons why some past invasions have not been stopped in time.

Many species also could be eradicated but due to difficult detection they are often detected too late to be rapidly eradicated or contained. This is true of all IAS but detection of aquatic invaders poses more difficulty since we cannot see them readily and they often get in invasive stage before detected (Zaiko at al., 2014). It is approximated that eradication campaigns should commence within four years of detection, on average, to achieve success (Pluess et al., 2012).

One of the best examples of lack of action in Europe is the case of *Caulerpa taxifolia*. The species was first detected in 1984 in Europe, few meters from the public aquarium in Monaco (Meinesz et al., 2001) and the decision to start an eradication was delayed, due to academic controversy and unclear repartition of roles (Genovesi, 2005), until it was too late. By the end of millennium, species have colonized approximately 131 km^2 of benthos in 103 independent areas along 191 km of coastline in six countries (Spain, France, Monaco, Italy, Croatia and Tunisia) (Meinesz et al., 2001). In contrast, the case of *Caulerpa taxifolia* in California, U.S.A., which was discovered on June 12 2000, can serve as a model of effective rapid response, since it took only 17 days in total to confirm the infestation, include relevant stakeholders in dialog, get adequate funding and mobilize a team working on the treatments (Anderson, 2005). The action costed approximately 7 million American dollars and no further *Caulerpa taxifolia* was seen after two years of intensive monitoring (Simberloff, 2009b).

Today, a wide array of available techniques for removal of the target organisms exists: traps, chemical sprays and baits, detection dogs, host removal, sterile male release, etc., which have varying levels of success. Most techniques in use are still fairly low-tech and outdated and there is a pressing need for development and adoption of innovative control techniques, especially for aquatic environments (Caffrey et al., 2014). Knowledge transfer between scientist and stakeholders engaged in policy and management is of the utmost importance for development of successful management initiatives, such as informing the general public via awareness raising campaigns, who are often keen to participate at the management level, once they understand the problem (Shackleton et al. 2019).

Several initiatives (e.g. Essl et al. 2015; Latombe et al. 2016; Lucy et al. 2016) and projects (e.g. DAISIE, IMPASSE, COST Action TD1209 Alien Challenge) have addressed diverse problems concerning IAS in Europe and worldwide (Tricarico et al., 2017). The Marie Skłodowska-Curie Innovative Training Network (MSCA ITN) "AQUAINVAD-ed" (grant agreement no 642197) was developed to tackle issues concerning vectors of introductions, pathways of despersal, early detection, management and impact of aquatic invaders in Europe. This research project aimed to assess the existing gaps and develop innovative solutions and methods in prevention of introduction and establishment of aquatic invasive species and in their management. The project brought together a multi-disciplinary consortium of experts working in universities, research centres, companies and non-governmental organisations from different European countries to train eight PhD students in UK, Spain and Italy (https://www.aquainvad-ed.com/).

1.2.1. Risk assessment

Given that management of established populations is proven more difficult and expensive, ecologists agree that focus on the prevention of introduction and establishment of IAS in marine, freshwater and terrestrial ecosystems would yield most effective results. CBD highlights the need for prioritization and management of priority species. Prioritization of species is of special importance since resources to manage IAS are often limited. With increasing globalization, the influx of potentially harmful organisms will only continue to increase and a strategy that facilitates the optimal allocation of resources to manage the most harmful IAS in a given area is unavoidable (Kumschick et al., 2012). Decision-support tools are becoming crucial in supporting decision-making processes in environmental management and science today (Copp et al., 2016).

Risk assessment (RA) of alien species is defined as the technical and objective process of evaluating biological or other scientific and economic evidence to identify potentially invasive alien species and determine the level of invasion risk associated with a species or pathway and specifically whether an alien species will become invasive (Roy et al., 2014). Risk assessments can be performed using qualitative, semi-quantitative and quantitative frameworks. The majority of RAs use a qualitative approach, through expert judgement and afterwards a semi-quantitative approach (Roy et al., 2014).

RA protocols are based on the generally accepted premise that species invasive in some parts of the world have an increased chance of being invasive in other areas with similar environmental conditions. RA generally assesses and scores several elements such as the biogeography and history of the species, the presence of 'undesirable traits', species biology and ecology, climate match, presence of potential predators and control methods. This evaluation is mandatory on two levels: to limit new IAS from entering a country and to manage species with high impacts, which already entered the country, in their new range (Kumschick et al., 2012). Attempts to evaluate and prioritize IAS have led to the development of risk screening systems and risk assessments. Most commonly risk screenings are used in risk identification step to evaluate whether the species in question should be subjected to more comprehensive risk assessment (Kolar & Lodge, 2002; Copp et al., 2016).

There is also a lot of different approaches worldwide to evaluate invasion risk: some protocols are more taxon-specific (e.g. FISK for freshwater fish, MFISK for marine fish, FI-ISK for freshwater invertebrates, MI-ISK for marine invertebrates, Amph-ISK for amphibians) and some more taxon-generic that are even above ecosystem level such as AS-ISK (Aquatic Species Invasiveness Screening Kit), which is applicable to virtually all climatic zones and all aquatic plants and animals (Copp et al., 2016). AS-ISK contains 49 basic questions on the taxon's biogeographical and historical traits and its biological and ecological interactions and it follows the "minimum requirements" for use with the new EU Regulation on invasive alien species of EU concern.

Roy et al. (2018) identified and delivered 14 minimum standards (requirements) that a risk assessment scheme should include with reference to the requirements of the EU Regulation on IAS (1143/2014) and international agreements, including the World Trade Organisation, CBD and International Plant Protection Convention, addressing also gaps in existing protocols. These minimum standards are: 1. Basic species description; 2. Likelihood of invasion; 3. Distribution, spread and impacts; 4. Assessment of introduction pathways; 5. Assessment of impacts on biodiversity and ecosystems; 6. Assessment of impact on ecosystem services; 7. Assessment of socio-economic impacts; 8. Consideration of status (threatened or protected) of species or habitat under threat; 9. Assessment of effects of future climate change; 10. Completion possible even when there is a lack of information; 11. Documents information sources; 12. Providing a summary in a consistent and interpretable form; 13. Including uncertainty; 14. Including quality assurance. Although this framework was developed within an EU context, because of necessity for risk assessment protocol to be representative, repeatable, reliable and robust, these standards should be applied globally (Roy et al., 2018).

Vanderhoeven et al. (2017) assessed shortcomings of currently available protocols and recommended improved quality control of risk assessments through formalized peer review to maximize the evidential basis, properly assess the manageability of invasive species and insure efficient conservation resource allocation.

1.2.2. Predicting species distribution and creating risk maps for management planning

Strategic decisions for the management of invasive alien species depend on accurate spatial and temporal characterizations of pest risk (Venette et al., 2010). Species distribution models may be particularly useful in risk analysis of harmful IAS that have not yet spread to all suitable habitats or even entered a country.

Risk maps are visual tools that describe where invasive alien species might arrive, establish, spread, or cause harmful impacts, according to the variables considered in the species distribution models (Venette et al., 2010). Summarizing suitability of novel areas for invading species can be valuable tools for planning of effective conservation management responses, e.g. preventing species' invasions or controlling their spread. The geographic potential of species' invasions can be estimated via distribution modelling procedures.

Species distribution models (SDMs) or niche-based models (NBMs) are correlational techniques aimed at identifying key suites of environmental conditions within which the species is present, based on occurrence data and environmental data stored in geographic information systems (Jiménez-Valverde et al., 2011). Once the SDMs have been considered valuable after evaluation (e.g., cross-validation), the outputs of SDMs can be used to generate risk maps. These final maps are delivered to decision-makers. The authorized risk managers, commonly government officials, take an active role in IAS prevention and control, which is why the information delivered to them has to be of the maximum quality. To ensure the quality and usability of risk maps decision makers and stakeholders should be involved throughout the whole process of modelling (Guisan et al., 2013). The quality of the map is also subjected to the constraints of available knowledge about the biology of the species and the environmental conditions in the area of interest (Venette et al., 2010). All risk maps have an associated error or uncertainty, which is important to account for the interpretation of the results (Jiménez-Valverde et al., 2011). Also, risk maps should be interpreted with a full understanding of what the map is showing, as well as the assumption in the modelling process.

Ensemble models provide a simple technique to deliver robust estimates of suitable habitat for a given species in a given time, combining predictions of single models and decreasing their predictive uncertainty. These models also provide insights to the extent of possible shifts in the range of invasive species and identify the geographic regions that will most likely be at risk, thus helping managers to make informed decisions and assuring most productive use of resources (Gama et al., 2017).

1.2.3. Control of non-indigenous crayfish species (NICS)

Some of the most damaging AIS currently present in Europe are crayfish species, causing massive environmental and economic damage. Burrowing species of NICS, e.g. *P. leniusculus* and *P. clarkii*, may have such an adverse impact on recipient ecosystem that in some cases they are thought to be preventing waterbodies from attaining good ecological status, as prescribed under the Water Framework Directive (WFD) (Holdich et al., 2009). Clearly, there is urgent need to manage NICS. So far, attempts to manage these populations mostly failed short of fulfilling objectives and none of the methods used has provided to be a definitive one, or 'silver bullet' method. Crayfish invasive species are so diverse and have occupied so many various habitat types that one single strategy will hardly be achievable (Freeman et al., 2010). Integrated pest management (IPM) is considered most effective and more likely to lead to successful control of the target crayfish population (Gherardi et al., 2011; Stebbing et al., 2014).

Several different methods have been used to control, contain and/or eradicate invasive populations of NICS. A review of current methods used in management of invasive species of crayfish was conducted. We can classify them in five broad categories as follows: (1) mechanical; (2) physical; (3) biological, (4) biocidal and (5) autocidal control methods (Table 1.3.).

1.2.3.1.Mechanical control

Mechanical management has been proven effective in decreasing density of NICS populations, but eradication with those methods is not achievable (Stebbing et al., 2014). Mechanical control includes the removal of crayfish from water bodies using traps (cylindrical funnel traps, Swedish ‘Trappy’ traps, Evo-traps, collapsible traps, artificial refuges traps, etc.), nets (fyke nets, seine nets, etc.), manual searching and/or electrofishing. These techniques are labour intensive and to be effective need to be used over extended period of time, potentially incurring considerable cost and manpower (Gherardi et al., 2011).

Trapping for crayfish was primarily used for commercial purposes and only later for control. Historical records of collapsed marine and freshwater fisheries due to overharvesting raised the idea to try to intentionally over-exploit invasive crayfish species using fishing techniques (Hein et al., 2007). Several authors note that crayfish traps are highly selective for dominant large adult males (Lodge et al., 1985; Gherardi et al., 2011), thus making it difficult to efficiently trap much of the reproductive population. Compensatory density-dependent processes have also been observed as a response to trapping: the removal of the dominant males consequently may reduce pressure on juveniles giving rise to larger populations (Gherardi et al., 2011), resulting in a reduction in the average size of animals in the population with earlier maturation (Freeman et al., 2010) and production of more eggs by remaining females (Holdich et al., 1999). Populations usually also compensate for the lost numbers with high breeding and high survival rates, due to newly available resources (Stebbing et al., 2014). To raise the efficiency of traps in catching wide reproductive population and eliminate the perceived bias to the removal of large adult males, Stuecheli (1991) recommended using several trap opening diameters for trapping crayfish. Decreasing the mesh size also resulted in traps catching a wider size range of animals (Wright & Williams, 2000). Trapping by using different types of traps would also affect different size classes and sexes; e.g. funnel traps (for catching large males) and artificial refuges traps (ART) (for catching smaller individuals of both genders and female individuals) (Green et al., 2018) could produce similar results. Studies assessing the use of traps for the management of populations of NICS reported decline of populations, but none achieved eradication (Stebbing et al., 2014).

Seine and fyke nets have also been used to catch crayfish. These methods involve greater costs and manpower and are only considered effective in still water sites, where they have been reported to be more efficient than trapping for removing juveniles and ovigerous females (Freeman et al., 2010). In any case, removing ovigerous females is not recommendable, since it may lead to density-dependent feedback mechanisms so that remaining crayfish would probably respond to low numbers in the population by producing more eggs and reaching maturity earlier (Holdich et al., 1999).

Electrofishing has been used as a method of sampling fish for a number of years and it can be used for capturing animals out in the open, but not those in burrows (Stebbing et al., 2014). Westman et al. (1978) noted that electrofishing was effective at removing all sizes of crayfish, but it is not feasible in deep or turbid waters and not efficient in streams with large stones or where crayfish hide in the banks (Freeman et al., 2010). Nevertheless, electrofishing has potential to harm non-target organisms and act as a vector in crayfish spread, since electrocuted crayfish will not all be netted but washed downstream (Freeman et al., 2010).

However, all mechanical control methods are long-term options and for this reason, among techniques, trapping can be considered most advantageous since it does not require any specialist training, can be conducted by anyone and used in variety of habitats.

1.2.3.2.Physical control

Physical control methods include: drainage of ponds, diversion of rivers, and construction of barriers. The principle behind it is to remove suitable habitat for the animals, exposing them to conditions that will cause mortality, e.g. desiccation or predation. In the literature information is limited in relation to this method and very little is known about its efficiency. For instance, drought cannot be effective with *P. clarkii*, which can survive out of water for long periods in their burrows (Gherardi et al. 2011). Rivers may be diverted via a channel or pipeline and the remaining water pumped out and isolated stretch can be thoroughly searched for crayfish and crayfish removed from their burrows by hand, or burrows can be treated by biocides. These techniques seem to have significant short-term impacts on invasive crayfish population, reducing their size, but they are not able to achieve a complete eradication (Stebbing et al., 2014). Additionally, they are generally considered costly and impractical in rivers and ineffective in small ponds and unacceptable because of damage to the local environment that they cause (Freeman et al., 2010).

Table 1.3. Synthesis of the different methods used to control invasive non-indigenous crayfish species and evaluation of their general efficacy adapted from Gherardi et al. 2011. Efficiency is noted as Low: +; medium: + +; high: + + +; irrelevant: - ; unknown: *

Methods	Population size	Area size	Applicability	Species- specificity	Selectivity	Impact	Time	Cost	Efficacy
Mechanical									
Trap	+ + +	+ +	+ + +	+	+ + +	+	+ + +	+ + +	+ + +
Electrofishing	+ +	+	+ +	+ +	+	+	+	+	+
By hand	+	+	+	+ + +	+ + +	+	+	+	+
Physical									
Drainage	-	+	+	+	+	+ + +	+ +	+ + +	+
Diversion of rivers	-	+	+	+	+	+ + +	+ +	+ + +	+
Barriers	-	+	+ +	+ +	+	+ +	+	+ + +	+ +
Electrical fences	-	+	+ +	+ +	+	+ +	+	+	+ +
Biological									
Predators	+ + +	+ +	+ +	+ +	+ + +	+	+ + +	+ +	+ +
Pathogens	-	-	+ + +	+ + +	+	*	+	+ +	+ + +
Biocides									
Chemical	-	+	+ +	+	+	+ + +	+	+ +	+ + +
Natural	-	+	+ +	+	+	+ +	+	+	+ + +
Autocidal									
SMRT (X-ray)	+	+	+ +	+ + +	+	+	+ + +	+ +	+ +
Sex pheromones	-	+ +	+ + +	+ + +	+ + +	+	+ + +	+	+

Large barriers, such as waterfalls, can be effective at limiting the movement of crayfish (Kerby et al., 2005) and have been successfully used to prevent the dispersion of crayfish populations. Even though barriers could potentially be a long-term, cheap and low-maintenance control measure, species can still be moved beyond them by human activities, predators or in occurrence of floods (Stebbing et al., 2014). Electric fences or barriers have also been successfully used to avoid migration of crayfish, but they have only been used as rapid response measure to stop the spread of infected crayfish with crayfish plague in rivers and showed satisfactory results in stopping the further spread of disease upstream (Hastein & Gladhaug 1973; Benejem et al., 2015).

1.2.3.3.Biological control

Biological control or biocontrol can be defined as the use of one species for the suppression of another (Molloy, 1998). Biological control methods include a variety of methods based on the use of natural enemies of the invader and the premise is based on ‘Ecological resistance hypothesis’ and 'Enemy release hypothesis'. More generally the ‘Ecological resistance hypothesis’ proposes that competition with native species and attack by native predators and pathogens keep invaders in check, while 'Enemy release hypothesis' states that the abundance or impact of alien species may be related to the absence of natural enemies controlling these species in their native range (Clay, 2003). Invasive species are successful in a new environment especially if they are introduced in the absence of constraints, such as pathogens or predators that would normally keep IAS population numbers under control, and control methods that use species natural controllers may be seen as a more natural approach to the control of pest species, compared to other control methods.

Biocontrol has successfully regulated pest populations in terrestrial ecosystems (Williams, 2004), but it has also caused negative unintended consequences for native species and in the last decades natural enemies have been assessed as biological control agents to be used in control of invasive species in the aquatic environments (Thresher et al., 2000; Thibaut et al., 2001); in parallel, the extent to which these imported natural enemies may impact populations of non-target species has been questioned (Messing & Wright, 2006). Although permanent, non-polluting and ethical, control that suggests introduction of another alien species to a habitat as a biological control agent remains controversial. Co-evolved natural enemies of invasive species are not always specific to the target organism and may instead attack native species of the same taxon and other organisms and cause more damage than good (Gherardi et al., 2011). For this reason, control agents should be thoroughly checked for specificity and non-target effects before releasing them into the wild.

Even though crayfish have many natural enemies, e.g. predatory bird and fish species, pathogens and microbes that produce toxins, e.g., the bacterium *Bacillus thuringiensis* (Gherardi et al., 2011), only fish are worth considering as control agents (Holdich et al., 1999). Several studies that have examined the potential of fish predation for management of crayfish populations (Table

1.4.) and suggested that fish predators that are well-known predators of crayfish, such as eel, perch, and pike (Westman, 1991) and zander (Reynolds, 2011) can have impact on crayfish populations.

Since impact of fish predation on crayfish population depends on the balance between recruitment and predation (Freeman et al., 2010), estimating such predation levels that would inevitably result in population control would be mandatory to properly use predators as control agents. Predation by fish is also size-selective, depending on the species of fish and, for example, pike predates on all sizes (Stebbing et al., 2014), whereas eel (Aquiloni et al., 2010), perch, carp and tench predate on smaller animals (Neveu, 2001a). Some species were found to be not so voracious feeders (e.g. eel, Aquiloni et al., 2010) as others (e.g. perch, Freeman et al., 2010).

Density of fish has been shown to affect density of crayfish under both experimental and field conditions, e.g. heavily stocked lakes with fish predatory species and continued predation may have long-term effects on population size and production (Holdich et al., 1999). The suitability of fish species as biological control agents will vary significantly depending on the targets' area habitat type and water body conditions, and management of introduced fish stocks will reflect on success of control attempts (Freeman et al., 2010). Moreover, some potential issues with a fish as a control agent need to be considered: fish might predate on non-target native species (Reynolds, 2011), especially once the crayfish population has been reduced, or migrate away from the target area, if used in an open water system (Stebbing et al., 2012).

Table 1.4. Effect of fish predators on adult (A) and juvenile (J) crayfish population size: *Astacus astacus*, *Orconectes* spp, *P. clarkii* and *P. leniuculus* (updated from Holdich et al., 1999).

Crayfish species	Crayfish stage	Predator	Type	Impact study	Authors
A. astacus	A	European eel	F	+	Svärdson (1972)
O. rusticus	A	Smallmouth	F	+	Mather & Stein (1993)
O. sanborni	A	& Rock bass			
O. rusticus	A	Largemouth	F	+	Lodge & Hill (1994)
O. propinquus	A	bass & Yellow			
O. virilis	A	perch			
O. nais	A	Largemouth bass	E	+	Ricket (1974)
O. virilis	A	Largemouth bass	E	+	Saiki & Tash (1979)
O. immunis	A	Largemouth bass	E	+	Rach & Bills (1989)
O. virilis	A	Brook trout	E	-	Gowing & Momot (1979)
O. limosus	A	European perch & Pike	F	+	Haertel-Borer et al. (2005)
P. clarkii	A	European eel	E	+	Aquiloni et al. (2010)
P. clarkii	A & J	European eel	F	+	Musseau et al. (2015)
P. leniusculus	J	European perch, Pike	E	+	Neveu (2001b)
P. clarkii	J	& Zander			
P. leniusculus	J	European perch & European eel	F & E	+	Blake & Hart (1993), Blake & Hart (1995)

E= experimental, F= field studies, – = no impact, + = impact

Most successes in the use of pathogens as control agents have been achieved in the terrestrial environment, against pest species of crops in agriculture (for a review see Georgis et al., 2006). Crayfish are susceptible to numerous microbial pathogens and parasites and some of them are well studied because of mortalities they cause in the context of aquaculture conditions, with high densities and easy transmission. Without doubt, the introduction of novel parasite or pathogen to a waterbody will elicit some opposition because of the well-known case of the oomycete *A. astaci* that was introduced to Europe from North America in 1860, which lead to decrease in native crayfish populations all around Europe, while the North American species are much more resistant on (Gherardi et al., 2011). Freeman et al. (2010) described the list of potential biocontrol agents: the intranuclear bacilliform viruses (IBVs), white spot syndrome virus (WSSV), fungi *Fusarium* spp. and burn spot disease; microsporidia including *Thelohania contejeani*; several species from two genera of Oomycetes: *Aphanomyces* and *Saprolegnia*; Rickettsia- like organisms (RLOs) and the protozoan *Psorospermium* spp. The problem in using most of them lies in that they usually lack host-specificity, have a long and relatively stable host–pathogen relationship, meaning also low virulence under normal circumstances, are not pathogenic but commensal, shown to cause infections only when associated with prior wounding. In the case of any pathogen or parasite that appears to be worthy candidate as a control agent, extensive testing will be necessary to elucidate transmission pathways and ensure that transmission to native species or non-target species will not occur.

1.2.3.4.Biocidal control

Biocides is the term that considers all chemicals used to control unwanted organisms. The use of chemicals as potential control mechanisms for any pest species is surrounded with major concerns, such as the collateral damage of non-target species, evolution of resistance, bioaccumulation and biomagnification in the food chain (Stebbing et al., 2012). To date, no biocide has been found that is specific to crayfish (Peay & Hiley, 2001). However, there are some biocides registered today that could be used to stop irreversible damage from an invasion: Pyblast, 3.0% pytherins plus piperonyl butoxide and alcohol ethoxylate, and BETAMAX VET, a synthetic pyrethroid (Gherardi et al., 2011). Pyblast rapidly breaks down in sunlight without harmful residues, but it is not recommendable for deep or turbid waters and is potentially toxic to fish, crustaceans and insects, but has a low toxicity to mammals and birds and is harmless to plants (Stebbing et al., 2014). BETAMAX VET is highly toxic to aquatic crustaceans and very similar in its effects to Pyblast (Stebbing et al., 2014).

Liming with agricultural limestone is commonly used in aquaculture to neutralize pond acidity and improve productivity. Using a CO_2 as a tool for aquatic invasive species management would also have negative impacts on a range of aquatic species, but this option could be preferable to traditional piscicides because it is naturally occurring and does not persist in the environment. Recent research indicates that CO_2 could be used in control of several aquatic species (Table 1.5.) (Treanor et al. 2017). Kozak and Policar (2003) carried out a chlorine treatment of a fish pond in Vodňany (Czech Republic) using 125 kg (780 kg ha-1) chlorinated lime on the water surface to supress signal crayfish population, but in field conditions chlorinated lime with an active chlorine content of more than 30% did not reach the target dosage and it was not successful.

Table 1.5. List of aquatic taxa that have been experimentally treated with CO_2 adapted from Treanor et al. (2017).

CO2 concentration (mg/L)	Aquatic species
1–100	Walleye *Sander vitreus*
101–200	
200-300	Rainbow trout *Oncorhynchus mykiss*, Nile tilapia *Oreochromis niloticus* hybrids, Westslope cutthroat trout *Oncorhynchus clarkii* lewisi
301-400	Eastern mosquitofish *Gambusia holbrooki*, African clawed frog *Xenopus laevis*
401-500	Channel catfish *Ictalurus punctatus*, Common carp *Cyprinus carpio*
501-600	American bullfrog *Lithobates catesbeianus*, Asian clam *Corbicula fluminea*
>600	New Zealand mudsnail *Potamopyrgus antipodarum*

1.2.3.5.Autocidal control

Autocidal approaches are based on the targets’ biology. Autocidal methods include the sterile male release technique (SMRT) and the use of chemical signalling molecules produced by animals: hormones and pheromones. SMRT has successfully been applied in insects (Klassen & Curtis, 2005) and sea lamprey, *Petromyzon marinus* (Twohey et al., 2003). SMRT is a technique whereby, in its traditional form, males are reared, sterilized and released in large numbers into the wild to mate with females, which will then produce non-viable eggs. The potential use of SMRT for the management of invasive crayfish was previously tested with two different techniques and species, X-ray sterilization with red-swamp crayfish and manual sterilization with signal crayfish.

Effects of X-ray irradiation were studied by Aquiloni et al. (2009); Aquiloni & Zanetti (2012); Duse (2015). Exposure of male *P. clarkii* to X-rays resulted in a reduction of the size of the testes and a significant reduction in spermatogenesis was leading up to 57% sterility with 40 Gy with no effect on their competitiveness (Aquiloni & Zanetti, 2012). This method involves combined effort with a trapping program. Drawbacks of the technique are that it requires highly trained and qualified staff to operate the irradiation equipment and transportation of animals away from the location of capture and back after the treatment, which results in high costs. The technique was applied to the small population of *P. clarkii* found at the site of Lake Casette (Italy) in 2012, with the average cost of 2.7 euros per treated specimen, and it led to a substantial reduction in this population. Reduction was calculated at 87% in just two years of activity (Aquiloni & Zanetti, 2012).

Stebbing et al. (2014) proposed a technique where males of *P. leniusculus* are sterilized manually, by removing the modified 1st and 2nd pleopods, which are used by males to deposit spermatophores into the females, as a form of sterilization. Preliminary trials showed that the removal of the pleopods did not impact the males' competitiveness, but spermatophore deposition was showed to be significantly impaired, resulting in functional male sterilization. The technique is considered cheap to apply and could be applied by anybody, without any need for specialist training or equipment, and it does not involve the transportation of animals away from their point of capture. The technique was estimated to be effective for approximately three years on *P. leniusculus.*

Semiochemicals or behavior-altering pheromones are commonly used in the management of insect pest populations (Byers, 2005) and attempts are also being made to implement it with crustaceans. Stebbing et al. (2004) tested pheromone traps on signal crayfish, but these traps showed less success than the normal food baited trap. Similar results were obtained by Aquiloni and Gherardi (2010) with red swamp crayfish. This demonstrated that crayfish utilize a number of other semiochemicals and further knowledge of this pheromones may provide the basis for methods of control using semiochemicals.

Masculinization through the insulin-like androgenic gland hormone (IAG) silencing has facilitated the production of non-breeding all-male monosex populations that are the progeny of sexually reversed males (Ventura & Sagi, 2012). This type of manipulations delivers another sustainable solution against established populations of alien crayfish that should be further explored.

Silencing of hormones through RNA interference (RNAi) is new autocidal methods based on neuro-endocrine disruptors that would allow selective disturbance of the target alien species. It refers to the process of injection of exogenous double-stranded RNA (dsRNA) to silence the corresponding hormone. Technique might be applied for the control and synchronization of the moult cycle, eradication of invasive species and immune defence, offering promising method for control of invasive crayfish species that is inexpensive and easily applicable year-round (Manfrin et al., 2019).

1.3. RESEARCH AIMS AND ORGANISATION OF THE RESEARCH

Invasion ecology is facing the "knowing-doing gap" between knowledge acquisition and its implementation, that reflects on translation of theoretical concepts into effective management actions. Body of research that could directly inform the rising applied challenges associated with invasion detection, control and management lags behind the body of research focusing on the definitions, concepts, mechanisms (Esler et al., 2010). Since biological invasions are continuing to increase and are generally irreversible, there is a need for closer integration between invasive species science and on-the-ground management to prevent the introduction of new species and control already established ones with most harmful impacts. This research addressed both the preventative and control issue of selected freshwater IAS by discussing the practicality, benefits and drawbacks of using several tools and methods with potential in invasive species management.

The main research goal was to assess available methods in IAS management, analyse the potential of present techniques and develop innovative methods for pre and post invasion management of AIS.

The first aim of my thesis was to develop predictive models for one of the AIS with a large invasive range, which is seriously threatening native European species. I selected American bullfrog *Lithobates catesbeianus* (Chapter 2), since several models have already been developed for red swamp crayfish at European and worldwide level (Capinha et al., 2011; Capinha & Anastácio, 2011; Liu et al., 2011; Capinha et al., 2013; Chucholl, 2016). American bullfrog has a large invasive range and is known for adverse impacts on native amphibian species across this range and often considered as one of the major contributors to amphibian decline. This research, conducted in collaboration with team of experts in species distribution modelling from University of Évora, should provide promising insights into the prediction of AIS in aquatic ecosystems under the impacts of global climate change and offer recommendations on how to use the gained information to improve conservation of native amphibian species at European scale.

The second aim of my research work was to assess the success of a four years long trapping activities on red swamp crayfish populations, through the C.P.U.E. index, in two wetlands in northern Tuscany (Chapter 3.). Activities concerning crayfish management at selected locations are part of the larger "SOS Tuscan Wetlands" project (http://www.life-sostuscanwetlands.eu/index.php/en/), funded by the European Union under the LIFE program, that started in June 2013 and ended in September 2017. Integrated pest management applied at project locations has involved the control of different invasive species: coypu (*Myocastor coypus*), crayfish (*P. clarkii*), plants *Amorpha fruticosa* and habitat restoration. The assessment of efficacy of trapping activities was conducted at two locations: Sibolla Lake and Ramone Marsh. The comparison between the two sites (differing in size, biodiversity rate and presence of indigenous predators of crayfish) offers interesting observations that might help in future attempts of red swamp crayfish control at local scale.

The third aim of my thesis was to develop a novel method for the control of red swamp crayfish and assess its efficiency (Chapter 3). Stebbing et al. (2014) proposed Sterile male release technique, where crayfish males are sterilized manually, by removing the gonopods, the modified 1st and 2nd pleopods, as a form of sterilization. Gonopods are appendages used by males to deposit sperm into the females. One of the main challenges in the development of SMRT is to reach the highest possible level of sterility in males, without reducing males' competitiveness and chances of successful copulation. Laboratory assessment of technique efficiency also included an extensive study of changes in sterilized male sexual and agonistic behaviours, testing and discussing the potential of application of this technique in the field. The study offered the first laboratory assessment of gonopod regeneration and provided a first proof of negative effects of gonopod removal on molting frequency in adult males of *P. clarkii*.

During my research I also contributed in the production of other publications and project deliverables, which are presented in Chapter 7. APPENDIX to this thesis. My contribution in these publications has been indicated per chapter in the following.

In chapters 7.1. and 7.3. the invasiveness of the North American channel catfish *Ictalurus punctatus* was assessed. In chapter 7.1. I contributed to idea development, wrote the part of manuscript concerning control and provided feedback on drafts of the chapter. In chapter 7.3. I contributed to idea development, helped in analysis of morphological analyses of the sampled specimens and provided feedback on drafts of the paper.

Chapter 7.2. focuses on cooling and freezing as methods to preserve specimens of the North American channel catfish *I. punctatus* for transport and the impacts these methods have on species' morphological characteristics. I helped in analysis of samples' length, body shape and colour and provided feedback on drafts of the paper.

Chapter 7.4. involves the publication of a paper concerning the presentation of Aquainvad-ED project. I contributed in the development of my research plan and delivered the segment concerning my part of project to the main author.

Chapter 7.5 includes Aquainvad-ED Deliverable 1.7. *A review on impact and management of AIS*. I contributed to idea development, wrote the part of report concerning the management of IAS and provided feedback on drafts of the deliverable.

Chapter 7.6. includes Aquainvad-ED Deliverable 3.3. I contributed in writing the report and provided feedback on drafts of the deliverable.

Chapter 7.7. includes Aquainvad-ED Deliverable 3.4. I contributed in writing the report and provided feedback on drafts of the deliverable.

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CHAPTER 2. Ensemble forecasting of the global distribution of the American bullfrog, *Lithobates catesbeianus*. A potential threat to amphibians in the European Natura 2000 network

-submitted

Running head: American Bullfrog invasion in Europe

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ABSTRACT

Freshwater biodiversity is declining due to climate change, and all scenarios indicate that the decline will continue throughout the 21st century. Processes such as the introduction and spread of invasive alien species will greatly affect the extent of future climate change impact on freshwater biodiversity. Recently, niche-based models have become an important tool in IAS risk assessment and conservation decision-making. In the present study, the current and future climatically suitable areas of the invasive bullfrog *Lithobates catesbeianus*, known for its adverse ecological impacts on amphibian species, were assessed at the global level, with special focus on Europe. An ensemble forecasting approach based on nine algorithms in the BIOMOD2 package was used. Six climatic variables, three timeframes (current, 2050, and 2070), and two increasing CO_2 emission scenarios were considered. The individual model performance was excellent according to the area under the receiver operating characteristic curve (AUC) and useful according to true skill statistics (TSS). The best performing individual model was random forest for both AUC and TSS. Temperature seasonality, minimum temperature of the coldest month, maximum temperature of the warmest month, and precipitation in the driest month were the most important variables predicting bullfrog occurrence. We estimated that 3.8% of surface land area globally is suitable for the bullfrog at current conditions and that an increase of up to 5.2% in 2070 is expected in a high-emissions scenario. An increase in suitable areas is expected at higher latitudes, especially in North America and throughout central Europe. Currently, 3.45% of total Natura 2000 area is suitable for the *L. catesbeianus,* and a maximum predicted range change gain of up to 355.93 % is expected in the highest greenhouse gas concentration scenario prediction. Conservation efforts should take place to stop future spread and to

minimize the negative impact of bullfrogs in areas with established populations, as well as to protect native amphibian species under threat.

Keywords: bullfrog, invasive species, habitat suitability, biodiversity, Natura 2000, management

1. INTRODUCTION

1.1. *Fresh waters and climate change*

Freshwater ecosystems support exceptional biodiversity – it is estimated that up to 12% of all species live in freshwater ecosystems, despite the small area it comprises globally (the paradox of freshwater biodiversity) (Balian, Harrison, Butchart, Chambers & Cordeiro, 2010). Currently their biodiversity is declining, due to global warming and changes in precipitation patterns related to climate change (Gitay, Suárez, Watson & Dokken, 2002), at far greater rates than most affected terrestrial ecosystems (Sala et al., 2000; Vaughn, 2010). The detrimental effects of climate change have become one of the most important topics for conservation biologists and the general public in the past few decades, thanks to rising media coverage. It is expected that climate change will have direct consequences on species' geographic ranges (Duan, Kong, Huang, Varela & Ji, 2016). Changes in distribution and an abundance of species linked to changes in climatic factors have already been observed on most continents and within major taxonomic groups (Gitay et al., 2002). Species migration will be limited by habitat fragmentation and the availability of habitats in new areas that are climatically suitable (European Commission, 2011). Climate change is expected to affect most communities of species that have poor dispersal abilities and narrow niches (Slatyer, Hirst & Sexton, 2013). Endemic species known to be range restricted are especially vulnerable (Malcolm, Liu, Neilson, Hansen & Hannah, 2006). Invasive species that are good dispersers with wide niches will be able to follow their optimal temperatures across the landscape and out-compete slow dispersers, causing their extinction (Urban, Tewksbury & Sheldon, 2012). Processes such as the introduction and spread of invasive alien species (IAS) will greatly affect the extent of the future impact of climate change on freshwater biodiversity.

1.2. *Studied species*

The American bullfrog, *Lithobates catesbeianus,* because of its widely recognized invasive character and adverse impact on native species across their invasive range, has been considered one of the 100 worst invaders in the world (GISD, 2015). Native to the eastern part of North America, from the southern USA to southern Canada, it has been introduced into approximately 40 countries on four continents, mostly via aquaculture and the aquarium trade (Lutz & Avery, 1999). The number of EU countries where this species of bullfrog is present has doubled in the last 20 years. This alien bullfrog was also recognized as a species of Union concern in Regulation (EU) No 1143/2014 of the European Parliament and of the Council “on the prevention and management of the introduction and spread of invasive alien species”. This obliges Member States to organize a system of surveillance for invasive bullfrogs in order to prevent their spread into or within the Union.

This introduced bullfrog has been identified as one of the most harmful invasive species in terms of negative impact on native European amphibians (Temple & Cox, 2009). Due to predation, competition for resources, and the spread of deadly amphibian pathogens such as the fungi *Batrachochytrium dendrobatidis* and *Saprolegnia ferax* (Nori, Urbina-Cardona, Loyola, Lescano, & Leynaud, 2011), it places additional pressure on already declining amphibian populations (Adriaens, Devisscher & Louette, 2013). The fungus *B. dendrobatidis* is the agent of chytridiomycosis, an infectious disease considered one of the main causes of ongoing global amphibian decline and extinction. American bullfrogs are carriers of *B. dendrobatidis* and are able to be infected by the fungus without developing the disease (Daszak et al., 2004; Ficetola, Thuiller & Miaud, 2007*b*).

1.3. *Protected areas and species in changing climate*

Protected areas are considered one of the most effective conservational strategies for biodiversity conservation (Bombi, 2010). It is expected that protected areas will also play a key role in mitigation and adaptation responses to climate change. Today, the most important conservation investment of the EU is Natura 2000, a highly fragmented network of protected areas comprising over 26,000 sites and 18% of the EU’s land mass (European Commission, 2011). The network contains a significant proportion of remaining semi-natural habitats of high conservation value in Europe, which are especially important as refuges for threatened species (Gitay et al., 2002). Although guiding principles for “Birds” and the “Habitats” EU Directives (BHD) implementation in a changing climate have been published (European Commission, 2013), climate change has not been addressed in the majority of the sites, mostly due to a lack of data, tools, or capacity to scale down global studies and *predict likely impacts at site level* (Stolton & Dudley, 2011). At the moment, adaptive resource management (ARM) is seen as the most practical tool to integrate climate adaptation measures into management and planning for all Natura 2000 sites (European Commission, 2013). Huge improvements should be made by better governance, management, funding, and inter-connecting protected areas to ensure their mitigation capacity (https://www.cbd.int/).

Guidance documents accompanying BHD, such as "Natura 2000 and climate change" (European Commission, 2013), express special concerns about regions with a high number of vulnerable species, e.g. the Mediterranean and Alpine regions. Climate change is expected to impact vulnerable species much more than species that have been assigned a favourable conservation status. Among Natura

2000 species, amphibians are considered highly vulnerable (Silva et al., 2009). Recent studies indicate that 32.5% of all amphibian species are likely to face a significant extinction risk, making them the most threatened vertebrate class (Stuart et al., 2004; Wang & Li, 2009). Two thirds of European amphibian species are endemic to Europe (Temple & Cox, 2009). In total, 53 (Silva et al., 2009) of 85 European amphibian species are listed in the Annexes to the “Habitats” Directive. The Iberian Peninsula, Apennine peninsula, Balkan coast, and several Mediterranean islands host the greatest concentration of threatened amphibian species (Temple & Cox, 2009). An extensive review of 21 European amphibian species found that 90% were already negatively affected by climate change, exhibiting population declines and reduced survival rates, habitat suitability, and range sizes (European Environment Agency, 2016).

1.4. *Study aims*

Here, we modelled the potential global distribution of American bullfrog. The aims of this study were: 1) to determine the potential global distribution of *L. catesbeianus* with a special focus on Europe, as well as to assess the projected range change in potentially suitable areas across different time periods and greenhouse gas emission scenarios; 2) to identify Natura 2000 areas at risk of *L. catesbeianus* invasion under current and future climatic conditions and to discuss managemental actions that could be taken to tackle the threat. We expect that bullfrog will be favoured by the climate change, causing more pressure on native amphibians.

2. MATERIALS AND METHODS

2.1. *Species and environmental data*

Individual records of 7,124 worldwide native and invasive occurrence data of *L. catesbeianus* were collected from different archives, such as: GBIF (http://www.gbif.org/), HerpNet (http://www.herpnet.org/), speciesLink (http://www.splink.org.br/), EASIN (https://easin.jrc.ec.europa.eu/), IUCN (http://www.iucnredlist.org/), NA2RE (http://na2re.ismai.pt/). To describe the environmental conditions that might influence habitat suitability, six climatic variables were selected, with a cell resolution of arc-min from the WorldClim datasets (Hijmans et al. 2005, version 1.4; http://www.worldclim.org; accessed February 2016). A pairwise Pearson correlation was performed and highly correlated variables ($|r|>0.80$) were excluded, to avoid collinearity in statistical models (Dormann et al., 2013). The climatic variables included in the model were: BIO13 = Precipitation of Wettest Month, BIO14 = Precipitation of Driest Month and BIO15 = Precipitation Seasonality, BIO4 = Temperature Seasonality, BIO5 = Max Temperature of Warmest Month and BIO6 = Min Temperature of Coldest Month, chosen from a set of 19 different climatic variables. Precipitation variables described water availability during the dry and wet periods of the year, since the availability of permanent water bodies is critical for the American bullfrog, especially for tadpoles that overwinter in water. This is thus related to the likelihood of bullfrog population establishment (X. Liu & Li, 2009). Temperature variables described the species' thermal tolerance. The latest projected climate data of spatial resolution 5 arc-min (~10 km) were acquired from the climate model data used in the IPCC Fifth Assessment Report (AR5) (2014), which holds information for both current and future conditions. Information on present-day distribution conditions (average 1950-2000) and projected climate scenarios for 2050 (average for 2041-2060) and 2070 (average for 2061-2080) were used and two contrast representative concentration pathways (RCPs) were selected, RCP2.6 (rcp26)- stringent mitigation scenario and RCP8.5 (rcp85)- scenario without additional efforts to constrain emissions with very high anthropogenic greenhouse gas emissions, that is also called business-as-usual scenario (Pal & Eltahir, 2016). RCPs were used to quantify the lowest and highest gas emissions scenarios for each timeframe in order to try and understand differences using the most conservative and worst scenario predicted.

2.2. *Modelling protocol*

To model the potential distribution of *L. catesbeianus* at a global scale, we used an ensemble forecasting approach similar to the one used in Gama et al. (2016, 2017). The algorithms were obtained from the BIOMOD2 package (Thuiller, Lafourcade, Engler & Araújo, 2009) in R software, version 2.14.0 (R Core Team, 2011). These included three regression algorithms {GLM (generalized linear models), GAM (generalized additive models), MARS (multivariate adaptive regression spline)}, two classification methods {CTA (classification tree analysis) and FDA (flexible discriminant analysis)}, three machine learning methods {ANN (artificial neural networks), RF (random forest for classification and regression), GBM (generalized boosted regression models), and one climate envelope method {SRE (surface range envelope)}. Ensemble forecasting is based on the knowledge that combined forecasts yield a lower mean error than any of the individual forecasts constituting the ensemble (Miguel B. Araújo & New, 2007). The evaluation of individual models is performed by calculating the measure of central tendency (e.g. the median or mean). In our case, the median was used as it proved more reliable in that it was less influenced by extreme output values than the mean (Miguel B. Araújo & New, 2007; Gama et al., 2016). Species occurrence data was coupled with an equal number of pseudo-absences randomly generated worldwide, since this has been shown to provide excellent results in predicting the distribution of other freshwater invaders (Capinha & Anastácio, 2011) and to avoid biasing predictions towards more prevailing responses (Capinha, Leung & Anastácio, 2011). Coupled data was then separated into two datasets using 80% of the data to build a model, while retaining the remaining 20% for evaluating predictions (Thuiller et al., 2009).

K-fold cross-validation (K-fold partitioning) was used to improve parameter estimates (Naimi & Araújo, 2016). A fivefold cross validation (CV) procedure for assessing the predictive quality of a model was implemented. The resulting area under the receiver operating characteristic curve (AUC) and true skill statistic (TSS) were calculated.

In the BIOMOD2 package, the importance of each variable is estimated through a randomization procedure (Thuiller et al., 2009). We assumed that the most important variables contributing to the model will be those with a relative importance above the mean of the predictor variables in the subsets (Allouche, Tsoar, & Kadmon, 2006) (Figure 1.).

Five ensemble models were produced using a weighted approach based on TSS values, for the present, 2050 RCP2.6, 2050 RCP8.5, 2070 RCP2.6, and 2070 RCP8.5. To reclassify the resulting continuous maps into binary maps (unsuitable and suitable areas), the sensitivity-specificity equality approach (http://r-forge.r-project.org/projects/biomod/) was used, wherein the absolute value of the difference between sensitivity and specificity was minimized (Liu, Berry, Dawson & Pearson, 2005).

All binary suitability maps, for the present and the two future time frames (2050 and 2070) under two RCP scenarios (+2.6 and +8.5), were "reclassified" using ArcMap Spatial Analyst tools, with absent data as "no data". The obtained maps were used as a "mask" to cut the Natura 2000 layer (https://www.eea.europa.eu/data-and-maps/data/natura-9/natura-2000-spatial-data/natura-2000-shapefile-1). The operation "extract by mask" was used for this purpose. To obtain the data (% of each area), the attribute table of each raster was checked and the "operator" statistic was used.

3. RESULTS

Table 1 shows the mean values of the AUC and TSS statistics and the respective standard deviation for each individual model used to compute the ensemble for current climatic conditions. Accuracy can vary between different models; AUC values varied from 0.823 (SRE) to 0.985 (RF). A comparison of model evaluations (mean ± S.D. = 0.932 ±0.007) indicated which models on average may be considered excellent; six out of nine models displayed accuracy exceeding this value. When evaluation by TSS models was assessed (mean ± S.D. = 0.773 ±0.017), mean accuracy was considered useful in predicting species distribution. With a mean of 0.907, Random Forest proved to be the best performing model, while BIOCLIM (SRE) showed to be the least predictive one (mean = 0.647). Here, two out of nine models can be considered good to excellent, while the remaining models were classified as useful (Table 1).

Minimum temperature of the coldest month (BIO6) (importance: 0.346), maximum temperature of the warmest month (BIO5) (importance: 0.299), temperature seasonality (BIO4) (importance: 0.289), and precipitation of the driest month (BIO14) (importance: 0.284) were the most important predictors, having values of relative importance higher than the mean importance value

(Figure 1.). The large standard deviation values observed indicate that the same variable has a very different influence in the predictions from the nine individual models.

Ensemble model performance was classified as excellent, based on the ensemble median TSS score of 0.895. The model correctly predicted 94.96% of *L. catesbeianus* presences (i.e. sensitivity) and correctly predicted 94.56% of its absences (i.e. specificity). The ensemble suitability models were projected onto the present time (Figure 2.a) and climate scenarios for 2050 and 2070. Binary (suitable–unsuitable) predictions were obtained using a threshold obtained in Biomod (Figure 2.b). In total, 3.8% of corresponding global continental area was predicted by the final ensemble model to be suitable for *L. catesbeianus* distribution under current conditions. When 2050 and 2070 climate scenarios were modelled, the percentages increased with elevating CO_2 emissions scenarios. The total continental area globally suitable for the American bullfrog increased to 4.76% and 5.01% for the 2050 projections and to 5.15% and 5.23% for the 2070 projections (Figure 3.), representing a maximum predicted increase in range change gain of 1.4%.

In total, the final ensemble model predicted 3.45% of the corresponding Natura 2000 area is suitable for *L. catesbeianus* under current conditions. When future climate scenarios were modelled, maximum predicted range change gain rose 323.36% (RCP 2.6) and 262.12% (RCP 8.5) for the 2050 projections and 317.52% (RCP 2.6) and 355.93% (RCP 8.5) for the 2070 projections (Figure 4.). Interestingly, the percentage of suitable area in the Natura 2000 area decreased when the higher RCP scenario was used to model species distribution in 2050; it was also higher in the lower RCP scenario for 2050 than in the lower RCP scenario for 2070.

4. DISCUSSION

4.1. *Current and future suitable areas*

Since risk maps visually describe where IAS might spread and establish, they can be used in strategical IAS management. The range expansion of *L. catesbeianus* will be favoured by climate change, which could greatly affect native amphibian communities.

The present conditions model predicted:

- global areas where the species has already been introduced (e.g. southern and south-eastern Brazil) and/or invasion is ongoing;
- areas in Europe where the species has been described in the literature, e.g. Belgium, France, Germany, Greece, Holland, Italy, Spain, the United Kingdom (Ficetola et al., 2007*a*), and Slovenia (Kirbiš et al., 2016);
- the largest globally suitable area outside its native range (e.g. North America);

The same model also predicted areas where the species has not yet been recorded globally (e.g. Bolivia) and European areas where the bullfrog has not yet been recorded (e.g. Croatia and Portugal).

There are already some current predictive models for *L. catesbeianus'* global or South American distribution (Giovanelli, Haddad & Alexandrino, 2008; Nori et al., 2011). Only Ficetola et al. (2007*b*) aimed to predict the potential distribution of the bullfrog in Europe, however only using data from the native range to describe the bullfrogs' climatic requirements. Despite different modelling approaches, indicated suitable areas under current conditions in Europe are comparable to, Ficetola et al. (2007b), especially in Balkan and Apennine peninsulas.

With regard to global climate change scenarios, the results show:

- the expansion of suitable areas occurred in all prediction scenarios, increasing with the time and RCP scenarios
- expansion is mostly expected at higher latitudes (e.g. North America and throughout central Europe). The predicted northern shift is in consensus with other work with aquatic invasive species (Araújo, Thuiller & Pearson, 2006; Banha, Gama & Anastácio, 2017; European Commission, 2013; Gama et al., 2017);
- a tendency towards a decrease in suitable area was shown in the Iberian Peninsula and south of the Balkan Peninsula. Dry conditions should gradually increase in these areas, making them less suitable for the American bullfrog and other amphibian species (Araújo et al., 2006).

Concerning amphibian biodiversity, the top five EU countries are: Italy, France, Spain, Germany, and Greece with 42, 38, 34, 23, and 22 amphibian species, respectively (Temple & Cox, 2009). Free-ranging populations of *L. catesbeianus* are also present in the majority of them, e.g. Italy, France, Germany and Greece (Ficetola et al., 2007*a*). Our model identified the Apennine peninsula and western Balkan coast (one of the areas with greatest concentration of threatened amphibian species in Europe (Red List of Threatened Species, www.iucnredlist.org; Temple & Cox, 2009) and central Europe as areas vulnerable to future bullfrog expansion (Figure 2.b).

4.2. *Bullfrog invasion in protected areas and potential impact on native species*

The American bullfrog is likely to colonize protected areas in the EU more efficiently in the future than today. Comparison of scenarios at Natura 2000 level showed that percentage of suitable protected area increased in all future models. Bullfrog range in Natura 2000 may raise 355.93% (2070 RCP 8.5 projection) when compared to the currently suitable areas (Figure 4.). The highest increase within suitable protected areas is expected in central Europe, the Apennine peninsula, and the western Balkan coast. The data set presenting the Emerald Network sites (https://www.coe.int/en/web/bern-convention/emerald-network) were not used; it would be advisable to include these in broader assessments on the pan-European level to better evaluate the threat the bullfrog poses to protected areas in the Balkan peninsula, Switzerland, Norway, and Eastern Europe. Climate change may enable a considerable expansion of the species in the Balkan part of the Emerald network, as shown in Figure 2.b. Moreover, our results showed great variation in percentages of suitable surfaces between different future time frames and RCP scenarios. The vulnerability of protected areas to bullfrog expansion will reflect climatic conditions at the small scale that will change as the climate does. The vulnerability of different habitats to climate change will depend on the degree of exposure, responsiveness, and adaptive capacity to adjust to these changes (Glick, Stein & Edelson, 2011). Consequently, habitats that will be under stress will be more vulnerable to invasive species.

L. catesbeianus negatively impacts amphibian species in the invaded range directly through predation and competition for resources and indirectly through the spread of deadly amphibian pathogens. Predation and competition of introduced bullfrogs with native frog species in North America caused a reduction in the range of foothill yellow-legged frog, *Rana boylii* and the disappearance of northern red-legged frog, *Rana aurora* and in California and the elimination of Vegas Valley leopard frog, *Rana pipiens fisheri* in Nevada (Moyle, 1973). Observational and experimental evidence shows that the bullfrog also competes for resources with other native ranid frogs in the western US, e.g. Oregon spotted frog *Rana pretiosa*, northern leopard frogs *R. pipiens*, California red-legged frog *R. draytonii* (Bomford, Kraus, Braysher, Walter, & Brown, 2005).

Currently, little is known about their impact on native species in Europe. The expansion of the American bullfrog through Europe may adversely affect species with similar ecological requirements, such as lentic breeding anurans (Adams, 1999). Native amphibian species that depend on a different kind of reproductive habitat, such as temporary or flowing waters, should be less impacted by bullfrog expansion. In Europe, the greatest niche overlap has been noticed with more common species like the green frog, *Pelophylax* kl. *esculentus* and common toad *Bufo bufo* (Adriaens et al., 2013). Adult bullfrog predation and competition from adult bullfrogs (Wu, Li, Wang & Adams, 2005) and tadpoles (Boone, Semlitsch, Little & Doyle, 2007) can have a strong direct negative effect on tadpoles and adults of local species. The risk posed by *L. catesbeianus* on the conservation status of amphibian species of conservation concern, such as IUCN red list species and species of Community interest (Annex IV(a) of the “Habitats” Directive) has not yet been properly assessed. Bullfrogs are much larger than any native European amphibian, therefore they can prey on and outcompete rare native species.

The case of Cretan frog *Pelophylax cretensis,* a threatened species endemic to Crete, is a good indicator of how bullfrog expansion may impact native European species. In 2000, the bullfrog was introduced into Agia Lake, Crete, which resulted in the local extinction of *P. cretensis* (Adriaens et al., 2013). Other threatened species from the genus *Pelophylax*, e.g. Epirus water frog *P. epeiroticus* and Albanian water frog *P. shqipericus*, could easily be subject to the same fate if bullfrogs are introduced in areas where these species are present. Since adult bullfrogs are generalist predators that eat any animal smaller than themselves (e.g. insects, crustaceans, fish, frogs, reptiles, birds, rodents, bats), they could impact more than just amphibian species. The presence of other IAS, e.g. co-evolved fish and crayfish species, could also accelerate the impact of bullfrogs on invaded freshwater ecosystems and amphibian species present in them (Bissattini & Vignoli, 2017).

American bullfrog also poses a significant threat as vector of the amphibian chytrid fungus *Batrachochytrium dendrobatidis* (Garner et al., 2006). Further spread of the fungus with a temperature shift towards its growth optimum could cause disease outbreaks (Pounds et al., 2006). This would additionally contribute to decline and extinction of amphibian species globally. This fungus currently occurs in six European countries: Italy, Portugal, Spain, Switzerland, United Kingdom (Garner et al., 2005) and France (Garnier et al., 2006). With climate change promoting spread of its important vector and facilitating infection, management of vector populations need to be improved to assure long-term conservation of European amphibian biodiversity.

4.3. *Management recommendations*

In accordance with "Three-stage hierarchical approach" set by Convention on Biological Diversity (Convention on Biological Diversity, 2002) in highly suitable Natura 2000 areas where the species has not yet been recorded, focus must be on prevention: most important pathways of introduction must be controlled, and early detection and rapid response protocols must be established. In areas where bullfrog has already been introduced and invasion is ongoing, the focus should be on minimising impacts and stopping future spread. Eradication programmes have succeeded four times in Europe: one in the UK and two in Germany (Ficetola, Thuiller, et al., 2007*b*) and one in Netherlands (Vane & Runhaar, 2016). These programmes involved killing both adults and juveniles by draining ponds or fencing the breeding pond and killing all individuals early when populations were still small (Ficetola et al., 2007*a*). This indicates that similar rapid response protocols should be prepared for areas with high suitability, where the impact of bullfrog establishment would significantly affect present native species of high value for the EU. There are ongoing programmes to control the populations of bullfrogs in Europe under several initiatives (e.g. Life CROAA, https://www.life-croaa.eu/en/actions/). The optimization and evaluation of the control methods will provide better recommendations on how to manage the bullfrog in nature and managed aquatic systems. Reintroduction of originally occurring native predators, e.g., pike showed to be efficient methods for controlling larval bullfrog (Louette, 2012). Reintroduction of native predators is also an important step after eradication of invasive species, leaving ecosystem less vulnerable to reinvasion. New techniques showing the potential effectiveness of cold and pressure shock in reducing male fertility (Descamps & De Vocht, 2017) foster hope in the prospects of adopting the Sterile Male Release Technique (SMRT) to control bullfrog populations.

Understanding how human activities can influence the invasion of species can also be helpful for stopping their spread into new habitats (Dudgeon et al., 2006): availability of permanent water bodies is critical to the bullfrog life cycle (tadpoles require two to three years to metamorphose (Wang & Li, 2009)) and certain modifications of freshwater habitats, such as changes in the hydroperiod of standing water bodies and changes in flowing water systems, can favour the invasion and spread of the bullfrog (Peterson, Richgels, Johnson & McKenzie, 2013). Therefore, special attention should be paid to the environmental screening and assessment of plans and programmes, e.g. EIA (Environmental Impact Assessment) and SEA (Strategic Environmental Assessment), suggesting modifications that could help the spread and establishment of bullfrog.

Some threatened amphibians are also specialized to a narrow portion of the water permanence gradient and allocation of resources and the preservation of more temporary freshwater habitats- not been well represented in the Natura 2000 network (Bagella, Caria, Farris & Filigheddu, 2009; Bagella, Caria & Filigheddu, 2013)- would directly benefit those species, as well as help fight populations of bullfrogs and alien fish species (Adams, 1999; Kats & Ferrer, 2003).

Climate change could reduce the relevance of the current protected areas for threatened species conservation (Hannah et al., 2007). SDMs can be useful tool to assess invasion risk and help in developing conservation strategies in such a scenario, e.g. addition of new protected areas. Also, a comparison between current and future areas of shared bioclimatic suitability and potential bullfrog-free refugia for threatened species can be made to help to prioritize the establishment of those new protected areas.

To conclude, this study showed that NBMs can help in understanding the combined threat posed by climate change and invasive species on the distribution of threatened species. Future scenarios suggest that the American bullfrog may strongly benefit from climate change, further spreading towards Northern and South-eastern Europe and occupying new protected areas. Ensemble forecasting provides insight into possible shifts in the range of invasive species and identifies the geographic regions most likely to be at risk. However, models involving more than simply environmental variables (e.g. compound topographical index, altitude, human footprint) have to be developed to further evaluate the risk of invasion with data collected on different spatial scales. These models will have better predictive power to assist authorities in making more informed decisions, re-examining and adapting their conservation strategies, and protecting native species under threat.

ACKNOWLEDGEMENTS

We would like to thank Wai-Tim Ng (BOKU) for his very helpful comments on an earlier version of this manuscript. Furthermore, we would like to thank Jeremy White (Terminus Translation) for language editing. This research was funded by the European Union's Horizon 2020 research and innovation programme under the Marie Sklodowska-Curie Aquainvad-ED project (H2020-MSCA-ITN-2014-ETN-642197).

CONFLICT OF INTEREST STATEMENT

All authors certify that they have no affiliations with or involvement in any organization or entity that could be considered a source of conflict of interest in the subject matter or materials discussed in this manuscript.

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Table 1. Area under the receiver operating characteristics curve (AUC) based on ROC determinations and true skill statistic (TSS) for each of the algorithms (see Methods section) used to predict *Lithobates catesbeianus* distribution

	TSS		AUC	
Algorithms	Mean	S.D.	Mean	S.D.
SRE	0.647	0.014	0.823	0.007
CTA	0.842	0.020	0.952	0.007
RF	0.907	0.029	0.985	0.006
MARS	0.774	0.013	0.948	0.004
FDA	0.746	0.015	0.934	0.004
GLM	0.721	0.012	0.927	0.006
GBM	0.799	0.011	0.958	0.004
GAM	0.766	0.012	0.946	0.005
ANN	0.753	0.023	0.912	0.021
Mean	0.773	0.017	0.932	0.007

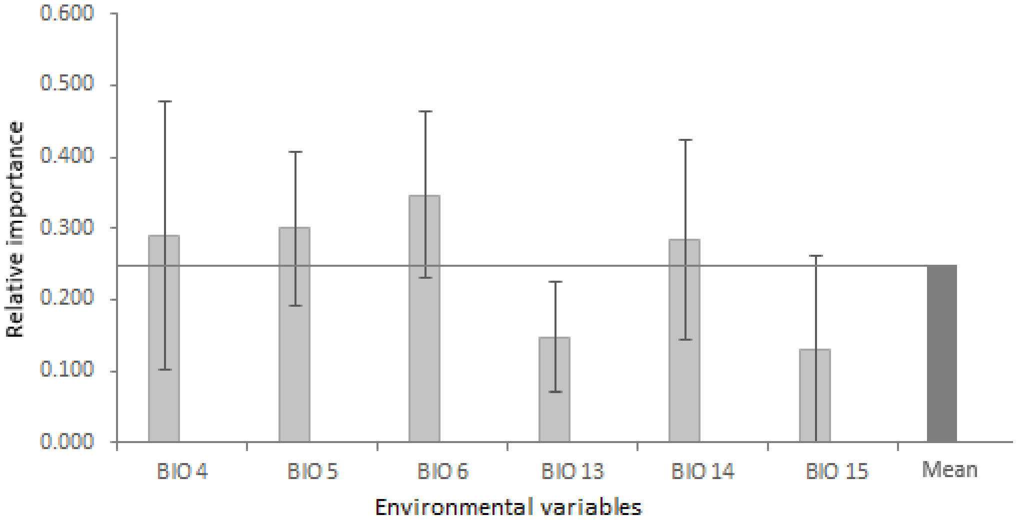

Figure 1. Relative importance of the six environmental variables used to predict the distribution of *Lithobates catesbeianus* with corresponding standard deviation values. Darker gray column and the horizontal line represent mean value of relative importance obtained from nine different modelling algorithms. Individual variables with relative importance above this horizontal line were assumed as important in determining *L. catesbaienus* suitability according to the models used. Environmental variables used were: BIO4 = Temperature Seasonality, BIO5 = Max Temperature of Warmest Month and BIO6 = Min Temperature of Coldest Month, BIO13 = Precipitation of Wettest Month, BIO14 = Precipitation of Driest Month and BIO15 = Precipitation Seasonality.

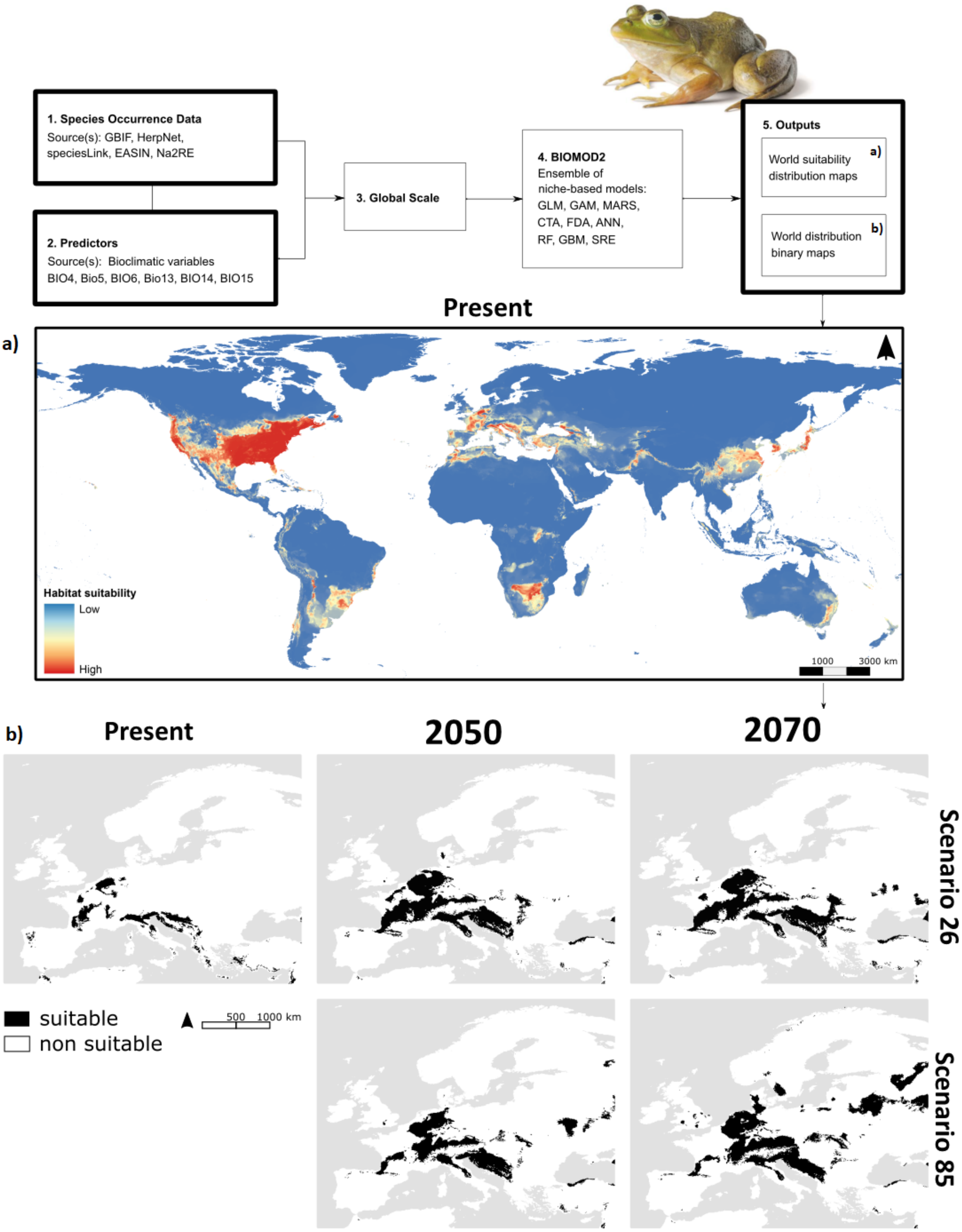

Figure 2. Conceptual diagram of the study and a) Worldwide projection for environmental suitability of *Lithobates catesbeianus* at the present time; b) Binary maps (suitable–unsuitable areas) of predicted distribution of *L. catesbeianus* in Europe for three time periods (present time, 2050 and 2070) and two RCP scenarios (rcp26 and rcp85).

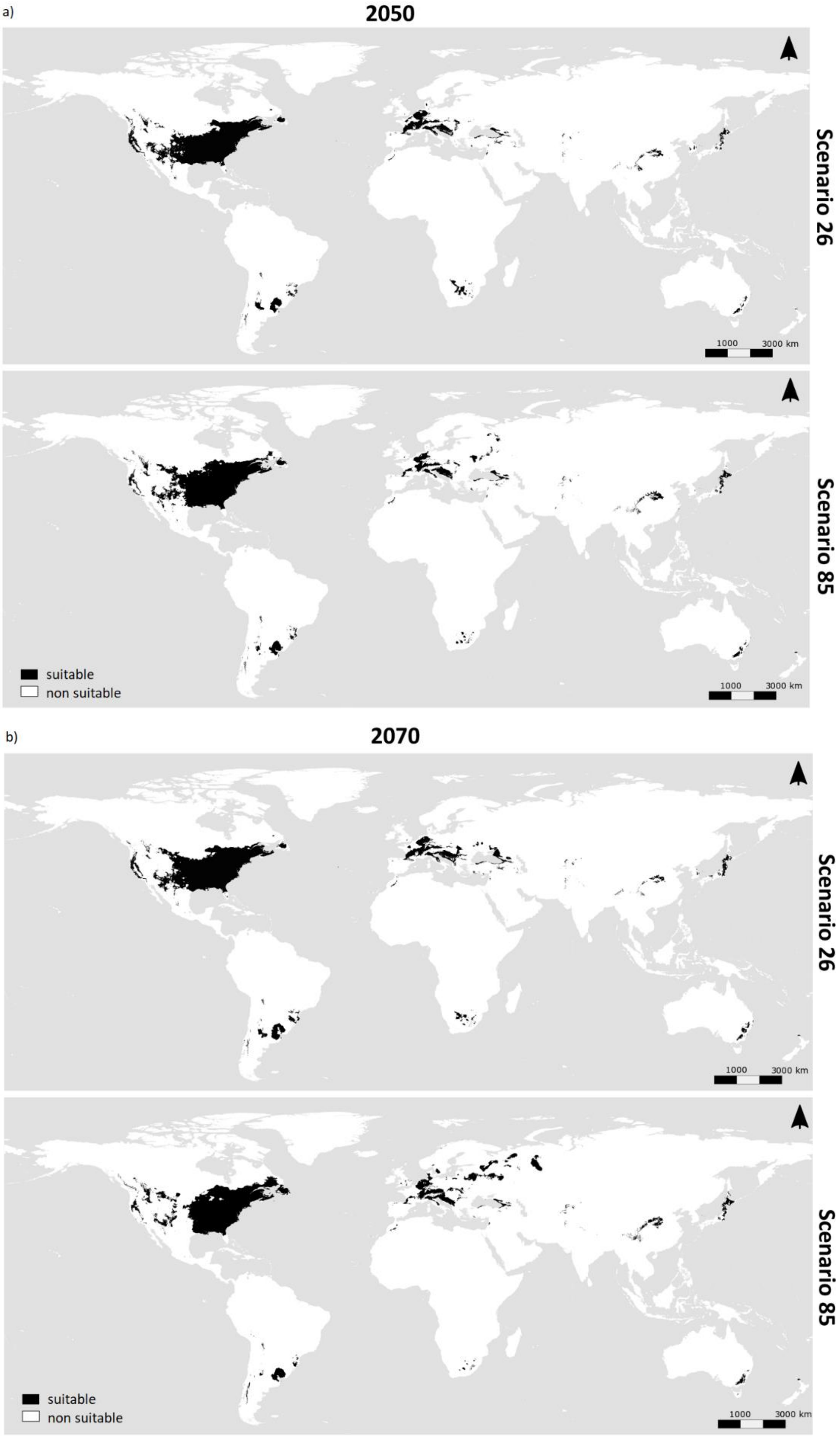

Figure 3. Binary maps (suitable–unsuitable areas) of predicted distribution of *L. catesbeianus* for two time periods 2050 a) and 2070 b) and two RCP scenarios (rcp26 and rcp85).

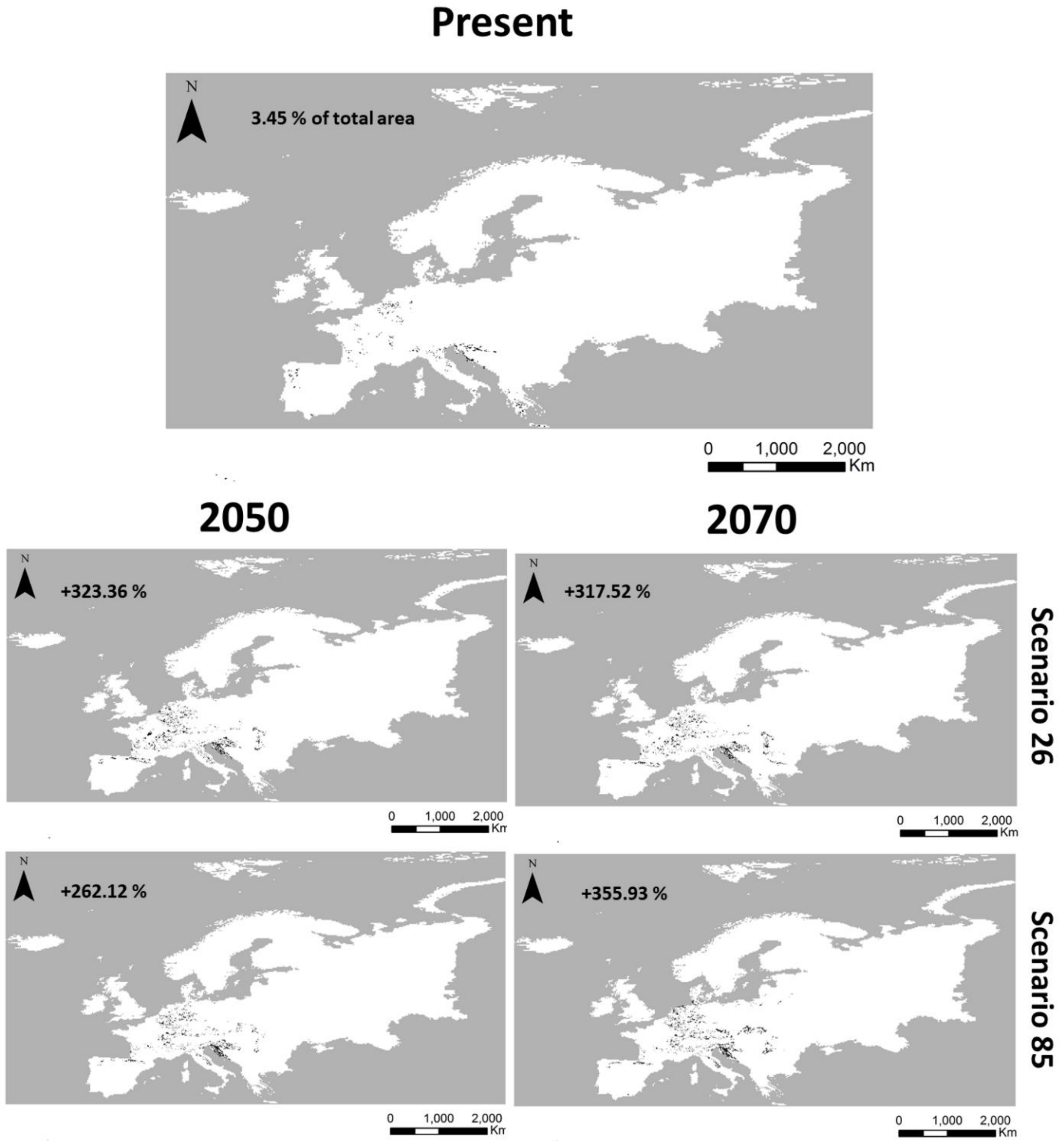

Figure 4. Natura 2000 areas suitability for *L. catesbeianus* at present time and two future time frames (2050 and 2070) and two RCP scenarios (rcp26 and rcp85) with calculated percentages of change in suitability

CHAPTER 3. The effectiveness of control activities on the invasive red swamp crayfish *Procambarus clarkii* in wetland areas of northern Tuscany, Italy

-submitted

Running Head: Control of the invasive red swamp crayfish in Italy

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ABSTRACT

The red swamp crayfish *Procambarus clarkii* is one of the most invasive species in Europe. For its heavy impacts on native species and ecosystems, it has been included in the list of invasive alien species of Union concern of the EU Regulation 1143/2014 on the prevention and management of the introduction and spread of invasive alien species. Member States are thus obliged to manage it. Within the project LIFE11NAT/IT/000094 SOS Tuscan Wetlands intensive trapping was used to control the population of *P. clarkii* for four years (2014-2017) in two protected wetland areas in Tuscany (Central Italy), Sibolla lake and Ramone marsh. Overall, 123,534 crayfish were removed during the four years. A significant reduction in Catch Per Unit Effort was achieved (55% in Ramone marsh, 74% in Sibolla lake) with a total of 171 trap nights. Large males were the most frequently trapped individuals. The presence of native predators (e.g. aquatic birds, fish), particularly in Sibolla lake, feeding on small crayfish complemented the activity of trapping. Management actions should be undertaken also after the project to maintain and decrease further the species abundance.

Keywords: trapping session, C.P.U.E., alien species, fresh waters

INTRODUCTION

Crayfish are the largest and amongst the longest-lived invertebrate organisms in temperate freshwater environments, and often are present at high densities. Most species are keystone consumers, feeding on benthic invertebrates, detritus, macrophytes, and algae in lotic and lentic waters (Nyström et al. 1996). They also constitute the main prey of several species, including otters, fishes, and birds (Gherardi 2007). Because of their ability to integrate into the food web at many levels and to persist on the substantial energy reserves of the detrital pool, alien crayfish species are good candidates for invading aquatic systems (Gherardi et al. 2011). The changes caused by their introduction usually affect all levels of ecological organization (Lodge et al. 2012; Twardochleb et al. 2013). The modes of resource acquisition by crayfish and their capacity to develop new trophic relationships, coupled with their action as bioturbator, may lead to dramatic and sometimes irreversible direct and indirect effects on the ecosystem. Since the end of the 1800s, a number of species, particularly from North America, have been introduced into Europe for aquaculture and restocking purposes (Souty-Grosset et al. 2006; Gherardi 2010). Currently, there are 11 established alien crayfish species in Europe (Kouba et al. 2014). Most of them are considered highly invasive species and have thus been included in the list of invasive alien species of Union concern of the EU Regulation 1143/2014 on the prevention and management of the introduction and spread of invasive alien species: spinycheek *Orconectes* (*Faxonius*) *limosus* (Rafinesque, 1817), virile crayfish *Orconectes* (*Faxonius*) *virilis* (Hagen, 1870), signal crayfish *Pacifastacus leniusculus* (Dana, 1852), the red swamp crayfish *Procambarus clarkii* (Girard, 1852), the marble crayfish *Procambarus fallax* f. *virginalis* (=*Procambarus virginalis* Lyko, 2017). According to the Regulation, management activities are mandatory for the species in the list.

According to Souty-Grosset et al. (2016), *P. clarkii* is currently recorded from 16 European territories. Due to its rapid life cycle, dispersal capacities, burrowing activities, and high population densities, it causes severe impacts on diverse aquatic ecosystems; it has even been recently discovered in caves (Mazza et al. 2014). This invasive crayfish is a polytrophic keystone species that can exert multiple pressures on ecosystems. It is responsible for the decline of macrophytes, increase of water turbidity, reduction of detritus biomass, and predation on several species (amphibians, molluscs, and macroinvertebrates); it is a devastating digger of the water drainage systems in southern and central Europe, and a threat to the restoration of water bodies in north-western Europe (Souty-Grosset et al. 2016). Several control methods are already known and applied to *P. clarkii*, including trapping, construction of physical barriers, biocontrol by native fish predators, male sterilization (Aquiloni & Zanetti, 2014), the use of pheromones as bait for traps and use of biocides (Gherardi et al. 2011). An integrated pest management (IPM) approach, using a range of control and containment techniques to suit different habitats and population status, is recommended to obtain positive results. However, few published studies are testing the effectiveness of control activities on this species (see Gherardi et al. 2011, Cecchinelli et al. 2012).

Established in 1992, the LIFE program is the EU's funding instrument for supporting environmental, nature conservation and climate action projects throughout the EU. In the last 20 years (even in absence of a specific regulation on invasive alien species, IAS), 256 projects (52% exclusively on IAS) were cofinanced to manage alien invasive species and mitigate their impacts in order to protect European native species and ecosystems (Silva et al. 2014). At least, eight LIFE projects deal with control of *P. clarkii*: seven out eight are/have been conducted in Italy, highlighting the importance to manage this species for protecting native habitat. Among the seven projects, LIFE11NAT/IT/000094 SOS Tuscan Wetlands ("Control of invasive alien species to restore threatened habitats inland wetlands of northern Tuscany", http://www.life-sostuscanwetlands.eu/en/home), co-funded by the European Union, aimed at decreasing the biodiversity loss in the wetlands of Northern Tuscany through habitat restoration and control of IAS established in the area, such as coypu *Myocastor coypu* (Molina, 1782), false-indigo bush *Amorpha fruticosa* L. and red swamp *crayfish P. clarkii*. Intensive trapping was used to control the population of *P. clarkii* for four years (2014-2017). Traps can capture non-target species as fish, amphibians, reptiles and small mammals (Gherardi et al. 2011; Stebbing et al. 2014) and are time-consuming because they should be checked daily. However, they are commonly employed as control methods because they enable data comparison with other studies (Larson and Olden 2016) and are easy to use also by non-experts. Here, we report the results of this four years management, showing how intensive trapping reduced *P. clarkii* population.

MATERIALS AND METHODS

The activities were carried out between 2014 and 2017, from May to September of each year, during the activity period of the species, in Tuscany (Central Italy) in two Sites of Community Importance (SCIs): Sibolla lake (SCI IT5120018; lake area: 2 km^2, average depth: 3 m) and in Ramone marsh (within the SCI IT5140010; marsh area: 1 km^2; average depth: 1 m). Every year, in May the population was monitored to assess the initial population abundance (in 2014) and the efficacy of the control activities of the previous years, while intensive trapping was conducted between June to September. Based on accessibility, eight and five subareas were identified for the activities in Sibolla lake and Ramone marsh, respectively (Fig. 1). For ten consecutive days per month, wire mesh double entrances cylindric traps (30 x 60 cm) were placed semi-submerged (to avoid any death of non-target species) along banks on accessible sites and regularly distanced (average 10 m). Traps were baited with cat food (monitoring session: 74 traps for Sibolla, 36 for Ramone; control: 118 traps for Sibolla, 62 for Ramone). Traps were checked daily and bait changed every day. Eventual native non-target species were removed and released, while alien species such as red-eared slider *Trachemys scripta* (Thunberg in Schoepff, 1792) were removed and transported in a dedicated center in Umbria (Central Italy). A severe drought reduced the number of used traps and stopped the activities in Ramone marsh for one month (September) in 2016 and three months (July-September) in 2017. During monitoring sessions, number, sex and size of all trapped individuals were annotated; during intensive trapping sessions, the number of trapped crayfish was reported for all the traps, while sex and size only for sample traps (eight in Sibolla, four in Ramone marsh). Cephalothorax length (CL, from the tip of the rostrum to the posterior edge of the carapace) was measured using a Vernier caliper to the nearest 0.1 mm. Trapped crayfish were divided into two size-classes: large with CL $\geq$ 35 and small with CL < 35 mm. Reproductive status of females was also checked. Sex-ratio was calculated as a percentage of males on the total number of trapped individuals. Trapped animals were humanely killed following the national legislation. C.P.U.E. index (Catch per Unit Effort, as a total number of caught crayfish per trap per day) was used to estimate population abundance; a decrease in C.P.U.E. was considered an indicator of successful control activities.

Statistical analyses

The frequency of size-classes and females/males was compared using a χ^2-test, while a regression analyses (R^2) was applied to assess the trend of C.P.U.E. index trough time. For Ramone marsh, data of size and sex collected in 2017 were not included in the analysis being recorded only for one month. The level of significance under which the null hypothesis was rejected is $\alpha = 0.05$.

RESULTS

Overall, 123,534 crayfish (44,997 in Sibolla lake, 78,537 in Ramone marsh) were removed during the four years.

In lake Sibolla, during monitoring sessions, C.P.U.E. significantly decreased through year by 67% (R^2=0.98, Fig. 2a), with sex-ratio almost biased towards males (on average 62%; $\chi2$= 115.44, P<0.0001; Fig. 3a) and large individuals being the most trapped (on average 73%; $\chi2$= 534.87, P<0.0001; Fig. 3a). During intensive trapping sessions, C.P.U.E. index linearly decreased every year (2014: R^2 = 0.89; 2015: R^2 = 0.83; 2016: R^2 = 0.92; 2017: R^2 = 0.52; Fig. 4a) with a total reduction of 74% (from 4.47 in 2014 to 1.18 in 2017; R^2=0.34; Fig. 4a). Sex-ratio was almost biased towards males (on average 58%; χ^2= 69.95, P<0.0001; Fig. 5a), with large individuals being the most trapped (on average 73%; χ2= 68.14, P<0.0001; Fig. 5a). No ovigerous females were found in the traps.

In Ramone marsh, during monitoring sessions, C.P.U.E. significantly decreased through year by 55% (R^2=0.66, Fig. 2b), with sex-ratio almost biased towards males (on average 57%; χ2= 223.14, P<0.0001; Fig. 3b) and large individuals being the most trapped (on average 76%; χ2= 784.75, P<0.0001; Fig. 3b). During intensive trapping sessions, C.P.U.E. index linearly decreased in 2015 (R^2 = 0.75) and 2016 (R^2 = 0.92), but not in 2014 (R^2 = 0.27) with a total reduction of 55% (from 20.56 in 2014 to 9.21 in 2017; R^2=0.36; Fig. 4b). Sex-ratio was almost biased towards males (on average 62%; χ2= 67.90, P<0.0001; Fig. 5b), with large individuals being the most trapped (on average 61%; χ2= 110.37, P<0.0001; Fig. 5b). No ovigerous females were found in the traps.

DISCUSSION

Our study showed how intensive trapping can successfully decrease the population of *P. clarkii*. Crayfish C.P.U.E. decreased through the four-year removal period in both areas, more in Sibolla lake than in Ramone marsh: the severe drought in 2016-2017 stopped the activities in Ramone marsh, slowing down the effect of control on this population. As expected, in agreement with studies on other crayfish species (see Gherardi et al. 2011 and Stebbing et al. 2014 for a review; Green et al. 2018), the majority of caught crayfish were large males. The large-biased catches might depend on mesh size, on the elusive and cryptic behaviour of juveniles that avoid being cannibalized by the adults (Guan and Wiles 1996), and on the less activity of females, in particular ovigerous ones, compared to males (Lowery 1988). Low numbers of females can thus be trapped, making up between 0% and 50% of catches (Cullen et al. 2003). Efficiency of traps can be improved by using several trap opening diameters and more attractive baits (generally freshwater or marine fish, either fresh or processed), decreasing the mesh size, emptying traps frequently (rate of escape is high, reaching 40%; Kozak and Policar 2003), and matching crayfish rhythms of activity (the number of trapped crayfish depends on both the time of the day and the season; Laurent 1995). Traps may be made more attractive by the use of sex pheromones as bait, but this technique is still ineffective (Gherardi et al. 2011), and by using different types of traps such as the recently developed artificial refuges traps (ARTs) for catching smaller individuals and females (Green et al. 2018).

A consequence of the larger trappability of big and dominant males could be the reduction of competition over juveniles, allowing the latter to grow and to reach maturity at smaller sizes, producing dense populations (Freeman et al. 2010). We did not observe a recovery of the population, despite *P. clarkii* having a short life span (usually two years) and high reproductive rates (a female can produce 500 eggs, also twice per year), and this could be due to two reasons. We conducted the activities during the activity period of the species, not allowing the full recovery of the species as during winter *P. clarkii* hibernates and does not reproduce (Gherardi et al. 2002). Moreover, control activities in Sibolla lake showed better results, although lake is wider and deeper, since it is inhabited by several native species, such as eel *Anguilla anguilla* (Linnaeus, 1758), pike *Esox Lucius* (Linnaeus, 1758), and aquatic birds: there is a big nesting site for herons, and numerous individuals of cattle egret *Bubulcus ibis* (Linnaeus, 1758), and cormorant *Phalacrocorax carbo* (Linnaeus, 1758), are present (Bartolini A., pers. comm.). These species are known to predate on juveniles of crayfish (Aquiloni et al. 2010; Gherardi et al. 2011), thus complementing the activity of trapping campaigns. Similarly, in Sparkling Lake (USA) intensive trapping on adults of *Orconectes* (*Faxoniu*s) *rusticus* (Girard, 1852) and restriction of harvesting fish predators (smallmouth bass *Micropterus dolomieu* (Lacépède, 1802) and rock bass *Ambloplites rupestris* (Rafinesque, 1817)) were used from 2001 to 2005 (Hein et al. 2007), leading to the removal of a substantial portion of the invasive population with a decrease of 95%, from 11 crayfish per trap per day in 2002 to 0.5 crayfish in 2005. Biodiversity rate is indeed greater in Sibolla lake than Ramone marsh that can be considered a hotspot of allodiversity for the several alien species found there (e.g. *Myocastor coypus* (Molina, 1782), *Trachemys scripta* (Thunberg *in* Schoepff, 1792) and several alien fish species); the resilience of Sibolla lake seems thus better than Ramone marsh.

In order to get some significant results, trapping should be conducted for an extended period of time, and this means considerable costs and manpower. However, in four years we achieved a significant reduction of 55-74% in C.P.U.E. with a total of 171 trap nights, highlighting the feasibility of the intervention. This effort (10 days per month from May to September) should continue after the project to maintain and improve the obtained results, avoiding returning to C.P.U.E. initial levels. To achieve this, protection of native fish and bird predators should be implemented, improving water and habitat quality and removing other invasive alien species present in the area (e.g. black bullhead *Ameiurus melas* Rafinesque, 1820, and channel catfish *Ictalurus punctatus* (Rafinesque, 1818)). This could increase efforts in terms of costs and time, but in long-term it would result in ecosystem recovery and maintenance of low abundances of crayfish.

Continuous trapping alone may produce significant effects on the population size, when conducted for an extended period of time and following given recommendations to maximise efficiency, but in most cases it will not be sufficient to eradicate the population. Integrated pest management (IPM) stands the best chance of achieving complete removal in isolated waters (e.g. trapping and reintroduction of natural predators, trapping and SMRT).

ACKNOWLEDGEMENTS

We thank people from the local association "Volpoca" and from the Consorzio Bonifica 4 Bassovaldarno (project coordinator) for logistic support and their participation during the activities. We warmly thanks Gianni Bettini, Elena Cecchinelli, Marco Pancino, Malvina Torrini, Vittorio Ruberti for their support in the field activities. The research was co-funded by the LIFE +11/NAT/IT 0094 project SOS TUSCAN WETLANDS. Participation of Iva Johović was supported by the AQUAINVAD-ed Project under the Marie Sklodowska- Curie grant agreement n° 642197.

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CHAPTER 4. Managing the invasive crayfish Procambarus clarkii: is manual sterilization the solution?

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Running head: Manual sterilization for managing *Procambarus clarkia*

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ABSTRACT

Management of invasive alien crayfish is challenging, as once they are established their eradication or control is difficult, even impossible in some areas. Sterile Male Release Technique has been previously assessed in crayfish with encouraging results, without, however, reaching the complete sterility of released competitive males. The present study explores whether manual removal of male gonopods, i.e. the appendages responsible for sperm transfer, as a sterilization technique, might affect male competitiveness and sexual behavior as well as reproductive potential in the red swamp crayfish *Procambarus clarkii.* Under controlled laboratory conditions, we analysed the agonistic and sexual behaviour of 64 treated and 64 control males both coupled with a female in single pairs, and 80 treated and 80 control males together with females in a natural-like social context. Removal of gonopods partly altered sexual behavior, affecting duration of copulation in the single pair and the social context experiment, and decreasing competitiveness in treated males. However, male readiness to initiate sexual interaction with females in both the single pair and the social context experiment was not affected by the treatment. Treated males needed to spend longer time in low intensity agonistic interactions with females in the single pair experiments and to initiate more low intensity agonistic interactions with females in the social context, probably to successfully dominate a female for the copulation to take place. Females coupled with treated males did not produce any offspring, compared to females coupled with control males. Treated males were able to regenerate removed gonopods, even if sometimes only partially or malformed gonopods. Females mated with 11 treated males having regenerated gonopods did not produce any juveniles. Since equal numbers of treated and non-treated males gained the opportunity to mate in both experiments and a majority of females showed no compensation for shorter and potentially unsuccessful copulation, but mated with one male as expected, further testing of the technique in the field is required.

Keywords: crayfish, invasive species, management, Sterile Male Release Technique

INTRODUCTION

For centuries, inland aquatic ecosystems have been subject to biological invasions (Havel, Kovalenko, Thomaz, Amalfitano, & Kats, 2015; Tricarico, Junqueira, & Dudgeon, 2016). Today, with increasing globalization, invasive alien species (IAS) are becoming an even greater threat to biodiversity of inland waters because of their capability to strongly affect the functions and services offered by these ecosystems across the globe (Aquiloni et al., 2010; Strayer, 2010). It is thus crucial to prevent new introduction and to control or eradicate already established IAS in order to halt and mitigate their negative impacts to ecosystems.

Some of the most harmful and widely distributed freshwater IAS are crayfish (Stebbing, Longshaw, & Scott, 2014). Compared to other aquatic species, the spread of this taxon is favored by several characteristics: uniquely large individual adult size, frequent high abundance and population biomass, and unusual trophic position (omnivorous—many species are capable of predation on other large organisms, including fish and amphibians - herbivory, and detritivory) (Lodge et al., 2012). Some species, such as the red swamp crayfish *Procambarus. clarkii*, can also survive for relatively long periods out of water and to travel long distances (Gherardi, 2007). Although the number of introduced species that become invasive is on average small, (e.g. Williamson, 1996), today 46% of all freshwater decapod species in Europe are invasive aliens (Hänfling, Edwards, & Gherardi, 2011; Vilà et al., 2010). Of the 644 currently known and described crayfish species, 28 have established viable populations outside of their native range (Gherardi, 2010). To understand which biological and ecological traits make some crayfish species prone to invasion, Larson & Olden (2010) performed trait analysis for 77 (involving 13 invasive) crayfish species native to the southeastern United States and discovered that all species that have previously invaded other regions had more general habitat requirements, larger adult size and higher fecundity compared to assessed non-invasive crayfish species.

The red swamp crayfish, *P. clarkii,* is considered one of the 100 worst invaders in Europe and is the most widely introduced crayfish today (Gherardi, 2006; Lodge et al., 2012; Souty-Grosset et al., 2016). It inhabits a wide variety of freshwater habitats, causing extremely negative impacts on ecosystems and services they provide such as reductions in valued edible native species, wide changes in ecological communities and increased costs to agriculture and water management (Souty-Grosset et al., 2016). A number of control methods have been tested to control *P. clarkii* and to mitigate its multiple impacts (Gherardi, Aquiloni, Diéguez-Uribeondo, & Tricarico, 2011). Previous attempts to control invasive crayfish have shown that 'silver bullets' are rare (Freeman, Turnbull, Yeomans, & Bean, 2010; Stebbing et al., 2014) and that integrated pest management (IPM), using a range of control techniques (e.g. trapping, natural predators, biocides), is the recommended approach likely to yield the best results (Gherardi et al., 2011). However, there is an urgent need for development of innovative control techniques that should maximize control efficacy and minimize economic and environmental management costs (Caffrey et al., 2014). Among innovative techniques, there is the Sterile Male Release Technique (SMRT), a highly species-specific and environmentally safe technique, that has successfully been applied in insects (Klassen & Curtis, 2005) and sea lamprey (Twohey et al., 2003). Its additional advantages are that the method ensures effective control even at low IAS densities and it could easily be used in IPM. SMRT is a technique whereby, in its traditional form, males are sterilized by a radiation source in the laboratory and then released in nature where they mate with wild females, thus impairing their reproductive capability. To be efficient, treated males should be able to compete with untreated males for mating and display behavior typical of untreated males (Whitten & Mahon, 2005). The success of the sterilization technique depends on some factors, such as the knowledge of the species biology, their mating behavior, the selected sterilization procedure, and the competitive ability of the animals that will be released in the wild (Duse, 2015). The potential of X-ray irradiation as SMRT for controlling population of *P. clarkii* has been assessed by Aquiloni at al. (2009), Aquiloni & Zanetti (2014) and Duse (2015). Using a dose of 20 and 40 Gy up to 57% sterility was achieved without behavioral changes in treated males. A higher dose was tested (60 Gy), but it caused an alteration of mating behavior of treated males, making it not recommendable for application in the field. Stebbing et al. (2014) suggested manual removal of the gonopods, specialized male appendages used in reproduction to facilitate sperm transfer, as a form of sterilization, hypothesizing it on the basis of preliminary experiments with signal crayfish (*Pacifastacus leniusculus*). The authors concluded that this technique could achieve almost 100% male sterilization, without effect on male competitiveness within the population or to find a mate. Manual sterilization would be cheap to apply (i.e. only requiring a pair of scissors) and could be applied by anyone, without the need for specialist training. Mass rearing of animals to supply substantial quantities, one of the principal steps in the process of applying the SMRT against insect species (Parker, 2005), would be time consuming and costly and trapping and sterilizing at location would be a more recommended option for applying the SMRT against crayfish species. Thus far, the potential of this manual sterilization technique was not assessed in *P. clarkii*. The main concern of control programs that integrate SMRT is that released sterile males should successfully compete with the wild males, being able to find a mate, compete for it and copulate. Careful assessment of the males' competitiveness through behavioral experiments is thus crucial to evaluate its efficacy.

The main aim of the present study was to assess the potential of manual sterilization in SMRT against *P. clarkii,* evaluating competitiveness and sexual behavior of sterilized and control males under controlled laboratory conditions, coupled with a female in single pairs, and together with females in a natural-like social context. A manually sterilized male may be less competitive than a wild male, because it may mate less frequently, or with fewer females, or for a shorter duration; or it may take more time, compared to control males, to start mating (Calkins & Parker, 2005; Mazza et al., 2016). Competitiveness is an important feature of released males and, when poor competitiveness occurs, it affects the successful application of the SMRT. Female reproductive outputs and the possibility of treated males to regenerate gonopods were also evaluated after the experimental phase. Manual sterilization should not affect competitiveness of crayfish and should lead to a decrease in female reproductive outputs.

METHODS

Collection of animals and holding conditions

To eliminate any factor that could induce an obvious bias to our experiments (e.g. mutilations, molt stage), only sexually mature males (Form I; Huner, Lindqvist, & Könönen, 1988) and mature females in good condition (no mutilations or visible diseases, symmetric chelae) for a total of 504 crayfish were selected from those collected using baited traps from Sibolla lake and Ramone marsh (Tuscany, Italy) in April and May 2016 and 2017, before the onset of reproduction (210 males and 182 females in 2016; 79 males and 33 females in 2017). Once the sex was determined in the laboratory, crayfish were separated by sex in PVC tanks, provided with refuges and maintained in a natural light/dark cycle at room temperature (28°C), and fed three times a week *ad libitum* with carrots. Water was changed weekly. Only hard-shelled, intact individuals were used for the experiments.

Prior to experiments the cephalothorax length (from the tip of the rostrum to the posterior edge of the carapace), and the width and length of both chelae were measured using a Vernier caliper to the nearest 0.1 mm. After three weeks of acclimation, crayfish were controlled for their sexual responsiveness by observing the behaviour of temporary couples, randomly composed by a male and female. Following Aquiloni et al. (2008), if a male tried to turn the female over for copulation, during 30 minutes of observation, the male was defined as sexually responsive, and then separated from the female before copulation took place. Overall, 208 sexually responsive males were paired with 208 females (80 males and 80 females for Experiment 1 -*Round 1* in 2016; 80 males and 80 females for

Experiment 2 in 2016; and 48 males and 48 females for Experiment 1-*Round 2* in 2017) were selected with a mean carapace length (±SE) of 45.39 ± 0.3 mm for males, and of 44.94 ± 0.3 mm for females. They were individually marked on their carapace with a waterproof paint and kept in isolation in opaque plastic aquaria (24×14×15 cm) for at least 2 weeks, which is sufficient time to reset any previous social experience (Aquiloni, Gonçalves, Inghilesi, & Gherardi, 2012).

Experimental design

Following the technique of (Stebbing et al., 2014), half of the males were randomly subjected to removal of gonopods (treated males) and half were left intact (control males) but subject to similar manipulation as treated males without cutting gonopods. Gonopods were manually removed with scissors 48 hours before the experiment took place. Preliminary observations showed that treated and control males behaved naturally, and that 48 hours were enough for the animals to recover from the treatment.

Experiment 1: single pair

Round 1

In June 2016, 40 treated (T) and 40 control (C) males were individually paired with one female each. Following Aquiloni, Gonçalves, Inghilesi & Gherardi (2012), each pair was kept in an experimental container (a circular opaque PVC container, d = 30 cm, h=17 cm) initially separated by an opaque PVC divider for 5 min to acclimatize. The experiment started with the removal of the divider and consisted of video-recording crayfish behavior for 30 minutes for subsequent analysis.

During behavioral observations, the following parameters were recorded:

1) latency time (i.e. the time elapsed between the removal of the separator and the moment in which first interaction occurs);
2) duration and type of interaction (i.e. sexual interactions: attempts, copulations; agonistic interactions: threats, weak and strong interactions);
3) abdominal (tail) movements: during copulation, male abdominal extensions were noticed in some couples and counted to compare them between control and treatment. An abdominal movement index (number of abdominal extensions per duration of copulation in minutes) was calculated for subsequent comparisons;
4) who initiated and who ended the interaction.

Following Gherardi & Daniels (2003) and Gherardi & Pieraccini (2004), agonistic interaction was classified as a simple threat (i.e. when chelipeds were raised above the plane of the carapace), weak contacts (i.e. simple touch, gentle pushing and antenna taps) and strong contacts (i.e. crayfish exchanging chela strikes, intensively pushing each other or interlocking their chelae). Any male attempt to grasp the female and turn her over was accounted as attempt for copulation, while copulation started when the male turned the female holding it by the claws and when they were in copulation position (ventral parts in contact). Since our focus was to evaluate the agonistic and sexual competitiveness, data for agonistic interactions were separated by intensity, as high intensity interactions (strong contacts) and low intensity interactions (threats + weak contacts), following Gherardi & Daniels (2003), while attempts and copulations were assessed together as sexual behavior. The majority of male - female interactions prior to copulation fell into low intensity agonistic interactions.

After the experiment, each couple was kept in the same aquarium for five consecutive days, a time sufficient for mating. Then, each female was isolated in an individual aquarium (24×14×15 cm) to allow spawning and hatching. After spawning, the number of eggs laid by each female was assessed by visual counts. The aborted eggs were counted and removed from the aquarium to prevent infection. The number of offspring was also recorded as soon as hatching took place. Treated males were kept after experimentation to assess molting and gonopod regeneration.

Round 2

All females were maintained in the laboratory until the subsequent year: females that moulted once after copulation in 2016 were considered virgins and taken into consideration for the new run in 2017. Although female decapods show high variation in duration and capacity to store sperm, *P. clarkii* females are able to store sperm until they moult (Conde & Domínguez, 2015), when the *annulus ventralis* or sperm receptacle is shed along with its content. Besides females from *Round 1*, an additional 79 males and 33 females were collected from the field in May 2017. New collected females, that moulted in the laboratory and whose exoskeletons were hardened in the time of preparation for the experiment, were considered when selecting females for *Round 2*. All animals were subject to the identical procedures as in 2016. 24 control males and 24 treated males, all collected in 2017, were paired with one female each. Reproductive output was assessed as in *Round 1*. To better assess behaviors, we prolonged observation time to one hour. To test functionality of regenerated gonopods of treated males from Round 1, the remaining virgin females were size-matched with sexually responsive treated males with regenerated gonopods from *Round 1* (n=11).

Treated and control males were also kept after experiment to assess molting and gonopod regeneration.

From 80 females used in the *Round 1*, three died during the experiment (two C, one T) and an additional 15 died before the end of the hatching season (eight C and seven T) and most of them, except for the females that died in the experiment, had laid and lost eggs or had eggs hatching prior to deceasing. From 48 females used in *Round 2*, three C and three T females died during the experiment and an additional 6 in weeks after experiment (two C and four T). None of these females had eggs hatching prior to their decease. From 11 females paired with treated males which regenerated gonopods from *Round 1*, three died in 48 hours after experiment.

Experiment 2: social context

Following Aquiloni & Zanetti (2014), in July 2016 ten groups of a balanced sex ratio, with 16 individuals (8 treated males, 8 control males, 8 females of similar size: maximum difference in cephalothorax length: 5-6%; width of both chelae: 2%; length of both chelae: 6%) were observed for 60 minutes interacting in circular arenas (d= 100 cm, h = 35 cm), containing 40 L of water, without shelters, with a population density comparable to that in the wild (20 individuals/ m^2, Gherardi et al., 1999). The ten groups were video-taped using a digital camera (Sony HDR-CX240E). Video-tapes were analyzed for the following parameters: (1) duration and type of interaction (sexual and agonistic interactions; see above for definitions); (2) initiator, receiver, and interrupter (if interruption occurred) of each interaction. Interruptions indicated any interactions between couples that were interrupted by the involvement of a third animal. Treated males were maintained after experiment to assess gonopod regeneration.

Statistical analyses

Data were first tested for normality and homogeneity of variance using the using the Kolmogorov–Smirnov and Levene tests, respectively, and transformed when necessary and possible. Since many data sets did not meet the assumptions for parametric tests, even after transformation, nonparametric tests were used. Statistical analyses were performed with SPSS (Statistical Package for Social Sciences) version 21.0 (IBM SPSS Inc., 2016) and R version 3.3 (R Core Team, 2016). The level of significance under which the null hypothesis was rejected is α = 0.05.

Experiment 1: single pair

The Mann-Whitney test (statistic: U) or Student's t-test (statistic: t) were used to compare control (C) and treatment (T) pairs. Kruskall-Wallis test (statistic: H) followed by post-hoc test was applied for comparison among control, treated and regenerated males. The parametric data were reported in the text and figures as a mean value ± standard error while for non-parametric data median values and 1st and 3rd interquartile were given.

Generalized Linear Models (GLM) with Poisson regression and log link function, (statistic: Z) were used to compare the proportion of sexual interaction in total interaction initiated by each male between control and treatment and *Round 1* and *2*, as the experimental time was different between the *Round 1.* (30 minutes) and *Round 2.* (60 minutes). The number of total interactions and number of males initiated male-female sexual interactions was considered and used to calculate the proportion of sexual interactions in total interactions initiated by each male.

Experiment 2: social context

NETDRAW within UCINET (Borgatti, 2002) was used for visualizing matrices of all the types of interactions. Matrices were composed by the number of interactions (initiated/received) per individual. We selected male emission of social interactions, which is an individual based measure, for closer assessment of competitiveness and we focused on the parameters that directly indicate competitiveness, readiness to initiate: 1. male to male agonistic interaction; 2. male - female sexual interaction (attempts and copulation) 3. male - female agonistic interaction, and 4. numbers of female partners males attempted to copulate and/or copulated with. Interaction interruptions caused by males were not considered here as indicators of competitiveness because they represented only 8.67% of the total interactions, and there was no significant difference in frequency of interruptions initiated by C or T males (U= 464.5, n=62, p=0.826). Data on general activity of males, represented as total number of social interactions initiated by each male and numbers of sexual and agonistic interactions initiated by the same male, were used to evaluate differences in behaviors between the control and treated males. Zero inflated GLMM with Poisson regression and log link functions was used (statistic: W), with group as a random effect and number of each interaction per type and total number of interactions as dependent variables.

A further evaluation of treated male competitiveness was performed by computing the Isolation Index, defined as ISI = (WW - SW)/(WW/ SW) (modified from Calkins & Parker, 2005, where WW means Wild males mated with Wild females and SW means Sterilized males mated with Wild females). This index ranges from -1 (complete negative assortative mating, i.e. in our case, all females mated with treated males) through 0 (random mating) to 1 (complete positive assortative mating, i.e. all females mated with control males). An ISI above 0.5 is considered a cause of concern as it suggests that treated males are not effective in competing with control males for females (Calkins & Parker, 2005).

RESULTS

Experiment 1: single pair

In *Round 1*, control and treatment pairs did not show any significant difference for duration of total interactions, duration of latency or number of interactions (Table 1). Likewise, the treated and control pairs did not differ in terms of duration of considered interaction types (Table 1). Frequency of males attempting at least one copulation (U=761.5, n= 80, p=0.664; out of 16 males attempting copulation in 40 C couples, 13 males attempted once, 2 twice and 1 three times; while out of 15 males in 40 T couples, 12 males attended once, 3 twice and 0 three times), and frequency of copulation (U=760,0, n=80, p= 0.579: C=9, T=7, all males copulated only once) showed also to be equal between control and treatment. Treated males showed a higher abdominal movement index compared to control ones (Table 1).

In *Round 2* (2017), control and treatment showed no significant difference for most behavioral variables, such as duration of total interactions, latency, attempts and high intensity agonistic interactions (Table 1). The number of interactions, on the contrary, showed significant difference between C and T, with more interactions observed in treatment couples (Table 1). In treatment couples, low intensity agonistic interactions lasted longer, while duration of copulation was shorter compared to control pairs (Table 1). Neither frequency of males attempting copulation (U=278.5, n=48, p=0.827) (from 11 males attempting copulation in 24 C couples, 6 males attempted once, 3 twice and 2 three times; from 10 males in 24 T couples, 5 males attempted once, 3 twice and 2 three times), nor frequency of copulation (U=271.0, n=48, p=0.653) (from 7 males copulating in 24 C couples, 3 males copulated once and 4 twice; from 6 males in 24 T couples, 4 males copulated once, 2 twice) showed significant difference between C and T. In total, 11 females copulated with control males and 8 females copulated with treated males during video recording. Similarly, to 2016, the abdominal movement index showed significantly different values between control and treated males (Table 1).

There was no significant difference in the proportion of sexual interaction initiated by control and treated males in both *Round 1* and *Round 2* (U=759.0, p=0.639 for *Round 1*, and U=260.5, p=0.532 for *Round 2*). When both *Round 1* and *Round 2* were compared for the proportion of sexual interaction in total interactions with a Poisson regression, there was a significant difference between the rounds (Z= 3.050, Pr(>|z|)=0.002), but not between C and T (Z=-1.793, Pr(>|z|)=0.073).

In *Round 1*, no significant differences in the number of females having eggs and juveniles were observed between control and treatment pairs (eggs: χ^2=2.360, n=76: C: 34, T: 32, p=0.193; juveniles: χ^2=0.059, n=66, p=0.495) (Table 2). From 37 females observed in *Round 2*, 14 out of 21 from control and 4 of 16 from treatment pairs had extruded eggs (χ^2=6.311, n=37, p=0.020) (Table 2) and only 5 females from control group had juveniles. From 11 females paired with treated males having regenerated gonopods from *Round 1*, seven mated during video recording (1 three times, 4 two times and 2 one time). From eight surviving females, only one extruded and then lost eggs.

Behavior of males with regenerated gonopods differed from behavior of treated and control males (Figure 1). Duration of copulation was equivalent to control males but significantly longer than in treated males (H=9.44, df=2, p=0.009; control=regenerated>treated). Mean abdominal movement index was significantly different than both control and treatment (H=13.75, df=2, p= 0.0014; treated>regenerated>control).

By April 2018, 47 males never molted, 21 molted once, 11 molted twice and only one three times (Figure 2a). The majority of molted males (76.5%, 26 males) regenerated all four gonopods (Figure 2b). In some cases, regenerated appendages were different in appearance from initial gonopods or gonopods of control males (Figure 3).

In *Round 2*, by April 2018 no treated male molted, while altogether 9 control males molted, proving a significant difference in molting frequency between control and treatment males (X^2=14.378, n=43, p<0.05).

Experiment 2: social context

From a total of 80 males across all 10 groups, only 50% of males were involved in sexual interactions and only 17.5% copulated during the video recording. Only 85 attempts, 50 attempts initiated by 24 control males and 35 initiated by 16 treated males, and 14 copulations, eight initiated by eight control males and six by six treated, were recorded. All males participated in agonistic interactions.

GLMM showed significant difference only for the total interaction among treated and control males and readiness to start low intensity agonistic male to female interaction (Table 3). Readiness to attempt copulation with females or males, and number of copulations, did not differ between C and T (Table 3). Heterosexual attempts accounted for 86% of all male-initiated attempts (C: 91%; T: 80%). Neither frequency of males attempting copulation with females (U=189.5, n=40, p=0.942) nor the number of females/males attempted copulation with (U=192.0, n=40, p=1.0) showed significant difference between C and T (Table 4). Treated males showed somewhat smaller values in frequency of attempts and number of the partners. While 24 control males attempted copulation 50 times with altogether 44 females, only 16 treated males attempted copulation 35 times with a total of 28 females.

Isolation index, with the value of 0.1428, confirmed random mating. Control males mated 8 times, with only one female each, while treated males mated 6 times, also with one female each. From 14 females who mated, 13 mated once and only 1 female mated twice, first with a treated and then with a control male. Duration of copulation in treated males again was significantly shorter (t=4.410, df=12, p=0.001; C: 660±64.74 and T: 299±36.61). Homosexual attempts accounted for 14% of all attempts initiated by males (C: 9%; T:20%). No difference between C and T was showed for the frequency of males attempting copulation with males (U=9, n=10, p=0.453).

DISCUSSION

The present work had the purpose of investigating the potential changes in male competitiveness and sexual behavior as a result of gonopod removal and the efficacy of this technique in impairing female reproduction. The results clearly showed that the sterilization technique used, while notably affecting the reproductive output, also led to some significant changes in male behavior.

Changes in male precopulatory behavior

Red swamp crayfish display a sequence of behaviors during male- female encounters prior to copulation, as described by Ameyaw-Akumfi (1981). On the first encounter chelae contacts are observed and short fights may also arise, finishing with male dropping its chelae in “refusal” to fight in majority of cases, while females that continue aggressive attacks have to be defeated by the male, in order to display non-aggressive postures on the next encounters. Later on, the male moves closer to the female in resting position, displaying movements of appendages for grooming, making antennule-to-antennule contacts. Next, the male may turn sideways, displaying his side presentation and, if the female remains passive, he will begin to mount her and later tries to turn her into the copulating position. Even relatively minor changes in the precopulatory and copulatory behavior could reduce male chances of successful copulation. Precopulatory behavior has several functions. It can reduce aggressive tendencies in either of the mates - the appeasement hypothesis - so that male and female can come close together and copulate; or it can favor sexual activity in individuals initially not interested in this activity - the arousal hypothesis (Barlow & Green, 1970). The sequence of interactions which follows the initial encounter between a male and a female decreases the aggressive tendencies and/or increases the sexual tendencies of the female, and occurrence of the mating evidences the success of such a display. In *P. clarkii* it can take up to three to six hours from first encounter until copulation (Ameyaw-Akumfi, 1981). In our experiments, treated males engaged in more and longer low intensity agonistic interactions than did control males. The degree of aggression and persistence are also major factors in other decapods, e.g. in lobsters, *Homarus americanus* (Waddy & Aiken, 1990), precopulatory behavior, where male lobsters must become dominant over the female in order to continue with sexual interactions. During these encounters, crayfish release chemical cues by urine signaling their motivation and physiological state. More readiness to initiate agonistic interactions, shown in treated males, is linked to an elevated aggressive state (Breithaupt & Eger, 2002), presumably induced by gonopod removal. Individuals with a high aggressive state release urine (which contains hormonal metabolites) for longer durations than crayfish with low aggression indices during precopulatory agonistic interactions. Those males are avoided by females in dense populations, in order to reduce the likelihood of suffering injuries from aggressive interactions (Berry, 2008). Therefore, it is likely that treated male, due to revealing of their aggressive state, had to initiate more interactions and invest more time to dominate the female and gain opportunity to mate. The occurrence of minor variation in sensory cues (e.g. visual, auditory, chemical and mechanical) increases the ability of females to discriminate between wild vs treated males and usually results with relatively low rates at which wild females will accept courtship overtures of treated males, as in Mediterranean fruit fly, *Ceratitis capitate* (Lance, Mcinnis, Rendon, & Jackson, 2000). Interestingly, the higher persistence that was necessary in treated males of *P. clarkii* to appease or arouse the female showed no influence on male success in gaining opportunity to reproduce, since equal number of treated and control males attempted copulation and copulated with wild females, resulting with random mating, confirmed through the isolation index in the social context.

Changes in male copulatory behavior

Treated males displayed an elevated number of abdominal extensions during copulation. Slow muscles (tonic) are responsible for slow changes in abdominal position, such as the one that we observed, and usually can be elicited by the loss of contact between one of the walking legs and a supporting substrate (Page, 1981). Presence of the same mechanism when contact between gonopod and *annulus ventralis* was not achievable possibly indicates that males are able to sense the lack of gonopods and/or lack of the contact. Bauer (1996) suggested the possible roles of petasma, a structure composed of endopods of pleopods 1 and *appendices masculinae* in copulation of penaeoid shrimp, *Sicyonia dorslis*, as a male sensory and stimulatory device, used to touch and prod the female, to connect with the genitalia and to adjust the copulatory position, in the same time providing information about the condition of the male. A similar mechanism could exist in the red swamp crayfish, and both males and females could be able to detect a lack of gonopods and/or lack of gonopod - *annulus ventralis* contact. The lack of a contact leads to a less stable copulatory position (fecundation is internal in the red swamp crayfish), inducing abdominal extensions (males trying to adjust position) and a shorter copulation time. Removal of gonopods could be responsible for this. Even though an equal number of treated and control males gained the opportunity to copulate, the effect of treatment on copulation duration is very important, since SMRT relies on absence of compensatory processes. Under natural conditions, *P. clarkii* females hide in burrows immediately after copulation, until the juveniles have reached independence (Aquiloni et al., 2009; Thiel, 2007) and in experimental conditions, with no refuges present, only one female copulated twice, the first time with a treated and second with a control male and compensated for potentially unsuccessful copulation. This observation is particularly interesting in relation to observed abdominal extensions during the copulation with treated males and raises the question of if this movement is just a reflex reaction or part of a more complex mechanism, and why more crayfish did not compensate for unsuccessful copulation with treated males. Although our results raise more questions than answers, one final remark is that, although the removal of gonopods did not affect male readiness to get involved in sexual interactions with females, it certainly affected the possibility of establishing the stable copulating position, resulting in a decreased sexual competitiveness.

Gonopod regeneration

The majority of the treated males (~ 60%) did not molt by the onset of the new reproductive season (Figure 3). Adult crayfish usually molt twice a year, in late spring and early fall (Jegla, 1966; Barki, Levi, Hulata, & Karplus, 1997), and removal of multiple limbs and consequent damage accumulation could have stimulated the attainment of the terminal molt in older animals (Juanes & Smith, 1995). Buřič, Kouba & Kozák (2010) found that for males close to maximum size, absence of molting arises naturally, because of the size reached, since growth, as a function of the number of molts decreases with increasing size and age, that also positively influences the survival of large males in the population. The initial values of carapace length of the treated non-molting males were not higher than those in treated molted males, therefore lack of molting is possibly due to the treatment. The majority of males that molted regenerated all four missing gonopods in the first molt after treatment; however, many regenerated gonopods appeared malformed (Figure 3) and virgin females that mated with those males did not produce offspring, suggesting that regenerated gonopods were not functional. All regenerated gonopods were photographed and changes in gonopod regeneration will be assessed elsewhere (Johović, pers. comm., October 10, 2018). Age of maturity in crayfish is temperature dependent and *P. clarkii* can reach sexual maturity in less than three months in their native Louisiana (Goyert, 1978) and live up to four to five years (Scalici & Gherardi, 2007), even if the average is two years. Additional studies identifying crayfish size-related technique efficiency would be a logical continuation of the technique assessment, that would help us standardize the method and insure maximum effectiveness when used for management of *P. clarkii*.

Reproductive output

In *Round 1* of the single pair experiment, females mated with control and treated males did not show any differences in extruding eggs and producing juveniles. This can be due to, despite having collected crayfish before the expected onset of the reproductive season, not all females were virgins, e.g. some crayfish may already have copulated or had sperm still stored from a previous season. Females of *P. clarkii* could have extruded the eggs fertilized with the sperm of males they mated with in the previous season or extruded unfertilized eggs if they had not copulated. In *Round 2* of the same experiment, only half of females observed mating with treated males released eggs and none had juveniles hatching. Also, only one of eleven females paired with sexually responsive treated males which regenerated gonopods produced eggs. Females can spontaneously release eggs at the end of the reproductive season, even if not mated, and, although eggs can also be lost due to several factors besides the lack of fertilization (e.g. spontaneous abortion, fungal infections, etc.; Duse, 2015) the significant difference in egg extrusion between control and treatment demonstrates that egg loss was probably related to lack of fertilization and that sperm transfer between males and females was probably not possible without gonopods or with regenerated gonopods.

CONCLUSIONS

One of the main challenges in the development of SMRT is to reach the highest possible level of sterility in males without affecting their competitiveness. When constraints such as poorer competitiveness occurs, it can contribute to an increased cost of the SMRT program, which must commit to producing and releasing more sterile males than would be required if released males were equal to wild males in their mating propensity and capability (Whitten & Mahon, 2005). Our research showed contrasting results: manual sterilization lead to a complete male sterility and a decrease in female reproductive output, but also partly altered male competitiveness and modified their precopulatory and copulatory behavior. Even though treated males had to be more persistent to get the opportunity to copulate with female, equal numbers of treated and control males gained the opportunity to mate. However, copulation with treated males was shorter and the majority of females did not copulate again with control males to compensate for a shorter and potentially unsuccessful copulation. There is no evidence of monandry in *P. clarkii* in natural conditions, but it has been shown that, in conditions where burrows are available, the *P. clarkii* females hide in them immediately after copulation, preventing them from copulating again (Aquiloni et al., 2009). This shows that despite the fact that competitiveness of treated males was shown to be impaired, this did not negatively influence male success in gaining an opportunity to reproduce and indicates that compensatory processes should not take place in natural conditions. Taken together, our results suggest the application of this technique. SMRT has been successfully used for almost 70 years against insect pests, but it will require substantial changes to be applied against crayfish species. Insects used in SMRT are usually reared in laboratory conditions, and then sterilized and released in large numbers. By contrast, crayfish should be taken from the target population to avoid an immediate increase in the density of the impacting individuals (Aquiloni et al., 2009) and sterilized at the field site (Stebbing et al., 2014), making this technique more convenient in comparison with SMRT against insects.

ACKNOWLEDGEMENTS

We would like to thanks Julian Reynolds (University of Dublin) for his very helpful comments on an earlier version of the manuscript and language edition. Furthermore, we would like to thanks Lorenzo Lazzaro (University of Florence) for his advice for the statistical analysis. This research was funded by the European Union's Horizon 2020 research and innovation programme under the project Marie Sklodowska-Curie Aquainvad-ED (H2020-MSCA-ITN-2014-ETN-642197).

CONFLICTS OF INTEREST STATEMENT

All authors certify that they have no affiliations with or involvement in any organization or entity that could be considered as source of conflict of interest in the subject matter or materials discussed in this manuscript.

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Table 1. Comparison of number (n) and duration (sec) of total interactions and duration (sec) of different types of interactions between control (C) and treatment (T) pairs in *Round 1* (2016) and *Round 2* (2017) using Mann-Whitney test (U) or Student's t-test. The table reports medians (with 1st and 3rd interquartile) for nonparametric data and mean values ± standard error for parametric data; significant p values are in bold.

	***Round 1* - 2016**					***Round 2* - 2017**				
Parameter	U/t	n/df	p	C	T	U/t	n/df	p	C	T
Total interactions (sec)	-.419	78	0.677	695.53 ± 67.64	T: 658.18 ± 58.16	-.676	46	0.503	1622.17 ± 205.10	1791.92 ±145.07
Latency (sec)	799.5	80	0.996	141.5 (49.3 - 505.3)	156.5 (43.3 - 403.8)	213.5	48	0.124	197 (83.3 - 299.8)	131.5 (23 - 246.8)
Attempt (sec)	-.274	29	0.786	30.5 ± 6.5	33.29 ± 7.8	40.00	21	0.314	38 (22 - 54)	67.5 (23 - 114.25)
Copulation (sec)	1.403	14	0.182	653 ± 167.6	366.4 ± 81.2	3.147	11	**0.009**	2055 ± 458.1	475.8 ± 68
Low intensity agonistic (sec)	53.7	75	0.706	562.3 ± 53.7	591.3 ± 54.4	184.0	48	**0.032**	600 (398.5 – 1225.5)	1285 (534.8 – 2006.8)
High intensity agonistic (sec)	40.0	23	0.118	3 (1-9)	11 (2-19.5)	128.0	33	0.800	261 (24 – 426)	191.5 (60.5 – 624.5)
Total interaction (n)	661.5	80	0.182	13.5 (9.25 – 21.75)	18.5 (11 – 25.5)	173.5	48	**0.018**	22 17.75 - 26.75)	27.5 (23 – 37)
Abdominal movement index (n/min)	8.5	14	**0.015**	0.21 (0.11-0.7)	2.02 (1.54 - 2.60)	9	11	**0.004**	0.18 (0.1 - 0.78)	1.97 (1.37 - 2.59)

Table 2. Comparisons for reproductive output between control (C) and treatment (T) pairs in *Round 1* (2016) and *Round 2* (2017) using Mann-Whitney test (U). The table reports medians (with 1st and 3rd interquartiles); significant p values are in bold.

	***Round 1* - 2016**				***Round 2* - 2017**				
Reproductive output	U	p	C	T	U	n	p	C	T
Eggs	652	0.469	427.5 (246.5 - 551.25)	190 (125 – 250)	78.5	37	**0.003**	130 (50 -595)	35 (6.5 – 59.75)
Juveniles	525	0.801	500 (270 - 550)	100 (50 - 250)	18	18	0.179	175 (75 - 500)	0 (0 – 0)

Table 3. Comparison of number of total interaction and of different types of interactions between control (C) and treated (T) males using Generalized Linear Models (GLMMs) with Poisson regression. The table reports estimate and standard errors, Wald Chi-square values and Pr(>Chisq) values associated with those estimates. Median (with 1st and 3rd interquartile) for nonparametric data is also presented for C and T; significant p values are in bold.

	C vs T		C	T
Parameter	W	Pr(>Chisq)		
Total interaction (n)	515.3	**2.20E-16**	24.5 (13 - 40)	28 (15.3 - 43.5)
Heterosexual attempt (n)	0.254	0.615	1 (0 - 2)	0 (0 - 1)
Homosexual attempt (n)	0.8146	0.367	0 (0 - 0)	0 (0 - 0)
Copulation (n)	0.284	0.594	0 (0 - 0)	0 (0 - 0)
Low intensity agonistic male to male(n)	0.204	0.651	6 (2 - 10)	6 (3 - 11.8)
Low intensity agonistic male to female(n)	6.004	**0.014**	14.5 (6 - 22.8)	14 (8 - 24.8)
High intensity agonistic male to male(n)	0.18	0.672	1 (0 - 3)	2 (1 - 4)
High intensity agonistic male to female(n)	0.727	0.394	1 (0 - 2.8)	1 (0 - 2)

Table 4. Comparison of number of total heterosexual attempts and female partners between control (C) and treated (T) males

Attempts (n)	C	T	Female partners (n)	C	T
1	10	7	**1**	12	8
2	7	4	**2**	7	4
3	4	2	**3**	3	4
4	2	2	**4**	1	0
5	0	0	**5**	1	0
6	1	1	**6**	0	0
Total	24	16	**Total**	24	16

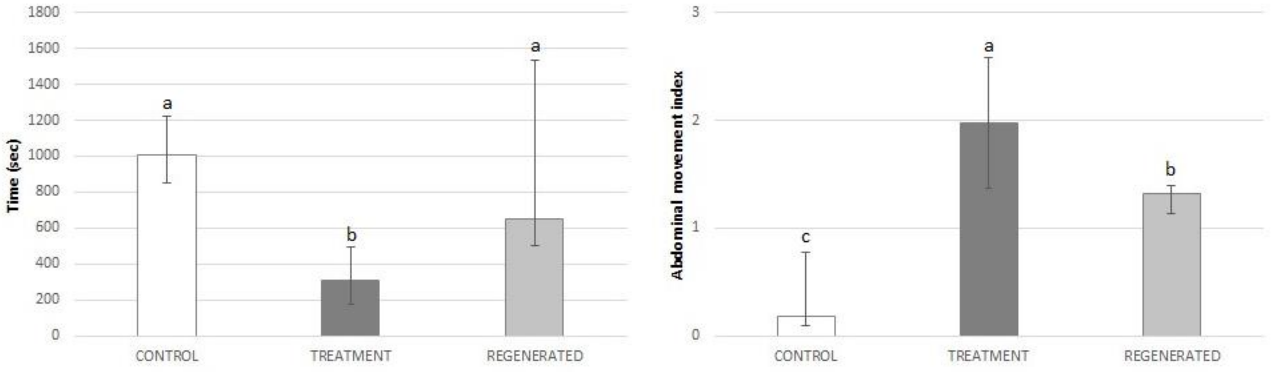

Figure 1. Comparison of copulation duration, a), and abdominal movement index, b), between control and treatment males from *Round 2* and males with regenerated gonopods. Letters over bars indicate post-hoc hierarchy.

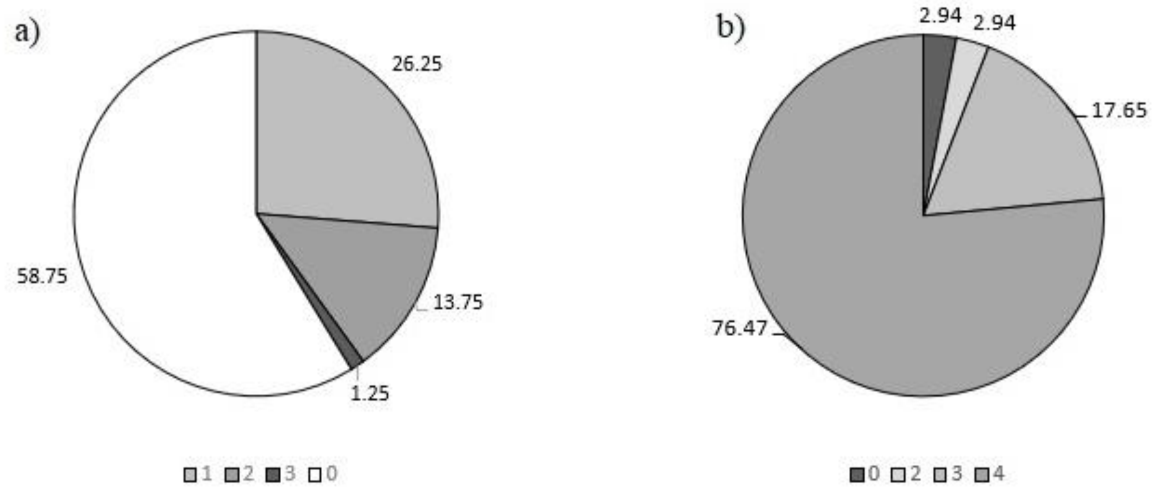

Figure 2. a) Number of molts for male individuals from *Round 1* (0 = 47 individuals, 1 = 21, 2 = 11, 3 = 1) by April 2018 and b) number of regenerated gonopods (0 = 1 individual, 2 = 1, 3 = 6, 4 = 26) by April 2018

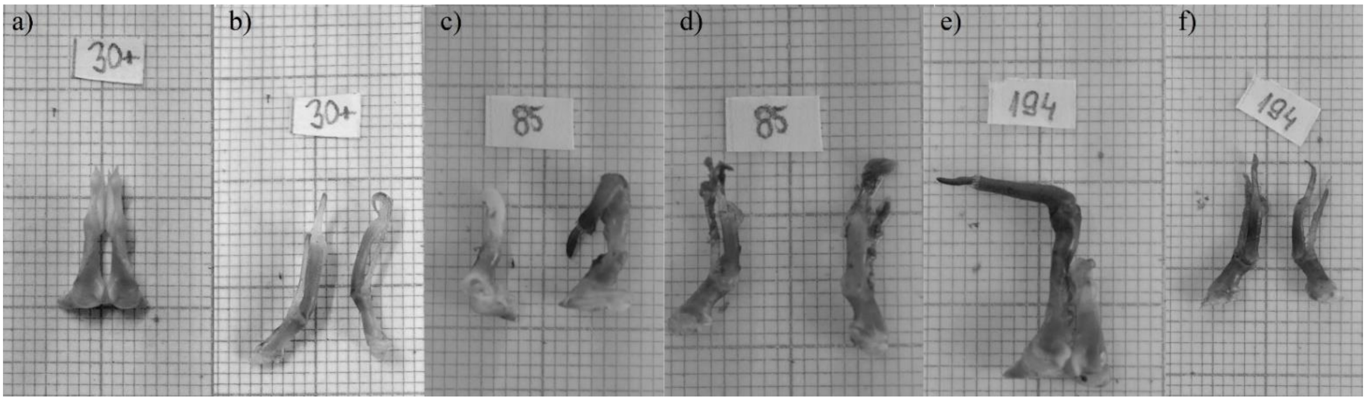

Figure 3. 1st and 2nd pairs of males gonopods; 30+ are gonopods from control male and 85 and 194 are regenerated gonopods from treated males. a), c) and e) are 1st and pairs and b), d) and f) are 2nd pairs. Right gonopod on photograph c) and left gonopod on photograph e) structurally resemble walking legs

CHAPTER 5. Malformed regeneration of gonopods in red swamp crayfish, *Procambarus clarkii*

-*in preparation*

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ABSTRACT

Gonopod regeneration was assessed in relation to gonopod removal treatment in the male red swamp crayfish *Procambarus clarkii*. Associated changes in gonopod size are described with new findings. Males were observed for 24 months (from April 2016 to April 2018). Most males 48.75% died without molting, 12.5% molted once and died, 1.25% molted twice and died, while 13.75% molted once, 12.5% molted twice and 1.25% molted three times, surviving till the end of observation period. In total 10% males survived and did not molt. The regenerated gonopods of males that molted only once were significantly shorter than gonopods of control males and not functional during copulation. It is possible that cutting position and generally cut instead of provoked autotomy has caused bigger damage, than autotomy does. This could explain the decrease in molting frequency and malformations we observed. Alternatively, the processes controlling regeneration of gonopods could be different from that controlling regeneration of other appendages that are usually damaged or lost in competitive interactions between animals.

INTRODUCTION

The red swamp crayfish, *Procambarus clarkii*, member of the family Cambaridae, is native to northeastern Mexico and south-central U.S.A, and is considered one of the 100 worst invaders in Europe (Gherardi, 2006). The life history of members of family Cambaridae differs from European crayfish, belonging to the Astacidae. In male of both crayfish families, first and second pleopods are modified in copulatory appendages (gonopods) (Castro et al., 2015). First gonopods have a groove through which sperm is passed to the female during copulation, while the second gonopods assist the first ones in transferring sperm to the opening of the female seminal receptacle along the groove of the first pleopod (Kato & Miyashita, 2003). Adult representatives of the family *Cambaridae* have secondary sexual features, i.e. “copulatory hooks” on the ischia of one or more pairs of the second through fourth pereiopods, and cyclic dimorphism. During copulation these hooks clasp the females (Oluoch, 1990). In species of the subfamily *Cambarinae*, two morphological types of adult males, form I (in breeding stage) and II (in non-breeding stage), occur and usually alternate during the life of an individual. In gonopods of form I, tips of each gonopod is corneous (Jegla, 1966). Gonopods are comprised of a basal two-part protopod (coxa plus basis) and terminal branches, exopod and endopod (Holdrich, 2009). Although crayfish continues to grow after having reached sexual maturity, the lengths of the first and second male pleopods have been under stabilizing selection, making males able to copulate with females of different sizes (Kato & Miyashita, 2003).

Adult crayfish usually molts twice a year, in late spring and early fall (Jegla, 1966; Barki, Levi, Hulata, & Karplus, 1997) and regenerate lost limbs with molting (Mykles, 2001). Most of the studies on limb regeneration in crustaceans have focused on the effects of damage of chelipeds or walking (pereiopods) legs, but not on gonopods.

Limb malformations usually result from abnormal healing of a wound after limb damage (Nakatani et al., 1997). To date there is number of studies reporting malformations of chelipeds and walking legs of crabs, chelipeds of crayfish and telson and eyes of prawns (Okamoto, 2006), all based on samples from different sites. In a laboratory study conducted by Johović et al. (2018), experiments have been performed to assess the effect of gonopod removal on male reproductive capability of *P. clarkii*. In treated males, the majority of gonopods in subsequent molt showed visible deformations. Functionality of these regenerated gonopods have been assessed and females that mated with males with regenerated gonopods did not produce offspring, suggesting that regenerated gonopods were not functional (Johović et al., 2018). The same study also suggested that removal of pleopods in adult males decreases molting frequency. The aim of present study was to assess the change in gonopod size and describe gonopod malformations caused by removal treatment.

MATERIALS AND METHODS

Collection of animals and holding conditions

Male specimens of *P. clarkii* (n=210) were captured with baited traps in Lake Sibolla and Ramone marsh (Tuscany, Italy) in April and May 2016. Male reproductive state was identified by its external secondary sexual features. A total of 80 sexually mature (form I; Huner, Lindqvist, & Könönen, 1988) and responsive males, with all the appendages, were selected and measured using a Vernier caliper (cephalothorax length and length of both chelae). Their gonopods were cut with scissors as part of the protocol to manually sterilize males (Johović et al., 2018). Treated individuals were maintained in laboratory conditions (isolated in opaque plastic aquaria 24×14×15 cm, fed 2-3 times/week with carrots and fish pellets, with water changed twice weekly, under natural photoperiod and ambient water temperature (20 °C). During the experimental period of 24 months, gonopod regeneration was visually assessed at every molting (form and number of regenerated gonopods). The majority of regenerated gonopods appeared to visually differ, from normal gonopods, especially in the subsequent molt after treatment. To compare regenerated gonopods of treated males that molted once in 24 months (11 males), to gonopods of untreated males, we selected 11 form I size-matching males, from 79 males collected from the same locations the year after, as control.

Data collection

Crayfish were measured in April 2018. Since carapace length (from the tip of the rostrum to the posterior edge of the cephalothorax) can vary between individuals (Buřič, Kouba, & Kozák, 2010) and rostrum can also be damaged, postorbital carapace length (POCL, from the edge of the eye socket to the posterior edge of the cephalothorax) was measured to the nearest 0.01 mm with Vernier caliper. The gonopods were photographed with a digital camera (Canon PowerShot SX510 HS) with millimeter paper placed beneath them. Measurements of the total length of the first pair of gonopods (GL, from the base to the tip of the copulatory stylet) were made to the nearest 0.01 mm using ImageJ 1.50i software (Rasband, 2017) which allowed us to use millimeter paper scale for calibration. The ratio GL/POCL was calculated and used to compare the gonopods of treated and control males.

Crayfish were individually euthanized by freezing and gonopods were dissected and fixed in 96% ethanol. Gonopods were analyzed under an electron microscope in the Laboratory of electron microscopy at the Institute of Parasitology of the University of South Bohemia, Czech Republic. For scanning electron microscopy, the samples were dehydrated through an ascending series of ethanol (90% ethanol, 100% ethanol, 100% ethanol : 100% aceton = 2:1, 100% ethanol : 100% aceton=1:1 and 100% ethanol : 100% aceton= 1:2, 100% aceton), and the dehydrated samples were critical-point dried (Pelco CPD2), mounted on aluminium stubs, and sputter-coated 3 minute time with 5 nm thick layer of gold. Preparations were examined using a FE-SEM JSM 7401- F (JEOL Ltd., Tokyo, Japan) at low accelerate voltage of 4 kV using GB-low mode. The accomplished topographic images provided a resolution of 1.5 nm by an efficient semi-in-lens detector.

Data analysis

Statistical analyses were performed with SPSS (Statistical Package for Social Sciences) version 21.0 (IBM SPSS Inc., 2016). Data were first tested for normality and homogeneity of variance using the using the Kolmogorov–Smirnov and Levene tests, respectively. The Mann-Whitney test (statistic: U) was used to compare gonopods of control (C) and treated (T) males. The level of significance under which the null hypothesis was rejected is $\alpha = 0.05$. The parametric data were reported in the text and figures as a mean value ± standard error while for non-parametric data median values and 1st and 3rd interquartile were given.

RESULTS

Control males had a significantly higher GL/POCL ratio compared to treated ones (U= 47.00, n=43, p=0.00, control: 0.29 ± 0.01, treated: 0.24 ± 0.13). GL differed between control and treated males (U= 47.00, n= 43, p= 0.00; control: 10.44 (10.02-11.25) mm; treated: 7.66 (6.57-8.27) mm), while POCL was similar (t= 0.26, df= 20, p= 0,799; control: 35.61 ± 0.75 mm; treated: 35.36 ± 0.64 mm) (Figure 1). Two treated crayfish had pereiopods instead of gonopods (Figure 2, 3).

DISCUSSION

During the observation period of 24 months 48.75% of treated males (39) died without molting, 12.5% molted once (10) and died, 1.25% molted twice (1) and died, while 13.75% molted once (11), 12.5% molted twice (10) and 1.25% molted three times (1), surviving till the end of observation period. In total, 10% males survived and did not molt. Over the half of males did not molt during observation period, while the highest percentage of males molted only once. The regenerated gonopods of males that molted only once were shorter than gonopods of control males and not functional during copulation (Johović et al., 2018). Regeneration potential and success decrease with size and age, and damage accumulation caused by removal of multiple limbs in older and bigger animals may have stimulated the attainment of the terminal molt in older animals (Juanes & Smith, 1995). Energy allocation during regenerative processes varies with limb type and sex (Juanes & Smith, 1995). In the red king crab, *Paralithodes camtschatica,* recovery rate at the first regenerative molt showed to be higher in the chelae than in the other walking legs (Kurata, 1963). Juanes & Smith (1995) suggested that, considering limb types, the greatest amount of energy is devoted to regenerate chelipeds, since they cover the greatest portion of the body compare to other limb types. Loss of chelipeds and/or walking legs increases molting frequency and decreases intermolt period in the *P. clarkii* with removal of all ten limbs proved to be more effective than removal of only eight walking legs (Bittner & Kopanda, 1973). Juanes & Smith (1995) hypothesized that limb types that are critical to overall fitness should be expected to regenerate more quickly than less important appendages. Johović et al. (2018) showed that removal of gonopods in adult males of *P. clarkii* decreases molting frequency, highlighting that gonopods are maybe harder to regenerate than claws or walking legs.

Autotomized limbs are the most favorable starting point for regeneration. Morgan (1902) showed that the limb of hermit crab regenerates most efficiently if the limb is removed at the breaking joint. Bittner & Kopanda (1973) showed that crayfish *P. clarkii* with autotomized cheliped and/or walking legs also had highest molting frequencies. The ability to regenerate entire limbs actually depends on the presence of the stump of the ganglionic root at the base of the regenerating limb (Cooper, 1998).

Limb malformations and outgrows are usually caused by chemical and/or mechanical perturbations during regeneration (Scholtz et al., 2014). Different limb types have also been reported growing in "wrong" places (Gordon 1963; Scholtz et al, 2014), often atrophied, e.g. atrophied pereiopod implanted on the abdomen of spiny lobster, *Palinurus argus* (Fausto & Da Costa, 1977).

It is possible that cutting position and generally cut instead of provoked autotomy has caused much bigger damage: this could explain the decrease in molting frequency and malformations we observed. Otherwise, the processes controlling regeneration of gonopods could be different from the one controlling regeneration of other appendages that are usually damaged or lost in competitive interactions between animals.

In conclusion, this study provides new findings about gonopod regeneration of cambarid crayfishes, which can contribute to optimization of sterilization method for Sterile Male Release Technique and help in management of *P. clarkii*, but also opens interesting questions about the regeneration process of limbs in crayfish.

ACKNOWLEDGMENTS

This research was funded by the European Union's Horizon 2020 research and innovation programme under the project Marie Sklodowska-Curie Aquainvad-ED (H2020-MSCA-ITN-2014-ETN-642197).

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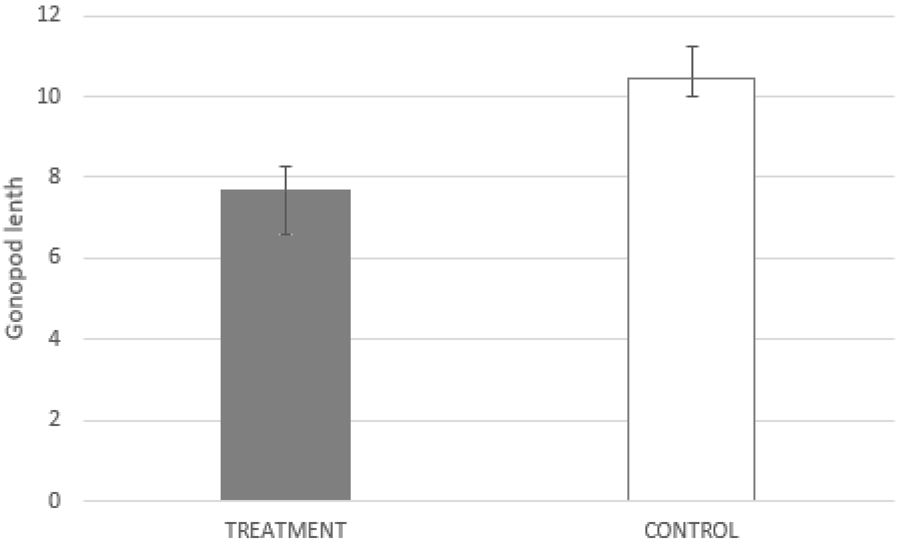

Figure 1. Comparison of first gonopods lengths in treated (n=11) and control (n= 11) males of *Procambarus clarkii*. Bars represent median ± interquartiles.

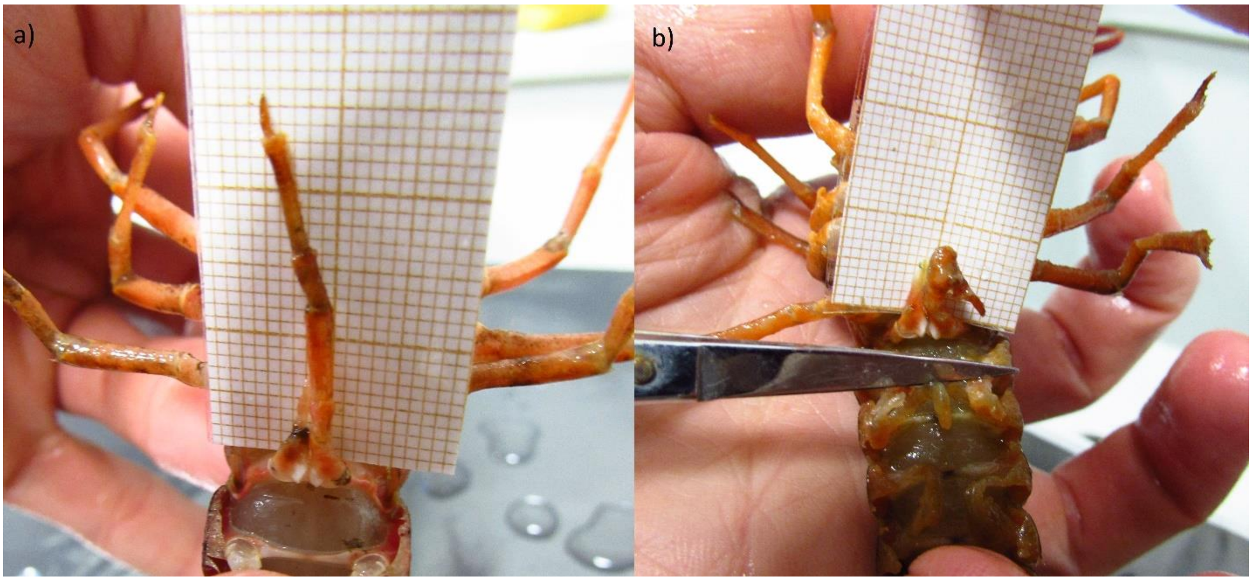

Figure 2. First gonopods of treated males; right gonopod on the photo a) (male 140) and left gonopod on the photo b) (male 85) resembling pereiopods

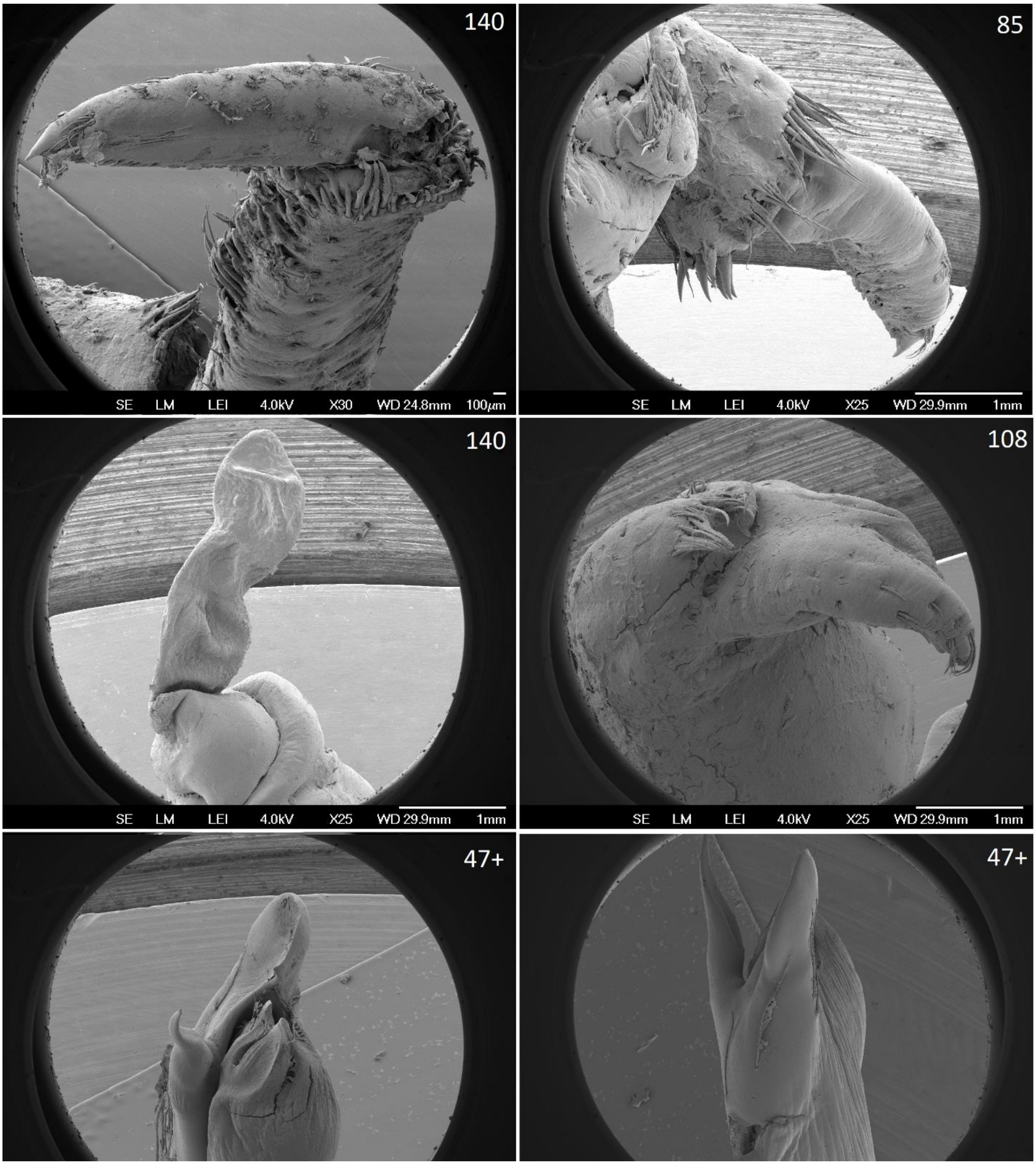

Figure 3. Terminal ends of first pair of gonopods of treated (male 85, 108 and 140) and control males (47+)

CHAPTER 6. GENERAL DISCUSSION

This PhD has provided new findings, highlighted recent progress in understanding of invasive species management and contributed to the growing field of invasion science. Moreover, it is hoped that the provided results will have useful applications in the management of invasive alien species.

The presented studies aimed at namely assessing available methods in IAS management, analyze the present and potential techniques as well as to develop innovative methods for pre and post invasion AIS management. The discussion which follows will summarize the findings of each chapter and make recommendations for future research.

The EU's IAS Regulation and CBD, both advocate prevention of introduction and establishment as the most environmentally preferable and cost-effective form of management of invasive alien species (IAS). Accurate spatial and temporal characterizations of suitable areas where invasive alien species might arrive, establish, spread and cause harmful impacts is fundamental in the prevention of invasive species introduction (Venette et al., 2010). As such, ensemble forecasting models provide insights about possible shifts in the range of invasive species and summarize suitability of not yet invaded areas for invasion Gama et al., 2017). The information extracted from these models can help authorities to make more informed decisions, re-examine and adapt their conservation strategies, and protect those native species under threat. For instance, comparison between currently suitable areas and areas that will be suitable in the future can provide information about where to allocate resources for monitoring. Alternatively, the comparison of suitable areas between invasive species and species vulnerable to their impacts under different time and CO_2 emission scenarios can help us to locate potential refugia for threatened species and help us create more effective strategies for the conservation of those species.

Within Chapter 2, the potential distribution of one AIS at the global scale based on different algorithms was modelled and summarized in an ensemble model to identify suitable areas for its introduction and establishment. Ensemble forecasting of American bullfrog, *Lithobates catesbeianus* distribution provided information on relative importance of used variables, algorithms, as well as ensemble model performance and located the areas suitable for invasion in different time periods and RCP scenarios. Gained information and modeling outputs were used to provide an example on how to assess this information in the planning of effective conservation management responses. The results revealed that more area will likely become climatically suitable in the future, increasing with time and elevating CO_2 emission scenarios from 3.8% suitable today to 5.23% for the 2070 (RCP 8.5) projection. Additionally, more areas will be suitable at higher latitudes, especially in North America and throughout central Europe. Moreover, more suitable areas will be available in EU protected areas, such as Natura 2000, underlying their vulnerability to bullfrog invasion. Even though a number of authors have developed models describing the potential global or South America distribution of bullfrogs (Ficetola et al., 2007; Giovanelli et al., 2008; Nori et al. 2011), we are not aware of similar studies, describing bullfrogs' climatic requirement using data from the whole range (invasive and native range). Beaumont et al. (2009), reported that the calibration of invasive species distribution models using native ranges leads to misrepresentation of potential invasive distributions. Despite different modeling approaches, identified suitable areas at current conditions are comparable to Ficetola et al. (2007) proposed global distribution, however the surface area with maximum suitability is larger in our model. In terms of water availability, our model showed that bullfrog presence is positively related to precipitation of the driest month. Presence of permanent waters is a commonly recognized feature related to bullfrog presence, since tadpoles overwinter in water (Ficetola et al., 2007; Liu & Li, 2009). The identified links between species and environment should be used to calibrate policies for frameworks intertwined with "the IAS Regulation", e.g. the river basin management plans (RBMPs) under the Water Framework Directive, "Birds" and the "Habitats" Directives, the Environmental Impact Assessment (EIA) and the Strategic Environmental Assessment Directives (SEA). For instance, EIA and SEA policy should ensure that any plan or project proposing modifications likely to have a significant effect on invasive species introduction and establishment is appropriately assessed, especially in areas where its impacts could contribute to the decrease and/or extinction of populations of species of Community interest (Annex IV(a) of the Habitats Directive). Thus, incorporating model outputs in management policies could help to control invasive species and in turn to create more accurate predictions of the future distribution of species at the finer scale, land cover and topography will have to be integrated in models to improve their predictive accuracy. Human activities are also vital factor influencing the likelihood of invasive species spread and establishment and use of variables that measure human influence on global surface, e.g. human footprint is recommendable to improve the prediction of invasion risk.

Once after introduction and establishment of the invasive species, eradication, containment and control are the only remaining management options. Due to biology of the invading organism, characteristics of the invaded ecosystem, high probability of subsequent introductions or potential non-target effects eradication is often unfeasible. Today, there are many examples of successful control efforts, which also proved to be less costly in the long term than the damages from unmanaged invasions (Wittenberg & Cock, 2005; Simberloff, 2009).

The core of the IAS Regulation is a list of "invasive alien species of Union concern", which includes five invasive crayfish species (marbled crayfish, red swamp crayfish, signal crayfish, spiny-cheek crayfish and virile crayfish). The Regulation obliges Member States to rapidly eradicate these species after being reported on the new territories and to manage ones already present in their territory (Article 19 of IAS regulation). Given that the list prioritizes actions on those species, strong strategies for management of the crayfish species on the list should be a priority for governments of all Member States. It concerns that definitive methodology for the control and eradication of invasive crayfish species is still not available (Gherardi et al. 2011) but it is hopeful that several novel techniques are under investigation and development. Since management options can be constrained by the status of the crayfish population and of the invaded area, it is often necessary to rely on traditional techniques, that can still achieve good results.

In the second part of this thesis, I evaluated the effectiveness of intensive trapping activities through the CPUE index, carried out within the project LIFE "SOS Tuscan Wetlands" in two wetlands in northern Tuscany (Chapter 3). Crayfish catch rates declined over the 4-year removal period in both areas. Sex-ratio was almost biased to males. Inactivity during egg incubation influences females catch rates (Hein et al., 2007). As expected, the majority of trapped males were large adult individuals. Standardized monitoring of the population control operations was carried out at the beginning of every control season to assess its effective impact. Overall, four seasons of intensive trapping activities led to the removal of 123,534 crayfish (44,997 in Sibolla lake, 78,537 in Ramone marsh). C.P.U.E. index in the Sibolla population was reduced by 74% (from 4.47 in 2014 to 1.18 in 2017). In Ramone marsh catches decrease was 55%, with a C.P.U.E. index of 20.56 in 2014 and 9.21 in 2017. Biodiversity rate is greater in Sibolla lake than Ramone marsh, which can be considered a hotspot of allodiversity for the several alien species present (e.g. coypu, red-eared turtle, black bullhead, pumpkinseed, western mosquitofish and eastern mosquitofish). Therefore, control activities in lake Sibolla showed better results, although the lake is wider and deeper, since it is inhabited by several indigenous predators of crayfish (eels and pikes) exerting a natural control on crayfish, especially on juvenile life stages.

Intensive trapping seems to be promising for the management of invasive crayfish populations. Efficiency of traps in catching wide reproductive population can be improved by using several trap opening diameters for trapping crayfish (Stuecheli, 1991), decreasing the mesh size (Wright & Williams, 2000) as well as by using different types of traps such as funnel traps to catch large males and artificial refuges traps (ARTs) to catch smaller individuals of both genders and female individuals (Green, et al. 2018). To obtain long-lasting results, intensive trapping should be combined with other management activities and control techniques (Gherardi et al., 2011), e.g. i) restriction of the size limits for the fisheries to limit the harvest of fish predator species known to consume crayfish; ii) reintroduction of the indigenous predatory fish species including restoration of their suitable habitats; iii) Sterile Male Release Technique. This combined approach could increase efforts in terms of costs and time, but in long-term it would result in ecosystem recovery and help in maintaining low abundances of crayfish (or even in achieving eradication) in at least isolated/confined waterbodies. Since traps select large animals and fish predates on crayfish that are too small to be trapped, fish predation combined with trapping works effectively in controlling invasive populations of crayfish (Hein et al., 2006). Hein et al. (2007) managed to achieve decline of 95% in rusty crayfish *(Orconectes rusticus)* population in Sparkling Lake, USA, over the course of five years combining smallmouth bass *(Micropterus dolomieu)* and rock bass *(Ambloplites rupestris)* predation and trapping. Dana et al. (2010) combined trapping, manual removal and electrofishing on signal crayfish population in Riofrío River in Spain, that over a 4-year period, resulted in a sharp decline in the population size with a catch rate (mean catch rate per worker and day) of 30 in the 1st year decreasing to 10 in the 4th year. Targeting all life stages equally is a key feature of combined approaches (Stebbing et al., 2014).

Recently, management activities integrating trapping and Sterile Male Release Technique have been carried out in Europe, but information about success of the actions is difficult to obtain, because it is scattered and often only available as part of project reports, if published at all. Aquiloni & Zanetti (2014) used this integrated approach on *P. clarkii* within the project LIFE RARITY. SMRT, where crayfish were captured with baited traps and sterilized with ionizing radiation with a dosage of 20 Gy, were released back to the lake of Casette, Italy. Two years of activities resulted with an impressive reduction in the Casette population of 87%. The biggest drawback of this technique is the average cost per *P. clarkii* specimen that was estimate at 2.7 euros (Laura Aquiloni & Zanetti, 2014). Under the scope of the River Barle Crayfish Project (2015-2017), conventional baited traps were used to collect larger adult males of *Pacifastacus leniusculus*, which were sterilized manually, using a technique described in (Stebbing et al., 2014), while artificial refuges traps were used for catching smaller individuals of both genders and larger adult females. Although the project report has not yet been published, but preliminary results were encouraging (pers. comm. with Nicky Green, October 2017), fostering hopes in the prospects of wider adoption of SMRT to control signal crayfish populations.

In Chapter 4 and Chapter 5, I conducted experiments to assess the effectiveness of manual removal of male gonopods, i.e. the appendages responsible for sperm transfer, as a sterilization technique to be used in control with SMRT against *Procambarus clarkii*. The aim of the work was to evaluate whether this treatment affects male competitiveness and sexual behavior as well as reproductive potential in *P. clarkii* in order to use this technique in the field. Removal of gonopods showed to partly alter male behavior. Treated males needed to initiate more interactions with females or needed to spend longer time in agonistic interactions to successfully dominate a female for the copulation to take place. Copulation showed to be also significantly shortened. However, male readiness to initiate sexual interaction with females was not affected by the treatment, resulting with equal number of treated males gaining opportunity to mate and significantly reduced reproductive success when compared to control males. Since females after copulation hide in burrows until the release of juveniles (Aquiloni et al., 2009), it is expected that they will not copulate with other males to compensate for unsuccessful copulations with treated males. Since changes in behavior of treated males do not prevent them from mating and results suggest that technique should be effective in decreasing the crayfish population, we recommend the further testing of this technique in the field. The next step would be to determine the number of sterile males to be released and the number of releases that would effectively reduce the reproduction rate, by means of mathematical modelling.

SMRT involving manual sterilization of males offers a control method fulfilling several criteria: it guarantees a good chance of success in terms of resources spent, respects the criteria of maximum precaution, is socially and it should be ethically more acceptable to the general public, and is safe for humans, the environment and other species (Holdich et al., 1999). Moreover, it is compatible with other techniques, is not particularly expensive and does not require long application times, as its effectiveness increases when the density of individuals is low, since probability of treated males mating with females is higher. The effectiveness of the use of SMRT in the control of insect pests motivates the further development of this technique with a hope for achievement of wider management strategy against invasive crayfish.

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CHAPTER 7. APPENDIX

7.1. The North American channel catfish, *Ictalurus punctatus*, in Europe: a neglected but potentially invasive species?

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Running title: The alien channel catfish in Europe

Abstract

Several alien species have not gained enough attention and an in-depth assessment on their potential invasiveness has not been carried out. The American channel catfish *Ictalurus punctatus*, despite having been highly introduced in Europe and Asia, is one of these neglected species, and its potential invasiveness as well as its distribution have not been sufficiently quantified and detailed. Literature review revealed the lack of studies in Europe assessing its potential impacts on invaded communities, although in the introduced North American area this species can cause relevant ecological damages, being highly prolific, tolerant to a wide range of conditions and an opportunistic feeder with few natural predators. Environmental Impact Classification of Alien Taxa (EICAT) was applied to evaluate the magnitude of its impacts for the invaded range in North America and in Europe. Interestingly, the scoring system assigned the species to the category "Major" for both areas, but with a high level of confidence for the North America, while a medium level of confidence for Europe. These results confirm the urgent need to carry out in-depth studies and update data on current presence in Europe to better understand its invasiveness in order to promote the most appropriate management measures.

Keywords: *freshwater, review, impact, management, assessment*

Introduction

In the last decades, the multiple impacts of the well-known and highly invasive alien fish species, such as topmouth gudgon *Pseudorasbora parva* (Temminck & Schlegel, 1846), Wels catfish *Silurus glanis* (Linneaus, 1758) or rainbow trout *Oncorhynchus mykiss* (Walbaum, 1792), have been the focus of an intensive scientific research. On the contrary, several other alien species have established remarkable and widespread populations, receiving, however, scanty or no attention by scientists and managers. The North American channel catfish *Ictalurus punctatus* (Rafinesque, 1818) (Figure 1.) is one of the most important, commercially cultured aquatic species in the United States (Brown, 1942; Appelget & Smith, 1951; Boyd et al., 2000; Rezk et al., 2003; Tucker & Hargreaves 2004; Simmons et al., 2006). It is supposedly native to the Gulf States and Mississippi Valley, from Canada to Mexico (Wellborn, 1988), but the exact native range is uncertain (Etnier & Stanes, 2001). Being a desired sport fishing species, its presence now outstretches across North America with populations being greatly sustained by increased annual stockings of even small impoundments (Scott & Crossman, 1973; Tucker & Hargreaves, 2004; Leonard et al., 2010). While scientific studies dealing with this species impact on invaded areas have focused mostly on the North American continent (e.g. Carlander, 1969; Minckley, 1973; Poe & Rieman, 1988; Townsend & Winterbourn, 1992; Jackson & Badame, 2002; Adams, 2007), several aspects of channel catfish biology outside its native range, such as diet, behaviour and reproduction, remain unassessed.

After its first introduction to Europe in the 19th century for angling and aquaculture, it has been recorded in at least 22 EU-countries, being considered established in seven countries (Welcomme, 1988; Elvira & Almodóvar, 2001; Copp et al., 2005; Olenin et al., 2008, CABI, 2016; Table 1). Even if it is so widespread, it is one of the least studied species (especially in terms of its potential invasiveness), most likely due to a shorter invasion history and lower “species profile” than other notable invaders such as *S. glanis* (Copp et al., 2009). This complete lack of data on its behaviour, ecology and potential impacts (Townsend & Winterbourn, 1992) has led to a neglect by policy makers and conservation efforts, favouring its broad distribution in European freshwater ecosystems. However, studying its introduction history is important to enable implementation of management and risk assessment strategies. Here, for the first time, we reviewed all the relevant available information on this species to provide a comprehensive overview on the existing knowledge and gaps to be addressed in future works. The overall aim was to retrieve data on its impacts in order to evaluate the invasiveness of the species in Europe. To achieve this, data on its introduction history were retrieved and the Environmental Impact Classification of Alien Taxa (EICAT; Blackburn et al., 2014) was applied to evaluate the magnitude of its impacts for the invaded range in North America and in Europe.

Introduction history

Although *Ictalurus punctatus* is mainly produced for aquaculture in the US, Canada and Russia, China and Brazil have recently established channel catfish farms (FAO Fishery statistics, 2002; Pool, 2007). Furthermore, it has been intentionally introduced in several countries for restocking, aquaculture, angling, ornamental or research purposes with further ongoing, accidental and intentional introductions having occurred (see Table 1 for the complete list of countries).

Invasiveness

Channel catfish have been described as one of the fastest expanding species (Tucker & Hargreaves, 2004; Olden & Poff, 2005). They have been shown to exhibit a variety of negative impacts in the Western US (Hawkings & Nesler, 1991) in the introduced range, especially on endangered species, as well as on fisheries and angling. Considering the data retrieved from North America, and the possibility of accidental escapes during natural events such as floods (Robinette & Knight, 1981; Townsend & Winterbourn, 1992; Orsi & Agostinho, 1999; Zanata et al., 2016), the introduction of channel catfish into New Zealand has been evaluated as highly risky for at least one native and other species of conservation concern, and it has not been allowed. Recorded impact mechanisms are manifold and include i) predation, ii) competition, iii) hybridization, iv) disease transmission, and v) modification of ecosystem services. Besides ecological impacts, also socio-economic impacts are considered.

i) Predation

Channel catfish are opportunistic omnivorous with a wide range of food items, being capable of exploiting new sources (see e.g. Heard, 1958; Bailey et al., 1945; Joyce & Chapman, 1987; Hawkins & Nesler, 1991), finding potential prey through their amino-acid sensitive taste buds. This ability gave them the nickname “swimming tongue” (Joyce & Chapman, 1987; Caprio et al., 1993). Usually, adult feeding takes place between nightfall and dawn. Juveniles are active during the entire day, although previous studies observed a complete stop in activity at water temperatures below 15.5 °C (Brown, 1942; Becker, 1983; Gerhardt & Hubert, 1991). Additionally, they are considerate to be fairly effective visual predators despite being primarily nocturnal foragers. In North America, the species can reach a maximum size of 915 mm and 13 kg (Pool, 2007; Smith, 1974), with a preference for environments with low turbidity, slow currents (Holland and Peters 1992) and well-structured, muddy or sandy bottoms (Buck & Cross, 1951; Finnell, 1954; Marzolf, 1957, Becker, 1983).

However, it is able to thrive in most freshwater habitats, including fast flowing currents, creeks, swamps or reservoirs (Etnier & Starnes, 2001), while even remarkable populations in brackish water with up to 11.4 ppm of dissolved salts are reported (Perry, 1968; Scott & Crossman, 1973; Wellborn 1988; Meisenheimer, 1988; Etnier & Starnes, 2001; Ross, 2001).

Beside algae and detritus, insects and other invertebrates found in reed areas make up the majority of the smaller sized specimens' diet (length < 102 mm) (Bailey & Harrison 1948; Walburg, 1975; Endo et al., 2015). Bigger sized individuals change to a wider diet, feeding also on larger insects such as mayflies (adult and larvae), crayfish, molluscs, nymphs, snakes, birds, spiders and plants (Menzel, 1945; Bailey & Harrison, 1948; Miller, 1966; Starostka & Nelson, 1974; Becker, 1983; Wellborn, 1988; Dagel et al., 2010; Endo et al., 2015). Predation on fish (especially on *Anguilla* spp.) and terrestrial preys (earthworms, crickets, mice and rats) is highly dependable on availability, but, when available, these can constitute the majority of the diet (Bailey & Harrison, 1948; Robinette & Knight, 1981). Particularly crayfish, a consistent food source (~ 25%) for juveniles and adults, are considered vulnerable to its predation (Poe & Rieman, 1988; Townsend & Winterbourne, 1992). This was confirmed in mesocosm experiments carried out by Adams (2007) in the US, leading to hypothesize that the spread of *I. punctatus* might be responsible for the decline of native crayfish. Furthermore, fish, amphibians in general (see e.g. Sartorius and Rosen 2000), and other benthic animals are heavily predated by the channel catfish in North America, with a consequent decline in their abundance (Menzel, 1945; Matsuzaki et al., 2011; Hill et al., 2015). This is bolstered by this species slow growth, favouring a demographic structure biased towards younger age groups (Holland & Peters, 1992). The life span is, indeed, about 6 - 10 years on average, but even up to 14 and 28 years old individuals have been recorded (Tyus & Nikirk, 1990; Bouska et al., 2011). According to its broad distribution, age of maturity is very variable. In its native range, maturity is reached in specimens with a total length of ~305 mm (Appelget & Smith, 1951) in approximately 2-5 years or later (DeRoth, 1965). In Southern regions of North America, where growing seasons are significantly longer, individuals mature at a smaller total length and with 5 years or earlier, while in Northern regions they mature with 6 or 7 years for males and females, respectively, or even later (Davis & Posey, 1958; Scott & Crossman, 1973; Starostka & Nelson, 1974; McMahon & Terrell,1982).

Nonetheless, as size increases, a preference for predation on fish becomes prevalent, threatening several species (McGovern & Olney, 1988; Hanzawa, 2004; Arayama, 2010; Matsuzakiet al., 2011). Boersma et al. (2006) observed, that *I. punctatus* gut contents showed 50% to 100% consumption of juvenile salmon, posing a considerable pressure on salmon populations. Even cannibalism is a common phenomenon in channel catfish populations (Unprasert et al., 1999). Predation and consumption of bigger fish occurs occasionally, as well as the consumption of dead fish: it has been observed that channel catfish can prey on adult specimens of *Cyprinus carpio* (Linnaeus, 1758), as revealed by the presence of their scales in *I. punctatus* stomach content (P. Haubrock, pers. obs.).

Another aspect, indirectly related to predation, is the presence of spines on lateral and dorsal fins. These are highly modified anatomical parts of fins, which can harm or kill predators as an anti-predator adaptation serving as a pure defensive purpose (Bosher et al., 2005). Additionally, spines are responsible for the stridulatory "distress-call" sound production through binding and spine locking (Fine et al., 1997; Bosher et al., 2005). While the juvenile and smaller life stages have somewhat softer spines and are therefore rapidly consumed by predators, adult become unpalatable. It has hence been repeatedly reported that predators choke on them due to these hardened pectoral and ventral spines (McAda, 1983; Pimental et al., 1985). Moreover, in contrast to its native range, channel catfish have barely any native predators in Europe except *Dytiscus sp.* for the juvenile stages (CABI 2016). Invasive alien species that are natural predators such as largemouth bass *Micropterus salmoides* (Lacépède, 1802), have been introduced to Europe, but there is no data on their effective predation on channel catfish.

ii) Competition

Species with wide feeding habits can overlap with native species (e.g. trout species), leading to significant impacts and severe threats for native species (Tyus & Nikirk, 1990; Townsend & Winterbourne, 1992). As populations of channel catfish rapidly grow (Hawkins & Nesler, 1995), it has been observed to compete with several native species in North America (Mitzner, 1989; Hawkins & Nesler 1991; Nesler, 1995). Individuals become increasingly piscivorous (Hubert, 1999) and can compete with other top predators such as largemouth bass for resources (Hackney, 1975). Competition can also occur when channel catfish occupy the same habitat of native species, particularly during periods of limited resource availability (Wick et al., 1985; Tyus & Karp, 1989; Nesler, 1995). Additionally, a recent study by Leonard et al. (2010) suggests that largemouth bass growth and suitable conditions are lowest if channel catfish stocking rates were highest, thus implying that with higher abundance of adult fish competition might increase proportionally. Although little is known about the size structure and growth characteristics among the different environments, back-calculations of fish lengths show that it takes 4-6 years to reach 300 mm total length on average (Holland & Peters, 1992). Thus, it is likely that increased growing seasons may affect annual growth, leading to an earlier maturity and capability to compete with other predators (Durham et al., 2005; Shephard & Jackson, 2006). Indeed, under optimal conditions, channel catfish can reach adult life stages in few years, showing a rapid egg, hatching and juvenile development, although displaying a diminishing growth rate between age groups (Holland & Peters, 1992). While the optimal growing temperature of adults is comprised between 26 and 29 °C (Shrable et al., 1969; Cheetham et al., 1976), with temperatures below 21 °C decreasing and below 18 °C completely stopping its growth (McCammon & LaFaunce, 1961; Macklin & Soule, 1964; Andrews & Stickney, 1972; Stickney & Andrews, 1972; Starostka & Nelson, 1974), spawning temperatures are broadly variable, between 21 and 29.5 °C with the spawning occurring in late spring or early summer (Appleget & Smith, 1951; Katz, 1954; McClellan, 1954; Smith, 1974; McMahon & Terrell, 1982; Minnesota Department of Natural Resources, 1988) or in captivity after the accumulation of 57 - 172 degree-days (Pawiroredjo et al., 2008). The species is claimed to be mating annually once in monogamous pairs (Tatarenkov et al., 2006) and has a high fecundity: in its native range, mature females spawn once a year producing up to 3,000 - 4,000 eggs per pound (~ 6,700 - 8,800 per kg) of body weight, when temperatures are suitable, producing up to 20.000 eggs in their native reproduction time between April and July. In addition, adults are usually inactive and do not feed during wintertime when temperatures drop below 15.5 °C. Variations in temperatures and conditions can result in more than one reproductive cycle (Appleget & Smith, 1951; Toole, 1951; McMahon & Terrell, 1982). According to the availability of spawning sites (such as rubble, stones, holes, covered areas), males dig the nest. During mating, male and female catfish align with each other in opposite directions, wrapping their tail around the head of the mate. Then, the male stimulates the female by “shivering”, who then releases the eggs. Males fertilize the eggs and in the following chase the female away (although McKaye et al. (1994) observed that females remain close) and remain to protect the up to 8,000 laid eggs, fanning oxygenated water towards them for 5- 10 days (Brown, 1942; Appleget & Smith, 1951; Mayhew, 1987; Pool, 2007). The developing eggs, which require water temperatures between 15.5 and 29.5 °C, hatch in 6 days at 25 °C or 10 days at 15.6 °C (Toole, 1951).

iii) Hybridization

In the North American invaded range, channel catfish have been observed to hybridize with the endangered congeneric *Ictalurus pricei* (Rutter, 1896) and *I. lupus* (Girard, 1858), threatening their populations where distributions overlap (Kelsch & Hendricks 1990; Sublette et al., 1990; Kelsch & Jensen 1997). Furthermore, *I. punctatus* females and male blue catfish *I. furcatus* (Valenciennes, 1840) can hybridize, producing faster growing (Sublette et al., 1990) and disease resistant offspring with a better food conversion ratio, tolerant to low oxygen (1.0 mg^{l-1}) and crowding (Masser & Dunham, 1998; Dunham & Brummett, 1999; Tucker, 2000; Dunham et al., 2008; Arias et al., 2012). As hybrids are fertile, back crossing with parental populations can represent a threat of genetic pollution accompanied by competition among hybrids and parental populations. Usually, hybridisation between *I. punctatus* and *I. furcatus* or other species of Ictaluridae is uncommon in the wild due to differences in reproductive cycles, different preferences for spawning environments, and size differences (*I. punctatus* being generally smaller). However, conditions may differ in closed or introduced environments. *I. punctatus* (female) x *I. furcatus* (male) (C x B) hybrids, which can produce large numbers of second-generation (F_2) hybrids (Dunham & Masser, 2012), are widely established in aquaculture. Additionally, the existence of hybrids in regions where *I. punctatus* and *I. furcatus* are introduced (e.g. Italy) could pose an increased threat to the communities and the environment for the aforementioned characteristics they have (Dunham & Masser, 2012).

iv) Disease transmission

American catfish species are well-known carrier of several potentially infectious parasite, viral or bacterial agents (Terhune et al., 2002; FishBase 2007; Pool, 2007). However, no impacts were reported in the North American invaded range, while potential transmission to other native/alien species has been reported in Europe. One recent discovered pathogen is the Myxosporidian *Sphaerospora* species (e.g. *S. ictaluri*), a so called "blood protozoan", which can be considered as the presporogonic stage of sphaerospores. It was described from infected *I. punctatus* in farm ponds in Italy (Molnár and Baska 1992), likely affecting cyprinid fish and causing "carp-dropsy complex". This disease is characterized by dropsy and exophthalmos (Lom et al., 1983; Dykova et al., 1986; Rukyani, 1990). Another frequent disease transmitted by channel catfish is the channel catfish virus (CCV) (Ictalurid herpesvirus 1), a member of the Alloherpesviridae family causing significant economic losses in catfish aquaculture. Although *S. glanis* fingerlings have been resistant to infections by the channel catfish virus in the lab (Plumb et al., 1985), no information is available about potential implications regarding other diseases affecting different live-stages of other species. Additionally, Ictaluridae are well-known carriers of several parasitic helminths, several being potentially host switching nematodes (Pérez-Ponce de Leon & Choudhury, 2002; Choudhury & Perryman, 2003), and *Flavobacterium columnare*, a pathogen known to cause columnaris disease, a serious condition affecting numerous freshwater fish species all over the world (Decostere, 2000; Welker et al., 2005).

v) Modification of ecosystem services

The presence of an invasive alien species can damage the services provided by the ecosystems (provisioning, regulating and maintenance, cultural: Haines-Young & Potschin 2013). For the channel catfish, it is likely that provisioning services are affected through direct predation on valued food items (as for the abovementioned Japanese lake). As excessive consumption of algae, detritus and aquatic plants by immature channel catfish has been observed (e.g. Menzel, 1945; Braun & Phelps 2016), *I. punctatus* can cause a decrease in water quality and change the primary production as well as the entire trophic chain, with bottom-up cascade effects, thus modifying provisioning, regulating and maintenance services. Additionally, cultural services are likely affected: angling and associated tourism can make up an important percentage of the regional income and they can be damaged by the feeding habits of the species (Aylward et al., 2005). On the other side, it is hence possible that economic impacts could be positive, drawing in tourism and that anglers increasingly enthusiastic about the fishing opportunities opening up with the abundance and presence of *I. punctatus* (P. Haubrock, pers. comm.). A study by Matsuzaki et al. (2012) showed that in a Japanese lake the presence of invasive channel catfish changed the native community composition and negatively influenced commercially important species. This led to a reduction in the provision of ecosystem goods, showing the great impact of *I. punctatus* also under suboptimal climatic conditions. In North American invaded range, economic losses are also reported for failed conservation actions on native amphibian and fish species that cannot re-establish because of the heavy predation by *I. punctatus* (Marsh & Brooks, 1989; Hawkins & Nesler, 1991; Rosen et al., 1995; Nelson et al., 1995; Lentsch et al., 1996; Tyus & Saunders, 1996, 2000; Hughes & Herlihy, 2012). Nonetheless, these impacts as well as a cost-benefit analysis have yet not been assessed and quantified.

vi) Socio-economic impacts

Although being a desired target species for angling and aquaculture activities, channel catfish were reported to prey upon other commercial sport and fishery species (Menzel, 1945; McGovern & Olney, 1988), causing a serious economic impact to commercial fisheries (Hanson et al., 2004; Hanzawa, 2004; Hanzawa & Arayama, 2007; Arayama, 2010; Matsuzaki et al., 2012), specially to angling (Tyus & Nikirk, 1990; Townsend & Winterbourn, 1992; Leonard et al., 2010). Finally, spines are poisonous and can inflict a burn similar to that of wasps without however being a significant threat for human well-being (Birkhead, 1967).

Environmental Impact Classification of Alien Taxa EICAT

As emerging from the literature analysis, most of the information for the impacts of the channel catfish are coming from the introduced range in North America, while for Europe data are really scanty. It is thus crucial to assess its potential invasiveness in European freshwaters to undertake consequent management actions. Here, we applied the classification system developed by Blackburn et al. (2014), the Environmental Impact Classification of Alien Taxa (EICAT), based on generic impact scoring system (GISS; Nentwig et al., 2010) and modified to align it to the impact scheme of IUCN (Kumschick et al., 2012). It evaluates the magnitude of species environmental impacts and uses five semi-quantitative scenarios (ranked 1-5) to assign species to different level of impacts, from Minimal (species is unlikely to have caused deleterious impacts on the native biota or abiotic environment) to Massive (species leads to the replacement and local extinction of native species, and produces irreversible changes in the structure of communities and the abiotic or biotic composition of ecosystems). The scheme measures the impact of species as environmental impact in twelve classes of impact mechanisms (Tab. 2). Each of these mechanisms is associated with one of a sequential series of five impact scenarios, describing increasing levels of impact exerted by species by that mechanism. It is based on scientific information available in peer-reviewed publications. For each impact mechanism, level of confidence is also considered: high confidence level is usually related to published scientific data on the study area, medium to some direct observational evidence to support the assessment, but with some information inferred from the literature coming from other invaded areas, and low to only inferred data used as supporting evidence. Species are assigned to impact category on the basis of the largest impact value recorded, while final confidence is considered as confidence of impact category with maximum score. EICAT is a simple, objective and transparent assessment process that can facilitate comparisons of the level of impact by alien taxa among regions and taxonomic groups, facilitate predictions of potential future impacts of taxa in the target region and elsewhere and aid in prioritisation of management actions. EICAT was used to evaluate the magnitude of impacts of catfish in North America by using available scientific and in Europe by evaluating existing literature on other areas and personal observations. The outcomes revealed that in North America the species can be classified under the level Major impacts with low uncertainty, while in Europe under the level Major with a medium uncertainty (Table 2.). So, the species can be considered invasive in Europe, but with a lower certainty as there are no published data on European situation, and impacts were inferred from literature related to North American invaded areas and, for some impact mechanisms (e.g. predation), from direct observations (e.g. stomach content analysis, Haubrock, pers. comm.).

Using the first developed version of GISS for environmental and economic impacts, van der Veer & Nentwig (2015) found a medium risk score for the species, while Copp et al. (2009) classified *I. punctatus* as potential pest for UK with the Freshwater Fish Invasiveness Scoring Kit (FISK). All these results highlight the urgent need of more research on the species in Europe to retrieve data in order to better support its invasiveness and promote some management plans.

Control

According to Pool (2007), there is very little effort to control channel catfish populations in introduced ranges, and, especially in the Pacific Northwest region, where this species has been present for over a hundred years, there is no specifically designed programme targeting its populations

(Davis, 2003). Chemical measures (Sodium sulfite, Guthion®, TFM (3-trifluoromethyl-4-nitrophenol) and Bayluscide®) could effectively remove Ictalurids, but they are highly tolerant to such chemical substances. Thus, high concentrations of these chemicals are required, and they potentially would harm endangered species, which occupy the same or neighbouring habitats (Dawson & Kolar, 2003). Mechanical removal is the most used technique for removal of invasive alien fish. Even if long-term fishing campaigns decrease channel catfish abundance, depending by the context, they can fail to totally remove this species from environment and can be very expensive. This technique has been used for longer than a decade in Lake Kasumigaura, the second largest natural lake in Japan, where Ibaraki Prefecture initiated an *I. punctatus* removal project in 2005 with the support of local anglers, which had to remove *I. punctatus* when caught as a by-product of stationary nets (Matsuzaki et al., 2011). Thus, the population appeared to have decreased (Craig, 2015), and it is likely that population will gradually decline if removal of the population will be increasing (Matsuzaki et al., 2011). Since chemical and mechanical measures are small-scale control options, for well-established and widely distributed populations, the only realistic options could be biological and autocidal control measures, which have barely been used with fish species in general (Brooks et al., 2000). Santos et al. (2009) assessed the potential of the native characid, *Brycon orbignyanus* (Valenciennes, 1850), to help controlling some invasive alien fish currently present in the Parana´ River basin through microcosm experiments. Since *B. orbignyanus* showed preference for two invasive species (*I. punctatus* and *Cichla piquiti*: Kullander & Ferreira, 2006), restoring its populations was recommended to limit the spread of *I. punctatus* which is currently not widespread.

Conclusions

From data retrieved from North American invaded areas, it is undeniable that *I. punctatus* is a threat for invaded environments. Its invasive capability is increased by its migratory activity during fall and spring, looking for suitable spawning sites or prey after increased river flow (Duncan & Meyers, 1978; Becker, 1983; Dames et al., 1989; Hanzawa & Arayama, 2007), and by the average equal sex distribution among mature fish (Appelget & Smith, 2011). Additionally, its opportunistic feeding habit, its broad adaptation to several different environments and the poisonous spines are an advantage in invaded areas when competing with the native fauna for food or shelter. Also, for Europe, the species resulted to be potentially invasive, even if up to now no published data are available for this area. Among the impact mechanisms the species could exert in Europe, predation on benthic species, insects, plants and macroinvertebrates would especially pose a high ecological pressure and potentially considerable environmental modification to European freshwater habitats, likely reducing native biodiversity. Particularly, with the high densities tending towards young age groups, behavioural interferences may result in decreasing abundances of native fish. In a study by Jenkins & Burkhead (1994), the extinction of *Percopsis omiscomaycus* (Walbaum, 1792) populations was explained as the result of *I. punctatus* predation. However, beside native fishes, other several already endangered species may be threatened by its presence. In Japan, Endo et al. (2015) showed that *I. punctatus* expressed a preference towards native crayfish over the invasive alien crayfish *Procambarus clarkii* (Girard, 1852), as this species, already overlapping its native distribution, is probably adapted to predation by channel catfish. A good example of a potentially affected species is the already threatened native trout species *Salmo trutta* (morpha *fario* Linnaeus, 1758) in low species rich ecosystems in Europe, already endangered by the introduction of the American rainbow trout Oncorhynchus mykiss (Lee et al., 1980; Rinne & Minckley, 1985; Page & Burr, 1991; Blinn et al. 1993; Thibault & Dodsen, 2013). Jenkins & Burkhead (1994) have shown that cold water adapted channel catfish, and especially young channel catfish that feed on aquatic plants, outcompete trout and perch for food and shelter. Thus, low species rich European ecosystems could offer catfish new opportunities to dominate entire ecosystems.

As spawning can be inhibited if suitable nesting cover is unavailable (Marzolf, 1957), it is particularly interesting to investigate whether European freshwater systems can offer suitable nesting cover and whether these overlap with native species. Furthermore, climatic differences can affect growth, spawning, and metabolism and eventually lead to unforeseeable changes in behavioural patterns altering its invasiveness (Wellborn 1988). Studies on the invasive alien sun perch *Lepomis gibbosus* (Linnaeus, 1758) have shown that, according to life-history traits (mortality rate, plasticity and reproductive strategy), maturity is reached earlier in Europe (Cucherousset et al. 2009). Furthermore, Europe acts as a “melting pot” for invasive alien species, as several species are already present. The additional impact caused by the hybridization between *I. punctatus* and *I. furcatus*, both already present in Europe, cannot be discarded.

Another very interesting aspect is the complete lack of natural predators since generally only young fish are consumed by predatory insects or birds (e.g. cormorants, herons and pelicans). Most of the species that naturally predate on *I. punctatus* are not present in Europe or, if present, considered invasive alien. The grey heron *Ardea cinerea* (Linnaeus, 1758) could be a potential European predator (Glahn et al., 1995a, b; King et al., 1995; Wywialowski, 1999; Glahn et al., 2000). However, potential predation by European or alien species has yet not been assessed. At last, the human exploitation rate through fishing activity, being lower than in the native region, has been neglected (Leonard et al., 2010), implying that no pressure is exerted on this species. Finally, the alteration of the environment through human activities could potentially increase this species invasiveness (Olden et al., 2005; Cucherousset et al., 2009, 2012) or impede it, as an establishment of *I. punctatus* was unsuccessful in Turkish waters (Innal, 2012). The impact on European freshwater habitats needs to be studied and quantified since *I. punctatus* is already present in at least 22 European countries. The high physiological tolerance and fecundity accompanied by parental care make this species a good candidate for future successful invasions throughout European waterbodies (Marchetti et al., 2004). By natural or human mediated spread, urban areas with rivers already disturbed and invaded by other species are prone to introductions and subsequently facilitate its population growth. Without no or only few scientific attentions on advancing spread of this potentially invasive species, heavy impacts to European freshwater systems could be caused and lead to an irreversible situation, as control or eradication methods do not exist.

Acknowledgements

We would like to thank Kit Magellan for many very helpful comments on an earlier version of the manuscript. This project has received funding from the European Union’s Horizon 2020 research and innovation programme under the project Marie Sklodowska-Curie Aquainvad-ED (H2020-MSCA-ITN-2014-ETN-642197).

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Table 1. Introduction history of the North American channel catfish *Ictalurus punctatus.* Adapted from CABI (2016) and Froese & Pauly (2013) and modified with further information.

Country	**Year of introduction**	**Pathway**	**Status**	**References**
Puerto Rico	1938	angling	established	Erdman 1984; Welcomme 1988; Burgess and Franz 1989; Neal et
Dominican Republic	1954-55	aquaculture, repopulation	unknown	Welcomme 1988; Chakalall 1993
Honduras	1960s	aquaculture	unknown	Matamoros 2009
Panama	1981	aquaculture	unknown	Welcomme 1988
Mexico	1933 and 1978	aquaculture	established	Contreras and Escalante 1984; FAO 1997; Zambrano and Macias-
Cuba	1979 and 1984	aquaculture	unknown	Welcomme1988
Costa Rica	1976	aquaculture	unknown	Cam 2011
Brazil	1971 and 1980	aquaculture	unknown	Welcomme 1988; Piedras 1990; Cruz et al. 2012
Chile	1995	aquaculture	established	Pérez et al. 2003
Paraguay	1971	unknown	unknown	Froese and Pauly 2013
Venezuela	1971	unknown	unknown	Froese and Pauly 2013
Nigeria	1970 and 1976	aquaculture	unknown	Welcomme 1988
Egypt	1982	aquaculture	not established	Welcomme 1988
Côte d'Ivoire	unknown	aquaculture	unknown	Welcomme 1988
Japan	1971	aquaculture	established	Welcomme 1988; Chiba et al. 1989; Japan Ministry of Environme
Republic of Korea	1972	aquaculture	unknown	Welcomme 1988; Jang et al. 2002
Philippines	1974	aquaculture, aquarium	not established	Juliano et al. 1989; ASAP 1996; FAO 1997
Taiwan	1975-76	aquaculture	probably established	Welcomme 1988; Liao and Lia 1989
China	1984	aquaculture, accidental	unknown	Tan and Tong 1989; FAO 1997; Ma et al. 2003
Indonesia	1986	experimental research	not established	Eidman 1989
India	1985-89	aquaculture	unknown	Csavas 1995; Molar and Walker 1998
Thailand	1989	aquaculture	not established	Csavas 1995; Vidthayanon 2005
Turkey	1989	aquaculture, angling	not established	Innal and Erk'akan 2006; Cowx and Nunn 2008
Uzbekistan	unknown	unknown	established	Salikhov and Kamilov 1995; Bartley 2006
Russia	unknown	aquaculture, escaped into	established	Bogutskaya and Naseka 2002; DAISIE 2013
Armenia	unknown	aquaculture	established	Gabrielyan 2001
Malaysia	unknown	aquaculture	unknown	Freshwater Fisheries Research Centre Malaysia (FFRC) 2001
Pakistan	2003	aquaculture	unknown	Rab et al. 2007
Hawaii	1953	aquaculture	established	Brock 1960; Maciolek 1984; Welcomme 1988
Guam	1966	aquaculture	not established	Welcomme 1988; Eldredge 1994
French Polynesia	1966	unknown	unknown	Eldredge 1994; Bartley 2006
UK	1968	angling, aquarium	established	Lever 1977; Welcomme 1988
Serbia	1971	aquaculture	not established	Welcomme 1988
Montenegro	1971	aquaculture	not established	Welcomme 1988
Cyprus	1975	aquaculture, angling	established	Welcomme 1988; Dill 1993
Italy	1976	aquaculture, angling	established	Amori et al. 1993; Bianco and Ketmaier 2001; Copp et al. 2005; L
Slovakia	1985	aquaculture	probably established	Welcomme 1988, Kosco et al. 2004
Czech Republic	1985	aquaculture, angling	established	Welcomme 1988; Lusk et al. 2010; Lusk et al. 2011
Belgium	1968 and 1984	aquaculture	established	Holcík and 1991; FAO 1997; Verreycken et al. 2009
Bulgaria	1975	aquaculture, angling	established	Uzunova and Zlatanova 2007; Hadjinikolova et al. 2010
Romania	1978 and 1991	aquaculture	probably not	FAO 1997; DAISIE 2013
Estonia	2002	unknown	unknown	NOBANIS 2005
Hungary	1975 ongoing	aquaculture	not established	Holcík 1991; FAO 1997
Lithuania	1975 ongoing	unknown	unknown	NOBANIS 2005; DAISIE 2013
Ukraine	unknown	aquaculture	not established	Welcomme 1988; Holcík 1991; FAO 2006; DAISIE 2013
Belarus	1979	aquaculture	established	Shumak and Mischenko 1989
Germany	unknown	unknown	unknown	Fishbase 2016
Netherlands	unknown	unknown	unknown	Cowx and Nunn 2008
France	unknown	aquaculture	not established	Welcomme 1988; Holcík 1991
Spain	unknown	unknown	probably established	Welcomme 1988; Doadrio 2002
Portugal	unknown	unknown	probably established	Pedro Anastácio, pers. comm.
Greece	unknown	unknown	unknown	Froese and Pauly 2013

Table 2: Outcomes of the Environmental Impact Classification of Alien Taxa (EICAT; Blackburn et al., 2014) applied to the channel catfish *Ictalurus punctatus* in the introduced range in North America and Europe.

	North America		**Europe**	
Ecological impacts	**Impact**	**Uncertainty**	**Impact**	**Uncertainty**
Competition	4	1	3	3
Predation	4	1	4	2
Hybridisation	4	1	1	2
Transmission diseases to native	1	3	1	2
Parasitism	1	3	1	3
Poisoning/toxicity	1	1	1	2
Bio-fouling	1	1	1	1
Herbivory	3	2	3	3
Physical, chemical, structural	1	2	2	3
Interaction	1	3	2	3
Final score	4		4	
Category	Major (MU)		Major (MU)	
Level of confidence	3 (High)		2 (Medium)	

Kingdom	*Animalia*	Animals
Phylum	*Chordata*	Chordates
Subphylum	*Vertebrata*	Vertebrates
Superclass	*Osteichthyes*	Bony fishes
Class	*Actinopterygii*	Ray-finned & spiney rayed fishes
Subclass	*Neopterygii*	Neopterygians
Infraclass	*Teleostei*	
Superorder	*Ostariophysi*	
Order	*Siluriformes*	Catfishes
Family	*Ictaluridae*	
Genus	*Ictalurus*	
Species	*Ictalurus punctatus*	Channel catfish

Figure 1. Diagnostic information of *Ictalurus punctatus* (Adapted from Pool, 2007)

- Upper jaw extending over the lower jaw
- Deeply forked caudal fin
- Dark points on greyish – golden skin
- Lacking scales
- 8 barbels
- Round anal fin with 24-29 rays
- Fleshy and long adipose fin separated from caudal fin

Figure 2. Basic means of identification – physical characteristics used for identification

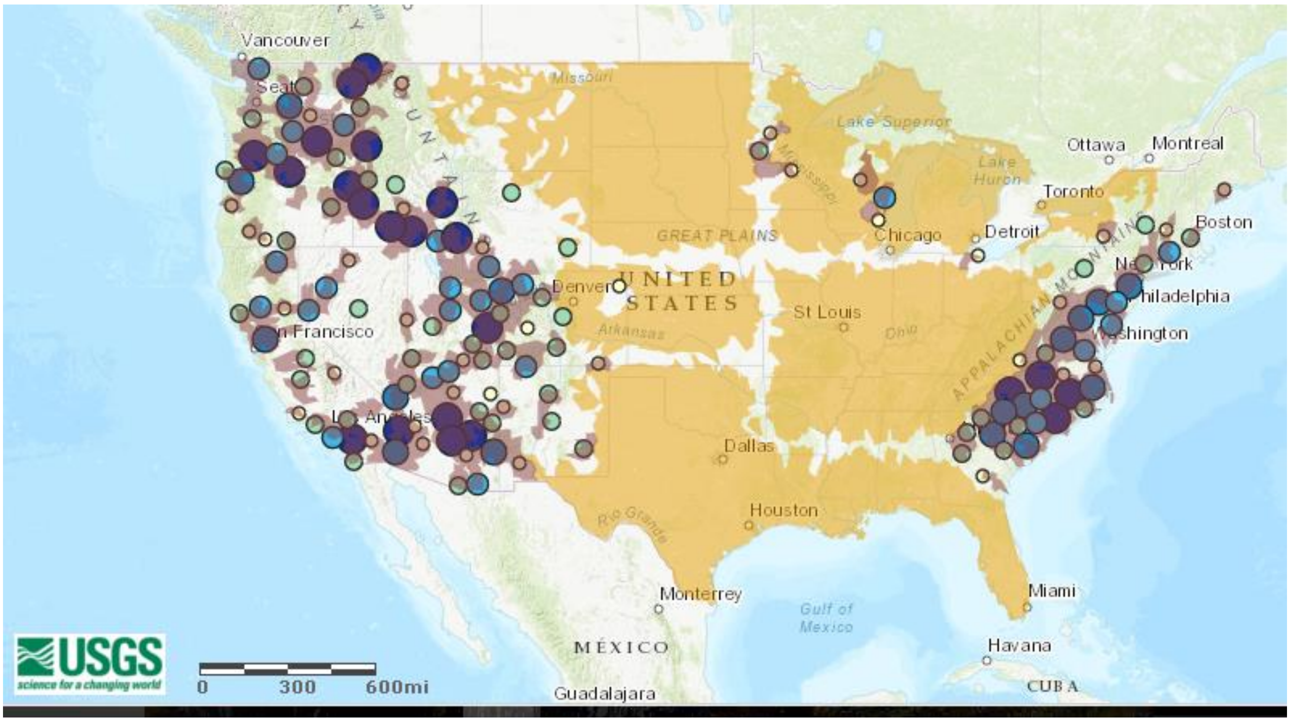

Figure 3. (Option 1) USA distribution of *Ictalurus punctatus*. Brown area: native distribution. Species observations: White dots = 1; green dots = 2-5; turquoise dots = 6-19; dark blue dots = 20 or more. (USGS https://nas.er.usgs.gov/queries/FactSheet.aspx?speciesID=2341)

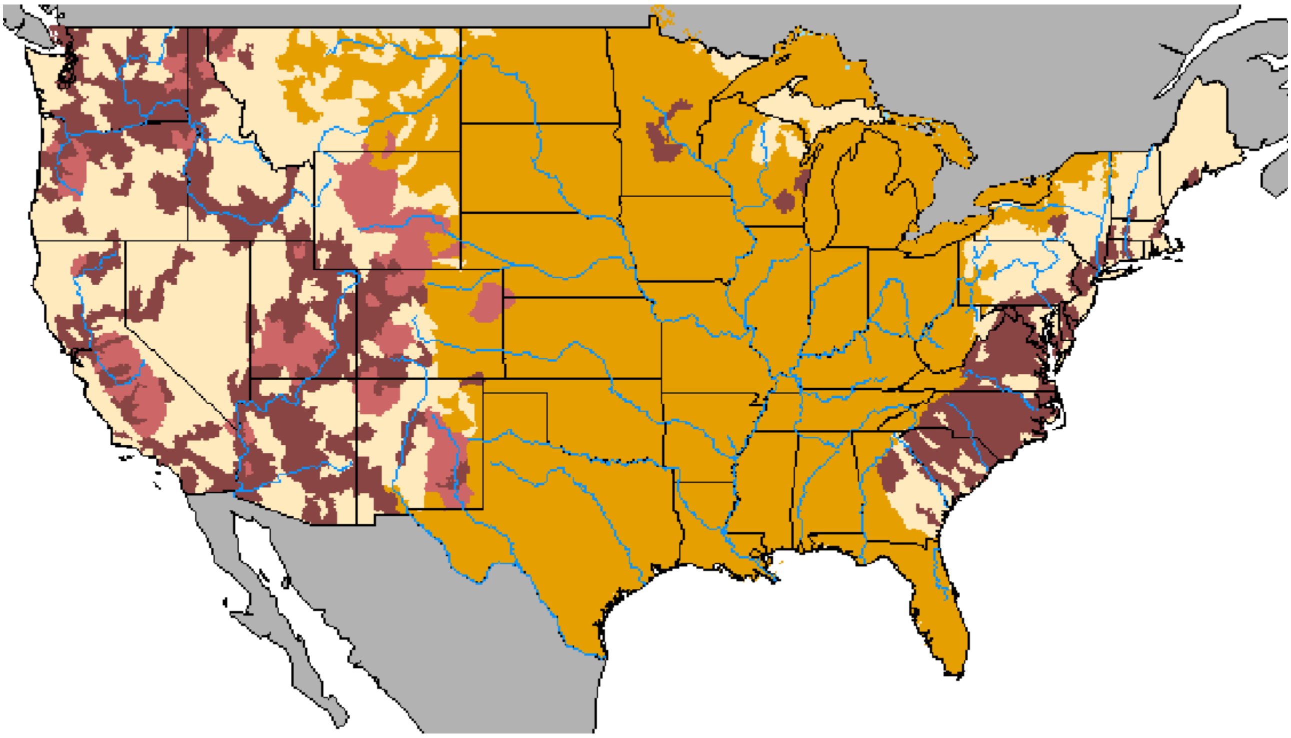

Figure 3. (Option 2) USA distribution of *Ictalurus punctatus*. Brown area: native distribution; purple: non-native range. (USGS https://nas.er.usgs.gov/queries/FactSheet.aspx?speciesID=2341)

7.2. The effects of two different preservation methods on morphological characteristics of the alien channel catfish Ictalurus punctatus (Ictaluridae, Siluriformes) in European freshwater.

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Running title: Preservation methods in channel catfish

Abstract

Different methods used to store specimens, even for short periods, can notably affect morphometric characteristics such as colour, length, and shape. As a consequence, in the case of samples that have to be analysed morphometrically, the right preservation method is crucial.

In this study, two comparably easy applicable methods, the effects of freezing and moderate cool storage on morphological traits, were studied on specimens of the scaleless North American channel catfish, *Ictalurus punctatus*, an alien species to European freshwaters of increasing concern. Results revealed that freezing, like preservation with alcohol or formalin, affected the generally considered characteristics (colour, standard length, fork length, total length, shape), while cool storage was shown to preserve most traits without notable alteration, an important difference when studying potentially adapting alien species. Implications are discussed.

Key Words: fish morphology, freezing, cooling, colour, alteration

Introduction

Samples of different taxonomic groups are generally preserved under varying conditions (Asahida et al., 1996; Gould, 1996; Ghaly et al., 2010). For fish specimens, ichthyologists usually use formalin, alcohol, or freezing (Ghaly et al., 2010). Storing samples in formalin and alcohol as a long-term measure is reasonable, but short-term storage of large quantities (e.g. transport) can be delicate and is not always the most efficient method. However, the availability, transportation, and storage of ice is not always a feasible option.

Different short and long-term storage techniques have been shown to affect taxonomically important morphometric characteristics of fish, such as length (e.g. Al-Hassan & Shawafi, 2003). Additionally, when considering the water content ratios of white and red muscles, the effect of storage and preservation method may cause variations between scale-less and scaled fish (Leslie & Moore, 1986).

Few studies deal with the effect of varying preservation methods on pigmentation and morphometric characteristics in different species of fish (see e.g. Jawad et al., 2001; Neave et al., 2006). This variability in morphology among different preserved species and samples can be due to several factors such as time elapsing until preservation, the applied preservation method, concentration of preservative, preservation duration and temperature, morphological variability among specimens, and species-specific factors (age, size, state, the osmoregulatory activity of the fish at the point of death; Yeh & Hodson 1975).

As the North American channel catfish, *Ictalurus punctatus*, is receiving an increasing scientific attention in Europe as an alien species (Banha et al., 2017; Haubrock et al., 2017) and due to the potential influx of further North American catfish species that are difficult to distinguish by the untrained eye, a preservation method that does not impact morphological characteristics is needed.

The present work aims at investigating the effect of two easy applicable methods, freezing and cooling, on morphological characteristics. These options were chosen as two commonly applied and feasible methods for transporting and storing freshly caught specimens.

Material and Methods

Specimens of *Ictalurus punctatus* were collected from the river Arno (Central Italy). Immediately after the capture, morphological characters of all samples (total length, standard length and fork length, body shape and colour) were measured or photographed. Subsequently, specimens were euthanized with a standard gill cut. Twenty samples per preservation method were stored in two batches of ten specimens each. Specimens in the freezing treatment, were directly laid on and covered with ice cubes and were then transported to a standard freezer within one hour and frozen at -20 °C. For the cooling treatment, cooling down without freezing was achieved by transferring specimens into a reefer-container and loosely placing them between cloth-wrapped cold packs. Cold packs were replaced with new replenished packs after eight hours to keep the temperature between 2 and 4 °C. After 96 hours, cooled and frozen specimens were measured again, and measurements were compared. In the case of frozen samples, defrosting was achieved at ambient temperature over a period of 2 - 4 hours. Specimens were straightened up and subsequently measured. Measurements from before and after preservation for both methods were analysed using paired t-test (statistic: t); changes in length before and after preservation were compared between the two methods using Mann-Whitney test (statistic: U). The level of significance under which the null hypothesis was rejected was $\alpha = 0.05$. Table 1 provides mean values or median values plus standard error or 1° - 3° interquartile. SPSS®, Statistical Package for Social Science 13.0 for Windows was used for the statistical analyses.

Results

Freezing and cooling led to different effects on morphological characters. In the case of freezing, a degradation in colour from goldish-brown to grey-blackish was visible in every case, while the body shape was unaffected overall, except for the belly being less elevated, soft, and pliable after defrosting. The standard length of frozen fish increased between 0.1 and 1.9 cm (~3.3%), the distance between standard length and fork length by 0.1 - 0.4 cm and total length respectively by 0.1- 0.5 cm. Results of statistical analysis are displayed in Tab. 1. Additionally, the changes in size of specimens is linked to increases in all length categories (Figure 1.). Comparably in cooled specimens, the increase in all length measures was between 0 and 0.2 cm, bellies kept their firm shape, and although colour change, it maintained a lighter tone (including the typical punctuation of *I. punctatus*) (Figure 2.).

Discussion

Differences in size, shape or size of morphological characters can be of outmost importance in studies assessing the morphology of a target species (Sayers, 1987; Al-Hassan & Shawafi, 2003). This is crucial when studying similar species with overlapping distributions, such as *I. punctatus*, *Ictalurus furcatus* and *Ameiurus catus*. These species can be morphologically variable, as for instance in the case of genetic introgression in *I. punctatus*, causing anal fin ray reduction up to 23 cm, making it morphologically more similar to *A. catus*. Another example might occur in the future, when alien species which genetically compatible and morphologically very similar species with almost identical juvenile life stages, such as *I. punctatus* and *I. furcatus*, hybridize, making morphological distinction crucial. Furthermore, to the best of our knowledge, there are no standard approaches to calculate the size of specimens while alive based on only the size of preserved specimens. There is no evidence of standard correlation factors between living length and preserved length (Yeh & Hodson 1975).

In the present study, both preservation by freezing and by cooling had a significant effect on fish size. However, freezing caused a significantly higher increase compared to cooled specimens. Furthermore, we observed a change in skin colour and pigmentation in frozen specimens, leading to a much darker tone and invisibility of the typical black dots of the species. This pigmentation change has also been similarly observed in rainbow trout (Chen et al., 1984, No & Storebakken, 1991). Conversely, cooling down preserved most traits in a more reliable way.

Hossaini et al. (2016) found that as an alternative to the described cooling and freezing, preservation with alcohol affected colour and body proportion of Zagros Tooth-Carp, *Aphanius vladykovi*. Additionally, Jawad et al. (2001) states there is shrinkage of multiple characteristics caused by different concentrations of formalin and alcohol, and also freezing, in sparid-fishes. In contrast, Al-Hassan & Abdullah (1982) observed the opposite, a slight increase in preserved specimen sizes. Although the impact of preservation using alcohol and formalin have not been the target of this study, differing results and the most often observed shrinkage in length can be explained by varying chemical concentrations in the body (e.g. a gradient in the tail), genetically based differences in white to red muscles ratios, and variable fluid content (Leslie & Moore, 1986; Hossaini et al., 2016). Hence, different preservatives may influence characteristics differently and thus, while alcohol may damage proteins and therefore the tissue, formalin will negatively affect the colour while sustaining colour pattern. Therefore, a standardized and repeatable preservation is of interest for the correct identification and analysis of morphology.

Subsequently, it can be stated that the applicability of preservation with alcohol, formalin and freezing can be considered as more difficult to apply considering the hardship of transporting sufficiently sized container and proper amounts of preservative when dealing with multiple bigger sized specimens. We therefore suggest that morphometric measures on channel catfish should be taken directly on fresh or cooled specimens, while the use of frozen and defrosted fishes should be avoided for potential misidentification of species.

Acknowledgements

This project has received funding from the European Union's Horizon 2020 research and innovation programme under the project Marie Sklodowska-Curie Aquainvad-ED (H2020-MSCA-ITN-2014-ETN-642197).

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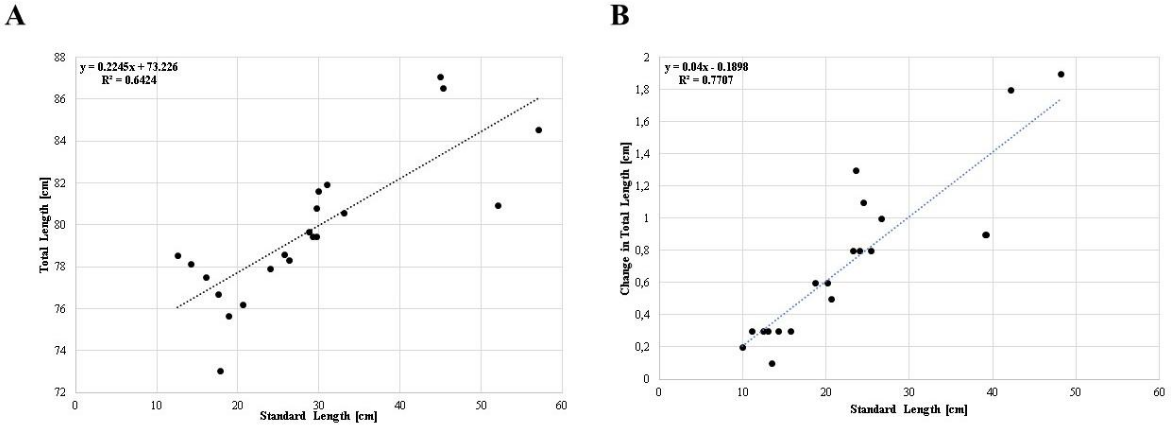

Figure 1. A) Standard length (X-Axis) in relation to total length (Y-Axis) before freezing. B) Standard length (X-Axis) in relation to increase of total length (Y-Axis) after 96 hours of freezing

Figure 2. Comparison between a specimen of *Ictalurus punctatus* after A) 96 hours of cooling and B) 96 hours of freezing.

Tab. 1: Comparisons of changes in Standard- Fork- and Total-Length between frozen and cooled specimens using Mann-Whitney-U test [U], and lengths before and after freezing and cooling using paired t-tests [t]. For paired t-tests, means ± standard errors and for Mann-Whitney-U Tests medians and 1°-3° interquartiles are reported. Significant values are highlighted in bold.

Cooling vs. Freezing

Parameter	**U**	**n**	**p**	**Freezing**	**Cooling**
Change Standard Length (cm)	208	20 20 40	**< 0.01**	0.74 (0.3 – 0.98)	0.1 (0.0 – 0.2))
Change Fork Length (cm)	207.5	20 20 40	**< 0.01**	0.64 (0.3 – 0.8)	0.1 (0.0 – 0.18)
Change Total Length (cm)	190	20 20 40	**< 0.01**	0.62 (0.3 – 0.7)	0.1 (0.0 – 0.1)

Before and After preservation

Preservation method	**Parameter**	**t**	**df**	**p**	**Before**	**After**
Freezing	Standard Length (cm)	-6.579	20	**< 0.01**	23.26 ± 2.47	24.00 ± 2.56
	Fork Length (cm)	-6.312	20	**< 0.01**	25.59 ± 2.70	26.22 ± 2.79
	Total Length (cm)	-7.132	20	**< 0.01**	28.77 ± 2.80	29.39 ± 2.88
Cooling	Standard Length (cm)	-5.667	20	**< 0.01**	26.25 ± 2.59	26.33 ± 2.60
	Fork Length (cm)	-5.596	20	**< 0.01**	28.42 ± 2.66	28.52 ± 2.68
	Total Length (cm)	[-5.6270	20	**< 0.01**	32. 40 ± 2.92	32.50 ± 2.93

7.3. The alien channel catfish *Ictalurus punctatus* in the Arno river (Central Italy): a first study on its diet

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Running title: Alien channel catfish in Central Italy

Abstract

The North American channel catfish *Ictalurus punctatus* has been widely introduced to Europe, but no in-depth studies on its ecology and potential impacts in the introduced European range have been carried out. In 2016, 248 specimens of *I. punctatus* were collected from the Arno river, Florence (Central Italy), and analysed for their length, weight, size, sex and stomach contents to assess their diet. Specimens < 30 cm TL represented the majority of the sampled population. In their diet, detritus and phytoplankton dominated, while in larger (≥ 30 cm TL) fish two invasive alien species, the topmouth gudgeon, *Pseudorasbora parva* and the red swamp crayfish *Procambarus clarkii*, prevailed. Diet composition of *I. punctatus* significantly varied among size classes, but not between sexes. The results indicate an opportunistic but gape size limited feeding behaviour, suggesting an intra-specific competition avoidance mechanism.

Keywords: *fisheries, freshwater, impact, feeding habit*

Introduction

Europe has been the centre of globalization for the past two centuries, thus, invasive species are an increasing concern (Schulz & Della Vedova, 2014). Not only those alien species that have already been classified as invasive are important in terms of scientific effort, but rather those, that have yet to be assessed and eventually be prioritized. This is important, as decision and policy makers must allocate efforts to minimize the threat of more species becoming invasive (Keller et al., 2011). Hence, the assessment of alien species, even if approaches are rudimentary, are important to explore the growing amount of alien species to determine those that pose potentially big threats (Dukes & Mooney, 1999; Walther et al., 2009; Vilà et al., 2010), especially with multiple cases of alien catfish species negatively impacting recipient European freshwater ecosystems (Benejam et al., 2007; Leunda et al., 2008).

The channel catfish, *Ictalurus punctatus* Rafinesque 1818 (Siluriformes: Ictaluridae), is native to North America, where it is extensively cultured (Appelget & Smith, 2011), widely distributed and a major sport- and appreciated food species (Tucker & Hargreaves, 2004; Leonard et al., 2010). This species inhabits a wide variety of ecosystem types and is highly tolerant towards extreme environmental conditions (Dunham & Masser, 2012), exhibiting an opportunistic feeding habit and high fecundity (Appleget & Smith, 1951; Toole, 1951; McMahon & Terrell, 1982). In North America, several studies on its life history, growth, and reproduction as well as on ecology and behaviour have been conducted, showing predictable differences in diet among juveniles and adults (see e.g. Townsend & Winterbourne 1992; Adams, 2007). In its native area, it is known for its opportunistic diet, negatively impacting populations of amphibians (Rosen et al., 1995) and threatened endemic fish species (Marsh & Douglas, 1997).

Despite having been introduced and being present in 22 European countries (Welcomme, 1988; Elvira & Almodóvar, 2001; Copp et al., 2005), data on introduction history are scanty, and no in-depth studies focusing on *I. punctatus*' potential impacts in Europe have been conducted (Welcomme, 1988; Elvira & Almodovar 2001; Copp et al., 2005). In Italy, it was first reported in 1986 in the river Oglio in the Northern part of the country (Gandolfi et al., 1987), while in the province of Florence in Central Italy, the area this study has been conducted, it was first recorded a decade later in 1998 (Nocita, 2001). The species is currently considered established in Italy (Nocita & Zerunian, 2007), but the already performed studies only report its presence, without any information on population structure and possible impacts. Adaptations in the life-history of alien species are increasingly studied (Fausch et al. 2001; Copp et al., 2004; Cucherousset et al., 2012). Investigating the feeding behaviour and ecology of *I. punctatus* in introduced areas could indicate such potential adaptations. Additionally, studying this species' impact on native communities could provide important information for management and conservation efforts rather than relying on behavioural studies from native areas.

Based on the opportunistic predation and specialisation of mature channel catfish recorded in its native area, it is potentially possible that alien catfishes such as *I. punctatus* exert a high pressure on native species. Hence, diet analysis can provide detailed information about species affected and show which food sources characterize different seasons. Additionally, combining these dietary data with information on population characteristics can not only reveal potential impact, but also identify which life stage is exerting the stronger predation impact due to its abundance. This assessment will be the first investigation of *I. punctatus*' diet in Italy as well as in Europe, determining if this species poses a potential threat for the studied ecosystem.

Materials and Methods

Sampling

Specimens were collected in the inner-city section of the Arno river in Florence, Central Italy. The Arno river is characterized by an irregular flow rate, a length of 241 km, a drainage basin of approximately 8.200 km² and a mean annual discharge of about 110 m³/s. It is the most important freshwater river in Central Italy after the Tiber river, and it is inhabited by several alien species such as *Silurus glanis*, *Lepomis gibbosus*, *Pseudorasbora parva* and *Procambarus clarkii*, while native species are considered rare in the studied ecosystem (see Nocita & Zerunian, 2002) for detailed information on the species community). Sampling was conducted two to three times per week at the same location (43°45'49.9"N, 11°18'04.2"E) within the inner-city stretch of the river Arno during the activity phase of the species from March to November 2016. To catch fish, standard fishing rods (2.20 – 3.90 m), 0.22 – 0.36 mm monofilament line and size 2 - 26 fishing hooks baited with a variety of baits (maggots, worms, and freshly cut liver or bait-fish) were placed on the bottom and in the middle water. Immediately after capture, fish were placed on ice before being transported to the laboratory of the Department of Biology and the Natural History Museum "La Specola" in Florence.

Morphometry and population structure

Total length (TL, mm) and net weight (g) were measured, and sex of all specimens was determined using the morphological feature of the pelvic fins, as female *I. punctatus* have two openings, whereas male fish develop just one (the anus) and a small fleshy flap (genital papillae) that sticks out (Norton et al., 1976). Maturity of all individuals was assessed. For males, maturity was identified with the presence of white milt within the gonads. In the case of females, ovary maturity was determined based on i) one or more eggs containing yolk, ii) red capillary arteries as part on the circulatory system around the outside of the ovaries (De Silva et al., 1973; Infa et al., 2015) as well as oocyte development, i.e. analysing if gonads demonstrate the capacity to ripen during the vegetative season (Gordon Copp pers. comm.). As maturity could not be determined for every specimen in our study due absence of gonads in some even large (>50cm TL) specimens, size classes were chosen as a proxy for maturity. As we found no mature gonads (i.e. gonads that would be identifiable as mature or that will potentially ripe during the season, according to De Silva et al., 1973 and Infa et al., 2015) in fish < 30 cm of total length, we set the 'maturity' threshold to 30 cm (corresponding to maturity thresholds set in Appelget & Smith, 1951; DeRoth, 1965; Perry & Carver, 1973; Stephen & Jackson, 2005). This resulted in size ranges of 7–29.9 cm TL for 'immature' (n = 175) and 30–52 cm for 'mature' individuals (n = 73).

As a characteristic of this studied population, the length-weight relationship was plotted. After testing the data for normality using the Shapiro-Wilk normality test, the distribution of length and weight of sampled specimens were compared between males and females using a non-parametric Mann-Whitney U-test to characterise population and enable comparisons with other populations. For the same purpose, frequencies of males/females and 'maturity' stage ('mature'/'immature') were compared using a chi-squared test to estimate if they significantly differ in numbers. The Fulton condition factor (K), generally used to compare populations, was applied using the formula $K = 100 * W / L^3$ (Nash et al., 2006) to better visualize length-weight relationship of this species outside its native region and to be available as a reference for future studies on *I. punctatus* outside its native area.

Dietary analysis

Stomachs were removed, and their content analysed. Prey items were identified to the lowest possible taxa with a standard stereo-microscope. Fragmented prey items were considered part of a whole organism and counted as such. Only numbers of prey items were recorded. Prey compositions were compared for sex, maturity and sampling season. The nine months of sampling in 2016 were merged into three groups (spring: March–May; summer: June–August; autumn: September–November), resulting in a distribution of individuals among spring (n= 42), summer (n= 131) and autumn (n= 75; no fish were caught in November). Diet was analysed as frequency of occurrence (F% = 100 * A_i / N) where A_i was the number of fish containing prey item i and N the total number of fish analysed (excluding those with empty stomachs). The diet breadth of the 'mature' and 'immature' subpopulation was estimated, also for all seasons, based on Levin's index formula (Whittaker et al., 1973): $B_i = \frac{1}{\sum p_i^2}$ where B_i is the standardized index of diet breadth for subpopulation i and $\sum p_i^2$ the sum of all squared proportions of individuals found with prey item i in their stomach, estimated by dividing the number of individuals containing prey item i with the total number of individuals sampled (Krebs, 1998). To determine if sample size was sufficient to describe the diet of non-native channel catfish in the Arno river, the cumulative number of prey taxa was plotted against the cumulative number of examined stomachs while analysed stomachs were randomized ten times (Ferry & Cailliet, 1997; Ferry et al., 1996). Cumulative curves were considered to be asymptotic, if ten previous values of the total number of prey taxa were ± 0.5 in the range of the asymptotic number of prey, indicating the minimum sample size required to describe the diet (Cailliet et al., 1986; Cortés, 1997; Huveneers et al., 2007). Because specimens yielded multiple prey taxa, prey composition was analysed using multivariate statistical methods. Fish without any stomach content (n=16) were excluded from the dataset and the analysis, resulting in 166 stomach contents (spring= 32; summer= 76; autumn= 58) from 'immature' and 66 (spring= 7; summer= 48; autumn= 11) from 'mature' specimens. A bivariate non-parametric Spearman-correlation was conducted between the total sum of different prey items found in the stomach and the total length of individual fish.

A presence/absence matrix was built including stomach content records from each sampled specimen and a Permutational Analysis of Variance (PERMANOVA; 3 orthogonal fixed factors: 'maturity' ['immature', 'mature'] (threshold TL=29.9 cm), 'sex' [male, female] and 'season' [spring, summer, autumn]; sums of squares: type III, partial; permutation of residuals under a reduced model) was used to test if the diet of the studied population differs according to the total length of specimens, the sex of individuals, the season or a combination of factors. Also, a post-hoc test (pair-wise test) was included to identify differences between couples of levels for each factor/factor interactions found significant by the PERMANOVA main test. Additionally, a Canonical Analysis of Principal Coordinates (CAP) for factors whose levels were found to be significantly different was applied, thus identifying the variables (i.e. prey items) contributing more consistently in differentiating the levels. Spearman correlations for each variable with CAP1 axis, the only one found informative in differentiating 'mature'/'immature' specimens and the three seasons considered, are reported. MDS, PERMANOVA and CAP were performed using PRIMER v. 6 (Clarke, 1993). For all tests, the level of significance under which the null hypothesis was rejected is $\alpha = 0.05$ and values are reported as median and interquartile range (i.e. the range between first and third quartile).

Results

Morphometry and population structure

Overall, 248 specimens of *I. punctatus* (females= 135, males= 113) ranging from 7 to 52 cm TL (median [Md] = 23 cm, interquartile range [Q1-Q3] = 17 – 31 cm) were analysed (Figure 1). Males and females differed for TL (Mann-Whitney U-test, U= 4.395; n= 248; p< 0.001; females: Md = 27 cm, Q1-Q3= 19 – 35 cm; males: Md = 20.8 cm, Q1-Q3 = 16 – 26.5 cm) and weight (Mann-Whitney U-test, U= 4.607; n= 248; p< 0.001; females: Md = 198 g, Q1-Q3 = 89 – 420 g; males: Md = 91 g, Q1-Q3 = 59 – 181.5 g). No significant difference for the overall number of sampled males and females was found (males = 45.5%; females = 54.5%; chi-square test, $\chi^2 = 0.9777$; n= 248; p > 0.5). The size of caught specimens was strongly biased towards 'immature' specimens under 30 cm, ('immature' = 70.6%; 'mature' = 29.4%; chi-square test, $\chi^2 = 21.9$; n = 248; p < 0.05). The Fulton condition factor for the entire population was estimated between 0.07 and 5.27 (overall: Md = 1.174, Q1-Q3 = 0.936 – 1.424; females: Md = 1.123, Q1-Q3 = 0.904 – 1.326; males: Md= 1.256, Q1-Q3 = 0.981 – 1.640) and showed the highest values in March (Md = 1.526, Q1-Q3 = 1.157 – 1.814) and the lowest in in September (Md = 0.887, Q1-Q3= 0.717 – 1.370) (Figure 2).

Dietary analysis

Overall, 24 prey taxa were identified (Table 1). The plotted cumulative prey curve for total number of prey taxa reached the asymptote after 74 stomachs (Figure 3). A significant correlation was found between the observed total sum of stomach contents observed in catfish stomachs and fish size (TL) (r_s= -0.129; n= 232; p< 0.043), showing that larger fish had generally fewer prey items in their stomach. On the other hand, the diet breath for 'immature' ($B_{\text{'immature'}}=10.8$) and for 'mature' fishes ($B_{\text{'mature'}}=10.7$) was almost identical. Seasonally, the diet breadth for 'mature' and 'immature' catfish was similar in spring ($B_{\text{'matureXspring'}}=9.4$; $B_{\text{'immatureXspring'}}=9.0$) and summer ($B_{\text{'matureXsummer'}}=8.6$; $B_{\text{'immatureXsummer'}}=8.1$), while immature fish expressed a higher diet breadth in autumn ($B_{\text{'matureXautumn'}}=5.3$; $B_{\text{'immatureXautumn'}}=9.7$). The most common prey items found in stomachs was detritus, followed by algae and plants. Generally, detritus, algae and plants were found to occur in higher percentages in 'immature' than in 'mature' specimens (Table 1). Most common animal preys in 'immature' fish were the topmouth gudgeon *Pseudorasbora parva* and Coleoptera, while in 'mature' fish *P. parva* and the red swamp crayfish *Procambarus clarkii*. Also, a high frequency of white "crumbs" with the consistence of customary soap products, visually and gustatorily detected as detergent (P. Haubrock, pers. obs.), occurred in the stomach content of 'immature' fish. Overall, *P. parva*, Coleoptera and *P. clarkii* majorly contributed to *I. punctatus* diet (Table 1).

The PERMANOVA main test confirmed supposed differences for the interaction between factor 'maturity' and 'season' (Table 2), with no inter-sex differences (i.e. no differences in item composition between males and females). In particular, post-hoc tests revealed that in 'immature' fish diet significantly differs according to season, while a significant difference for 'mature' fish is pointed out only between summer and autumn (Table 3). Moreover, focusing on each season level, significant differences in diet (i.e. item composition) of 'immature' and 'mature' fish were found in summer and autumn, but not in spring (Table 2). The CAP1 and 2 axes (squared canonical correlation of δ_1^2=0.484 and δ_2^2=0.183, respectively), with an overall mis-classification error of 48.28% (i.e. reallocation of each sample to the right level of the factor), only partially separated the groups of specimens identified by the combination of factor 'maturity' and 'season' (Figure 4). Correlation of variables' scores on CAP1 axis revealed detritus (r_s = -0.63), detergent (r_s = -0.56) and Coleoptera (r_s = -0.39) as major variables in characterizing the 'immature' specimen diet (negative sector of CAP1 axis), especially in summer and autumn, while *P. parva* (r_s = 0.66) and *P. clarkii* (r_s = 0.40) highly characterize 'mature' specimen diet in Summer and Spring (positive sector of CAP1 axis). Correlations with CAP1 and the other variables ranged from -0.23 to 0.14, thus not contributing to clearly characterize identified groups, and are not reported in the text (Supplement 1). CAP2 axis was not considered for further analysis, due to its low power in discriminating groups and inconsistent correlations with variables, despite a unique high inverse correlation with algae (r_s = -0.86), that characterize specimens from the negative sector of the CAP2 axis (Figure 5).

Discussion

Our study revealed a diet difference between life stages and showed a population structure unbalanced towards 'immature' individuals, suggesting a stable reproduction, high fecundity and birth rates of the species, similar to observations from native populations in North America made by Holland & Peters (1992). Also, sampled females were significantly longer and heavier than males, which is matching the sexual dimorphism described in native populations (Wang, 1986). The Fulton conditional index for both sexes appeared lower compared to specimens analysed in other North American studies (see e.g. Holland & Peters, 1992; Mesa & Rose, 2014). This could be due to an increased linear length growth (without a simultaneous rapid weight growth) or a fundamentally different growth in the introduced habitat, possibly due to environmental factors (availability of prey, longer activity time, temperature etc.). A link between growth and water temperature has been also suggested by Endo et al., (2017) for *I. punctatus* in Japan and was observed in non-native populations of the black bullhead *Ameiurus* melas (Copp et al., 2016). Nonetheless, the Fulton index has also been identified as an indicator for migratory behaviour (Gillanders et al., 2015). Because channel catfish are known to reduce their feeding activity in relation to falling ambient temperatures, the observed low Fulton index values in September may indicate a potential fall migration for adult and sub-adult catfish (Pellett et al., 1998), while low values between April and July but also in September could relate to periods of breeding (Peters et al., 1992). An adaptation towards secondary reproductive phases has been described for several non- native fish species (Copp & Fox, 2007), but never from North American Siluriformes in Europe.

The stomach content analysis showed a wide and opportunistic feeding habit of channel catfish, without any differences between sexes. With increasing size, the total sum of stomach contents decreased, likely due to a changing prey composition. Also, ‘mature’ fish expressed a more piscivorous diet typical for this species (Hubert, 1999). Detritus with algae and Coleoptera represented the most frequent non-animal and animal portion of the diet in ‘immature’ fish, respectively, confirming that they forage close to the river bank in reed areas (Endo et al., 2015). In ‘mature’ fish, detritus and algae were less frequent, while *P. clarkii*, which is generally found on the bottom of an aquatic body, and *P. parva*, a prey item occupying the centre and upper water layer (Gozlan et al., 2010; Čech & Čech, 2011; Annamaria Nocita pers. comm.), majorly contributed to the diet. Predation close to the ground and throughout the water column was shown for this species before (Heard, 1958; Poe & Rieman, 1988; Townsend & Winterbourne, 1992; Matsuzaki et al., 2011). Diet variations according to age and size classes are commonly related to idealized energy intake, especially as the diets of fish are partitioned by life history to lower potential intra-specific competitions (Flecker, 1999; Couture & Pyle, 2015). This may be especially true for opportunistic channel catfish (Busbee, 1968), which exhibit varying predation according to predator size, higher mobility, and predation in open water by adult specimens (Robinette & Knight, 1981; Boersma et al., 2006; Matsuzaki et al., 2011) and secondarily availability of prey. In our case, dietary differences and proposed implications about the frequented habitat are indeed potentially linked to gape-limitation (Johnson et al. 2008). *Pseudorasbora parva* occurred also in ‘immature’ specimens (although with low frequency), which can be explained by dietary opportunism in *I. punctatus* and their capability to predate small sized or ailing fish (~3cm) along with a possible mismatch between 'maturity' threshold and the threshold for gape size being increased enough to consume *P. parva*. Indeed, the observed diet change in 'mature' specimens would not be observable without the presence of *P. clarkii* and *P. parva*, both being highly invasive and frequently abundant species (Gherardi & Acquistapace, 2007; Nocita & Zerunian, 2007; Britton et al., 2010).

The applied CAP analysis underlines the importance of *P. clarkii* and *P. parva* for the diet of *I. punctatus*, as both these prey items characterize the diet of 'mature' specimens while also contributing to the diet of 'immature' catfish. The importance *P. clarkii* and *P. parva* for 'mature' specimens was apparent especially in spring and summer, the activity peak of these two species, although they were less frequent in the diet of 'immature' individuals during summer and autumn. Especially in summer, populations of *P. parva* and *P. clarkii* can reach high densities in the Arno river, although considerably lower than observed in standing water bodies (Correia & Ferreira, 1995; Gozlan et al., 2010; Gherardi & Acquistapace, 2007). Therefore, it is no surprise that both prey items were frequently consumed by 'mature' catfish in summer and in the case of *P. parva* also in autumn. These results are furthermore consistent with studies from lake Kitaura, Japan, where it was shown that invasive channel catfish exerted a heavy predation on *P. clarkii* and *P. parva* opposed to available native prey (Endo et al., 2015). The highest frequency of *P. parva* in 'immature' *I. punctatus* was observed in spring, coinciding with the lowest frequency of this prey items in the diet of 'mature' fish. This can be potentially explained by spring habitat use shift in *P. parva*, which in this time search for suitable spawning substrate such as plants or structure close to the river banks, i.e. the parts of the river, where most of other prey items of 'immature' *I. punctatus* occur. *P. clarkii* was less frequently found in the diet of both 'mature' and 'immature' catfish caught in autumn, which is possibly linked to a changing habitat use of *P. clarkii* in autumn (Correia & Ferreira, 1995).

The only season, in which PERMANOVA detected similar diet of the 'mature' and 'immature' *I. punctatus* was spring, which are likely based on the high occurrences of detritus and algae in both subpopulations as revealed by the post-hoc PERMANOVA. Although fish generally tend to prey on the most available and easily to obtain as well as energetically valuable prey (Vanni, 1987; Gill, 2003), it is unlikely that this similar diets of 'immature' and 'mature' fish during spring can be based solely on the availability of prey items. More likely, it can be assumed that higher levels of water and oxygen (respectively increasing water temperature) in spring lead to higher activities of all catfish and thus, lower selectivity in all catfish. Additionally, 'mature' catfish are likely to increase their energy intake after decreased rates of physiological processes due to colder temperatures during the winter and in preparation for the reproductive season (Kim & Lovell, 1995), potentially explaining the high occurrences of algae and detritus, as both items that are present in high quantities and are easily accessible. Although a regular consumption of algae with potentially positive physiological effects on weight gain were observed in this species (Lilyestrom et al., 1987; Tyus & Nikirk, 1990; Menghe et al., 2009), it cannot be excluded that catfish consume algae and detritus because of the lack of other, more energetically valuable prey or in search for any potential energy source prior to spawning. Lastly, changes in the flow regime, i.e. changes from high water level and strong current in winter towards low water level and almost stagnation in between weirs during summer, as well as an increasing water temperature lead to an increasing activity and abundance of potential prey, resulting in an increased exposure for predators. Note, however, that the spring similarity between 'mature' and 'immature' *I. punctatus*, as well as the difference in diet breadth in autumn, are potentially affected by relatively small datasets for 'mature' catfish (see Table 1).

Our study furthermore shows an unusual high frequency of detergent in summer and autumn, likely remains of water pollution or filter attempts by sewage disposal facilities (Annamaria Nocita pers. comm.) undermining the dominant bottom feeding. Detergent in the stomach content could generally be described as "accidental ingestion" that could potentially occur also in other fish species feeding on the river bottom (e.g. the common carp *Cyprinius carpio*). Nonetheless, the ingestion of detergent, as observed by Mahajan & Singh (1973) leads to pronounced "spitting" and decreasing appetite, resulting in fish to rather starve than feed on detergent treated prey items (Gupta et al., 1983). However, commercially available soap has been successfully used to catch *I. punctatus* in its native range and the Arno river (P. Haubrock, pers. comm.) but was not described for any species so far, raising the question if *I. punctatus* willingly ingests detergents found on the river bottom and how it might be affected by it. The occurrence of detergent in the diet of catfish clearly needs further investigation.

In conclusion, channel catfish and especially the 'mature' fish frequently feed on invasive alien species during summer and autumn in this highly-invaded environment, which is seemingly a common behaviour in alien fish species (Copp et al., 2009). Interestingly, these prey species are also considerable prey items of other predators such as the Wels catfish *Silurus glanis* (Carol-Bruguera, 2008; Copp et al., 2009). The wide feeding habit, with 'immature' specimens feeding on algae and detritus and 'mature' specimens feeding on higher invertebrates (and to some degree on highly abundant *P. clarkii* and *P. parva*), potentially lowers intra- and inter-specific competition between co-occurring species and other life stages (Tyus & Nikirk, 1990; Townsend & Winterbourne, 1992; Copp et al., 2009). Therefore, it would be interesting to investigate the relationship between *I. punctatus* and competitors such as *S. glanis* in respect to possible overlaps in feeding niches, especially with respect to observed declines in *S. glanis* populations after introductions of *I. punctatus* (Annamaria Nocita, pers. comm.). Additional studies considering more data from different European ecosystems and a more detailed assessment on the reproduction are needed. Furthermore, potential relationships with other alien species and thus, varying feeding dynamics and the occupied ecological role in invaded communities should be analysed, in order to better understand this species' potential impacts on recipient ecosystems.

Acknowledgements

We would like to thank Dr. Kit Magellan for initial improving comments on an earlier version of the manuscript and Dr. Michal Janáč his time and dedication to improve the quality of this work. Lastly, we thank Lauren Tonelli for proofreading. Funding was provided by the Aquainvad-ED project (2020 Marie Sklodowska-Curie ITN-2014-ETN-642197).

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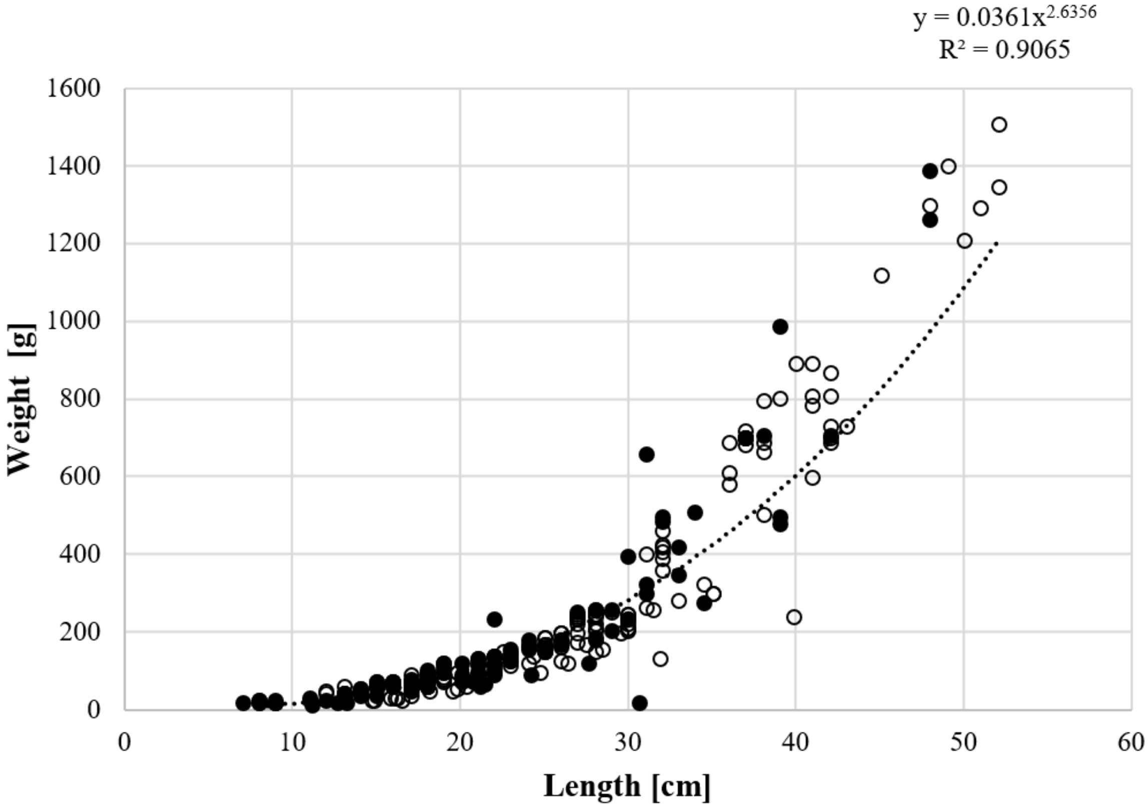


Figure 1. Growth curve for the overall sampled population of channel catfish, differentiating female (n=135, open circles) and male (n=113, filled circles) specimens.

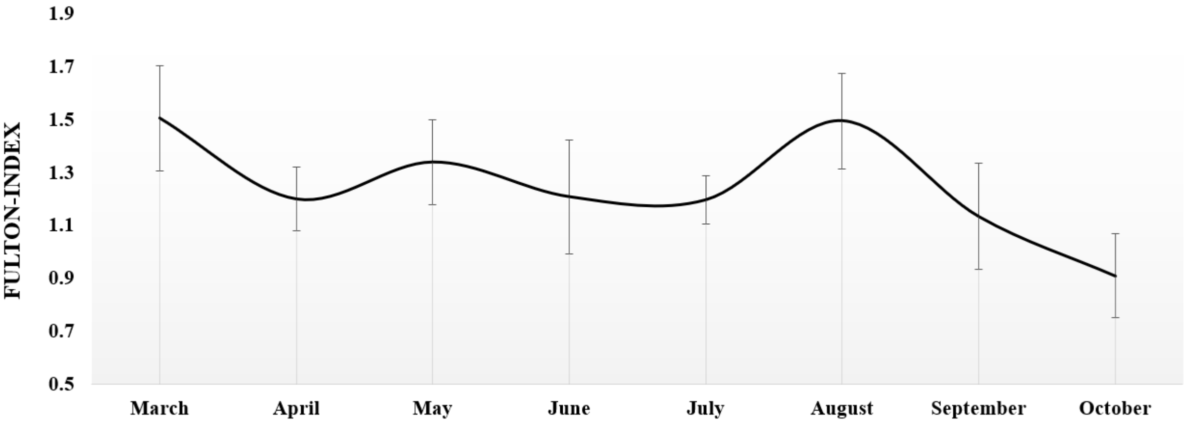


Figure 2. Development of the length-weight relationship in form of the estimated monthly Fulton-Index (mean±SD) over the activity period of caught specimens.

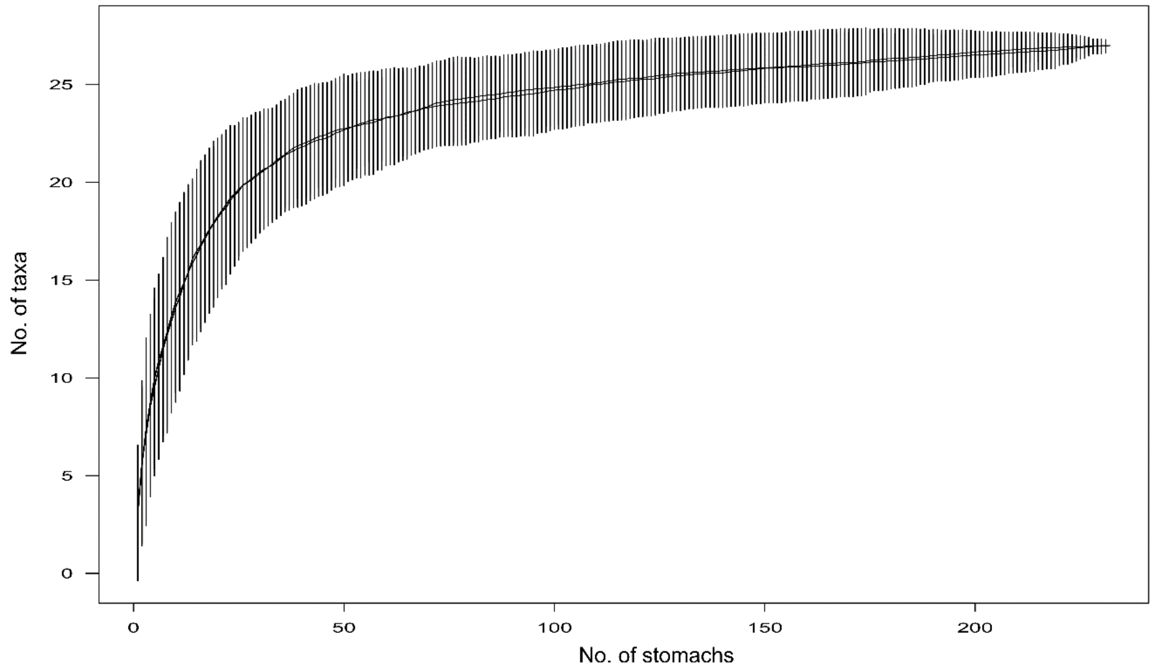
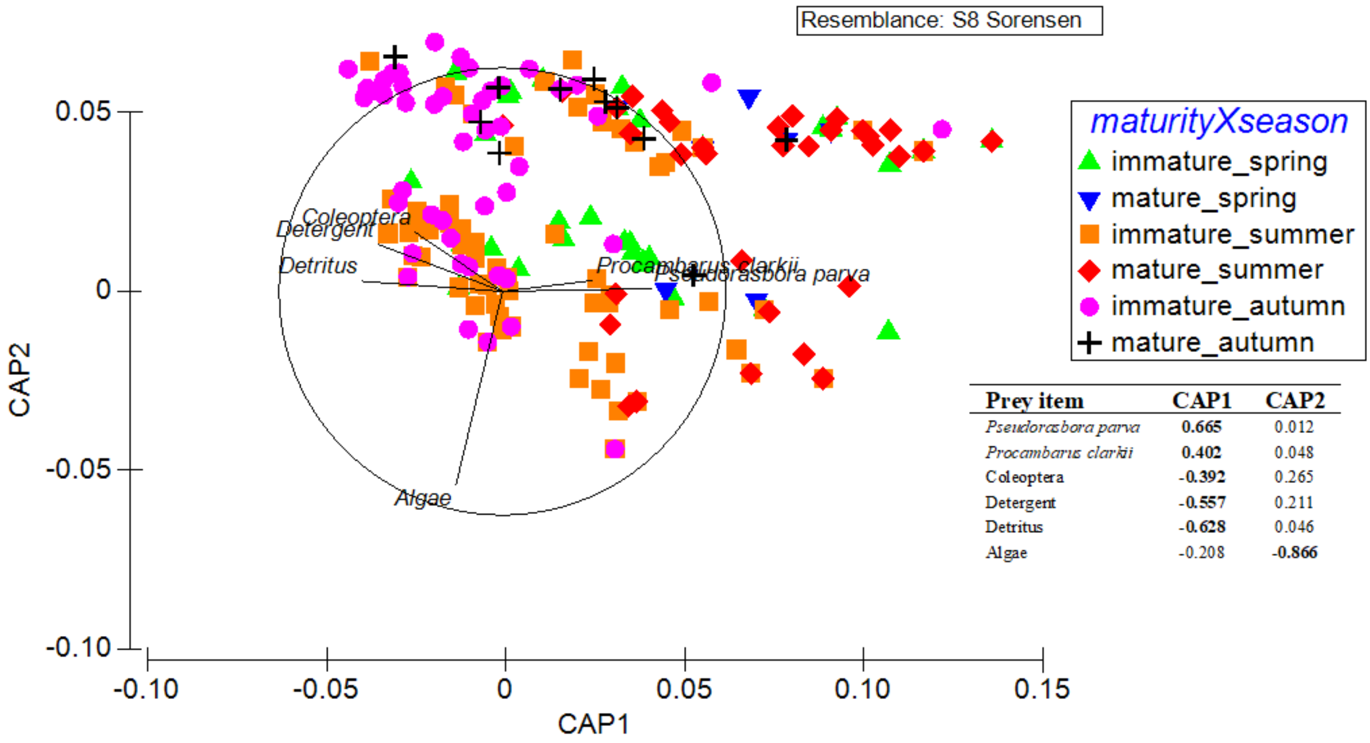


Figure 3. Cumulative prey curve displaying prey taxa per stomach for *Ictalurus punctatus* collected in Arno river in Florence, Central Italy (n = 232). Error bars represent standard deviations.



Prey item	**CAP1**	**CAP2**
Pseudorasbora parva	**0.665**	0.012
Procambarus clarkii	**0.402**	0.048
Coleoptera	**-0.392**	0.265
Detergent	**-0.557**	0.211
Detritus	**-0.628**	0.046
Algae	-0.208	**-0.866**

Figure 4. Two-dimensional scatter plot of the first and second principal coordinates axis (after resemblance matrix with Sørensen distance on presence/absence matrix of data, n samples= 232, n variables = 26) for 'maturityXseason', a combination of factor 'maturity' ('immature', 'mature') and 'season' (spring, summer, autumn). Vectors of the linear correlations between individual variables are

superimposed on the graph (only those with Spearman correlation index >0.4 are shown) and are listed in the lower right of the figure.

Table 1. Frequency of occurrence (%) for observed prey items according to season in ‘immature’ specimens (n=166), ‘mature’ specimens (n=66) and overall sampled specimens (n=232).

Prey	**Total**	**‚Immature‘**				**‚Mature‘**			
	n=232	Overall n=166	Spring n=32	Summer n=76	Autumn n=58	Overall n=66	Spring n=7	Summer n=48	Autumn n=11
Fish									
Pseudorasbora parva	29.7	19.0	56.3	11.8	8.6	56.0	14.3	58.3	81.8
Unid. Fish larvae	8.2	8.4	6.3	9.2	8.6	7.5	28.6	6.3	0.0
Cyprinus carpio	6.0	1.8	3.1	1.3	3.4	16.4	28.6	6.3	27.3
Lepomis gibbosus	4.7	1.8	6.3	0.0	1.7	11.9	0.0	14.6	0.0
Gobio gobio	0.4	0.0	0.0	0.0	0.0	1.5	0.0	2.1	0.0
Crustacean									
Procambarus clarkii	17.6	10.2	15.6	11.8	5.2	35.8	28.6	39.6	9.1
Paleomonethes	10.3	12.0	21.9	10.5	10.3	6.0	0.0	4.2	9.1
Dikerogammarus villosus	6.9	8.4	12.5	3.9	10.3	3.0	0.0	2.1	0.0
Amphibian									
Tadpoles	1.7	0.6	0.0	1.3	0.0	4.5	0.0	6.3	0.0
Molluscs									
Sinanodonta woodiana	0.4	0.6	0.0	0.0	1.7	0.0	0.0	0.0	0.0
Radix auricularia	0.4	0.6	0.0	0.0	1.7	0.0	0.0	0.0	0.0
Insects									
Coleoptera	18.5	22.9	15.6	17.1	34.5	7.5	14.3	6.3	0.0
Unid. Insect larvae	10.3	13.9	3.1	14.5	19.0	1.5	0.0	2.1	0.0
Heteroptera	8.6	10.8	15.6	10.5	8.6	3.0	0.0	2.1	9.1
Diptera	5.6	6.6	0.0	3.9	13.8	1.5	14.3	0.0	0.0
Hymenoptera	5.6	6.0	6.3	6.6	5.2	4.5	0.0	6.3	0.0
Fragments of insects	3.9	5.4	3.1	9.2	1.7	0.0	0.0	0.0	0.0
Odonata	3.4	3.0	6.3	3.9	0.0	4.5	0.0	4.2	9.1
Dermaptera	0.9	0.6	0.0	0.0	1.7	1.5	0.0	2.1	0.0
Other									
Detritus	55.8	66.9	59.4	63.2	75.9	28.4	42.9	22.9	36.4
Algae	48.5	58.4	53.1	75.0	37.9	23.9	42.9	18.8	36.4
Phytoplancton and terrestrial plants	28.8	32.5	50.0	22.4	37.9	19.4	14.3	20.8	9.1
Detergent	25.3	31.3	6.3	7.9	6.9	10.4	14.3	10.4	9.1
Plantseeds	7.3	9.0	18.8	22.4	50.0	3.0	0.0	4.2	0.0

Table 2. Results of PERMANOVA on stomach content of collected specimens. The value of F statistic and its probability values P (after Monte Carlo correction) are shown together with degrees of freedom (df) and mean squares (MS). Significant differences are marked with (*).

Source	df	MS	Pseudo-F	P(perm)
Season	2	13001	5.1548	0.001*
Sex	1	3595.4	1.4255	0.221
Maturity	1	19933	7.9032	0.001*
Season x sex	2	3326.5	1.3189	0.211
Season x Maturity	2	5423.1	2.1502	0.012*
Sex x Maturity	1	5399.2	2.1407	0.068
Season x sex x Maturity	2	1992.9	0.79017	0.647
Res	220	2522.2		
Total	231			

Table 3. Results of pairwise comparison (PERMANOVA) after significant differences founded in the interaction between TL and season on stomach content of collected specimens. The value of t and its probability values P (after Monte Carlo correction) are shown. Significant differences are marked with (*).

Source	t	P(perm)
Within level ‘immature’ of factor ‘TL’		
spring, summer	2.4849	0.001*
spring, autumn	2.9868	0.001*
summer, autumn	2.7633	0.001*
Within level ‘mature’ of factor ‘TL’		
spring, summer	0.66594	0.751
spring, autumn	1.2633	0.171
summer, autumn	2.1626	0.001*
Within level ‘spring’ of factor ‘season’		
‘immature vs. mature’	0.68573	0.439
Within level ‘summer’ of factor ‘season’		
‘immature vs. mature’	4.388	0.001*
Within level ‘autumn’ of factor ‘season’		
‘immature vs. mature’	1.634	0.031*

Supplement 1: List of variables (n=26) used for PERMANOVA tests, along with the correlation (Spearman rank correlation r_s) with CAP1 and CAP2 axis of the Canonical Analysis of Principal Coordinates performed.

PREY	**CAP1**	**CAP2**
Fish		
Pseudorasbora parva	0.66456	0.01213
Unid. fish larvae	-0.00411	-0.12922
Cyprinus carpio	0.13731	-0.02595
Lepomis gibbosus	0.08647	0.19368
Gobio gobio	0.07614	0.05944
Crustacean		
Procambarus clarkii	0.40221	0.04851
Paleomonetes antennarius	0.00845	0.13759
Dikerogammarus villosus	-0.10137	0.14835
Amphibian		
Tadpoles	0.11769	-0.09642
Molluscs		
Radix auricularia	-0.04667	-0.01130
Insects		
Coleoptera	-0.39183	0.26529
Unid. insect larvae	-0.23058	0.14055
Heteroptera	-0.17177	0.09792
Diptera	-0.18775	0.16043
Hymenoptera	-0.09166	0.06703
Fragments of insects	0.00450	-0.14350
Odonata	0.05821	-0.08113
Dermaptera	-0.03063	0.12532
Sinanodonta woodiana	-0.02997	-0.04863
Other		
Detritus	-0.62772	0.04554
Algae	-0.20841	-0.86559
Aquatic and terrestrial plants	-0.15118	0.18044
Detergent	-0.55671	0.21055
Plant seeds	-0.04712	0.06861

7.4. Developing innovative methods to face aquatic invasions in Europe: the Aquainvad-ED project

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Abstract

Aquatic Invasive Alien Species (AIAS) are increasing due to the synergistic effects of climate change and habitat destruction. AIAS can heavily impact biodiversity and human health, causing a loss of ecosystem services; therefore, their control and management have now become a priority, particularly in the light of the new EU regulation 1143/2014 on invasive alien species. The main research goal of the Innovative Training Network Marie Skłodowska-Curie Aquainvad-ED is to exploit the application of innovative tools and the power of citizen science for early detection, control and management of AIAS. Eight early stage researchers are involved in Aquainvad-ED, engaged in four main research themes: (1) development and application of novel methods for early detection of AIAS; (2) identification of vectors of introduction and pathways of dispersal; (3) impacts of freshwater and marine

invaders; and (4) risk assessment and control of AIAS. In order to develop multidisciplinary approaches to address these issues, the fellows are working within an international consortium (UK, Spain, Italy) composed of scientists and conservation practitioners from three universities (Swansea University, Universidad de Oviedo, Università degli Studi di Firenze), one technological institute (AZTI), two governmental agencies (Natural Resources Wales and Cardiff Harbour Authority), one NGO (Wye & Usk Foundation) and five SMEs working in fundamental and applied aspects of AIAS (Neoalgae, Natural Applications, NEMO, Ecohydros, and Itinera C.E.R.T.A).

Keywords: *alien species, invasive, early detection, control, pathway*

Introduction

Globally, as a result of the advancing breakdown of biogeographic barriers, the introduction of alien invasive species is greatly contributing to biodiversity decline, ecosystem homogenization, and loss of ecosystem services, heavily impacting human health and economic activities (Kettunen et al. 2009; Simberloff et al. 2013; Jeschke et al. 2014; Mazza et al. 2014; Roy et al. 2016). The problems posed by aquatic invasive alien species (AIAS) are particularly dramatic due to the synergistic effects of climate change and habitat destruction. Aquatic ecosystems, especially freshwater ones, are vulnerable to biological invasions due to the strong affinity of humans to water (e.g. alteration, exploitation, utilization) and the intrinsic dispersal ability of aquatic species compared to terrestrial ones (Gherardi et al. 2009; Strayer 2010; Havel et al. 2015; Tricarico et al. 2016).

Over the last centuries, aquatic ecosystems in Europe have been colonized by highly invasive alien species. A total of 1,369 alien species have been reported in the European seas (Katsanevakis et al. 2013), mostly introduced through the Suez Canal (with an increase since 1990s) that was recently enlarged, facilitating the arrival of new species (Zenetos et al. 2012; Galil et al. 2015). There are 756 alien species in European fresh waters (Nunes et al. 2015), introduced mainly through aquaculture, the pet/aquarium trade (an emerging important pathway since 2000s: Maceda-Veiga et al. 2013; Mazza et al. 2015) and through sport fishing (Nunes et al. 2015). In both aquatic realms, many notable invaders are present, causing substantial damage to invaded ecosystems (Katsanevakis et al. 2013; Nunes et al. 2015).

The management of AIAS has become a priority, particularly in the light of the new EU regulation 1143/2014 (EU 2014) on the prevention and management of the introduction and spread of invasive alien species (art. 25). More than half (57%) of invasive alien species included in the EU concern list are freshwater species (Implementing Regulation EU 2016/1141 of 13 July 2016). Successful management of AIAS requires several steps: early detection, identification of introduction routes and dispersal pathways, and development of efficient control measures (CBD 2002). Public awareness and stakeholder involvement are also critical for preventing new introductions and for mitigating the impact of existing ones (CBD 2002).

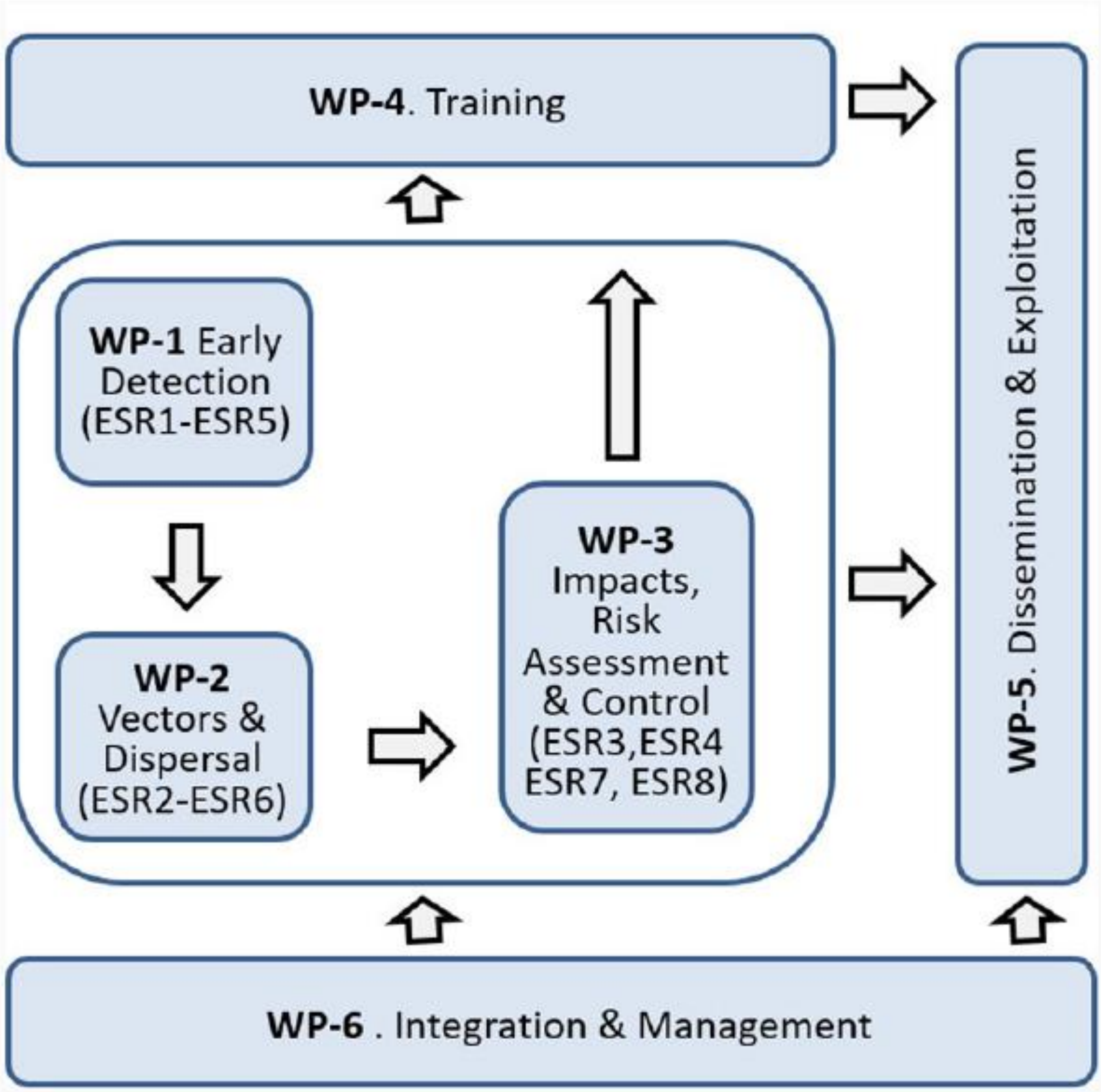
Several projects (e.g. DAISIE, IMPASSE, COST Action TD1209 Alien Challenge) and initiatives (e.g. Essl et al. 2015; Latombe et al. 2016; Lucy et al. 2016) have addressed and are addressing different issues concerning invasive alien species in Europe and worldwide in order to harmonize terminology and optimize actions (databases, pathways, monitoring process, fostering collaboration). In this context, the project Marie Sklodowska Curie 2014 ITN (Innovative Training Network) H2020 Aquainvad-ED (AQUAtic INVaders: Early Detection, Control and Management; 2015–2019; http://www.aquainvad-ed.com/) was developed to tackle AIAS in Europe and to harmonize with the Marine Strategy Framework Directive (2008) and the Water Framework Directive (2000). ITN projects bring together universities, research centres and companies from different European countries to train a new generation of researchers. The funding boosts scientific excellence and business innovation, and enhances researchers' career prospects through developing their skills in entrepreneurship, creativity and innovation. The main research goal of Aquainvad-ED is to exploit novel tools combined with the power of crowd data sourcing (citizen science) to develop innovative methods for early detection, control and management of AIAS.

The project

In order to develop multidisciplinary approaches to address AIAS issues, Aquainvad-ED involves an international consortium of three European countries (UK, Spain, Italy), composed by scientists and professionals from three universities (Swansea University, project leader, Universidad de Oviedo, Università degli Studi di Firenze); one technological institute (AZTI); two governmental agencies (Natural Resources Wales and Cardiff Harbour Authority); one NGO (Wye & Usk Foundation) and five SMEs (Small and medium-sized enterprises) working in fundamental and applied aspects of AIAS (Neoalgae, Natural Applications, NEMO srl, Ecohydros and Itinera C.E.R.T.A scarl). Together, the Aquainvad-ED network offers a multidisciplinary approach (genetics, behaviour, ecology, citizen science, risk assessment) and the expertise of academic and non-academic partners to the assessment and management of biological invasions in aquatic habitats, through the enhancement of unique skills (e.g. technical, research and analytical competences), knowledge-sharing and capacity building.

The specific goals of the project are: i) developing, optimizing and trialing innovative methods for early detection of freshwater and marine invaders; ii) identifying ecological and demographic factors determining AIAS establishment and spread; iii) recommending novel procedures for AIAS control which are applicable to natural and managed aquatic systems (e.g. rivers, estuaries, artificial reservoirs); iv) integrating information on location, dispersion and

Figure 1. The six Aquainvad-ED Work Packages (WPs) along with the corresponding Early Stage Researchers (ESRs).



control measures into management plans in order to prevent further AIAS introductions and dispersal in Europe, and v) raising public awareness about introduction routes and dispersal pathways, as well as about ecological and socio-economic impacts caused by AIAS.

In order to accomplish these goals, Aquainvad-ED is structured into six complementary Work Packages (WPs; Figure 1): WP1, dealing with the development and application of novel methods for early detection for AIAS; WP2, focusing on identification of introduction vectors and dispersal pathways; WP3, concerning the assessment of impacts of selected freshwater and marine invaders, as well as risk assessment and control of AIAS; WP4 on organizing training activities devoted for the recruited fellows; WP5 on dissemination and exploitation; and WP6 dedicated to the integration and management of the whole project.

The Fellows

Eight Early Stage Researchers (ESRs) are involved in Aquainvad-ED, and are dedicated to four projects linked to WP1, WP2 and WP3 (Figure 1): (1) development and application of novel methods for early detection AIAS; (2) identification of introduction vectors and dispersal pathways; (3) impacts of aquatic invaders; and (4) risk assessment and control of AIAS. Each ESR has academic and non-academic supervisors, and two planned secondments within the consortium partners in order to acquire multidisciplinary and multi-sectorial skills. WPs 4, 5 and 6 are dealing with training activities, dissemination and project management, involving the supervisors coordinated by Swansea University (WPS 4, 6) and by Wye & Usk Foundation (WP5).

Development and application of novel methods for early detection AIAS

Teja Muha (Swansea University, UK) and Anaïs Rey (AZTI, Spain) are developing molecular methods based on metabarcoding, able to detect the overall community, and qPCR, suitable for detecting specific species in freshwater [for detection of the killer *shrimp Dikerogammarus villosus* (Sowinsky, 1894), the zebra mussel *Dreissena polymorpha* (Pallas, 1771), alien macrophytes and fish] and marine environments (for detection of invaders as required by the "Ballast Water Convention"). To achieve this, laboratory and field calibration of molecular methods are applied to a range of freshwater and marine systems in the UK, Spain and Italy. As part of a citizen science programme, a smartphone app (AquaInvaders) is being used to promote citizen science programs for the early detection of AIAS.

Identification of vectors of introduction and pathways of dispersal

Marta Rodríguez-Rey (Swansea University, UK) and Sabine Rech (Universidad de Oviedo, Spain) are working on the identification of physical and ecological constraints for the survival of AIAS. Rodríguez-Rey is mainly addressing the different role of natural vs. anthropic variables in the dispersal of non-native invasive species, as well as the social perception towards alien species. Rech is focusing on floating objects and marine litter as potential vectors of AIAS (Rech et al. 2016a). The fellows will compile an inventory of AIAS arriving to selected freshwater and marine systems in the three project countries. They will estimate optimal conditions, potential floating vectors, and high-risk activities and source and sink areas for invasion and dispersal of AIAS, before mapping the main routes of introduction and dispersal, based on floating and stranded samples of rafting biota (Rech et al. 2016b), traffic research, meta-barcoding profiles (deriving from Teja and Anaïs), fouling experiments, using eDNA and experiments under controlled conditions. In this way, it will be possible to develop recommendations and guidelines for identifying vectors of introduction and pathways of spread of key aquatic invaders.

Impacts of aquatic invaders

To quantify current ecological and socio-economic impacts and the relationships among invaders, Matteo Rolla (Swansea University, UK) and Phillip J. Haubrock (NEMO srl, Italy) are studying selected freshwater invaders [such as *D. villosus*, *D. polymorpha*, the red swamp crayfish *Procambarus clarkii* (Girard, 1852), the channel catfish *Ictalurus punctatus* (Rafinesque, 1818), the bullfrog *Lithobates catesbeianus* (Shaw, 1802); Haubrock et al. 2016a] through laboratory and field experiments (Haubrock et al. 2016b; Rolla et al. 2016). Moreover, they are assessing the ecosystem services affected by these AIAS in order to quantify the economic costs. These fellows will develop guidelines for estimating current and future AIAS impacts in aquatic environments under a range of future climate and environmental scenarios.

Risk assessment and control of AIAS

To accomplish the last step of AIAS management, Iva Johović (Università degli Studi di Firenze, Italy) and Roberta Skukan (Neoalgae, Spain) are modelling and assessing the risk of invasion for a range of freshwater (e.g. *P. clarkii, L. catesbeianus*) and marine invaders [the invasive seaweeds *Codium* spp., *Sargassum muticum* (Yendo) Fensholt, 1955, and *Undaria pinnatifida,* Harvey (Suringar), 1873], respectively (Haubrock et al. 2016a). They are also testing different control techniques and mitigation measures to prevent the spread of selected AIAS (Johović et al. 2016). In order to assess the risk of marine invasion, molecular species identifications and biogeography data will also be integrated in Roberta's research as a valuable tool for effective management strategies (Skukan et al. 2016a). Citizen science programs as a useful tool for early detections and prevention of algae invasions will be also implemented (i.e. Skukan et al. 2016b). Their final aim will be to develop guidelines for mitigating biological and socio-economic impacts caused by freshwater and marine invaders, as well as predictive models for the identification of vulnerable areas under current and future climate change.

Network and training activities

Aquainvad-ED partners meet annually to review progress and provide an update on project status. They also engage in specific training activities to enable ESRs to develop new skills. For example, in December 2015, they attended the Inaugural Training Event on Entrepreneurship Skills at Swansea (UK), and the Rivers Trust Spring Conference at Hay-on- Wye (UK) in May 2016, where they also participated in a training event on Citizen Science and Communication. In April 2017, they attended a training workshop in Spain on early detection methods for aquatic invaders, and in spring 2018 they will attend a training workshop on strategies and methods for AIAS management in Italy.

Conclusion

As introductions of alien species in Europe increases, new legislation requires more efficient management tools for AIAS. The Aquainvad-ED project will contribute to this task, not only through the production of science-based guidelines and deliverables, but also through the training of a new generation of multidisciplinary researchers who will be able to face biological invasions from different perspectives. The project will benefit from the outputs of the previous cited projects and initiatives, and will surely establish a collaboration with the recently developed INVASIVESNET network (http://www.invasivesnet.org) (ET is part of COST Action Alien Challenge and INVASIVESNET).

Acknowledgements

This project has received funding from the European Union's Horizon 2020 research and innovation programme under the Marie Sklodowska-Curie grant agreement n° 642197. We warmly thank Frank Collas and an anonymous referee for their valuable suggestions on the first version of the manuscript.

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7.5. Aquainvad-ED Deliverable 1.7. A review on impact and management of AIS

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Introduction

Aquatic environments and the ecosystem services they provide are a widespread resource of utmost importance for human well-being. However, today invasive alien species are a major threat to them. In the European DAISIE database (DAISIE 2009), over 14 000 alien species, of which economic impacts are documented for 13% (1347) and ecological impacts for 11% (1094), are recorded. Most alien species are terrestrial with a smaller fraction in marine and freshwater environments (Vilà et al. 2009). Despite aquatic alien species being less then terrestrial ones, they are causing great damages, particularly in fresh waters (Ricciardi & Rasmussen 1999; Sala et al. 2000; Gherardi 2007).

Indeed, beside anthropogenic disturbances such as water quality deterioration, habitat destruction or exploitation of fragmented regions, the introduction of NIS (non – indigenous species) to freshwater and marine ecosystems has been considered as the main driver of biodiversity loss (Sala et al. 2000; Clavero & García-Berthou 2005; Clavero & García-Berthou 2006; Millennium Ecosystem Assessment 2005; Gherardi 2007; IUCN 2009) and homogenization (Rahel 2002). Thus, drawing on ecology and economics to incorporate the impacts of invasive species on ecosystem services into decision-making is a crucial key to reestablishing and sustaining those life-support services that all organisms depend upon (Pejchar et al. 2009).

Invasive alien species

In contrast to a native or indigenous species, the term alien refers to a species that has previously not been present in a certain area where it was accidentally or voluntary introduced by humans (IUCN 2000), whereas both, an indigenous or NIS can become invasive by exhibiting negative impacts on ecosystems. In the present report, we will use the term invasive referring to alien species causing ecological and socio-economic impacts (Gherardi 2006).

These impacts can vary for species and region depending on abiotic and biotic factors (Hulme et al. 2006; Kestrup & Ricciardi 2009; Ricciardi et al. 2013). Importantly, NIS can cause a loss of biodiversity in a relatively short time, resulting in the likely extinction of e.g. endemic cave species to go unnoticed (Mazza et al. 2014). Although the scientific field of invasion biology gained much attention due to the great impacts caused by AIS (aquatic invasive species) such as *Dreissena polimorpha* or *Lates niloticus* (Ricciardi et al. 1998; Ogutu-Ohwayo 1990; Zambrano et al. 2006), the natural spread and non-natural distribution of a mammal like Sus scrofa into a foreign terrestrial ecosystem or the presence of alien insect pests (Paine & Miller 2002; Dukes et al. 2009) are most often accompanied by generally higher public attention (Cruz et al. 2005; Sáaez-Royuella & Telleríia 2008; Merino et al. 2009; Cuevas et al. 2010), resulting in aquatic habitats often being altered to a certain degree without public or scientific notice. Freshwater ecosystems have shown to be more vulnerable than marine or terrestrial ecosystems (Ricciardi & Rasmussen 1999; Sala et al. 2000; Gherardi 2007; Strayer 2010) due to higher intrinsic dispersal abilities of freshwater organisms (Ricciardi & Rasmussen 1999; Jenkins 2003), geographic isolation of e.g. lakes (Lodge 1994) and intensive human use. While the spreading of species into a foreign region can be a natural occurrence, the increased intensity with which humankind has exploited these ecosystems (Rahel 2000, 2001; Gherardi 2007), paired with rising global climate (e.g. Capinha et al. 2012), is likely to increase the frequency of global invasions and range expansions (Capinha et al. 2012; Capinha et al. 2013a, b; Mazza et al. 2014).

According to Olenin et al. (2010), in aquatic ecosystems introduction can be divided in Primary introductions - appearance of a NIS in a new area (called introduced area) directly from its native range and Secondary introductions – consequential spread of NIS from the introduced area. Nonetheless, not all introduced alien species become invasive. Geographical and environmental barriers can limit the establishment and spread. Local environment conditions (e.g. climate, water chemistry, current), ecological and behavioural traits and the life history are last defining components of alien species establishments determining the efficacy of some species to become invasive (Gallardo & Aldridge 2013). Human activities play a key role as a major forecaster for the future distribution of alien species overcoming geographical borders and indirect cause of the invasion process (Pyšek et al. 2010; Pyšek & Richardson 2010). These human activities are mostly linked to recreation, food sources or commercial purposes with an intentional trend to farm mostly alien species (De Silva & Turchini 2008). Ornamental/aquarium trade (e.g. Mrugala et al. 2014) as well as aquaculture and recreational angling (Cambray 2003; De Silva et al. 2009) are indeed recognised as major drivers of AIS introductions. In England, in the highly commercially used River Thames, 96 established alien species have been reported and approximately 55% of them were intentionally introduced (Jackson & Grey 2013). This exploitation and use of aquatic ecosystems has caused a vast amount of accidental contaminations and numerous introductions with often irreversible ecological damage (Gozlan 2008, 2009). Particularly, unintentional introductions via e.g. ballast water, fishing gear, hull fouling etc. (GISP 2008) are currently facilitating the growing spread of invasive species into freshwater (Gherardi et al. 2009) and marine environments (approx. 7000 coastal and marine species travelling through ballast water in tanks every day unnoticed, WWF 2009). For example, the EU project “ALIENS” dealt with the invasive seaweed distribution along the coastline of six European countries and detected 15 seaweed taxa in 90 l of ballast water (http://cordis.europa.eu/result/rcn/84185_en.html). Other important pathways for accidental introductions are the artificial canals, which connect previously isolated water bodies enabling species to spread beyond natural borders (Panov et al. 2009). The spread of the killer shrimp was indeed directly facilitated through the opening of the newly constructed Danube-Main-Rhine canal in 1992 (Kinzelbach 1995). The Suez Canal, an artificial connection between the Mediterranean Sea and the Red Sea, led to the introduction of over 440 NIS in the Mediterranean with a variety of ecological effects on native species (Galil et al. 2015).

Another key player in invasive species biology with uncertain outcomes due to its less predictability will be global change. For invasive species, it is generally accepted that, besides ways of transport and introduction facilitated by anthropogenic activities, an increase in temperature will change the establishment, impact and distribution (Hellmann et al. 2008).

As the establishment and spread of a NIS likely varies with physical environmental gradients (Ricciardi 2003; Jokela & Ricciardi 2008; Gallardo & Aldridge 2013), a rising global temperature will potentially clear constraints by altering ecosystems and making them less resistant to invasions. The altered state will potentially increase the persistence of

NIS thriving in warmer environmental conditions (Dukes & Mooney 1999; Walther et al. 2009) and facilitate the colonization of new areas (Lee & Chown 2007) by enabling NIS to dominate indigenous species due to a tolerance towards higher temperatures (Byers 2002; Hellmann et al. 2008) while simultaneously exercising negative influence on alien species that require colder habitats. Together, invasive species and climate change are the double trouble for ecosystems (Dukes 2011).

Management of aquatic invasive species

Since AIS are rather difficult to manage after their establishment, model organisms have been used to approach posed threats and to understand the process of invasions in order to prevent further introductions. Furthermore, especially in the last years, NIS have received more attention due to rising interest and focus on protection and prevention measures accompanied by increasing ecological awareness (e.g. from the public). Due to the high potential for NIS to disperse in aquatic environments, identifying so-called pathways, ways of introduction and spread became of integral importance to the understanding of invasive species behaviour and threats (Gherardi et al. 2008; Hulme et al. 2008; Mazza et al. 2014). However, because pathways may differ among species and countries of introduction (Gherardi et al 2009), detailed assessments have to be made on a case-by-case basis (Gherardi et al. 2008) and have shown to be specific for target regions (Andersen et al. 2004; Correia et al. 2005). With increasing scientific and public awareness, several risk assessment protocols and methods to control the presence and spread of invasive species were created (see e.g. Tricarico et al. 2010). While effective risk-assessment management tools for several high-profile species exist (Tricarico et al. 2010; Blackburn et al. 2014), eradication success is variable (Gherardi et al. 2011a), sometimes having negative effects on indigenous species. However, there is an urgent call for the development of predictive management tools to identify the likelihood of introductions and consequences of alien marine and freshwater species (Hewitt & Campbell 2007; Campbell 2009). In 2002, the Convention on Biological Diversity (CBD), during its sixth meeting, proposed a hierarchical approach for invasive species. It stated that priority should be given to preventing the introduction of invasive alien species and, if this fails, early detection and rapid action are crucial to prevent the establishment of new introduced species. An immediate eradication is considered the preferable solution. If not feasible, containment and long-term control measures should be implemented together with mitigation actions. Furthermore, a long-term benefits and costs analysis (environmental, economic and social) should be planned (COP 6, Decision VI, 23).

Current regulation of invasive species

The growing concern of governmental institutions on NIS is not only due to the ecological impacts, but also for economic reasons such as high costs of management and control. Therefore, it was inevitable to introduce and enforce legislative measures to target and prevent the occurrence of AIS while simultaneously managing them. As in the world Biodiversity Strategy, the EU Strategic Plan for Biodiversity 2011-2020 puts emphasis on IAS, encouraged by the EU-Commission and participating member states to establish measures of prevention, monitoring, eradication and management for invasive plants and animals. While all measures are equally important in a globalized world, the prevention of invasions was recognized as the most cost-effective and environmentally desirable measure (European Commission 2014). Especially, the recent EU Regulation No 1143/2014 on invasive alien species is the first EU regulation completely dedicated to IAS and associated management, based on the hierarchical approach suggested by the CBD in 2002. The Regulation underlines the high importance of contributing „an adequate knowledge to address the problems raised by invasive species" and, among other measures, to carry out a risk assessment (assessment of potential pathways, introduction, establishment and spread of IAS in relevant biogeographical regions). UK government paid particular attention to the problem of NIS since 2003 with a policy review that has been the starting point for writing the first "GB Invasive Non-indigenous Species Strategy" (GB INNS) in 2008. This document was an important step for the IAS management. It encompasses the (1) establishment of a non-indigenous Species Information Portal (NNSIP), providing a central repository for non-indigenous species information and distribution data; (2) risk assessments for 60 species; (3) raise of awareness through two campaigns focused on aquatic plants ("Be Plant Wise") and recreational water users ("Check Clean Dry"); and (4) assessment of the economic impact of IAS (Department for Environment Food and Rural Affairs et al. 2008).

Where are we now? – What needs to be done!

Taking action is mandatory in order to avoid that loss of biodiversity, economic costs (Pimental et al. 2001; Kettunen et al. 2009) and socio-economic damage could increase because of biological invasions. The total number of NIS has indeed increased by 76 % between 1970 and 2007 (Butchart et al. 2010). The difficulty to increase awareness has led to this increase.). Given limited financial resources, species have to be ranked according to their overall impact for setting management priorities (McNeely 2001). However, it is important that applied ranks be not only based on economic interests. Species without or with low economic impact and invertebrates in general tend to draw less attention and hence receive less scientific focus. With this unbalanced distribution, lacking knowledge on potential threats will most likely result in actions being taken too late for any potential measure. Furthermore, the general understanding of invasive species biology has to go further than prevention, eradication and control (Engelmann et al 2000). Behavioural and ecological studies as well as predictive measures (considering ecological modelling, human-mediated vectors and ongoing climate change) can provide a lot of information about the future spreading of invasive species. Once a species is introduced and becomes invasive, early detection of further spreading is crucial for possible control measure. Modern molecular diagnostic marker (such as DNA barcoding) will be of great importance and a great advantage in the assessment and control (Trivedi et al 2015). Training local authorities in easily sampling methods can provide a lot of valuable information of IAS dispersion (Le Roux & Wieczorek 2008). For that account, scientific approaches such as the Aquainvad-ED Marie Skłodowska-Curie Innovative Training Network (funded by H2020-MSCA-ITN-2014-ETN-642197), which focuses on early detection, control and management of AIS by conducting eight PhD fellowships across Europe in an cooperative network involving academic and non-academic partners from a cross-sectorial programme of research, can be of great future value.

This document shall serve as a short résumé on the current state of the art research conducted on the model AIS used by Aquainvad-ED. We will outline the current knowledge on all the types of known impacts they cause and summarize the methods used for their control and risk assessments.

Impacts

For centuries, the spread of alien species facilitated by anthropogenic influence has broken biogeographic barriers through the creation of new pathways (Ricciardi 2003; Mooney & Cleland 2001). Biotas isolated for millions of years are affected along their evolutionary path by this biotic rearrangement. These evolutionary influences are manifold and can vary among niche displacement, hybridization, introgression, predation or even extinction. Nevertheless, not only indigenous species are being influenced. Alien species are also affected because of their interaction with indigenous species and the new environment (Mooney & Cleland 2001).

How to define impacts

Negative effects of invasive species have shown to significantly overweigh the benefits (McCarthy et al. 2006; Skelton et al. 2013), but several examples where alien species have proven to be beneficial do exist (Gherardi 2007). However, all of the reported positive effects an organism has on human society is valued from the anthropogenic perspective and can vary depending on emphasis. The introduction of the common carp by the Romans, which were harvested for human and as an alternative pet food, was beneficial for the Roman Empire, while monks facilitated the aquaculture of carp for the Lent in the 13th and 15th century (Fagan 2008). One further example is *Lasioglossum leucozonium*, a solitary sweat bee, introduced in North America a long time ago, which seemed to have found its own ecological niche, becoming an important pollinator of caneberries and other cultivated plants (Adamson 2011). The introduction of a NIS can have some beneficials, e.g. by restoring traditions proper to the cultural heritage of a country (e.g. crayfishing in Sweden and Finland) or by providing income for families in poorly developed areas (in Andalusia, Spain: Geiger et al. 2005), even if negative impacts on native ecosystems are reported for the aforementioned cases and, thus, a cost-benefit analyses should be undertaken.

Introductions were also beneficial as in the case for the introduction and use of new species to diversify agriculture and aquaculture e.g. astaciculture. Upcoming crayfish farming in Britain and in Spain (Holdich 1999) affected the inner-European market as well as between European and extra-European countries (Ackefors 1999). The ways and means of how NIS affect indigenous species and ecosystems has been the focus of several studies in the last decades. Even though impacts are usually considered as the last stage of an invasion process (after species establishment and spread), invasive species can exhibit impacts at any point after their introduction (Ricciardi et al. 2013), but quantified impact assessments do only exist for a few chosen species (e.g. Capinha et al. 2013B; Roy et al. 2014). Potential impacts need to be quantified in order to be properly recorded. These impacts can hence affect Economy, Socio-Economical aspects and Ecosystem Services and are strongly dependent on the invading organisms and their surrounding environments.

Ecological:

NIS can directly affect indigenous species by competition, niche displacement or hybridisation potentially driving them extinct, or indirectly by changing the composition and structure of habitats. Being generally accepted as one of the leading causes of animal extinctions and biodiversity loss (Sala et al. 2000; Gurevitch & Padilla 2004a, b), impacts of AIS can even cause biotic homogenization (Clavero & Garciá-Berthou 2005), i.e. the presence of same NIS in several ecosystems associated with the decline of indigenous species. By altering the distribution of biota, a homogenisation will substantially facilitate the decline in diversity. An important aspect of this loss in diversity is the possibility of genetic pollution. With the possibility of hybridization and introgression between closely related species, genotypes are replaced and homogenized resulting in the pollution of gene pools strongly affecting the less abundant species (Meilink et al. 2015). One example is the spread of closely related species of the genus *Triturus*, which, when populations overlap, show the potential to hybridize, causing a decline in the genetic identity and health of these species (Rhymer & Simberloff 1996; Meilink et al. 2015). Beside this direct causality, another possibility to explain the loss of biodiversity is the indirect consequence of habitat modification by anthropogenic and AIS influence (Didham et al. 2005). Through habitat alteration and exploitation, ecosystems have become more accessible for invading species. Furthermore is the presence of an invasive species a potential gateway for several successive introductions by lowering the ecosystems resistance to invasions (Ricciardi et al. 2013). NIS can change the functions of ecosystems: for example, the invasive grass *Bromus tectorum*, when dried, alters the fire regime. Several invasive species such as the Louisiana red swamp crayfish or the common carp can alter the ecosystem with their natural behaviour affecting abiotic factors of the environments (increase of water turbidity, decrease of oxygen and primary production, damages to bank stability, etc.) (Lougheed et al. 1998; Barbaresi et al. 2004; Scalici & Gherardi 2007). One of the rather infamous examples is the largest true freshwater fish in Europe, the wels catfish, Silurus glanis. Based on its high reproduction rate of approximately 30.000 eggs per kilogram of body weight, enormous size of up to 4 m and plastic feeding habit, it can cause the extinction of indigenous fish and especially threatened crayfish species in its introduction areas (Blanc 1997; Carol Bruguera 2007).

Economic:

Managing established populations of NIS can cause high economic costs (Pimentel et al. 2005). Although national approaches to quantify costs have been more anecdotal and vague (Pejchar & Mooney 2009), the high costs and enormous economic damage outgoing from invasive species is undeniable (Lovell &Stone 2005; Cecchinelli et al. 2012). The high estimated economical damage generated by invasive species (including costs of control measures) has been estimated to be higher than $138 billion per year just for the US (Pimentel et al. 2005) and exceeding €12 billion per year in Europe (Kettunen et al. 2009). This last amount might seem very low compared to general costs in the US, but it has to be considered that only a handful cost-efficiency analyses have been applied and that there have been only a few cross taxa estimations at the national level (which differ from the North American perspective). In Europe, most expenses generated by invasive species are due to management costs including eradication, control and environmental education programs targeting some particular natural areas (Vilà et al. 2009). Particularly, the Great Britain-wide costs of controlling invasive freshwater species were estimated to be approximately £26.5 million per year. However, the costs of control could aggregate to approximately £43.5 million per year if management efforts were undertaken at all invade locations (Oreska & Aldridge 2011). Furthermore, an assessment of the economic impact of invasive non-indigenous species by the Department of Environment Food and Rural Affairs (2008) indicated annual costs of £1.7 billion to the British economy. Even if economic impacts are usually direct as a loss of production in agriculture or management costs, there are also losses of recreational and tourism revenues which can be considered as passive economic impacts. Since environmental damage, loss of biodiversity and loss of ecosystem services (the benefits provided to human society by natural ecosystems) are often not considered among production loss or management costs, the general economic costs are probably far beyond estimated values (Pimentel et al. 2005). The major position among economic costs falls to the plant industry related to accidental introductions of pest plant species that accompany seeds for agricultural/ornamental use. These can be a threat to young cattle (e.g. *Euphorbia esula*) or unpalatable (*Centaurea solstitialis*) (Pimental et al. 2005). Additionally, the economic impact on tourism should not be underestimated. Imported fish species such as the bighead carp (*Hypophthalmichthys nobilis*) and silver carp (*Hypophthalmichthys molitrix*) in the Chicago Area Waterway System are listed as invasive and considered a threat to recreational fisheries in the Canadian Great Lake systems (Cudmore et al. 2011). Therefore, different species cause varying economic damage. For instance, invasive crayfish or the green crab have shown the ability to greatly damage agriculture and fisheries reducing annual production while also causing high regulatory costs (Lovell & Stone 2005; Cecchinelli et al. 2012; Lodge et al. 2012). Other examples are the introduced frog species common coquí (*Eleurherodactylus coqui)* and American bullfrog (*L. catesbeiana*) whose populations, once established, can reduce real estate values with their loud call (Sin & Radford 2007; Orchard 2011). The introduction of alien crayfish facilitated the spread of the crayfish plague, which subsequently diminished the abundance of indigenous European species and resulted in a loss of festivals to celebrate indigenous crayfish services (Lodge et al. 2012). Although the introduction of invasive species has shown to restore traditions proper to the cultural heritage of a country (e.g. crayfishing in Sweden and Finland), which were previously threatened due to the decline of crayfish abundance, the introduction of invasive species for a certain advantage implies generally negative consequential problems – a relation called Frankenstein effect. However, whenever the economic costs of an invasive species were quantified, the affected ecosystem services have been often partially not considered or completely neglected (Charles & Dukes 2007).

Ecosystem-services:

Another often neglected aspect is the potential change to ecosystem characteristics caused by invasive species (Ehrenfeld 2010), including the economic impact of invasive species on ecosystem services (Pejchar & Mooney 2009). Ecosystem services can be defined as the benefits provided to human society by natural ecosystems. They can be divided into 1) supporting services, 2) provisioning services, 3) regulating services and 4) cultural services (Millennium Ecosystem Assessment 2005). While most NIS negatively affects ecosystems, the vulnerability of ecosystems may vary. The disruption of services can have socio-economic and cultural impacts as in the case of the invasive plant *Heracleum antagarrianum* (Tiley et al. 1996). Introduced carp populations can alter the habitat structure and ecosystem composition in a way that can change the region, making it unattractive for sport fishing while also decreasing the water quality. Furthermore, can invasive species be potential carriers for diseases such as the Chinese mitten crab (*Eriocheir sinensis*) carrying the Asian lung fluke (Lerner & Heimowitz 2000) or illnesses such as malaria, typhus and yellow fever which are all carried by several species (Elton 2000). Additionally, invasive species used as a food source can become a problem, such as in the case of the crayfish *P. clarkii*, possibly causing severe problems due to its ability to accumulate toxins in its body (Tricarico et al. 2008; Lodge et al 2012).

While this classification of impacts is kept fairly general, only few invasive species tend to impact all five categories. Those that outstretch their impacts in several fields were depending on research question established as model organisms and used by scientists to understand impacts on the food web, environment, other species, economy and many more. While species with a broad spectrum of impacts and high associated economic costs tend to get broad attention by public and science, the ecological and economic impacts of the other invasive species are often overlooked. Invasive plant species impacts are spreading undetected being heterogeneous and changing nutrient cycles, affecting other plant species and communities (Vilà et al. 2011) but, since non-indigenous plant and mammal species are often economically beneficial and have had a history of cultural integration, it is easy to understand why the general focus was more towards them.

Below we are going to present the cases of several chosen freshwater and marine invasive species, which will serve Aquainvad-ED as a model organism. They are some of the highest alert invasive aquatic species and the prevention of further spreading as well as their control should be a priority for authorities.

Marine invasive species

Green Sea Fingers (Codium fragile subsp. *fragile* „Suringar“ Hariot 1889*)*

C. fragile subsp. *fragile*, invasive Japanese green macroalgae (Figure 1.), was first reported in Europe ca. 1900, in the Netherlands (Trowbridge 1999). In the coastal rocky shores of the marine environment (Figure 2.), *C. fragile* is a highly notorious invader with the reputation to repress indigenous algae communities. Its shading influences eelgrass growing in manipulative seminatural experiments, but this observation was not shown in natural environmental conditions. Future assessments to analyse *C. fragile* long-term impacts on eelgrass communities are needed (Drouin et al. 2011) to understand future effects of climate change on environmental conditions and future invasive algae spread. The DAISIE project included *C. fragile* on the list of 100 worst invading species in Europe for a variety of reasions. It shows competitive advantages by settling in the gaps of indigenous seaweed communities. Once established, it inhibits re-colonization of indigenous seaweed (Scheibling & Gagnon, 2006). In manipulative laboratory experiments and niche modelling approaches with invasive ascidian *Ciona intestinalis* and invasive seaweed *C. fragile* Madariaga et al. (2014) concluded that *C. fragile* way of reproduction (sexual and asexual), thalli buoynacy and low palatability by predators makes its invasion risk of spread higher then the risk of *C. intestinalis* spread. Although *C. fragile* is mostly associated with suppressing mussel and shellfish by high density, Bulleri et al. (2006) states reverse effect: *C. fragile* canopy could be negatively affected by the highly developed density of mussels. The Atlantic coast of Canada is inhabited by many cold-temperate species of indigenous seaweed but under effects of climate change, this communities could change significantly. In a laboratory experiment, by increasing sea surface temperature, two indigenous communities of algae didn't survive while one indigenous community of seaweed, along with *C. fragile*, exhibited high survival rates. This can indicate a future species community change with altering effects on ecosystem services (Wilson et al. 2015).

Figure 1. Photo of green sea finger, taken by Skukan, R.

Figure 2. Photo of rockey shores of Asturias Coast (Playa de la Concha de Artedo en Cudillero, December 2015), taken by Skukan, R.

Japanese Wireweed (Sargassum muticum, Yendo“ Fesholt 1995*)*

Brown macroalgae, indigenous for Japan, inhabits shallow subtidal zones causing negative effects by decreasing the biomass of indigenous seaweed species and altering food web structures (Salvaterra et al. 2013). Some of the negative effects this species is showing, are potential reversal effects on indigenous communities of seaweed (Olabbaria et al. 2009) by reducing the abundance of sea urchins which don't feed on it (Britton-Simmons 2004) and inhibiting recreational swimming and commercial fishing (Eno et al. 1997). In a long-term invasion process, *S. muticum* will lead to pauperization of the environmental and biotic homogenization, albeit *S. muticum* can increase habitat (spatial) heterogeneity, i. e the species richness of animals is directly related to the species richness of plants in a certain environment. This feature is limited to the time of the year during which natural growth of *S. muticum* and maintenance of organism that live attached on indigenous seaweed may not be possible. With the displacement of the seagrass *Zostera marina* it could lead to habitat loss and overall primary productivity reduction (DeAmicis & Foggo 2015). However, in the case of the well-established population of the snake pipefish (*Entelurus aequoreus)*, *S. muticum* can serve as habitat and food source (Polte & Buschbaum 2008). The fauna composition can be also affected by seaweed invasions. In studies conducted along the west and south coast of Portugal, Engelen et al. (2013) found out that the fauna in *S. muticum* communities was rating among lowest or highest diversity composition, depending on location. While comparing their data with existing literature they concluded that *S. muticum* does not have severe negative impacts on local seaweed-associated assemblages. Along the Galician coast (with two indigenous seaweeds *Bifurcaria bifurcata*, *Saccorhiza polychides* and invasive *S. muticum*), heterogeneity of seaweed community seems to provide a suitable habitat for many invertebrates, especially gastropods and isopods. Nonetheless, the consequences of these fauna changes are unknown, as they could alter the trophic dynamics of omnivorous fishes and decapods (Gestoso et al. 2012). In the North coast of Asturias (Spain), Sanchez-Fernández (2005) noted that there has been significant change in spatial distribution of seaweed community. From 1990 to 2005, the native red macrolaga *Gelidium spinosum* (Figure 4.) was displaced by *S. muticum* (Figure 3.).

Figure 3. Photo of Japanese wireweed, taken by Skukan, R.

Figure 4. *G. spinosum* (1990) displaced by *S. muticum* (2005), photos taken from Sanchez-Fernández (2005)

In Figure 5, the total biomass of seaweed communities changed and showed population decline during sampling years (1997, 1985, 2002) (Figure 5).

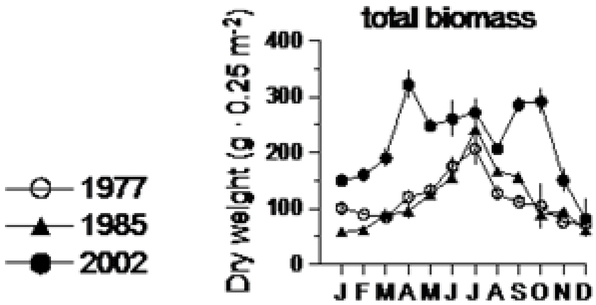


Figure 5. Total biomass of seaweed community in Armar (Asturias) (Sanchez-Fernández, 2005)

Japanese seaweed was the only invasive marine seaweed present in sampling done by Sanchez-Fernández (2005). He concluded that the abundance of *S. muticum* affects native community distribution. Some native seaweeds (such as *B. bifurcata*) and epiphytic opportunistic species increased their abundance due to decay of invasive *S. muticum*. However, he suggested that longer temporal scale research is needed to identify these changes (Figure 6.).

Fig.
4.

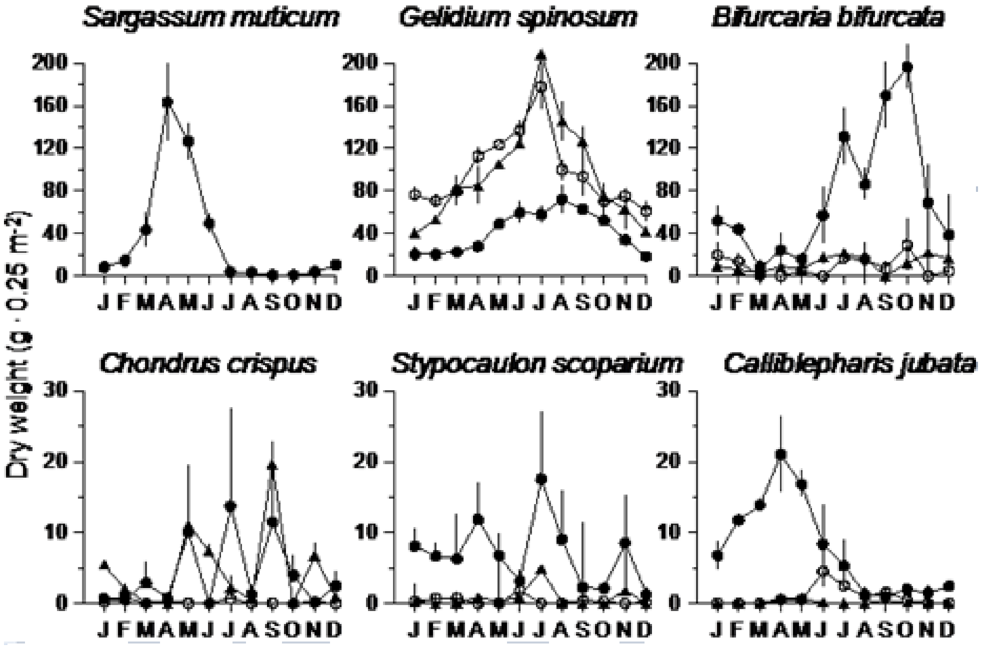


Figure 6. Seasonal changes in the species abundance in Aramar (Asturias) in 1997, 1985 and 2002. (Sanchez-Fernández 2005)

Freshwater invasive species

Zebra Mussel (Dreissena polymorpha Pallas, 1771*)*

The zebra mussel, *Dreissena polymorpha* (Figure 7.) is indigenous to Danube, Dniestr, Berezan, Southern Bug, Dniepr, Molochnaya, Don, Kuban, Kamchia, and Veleca river basins from where it started to spread throughout Europe during the nineteenth century (Son 2007). Zebra mussels are filter feeders that can attach to any stable substrate in the water column or benthos: rock, macrophytes, artificial surfaces (cement, steel, rope, etc.), crayfish, unionid clams, and each other, forming dense colonies called druses. Due to their temperature tolerance and their high spawning ability, zebra mussel spread in Europe and North America outnumbering the indigenous mussels and causing great environmental changes. The first zebra mussel record in the UK dates back to 1824. Since then, the species started spreading, but between 2000 and 2002, both its population size and impacts notably increased (Aldridge et al. 2004). However, it is not clear whether this increase is due to a real increase in and the population of zebra mussel or to an increased awareness of its presence causing considerable concern (Aldridge et al. 2004).

Zebra mussel ecological impacts involve are numerous. Since they are able to filter particles smaller than 1µm in diameter, all suspended material including bacteria, protozoan, zebra mussel veliger, and silt is filtered from the water column, even if they primarily feed on phytoplankton. The feeding of zebra mussel can change the phytoplankton community composition through differential grazing: some phytoplankton species are rejected or less digested by zebra mussel and the stay trapped in the pseudofaeces so when the excretions are resuspended it can return to the pelagia and survive (Bastviken et al. 1998). Overall, they can significantly reduce the amount of phytoplankton biomass, augmenting water transparency (Holland 1993; Fahnenstiel et al. 1993). Zebra mussels compete with indigenous mussel where they are present. They compete for food and space, but the main threat is the extinction of indigenous unionids through epizootic colonization (Schloesser et al. 1996; Baker and Hornbach 1997). Zebra mussels impede valve functionality, causing shell deformity, smother siphons, impair movement, and deposit metabolic waste onto unionid clams. Ricciardi et al. (1995) found a strong relation between zebra mussel density in the wild and indigenous unionids mortality, elaborating a model to predict it.

The damage caused by zebra mussels is not only ecological, but also economical when this species colonizes human structures, causing problems to different human activities. They can colonize water supply, pipes of hydroelectric and nuclear power plants, public water supply plants, and industrial facilities. The small mussels can get into boats engine cooling systems causing overheating and damage. Navigational buoys can been sunk under the weight of attached zebra mussels and even deterioration of dock pilings increases when zebra mussels encrust them. Direct economic costs have resulted from the invasion of zebra mussels due to the maintenance and repair of power plants, industrial facilities, and other businesses, as well as research, monitoring, and control. In the US, a wide variety of estimations have been made regarding zebra mussel-related expenses, ranging from $92,000 per hydroelectric plant per year to $6.5 billion in total costs over 10 years (Lovell et al. 2006).

Figure 7. Photo of zebra mussel colony, taken by Matteo Rolla

Louisiana Red Swamp Crayfish (*Procambarus clarkii* Girard, 1852*)*

One model organism, which has been used to understand invasive biology, is the invasive red swamp crayfish (*Procambarus clarkii*). Native to North-America. It has been identified as one of the most invasive and dangerous species in Europe (Holdich et al. 2009; Gherardi 2011), for a variety of scientists in the last decades (Lodge et al. 1994; Nyström et al. 1996; Tricarico et al. 2008; Gherardi et al. 2011b). In 1859, infected North American crayfish species present in fish batches imported from North America into Italy exterminated all indigenous crayfish populations in Lombardy. After 1860, the export of indigenous crayfish species drastically sank by up to 90% in Scandinavia, Germany, Spain and Turkey, causing an enormous economic damage (Köksal 1988; Holdich 1999), and inducing the introduction of alien crayfish in Europe. The red swamp crayfish was first introduced in Spain in 1977 and then in Italy in 1989 where it became a highly invasive species (Gherardi et al. 1999; Aquiloni et al. 2010). It is one of the most introduced species worldwide for aquaculture purpose. This species is among the largest and longest living invertebrates (Nyström et al. 1996), feeding on benthic invertebrates, detritus, algae, hydrophytes (Whitledge & Rabeni 1997), fish and eggs (Lowery & Mendes 1977) and being the main prey for various species including birds, fishes and otter (Slater & Rayner 1993). While most other crayfish in its invaded area mainly belong to K-selected species with a slow growth rate, long life span and 2-3 years until maturity, *P. clarkii* is classified as an R-rated species found to carry up to 724 eggs (Chucholl & Pfeiffer 2010). With its elevated growth rate of 50 g in 3-5 months and maturity with approximately 10 g, it reproduces faster than indigenous species, out competing or preying upon them (Gherardi 2011a). Experiments showed that *P. clarkii* is able to evict indigenous crayfish and fish species from shelters making them more susceptible for predation while simultaneously preying on eggs (Gherardi 2011b). Beyond this, *P. clarkii* diminished populations of indigenous crayfish in Europe (see e.g. Gil-Sanchez & Alba-Tercedor 2002; Gherardi 2011a) being a vector for the oomycete *Aphanomyces astaci*, the cause of the crayfish plague which decimated populations of *Astacus astacus* and *Astacus leptodactylus*.

P. clarkii is a strong and capable invader due to its ability to burrow and survive for long periods out of water, in brackish or deoxygenated water, enabling it to travel long distances even overland (Gherardi et al. 2000; Scalici et al. 2009). It tolerates elevated turbidity and wide ranges of water temperatures or salinity, while it is resistant to pollution and diseases (Angeler et al. 2001; Gherardi 2011a).

The burrows dug by *P. clarkii* are rarely constructed for permanent use, meaning that crayfish do not tend to return to previously dug holes. This burrowing activity of *P. clarkii* increases with the amount of fine sediments, causing extensive ecological damage to sandbank (Barbaresi et al. 2004), erosion of littoral zone sediments and reducing coastal protection from storms and sea level rise (Lodge et al. 2012). *P. clarkii* shows non-consumptive plant clipping and uprooting behaviour while choosing seedlings over older plants (Nyström & Strand 1996). Additionally, it is a selective feeder depending on the availability of food (Gherardi 2006). Young *P. clarkii* seem to prefer macro invertebrates to a herbivore or detrivore diet (Alcorlo et al. 2004; Banha & Anastácio 2011), but adult specimens are rather omnivorous (Correia 2005; Gherardi 2006) with organic plant detritus as the principal food source (Lorman & Magnuson 1978). Therefore, with establishment and increasing population density, *P. clarkii* is actively changing the water from a transparent to a turbid eutrophic state (Nyström et al 1996; Alcorlo et al. 2004) accompanied by a significant increase of dissolved inorganic nutrients, bioturbation and a general impoverishment of water quality (Angeler et al. 2001; Rodriguez et al. 2003). It becomes an integral part of the food web wherever *P. clarkii* establishes a population, posing a threat to human health when consumed due to its ability to survive in areas with contaminated water (Geiger et al. 2005) and accumulating higher concentrations of toxins in its organs (Tricarico et al. 2008). Due to its abundance, it became the main prey for Eurasian otter (*Lutra lutra*) in Spain (Adrian & Delibes 1987) and African clawless otter (*Aonyx capensis*) (Ogada et al. 2009). Toxic substances like arsenic or BMAA (ß-N-menthylamino-L-alanine) produced by cyanobacteria which are accumulated by crayfish can thereby enter the food chain and magnified along it (Geiger et al. 2005).

Amphibian populations are especially threatened. *P. clarkii* was found to prey upon larvae of up to 13 amphibian species (Cruz & Rebelo 2005), being more efficient than indigenous crayfish species in capturing larvae of e.g. the smooth newt (*Lissotriton vulgaris*) (Renai & Gherardi 2004). The presence of *P. clarkii* can therefore spoil valuable breeding ponds for several amphibian species (Nunes et al. 2010). Furthermore, the economic damage posed by this invasive crayfish cannot be underestimated. Crayfish caught in gillnets damage valuable fish (tilapia and largemouth bass) in quantities of nearly 30% of the catch and fish nets (De Moor 2002). Another major economic damages caused by *P. clarkii* is the destruction of valuable rice in china (Yue et al. 2010), Portugal (Anastácio et al. 2005) and Kenya (Rosenthal et al. 2005) causing economic losses exceeding millions of Euro.

It is questionable how other invasive species can facilitate or hinder the establishment of *P. clarkii* such as *Silurus glanis*. The presence of the invasive but naturalized mosquitofish *Gambusia holbrooki* or *P. clarkii* was found to facilitate the establishment of either one due to a mutual benefit (Anastácio et al. 2011). Finally, all exerted effects are strictly density dependent but even a small abundance of *P. clarkii* can reduce the biomass and species richness of macrophytes, invertebrates (Lodge et al. 1994) and rooted phyto-vegetation while increasing phytoplankton density (DeMoor 2002). Additionally, with increasing global climate, *P. clarkii* will most likely expand its range and spread even faster (Capinha et al. 2013). For these reasons, *P. clarkii* is listed among the 100 worst invasive species in Europe (DAISIE) and further spread needs to be contained whenever possible. Extrapolations of applied climate models showed that with rising global temperature, *P. clarkii*'s distribution in Europe would be favoured and the species would increase its invasive potency (Gherardi & Panov 2009) due to the ability to burrow and tolerate desiccation and low water quality

(Gherardi 2006). However, many aspects regarding interaction and threats posed by invasive species are still unknown (Reynolds 2011).

To overcome these gaps, we will try quantifiyng the economical, ecological and health-related impacts caused by *P. clarkii*. The burrowing activity will be particularly investigated in the laboratory to analyse the effect of burrowing activity on bank stability and permeability at different crayfish density and sex.

*Killer Shrimp (*Dikerogammarus villosus Sowinsky, 1894*)*

The killer shrimp, *Dikerogammarus villosus* (Figure 8.), is an amphipod indigenous to the Ponto-Caspian region that has recently invaded and spread throughout Western Europe (Tricarico et al. 2010). Killer shrimp inhabits fresh/brackish water, lakes, rivers, and canals in areas with low velocity currents. It can adapt to a wide variety of substrates as well as a wide range of temperature, salinity, and oxygen levels (Devin & Beisel 2006). Killer shrimps are omnivorous predators preying on many macroinvertebrates, including other gammarids, and are also able to collect detritus and to filter out suspended algae (Mayer et al. 2008). The name “killer” shrimp originates from its cannibalistic nature by occasionally eating nonspecific newborns and weak adults (Devin and Beisel 2006; Dick & Platvoet 2000; Dick et al. 2002; Platvoet et al. 2009). Moreover, killer shrimp have been observed to kill or injure potential prey without consuming it (Dick et al. 2002). Its populations have caused significant ecological disruption, including reduced biodiversity and local species extinctions. It started to spread in Western Europe in 1992 after the opening of the Danube-Main-Rhine canal (Germany) and reached UK in 2010. The arrival of killer shrimp in the UK may have implications for river water quality monitoring being the macroinvertebrate taxon, which contributes to biotic indices of water quality, possibly impacted by the invader (MacNeil et al. 2010).

Due to its ecological traits, killer shrimps have strong impacts on the indigenous macroinvertebrate communities. Killer shrimp predation affects many taxa that have a high score in the River Invertebrates Classification Tool (RICT). A reduction in the number of high scoring families and little or no effect on low scoring families will result in both lowering of the Average Score per Taxon (ASPT) and a lowering in the number of scoring taxa in water of high and good quality. Additionally, the presence of D. villosus within the macroinvertebrate community may affect the RICT, as D. villosus individuals would score as Gammaridae under the RICT. Emerging evidence is that D. villosus has the capacity to withstand greater environmental extremes compared with indigenous gammarids (Devin &

Beisel 2007). Furthermore, interactions between killer shrimp and indigenous gammarid species can result in displacement or local extinction of indigenous species, thereby reducing biodiversity (Dick & Platvoet 2000).

The socio-economic impact of this species on invaded areas of Western Europe is largely unknown. However, the ability of this species to consume eggs or juvenile stages of small fish creates a potential concern for fishery populations (Devin and Beisel 2006).

Figure 8. Photos of killer shrimp, taken by Jean-Françosi Cart (https://www.flickr.com/photos/jfcart/)

To actively improve scientific knowledge on the partially studied killer shrimp and zebra mussel interactions (Ricciardi et al. 1997; Gergs & Rothhaupt 2008a,b), three laboratory experiments are planned. We will investigate why killer shrimp move to zebra mussel substrate by testing the possibility of chemical attraction through habitat choice experiments in flowing water systems. Hence, this attraction of killer shrimp towards substrates with different zebra mussel densities, it will be of significant importance to test which density is favourable for killer shrimp settlements by applying different artificial setups. In the last laboratory experiment, we will investigate if and how the presence or the scent of killer shrimp can modify zebra mussel valves closure rate affecting the feeding activity. Regarding the ecology of killer shrimp, we will additionally test survivability of killer shrimp to environmental stress factors such as: **(1)** responses to anoxia/hypoxia, **(2)** desiccation and freezing resistance and **(3)** response to starvation and furthermore to test its potential ability to feed on eggs of economically valuable fish.

American Bullfrog (Lithobates catesbeianus Shaw, 1802*)*

It is the most commonly farmed amphibian (Garner et al. 2006) and therefore introduced worldwide as a food source in over 40 countries on four continents over the last century with at least 25 introductions in Europe (Figure 9.). It is capable of establishing dense populations in a short period due to its adaptable, prolific, competitive loud and predatory nature (Moyle et al. 1973; Wu et al. 2005; Ficetola et al. 2007). Additionally, post-metamorphic stages are capable of long-distance dispersal (> 1200 m) and females possess the ability to produce 1000 – 25.000 eggs. Once established, a population can reach densities of up to > 780 adults per hectare (Adams & Pearl 2007). As a result, the American bullfrog is listed among one of the 100 worst alien invasive species in Europe (Orchard 2011). Juvenile tadpoles readily become part of the food chain but have a comparably much higher survival rate than other amphibian species thanks to the much shorter time until metamorphosis (Cecil & Just 1979). Furthermore, this species shows the ability to digest bloom-forming algae, such as *Anabaena*, considerably affecting the nutrient cycle and primary production in freshwater ecosystems tackling the process of eutrophication (Pryor 2003). While research has focused mostly on juvenile interactions and the impact of the species on indigenous ranid frogs in terms of competition and predation (Kupferberg 1997, Lawler et al. 1999, Wang et al. 2007), the possibility of breeding interference interactions of adult specimens with indigenous species and the dispersal ability of female specimens has been mostly ignored (Pearl et al. 2005). Furthermore, the presence of *L. catesbeianus* can significantly increase indigenous species susceptibility of other predators (Kiesecker & Blaustein 1998) and decrease the abundance of all aquatic insects (Govindarajulu et al. 2006). Another interesting impact is the ability of adults to prey upon indigenous vertebrates and of its opportunistic ecological footprint (Jancowski & Orchard 2013). Many fish species avoid feeding on bullfrog tadpoles as being mostly unpalatable, passively increasing the feeding pressure on macroinvertebrates, possible predators for bullfrog larvae (Adams & Pearl 2007).

Infectious diseases are increasingly recognized as key threats to indigenous species (Kilpatrick et al. 2010). The American bullfrog is a known carrier of Chytrodiomycosis, causing *Batrachochytrium dendrobatidis* with implications of linked amphibian declines worldwide but being immune to most strains at the same time (Hanselmann et al. 2004; Gervasi et al. 2013; Eskew et al. 2015). At the same time, the spread of Chytrodiomycosis is facilitated by the trade value of ranid species in the US economy. Between 2000 and 2005, 28 million individuals were imported into the US, showing an overall infection prevalence of 62% and 8.5% by *B. dendrobatidis* (Schloegel et al. 2009). The advancing habitat loss

(Schloegel et al. 2009) combined with the rising threat of infectious diseases lead to 32.5% of amphibian species being threatened (Kilpatrick et al 2010). Especially those species that are critically endangered seem to suffer significant losses linked to *B. dendrobatidis* being the plausible reason for the extinction in 30 of the 113 species of the Harlequin toads *Atelopus* (Kilpatrick et al. 2010).

Being generally understudied, the assessment of the impacts caused by tadpoles and adult specimens of the American bullfrog on the native fauna will be performed. We will thus conduct gut content analyses to investigate the feeding habits in Italian ecosystems and the survivorship under different temperatures and in the presence of natural predators or already established AIS.

Figure 9. Picture of American bullfrog, taken by don.white55 [flickr]

Chinese Mitten Crab (Eriocheir sinensis Edwards, 1854*)*

The Chinese mitten crab, *Eriocheir sinensis* is a catadromous species indigenous to the Far East, with a native distribution from the Province of Fukien, China ~26 N° northwards to the Korea Peninsula ~40 N°, from where it spread throughout Europe and into North America (Clark et al. 1998). Throughout its life, the Chinese mitten crab occupies different ecosystems depending on its life stage (Veilleux & de Lafontaine 2007), moving from freshwater habitats where it spends its juvenile years to saltwater habitats in order to reproduce (Rudnick et al. 2000). Adult crabs are found in fresh, brackish and salt waters, but oviparous females are normally found in greatest number in saltwater while larval stages are found in the open water of bays and estuaries (Rudnick et al. 2003, Veilleux & de Lafontaine 2007). Chinese mitten crab first record in Europe dates back to 1912 in River Aller, Germany (Clark et al. 1998). From Germany, it has subsequently spread throughout northern Europe.

It was found in the UK in 1935 but became established in 1973 (Harold 1935, Clark et al. 1998). The study of *E. sinensis* spread in the UK suggests that when the population increases, exceeding the home range carrying capacity, the migration of partial populations to other upstream habitats becomes more probable. Now in the UK there is a concern that Chinese mitten crab will be spreading along the west coast and possibly further inland (Clark et al. 1998).

Occupying different ecosystems, the damages caused by its presence can affect rivers, estuarine and saltwater ecosystems (Rudnick et al. 2003, Veilleux & de Lafontaine 2007). The ecological impacts are represented by: (1) predation: due to its opportunistic omnivore nature consuming aquatic plants, algae, detritus and a variety of macroinvertebrates (Gollasch 1999; Rudnick et al. 2003) while also preying on fish eggs (Veilleux & de Lafontaine 2007); (2) competition: indigenous crayfish species could be negatively affected by very abundant crab populations due to common freshwater habitats and diets (Veldhuizen & Stanish 1999; Rudnick et al. 2000; Veilleux & de Lafontaine 2007); and (3) biomass transfer from freshwater to estuaries: adult crabs migration out of freshwater systems to reproduce and die in estuaries indicates may impact the food web (Rudnick & Resh 2005).

Chinese mitten crab's economic impacts involve mainly the fish industry, since they can be the cause of profit losses for commercial fisheries and aquaculture facilities, feeding on the fish trapped in nets or on the farmed ones. Another relevant effect of *E. sinensis* presence is the erosion of dikes and riverbanks caused by its burrowing activities (Gollasch 2006). After juvenile crabs having migrated into channels and dikes, they dig burrows that provide a refuge and protection from desiccation on the banks between the high and low tide lines. The significant amount of sediment removed in areas with high densities of burrows can cause weakening of the bank, accelerate erosion and even cause banks to collapse (Rudnick et al. 2000).

Our first objective is to quantify the impact of Chinese mitten crab on the bank assessing the erosion caused by this species in relation to its density and time of invasion to understand when a Chinese mitten crab population becomes dangerous for the safety of the structural integrity and ecology of riverbanks.

Topmouth Gudgeon (Pseudorasbora parva, Temminck & Schlegel, 1846*)*

The topmouth gudgeon is a small cyprinid species indigenous to Japan, China, Korea and the Amur river basin. Today, it is widespread and locally abundant within favourable habitats throughout much of Europe and Russia (Perdices & Doadrio 1992; Wildekamp et al. 1997). Its life history is characterized by early maturity (sexually mature with 1 year); batch spawning, nest guarding and broad environmental tolerance limits. These traits enabled topmouth gudgeons to succeed in invading new water bodies (Ricciardi & Rasmussen 1998), facilitating its rapid invasion in European water bodies (Gozlan et al. 2005). This species is indeed now considered the most invasive fish species in Europe (Gozlan et al. 2010).

Due to its ecological traits, topmouth gudgeons ecological impacts are yet visible after few years from its introduction. Britton et al. (2007) found that in a lake where the species was introduced in 2000, it rapidly established a breeding population that, by 2003, was the dominant species in size classes <70 mm and in 2004 it was the only species in the lake that produced young-of-the-year. *P. parva* can impede the recruitment of indigenous fish and become a potential threat for fisheries. *P. parva* can dominate fish communities and change the trophic web structure: a study comparing ponds with and without populations of *P. parva* shows how this invasive species overlaps with resident fishes resulted in depressed growth rates (and also production) and shifts in trophic position. For example, in the ponds with populations of *P. parva*, *Scardinius erythrophthalmus* has been observed to assimilate energy at a higher trophic level than in control ponds, probably by consuming *P. parva* (Britton et al. 2010).

A very important threat related to topmouth gudgeon is its potential ability to carry non-specific pathogens such as *Leucaspius delineatus* (Gozlan et al. 2005) and *Sphaerothecum destruens* that can cause diseases and mortalities in cultured North American Chinook salmon *Oncorhynchus tshawytscha* and Atlantic salmon *Salmo salar* (Andreou et al. 2011). For that reason, the spread of *P. parva* can lead to enormous damages in terms of indigenous biodiversity loss and economical costs for fish farming.

Rainbow Trout (Oncorhynchus mykiss, Walbaum, 1792*)*

Today, rainbow trout is the most widely introduced salmonid worldwide and one of the most widely introduced fish species in general (Crawford & Muir 2008). Since 1870, when the California Acclimatization Society conducted the first artificial propagation of rainbow trout from the San Francisco Bay area (Behnke 2002), this species has been introduced into at least 99 countries, with populations established in at least 53 of them (Gherardi 2010).

Rainbow trout (Figure 10.) introductions have strong impacts on indigenous salmonid species with problems related to red superimposition and competition for space and food (Scott & Irvine 2000; Seiler & Keeley 2009; Van Zwol et al. 2012) but also hybridization with conspecific species, leading to consequent loss of genetic diversity and integrity (Pearse et al. 2010; Simmons et al. 2010; Finger et al. 2011). Rainbow trout are also able to modify the indigenous macroinvertebrate community, changing invertebrate behaviour, drift and spatial distributions (McIntosh & Townsend 1994). They have also been observed to change the mean body size of macroinvertebrate populations through selective predation on the biggest specimens (Buria et al. 2007).

Due to its economic value as food resource and as valued game fish, rainbow trout are extensively farmed at the global level (Stankovic et al. 2015) and, as well as wild populations, trout farms can have a heavy impact on the surrounding wildlife. Trout raceway farms can modify the biodiversity in the outgoing stream, affecting the richness of *Ephemeroptera*, *Plecoptera* and *Trichoptera* by reducing the water quality (Loch et al. 1996). Trout cage farms tend to attract wild fish, changing also wild predator distribution (e.g. shags), since for them trout farms guarantee an easy access food resource that is constant during the year (Carss et al. 1993). The presence of wild predators often leads to conflicts with the fish industry because they are considered responsible of big economic loss and in the past years cormorants have become a highly politicised and unifying symbol of this problem (Buller 2008). The European Community is aware of the problem and in 2010 the European Commission has funded a Service Contract (No. 07-0307/2010/ 575579/SER/B3 Sustainable Management of Cormorant Populations, seehttp://ec.europa.eu/environment/nature/cormorants/Background-and-Activities.htm (last accessed January 2016) to **(1)** disseminate relevant information about cormorants through a Cormorant Platform, and **(2)** organise counts of cormorants in Europe during the breeding season and in winter.

Figure 10. Photo of rainbow trout, taken by Anja Stettin (www.flickr.com/nien0r/)

Risk assessment

Only approximately ten percent of alien species entering a country or region outside their native area are becoming highly invasive in marine and freshwater systems (Ricciardi & Kipp 2008). The damage they cause to ecosystems reflects on the economy, agriculture and human health. High impacts of most of the invaders are reported only in a minority of regions they invade, e.g. alien fish invaders in about 19% of the total regions (Ricciardi & Kipp 2008).

Risk assessments are tools made for the identification of species that are likely to become invasive and may cause significant negative impacts. In the last decades, they have gained much interest as an instrument to support policy makers in developing legislation, policy and management strategies (Reis et al. 2013).

Several European countries have developed their own national risk assessment protocols (e.g. Austria, Belgium, Germany, Ireland, Switzerland and the United Kingdom). There are furthermore numerous specialized protocols for certain taxons such as Weed Risk Assessment (WRA) that served as the basis for others: Freshwater Fish Invasiveness Scoring Kit (FISK), Freshwater Invertebrate Invasiveness Scoring Kit (FI-ISK), Marine Fish Invasiveness Screening Kit (MFISK), Marine Invertebrate Invasiveness Screening Kit (MI-ISK) and Amphibian Invasiveness Screening Kit (Amph-ISK). Today all of these, taxon-specific ones, have been replaced by a single risk identification tool, the Aquatic Species Invasiveness Screening Kit (AS-ISK), which is applicable to all aquatic plants and animals from any type of aquatic system (marine, brackish, freshwater) (available on: https://www.cefas.co.uk/).

The creation of a unique and transparent risk assessment tool like the AS-ISK is necessary but we will know more about its performance after the completion of a test phase. In accordance with this, we will see what our next steps within AQUAINVAD-ed project concerning risk assessment will be.

Marine invasive species

As economic activites (shipping) are increasing rising number of alien marine species are entering via ballast water and hull fouling (Hewitt & Campbell 2007). Voyage duration (local, regional or national shipping) needs to be taken into account when discussing the spread and overcoming of geographical barriers by potential invasive species. Secondary introductions (through recreational boating and shipping) exceed geographical limitation and contribute to marine invaders propagation. Although temperature and salinity play an

important role in the spread of alien marine species, it is not always possible to assume that these factors will limit their establishment and dispersal (Gollasch & Leppäkoski 2007). In addition, International Maritime Organization (IMO 2011) issued the Guidelines for the Control and Management of Biofouling to minimize invasive species transfer, while the International Convention on the Control and Management of Ship's Ballast water and Sediments (BWM Convention 2005) noted that monitoring of ballast water should be under scientific and technical jurisdiction (Article 6).

A difficult task is to make quantitative or qualitative estimations for potential harmful species, which is particularly evident with invasive alien marine species. Unlike freshwater and terrestrial, once established, marine invaders exceed geopolitical boundaries and cannot be managed without considering propagation on neighbouring states (Hewitt et al. 2009), thus risk assessments of marine invasive species (MIS) should begin on a local scale to detect areas (hot spots) of potential introductions (marinas, port areas). In addition, the local scale can further be unified into the next sub-regional or regional scale (Olenin et al. 2010). It means using existing and ongoing data on transport networks (shipping) and human disturbances of ecosystems as resources to provide more accurate assessments of invasion risks (Gallardo 2014).

In this project, during 2015-2018, seasonal sampling of *Codium fragile* ssp. *fragile*, *Sargassum muticum* and *Grateloupia imbricate* along Asturias, Galician and Basque coast will be actively performed. The samples from the coast of France (sub-regional scale of Bay of Biscay) and the UK will also be included in this study. Spatial and temporal distribution of seaweed is of great importance due to knowledge of environmental features of the invaded area.

Quarantine endpoints (preventing the introduction and spreading of alien marine species) and public education are probably the most effective methods for invasive seaweed risk assessment since seaweeds are mostly introduced unintentionally (DAISIE 2009). Prediction of a high/low risk of invasion can be a low-cost and beneficial approach. If the result cannot enable risk levels, then the risk tool is unsuccessful (Barry et al. 2008). The ballast water and hull-fouling risk assessments are a first important step in quarantine endpoints.

There are two major approaches to ballast water risk assessment (BWRA) (Barry et al. 2008):

1. species-specific risk assessment - estimation of MIS survival potential in introduced areas based on physiological traits; and
2. environmental similarity risk assessment - comparing physical conditions of the source and introduced area.

Ballast water risk assessment (BWRA) can be grouped in three categories (Clarke et al. 2003):

1. qualitative: simplest approach with „low“, „medium“ and „high“ risk category, based on subjective parameters are drawn from previous experience (e.g. Haugom et al. in Leppäkoski et al. 2002);
2. semi-quantitative: lowering the subjective risk perception (of individuals) by using quantitative data and hence cannot be considered as a qualitative approach; and
3. quantitative: analyzing the risk of BW (ballast water) introduction with combining physicochemical, biological, pathway data, environmental tolerance data for every alien marine species of risk and port environmental conditions.

Green sea fingers (*Codium fragile* subsp. *fragile*)

Australian BWRA is a modular quantitative system that emphasizes on the invasion process in four steps:

1. the probability that the donor port (native range) is infected by target species,
2. the probability that the vessel (vector) becomes infected,
3. the probability that the species survives the vessel’s journey,
4. the probability that the species can complete its life-cycle in the recipient port (introduced place).

Herein, the Coordinating Committee for Introduced Marine Pest Emergencies (CCIMPE) is in charge for initiating actions in response to any new or suspected new incursions of marine pests. According to Hayes et al. (2008), they divide species as:

1. target- harmful alien species that are known to be established in Australian waters, and
2. trigger- harmful alien species that have a ballast-mediated invasion history, can be managed by ballast water exchange and are either not established in Australian waters or are established but not widespread.

Green sea fingers is on a trigger species list (Hayes et al. 2008). Although it is mentioned as trigger species by CCIMPE, Australian pest marine monitoring states that it is a target species. This implicates that green sea finger distribution is probably less than previously thought or it is becoming widely distributed in Australia. Therefore, it needs to be monitored (DAFF 2009.). The Gulf of St. Lawrence (Canada) served as a case study for estimations of green sea finger establishments and spread. Using quantitative risk assessment tools, output data revealed that green sea finger is already well-established in the Gulf of St. Lawrence and is expected to considerably spread. As a result, the literature review and expert survey suggests that *C. fragile* will have a considerable effect on the ecosystem but less on economic and social sectors (Drouin & McKindsey 2007).

Although BWRA can be estimated prior to MIS introduction, hull fouling still presents a great risk of undetected invasion (Barry et al. 2008). Under IMO Guideline, management of ship fouling should have following characteristics: biological risk of being removed from the ship, factors that may influence biofouling accumulation, geographical area that was the source of the biofouling on the ship and toxic effects related to substances within the anti-fouling coating system that could be released during the cleaning action. Ashton et al. (2006) used a quantitative approach to determine the importance of recreational boating as a vector for distribution of marine alien species in ports of Scotland where green sea finger was described as one of the four alien species. He concluded that long stationary periods and reduced sailing activity increases the risk of macrofouling species (including seaweed) attaching to a hull. Recreational boating must be considered as a high-risk vector for alien marine species spread.

Japanese wireweed (Sargassum muticum)

Australian BWRA placed Japanese seaweed on the list of trigger species (Hayes et al. 2008) but it is a target species according to the Australian marine pest monitoring guideline (DAFF 2009). It is also one of the four alien species in ports of Scotland, along with green sea finger, and can be transported by long-time anchored recreational boats (Asthon et al. 2006).

Genetic variability and its approach to risk assessment in invasive seaweeds

Alien seaweeds exhibit "cryptic invasion" meaning that two or more morphologically inconspicuous species are genetically disparate and thus different species. The consequences of invasive on native species can be viewed from two points: the first is a change of the genetic structure (genetic-pool composition) in a population and the second change in the genome due to interbreeding of native and invasive species (Booth et al. 2007). The source (native range) and diversity of MIS need to be assessed for effective management strategies. Hence, this data can be provided from phylogeographic data and population genetic analyses accompanied with molecular markers (nuclear, mitochondrial DNA, chloroplast, microsatellite). Likewise, it is of great importance to distinguish cryptic species because it could lead to unserviceable risk assessments and misassumptions about ecological feature and consequences of invasive species (Geller et al. 2009). *Codium fragile* is an invasive species in Europe and morphologically the same as *C. tomentosum*, native to Europe. Only with the use of molecular genetic techniques these differences can be detected.

Unlike *C. fragile*, *Sargassum muticum* is not a cryptic species in Europe (Cheang et al. 2010). Using DNA barcoding, effective taxonomic molecular method enables the possibility to reduce the time usually needed to distinguish cryptic species in the lab. It is proved to be highly effective in brown (McDewitt & Saunders 2009), red (Saunders 2005) and green (Saunders & Kucera 2010) seaweed detection and has proven to be highly efficacious in distinguishing subspecies of the genus *Codium* (Woo & Sook 2015). Regardless of prevention efforts, invasive species do occur outside of their native range and it is important to detect them early and quickly (Kaiser & Burnett 2010). Instead of late intervention, the advantage of early detection is a cost-effective removal and a low risk of competition with native populations (Simberloff et al. 2013).

Due to mentioned difficulties in identifying species of the genus *Codium* by morphology, taxonomic distinction will be made in this project on a wide array of molecular markers. By collecting samples from various spatial scales during the first year, population genetic analyses and phylogeographic data on *C. fragile, S. muticum* and *G. imbricata* will be assessed for effective management tools in further prevention of MIS spreading.

Beside DNA barcoding, environmental DNA is a technique that allows detection of semi-aquatic and aquatic species, which release DNA into the environment through mucus, feces, urine and remains and can, therefore, be detected at low concentrations (Ficetola et al. 2008).

For example, in ballast water experiments with two invasive species, the zebra mussel (*D. polymorpha*) and quagga mussel (*D.buggensis*), in unmanipulated waters (harbor and ballast water samples) quagga was detected though not zebra. In manipulated waters (with small fragments of added tissue), both species were detected. Thus, in the future researche, environmental DNA could enable an early detection of AIS in the ballast water (Egan et al. 2015).

Why is modelling important in risk assessment?

Mathematical modelling is a tool that combines various data from field, laboratory experiments, genetic analysis along with natural history observation and therefore provides insight into the biological process (Wonham & Lewis 2009). Modelling can fill the gaps in the prediction of invasive species spatial distribution and furthermore define effective management and monitoring strategies (Williams & Grosholz 2008). Various environmental and morphological traits can play a role in the process of spread of invasive species: for example, larger and adult ripped fragments of green sea fingers generally have low dispersal potential but through fast water currents and storms they can be spread over a long-distance (Watanabe et al. 2009). Natural dispersion of warm-temperate green sea fingers along the East coast of Canada can reach a maximum of only 4 km y-1 but increasing temperature could lead to long-distance spread (Gagnon et al. 2014).

Modelling potential areas at risk of invasion by the red swamp crayfish in the northwest of Portugal proved the importance of using analyses at both local and regional/continental scales when estimating the invasion of an area since the current distribution is clearly the result of the interaction between regional and local variables (Moreira et al. 2014). For that reason, the inclusion of local variables refines the predictions at finer scales and helps a better identification where to concentrate conservation efforts (Moreira et al. 2014). The minimum temperature of the coldest month amongst climate factors and water chemistry (pH, alkalinity and the concentration of nitrate) amongst habitat factors showed as one of the most important predictors of the occurrence for serveral freshwater invaders (Gallardo 2014).

Species distribution models, matching donor and recipient environment conditions are playing a leading role in forecasting range expansions of introduced alien species and help to predict the effects of climate change on species distribution (Verbruggen et al. 2013). Both natural and human-mediated dispersions need to be evaluated along with existing data on climate change (increasing temperature) influencing the spread of AIS. The invasive moon jellyfish *Aurelia aurita* is assumed to show a high natural dispersal ability, but, based on genetic analysis and global biophysical model tools, it seems that it was affected by anthropogenic translocation (Dawson et al. 2005). Although there is a lack of information of human impact on the distribution of some invasive seaweeds (Gallardo 2014), Japanese kelp (*Unidaria pinnatifida*) is known to spread in human-disturbed areas, similar to *C. fragile* (in a shallow area of commercial ports). Application of risk modelling approach can overcome the unclear subjective expert opinions because of its objective nature, as showed by Acosta et al. (2010), who applied a modelling tool to assess the risk of invasion of hull fouling. He concluded that hull fouling on recreational boating represents the highest infection threat for the marine environment. As contrary opinion, Mineur et al. (2008) did a sampling at Mediterranean harbours and stated that hull fouling does not present a siginificant risk for new seaweed introduction, but it has to be considered that out of twenty-two sampled ships only one did not had antifouling coating.

Field and experimental (mesocosmos) studies will be performed on invasive and indigenous seaweed communities to assess how temperature variation (according to ongoing climate change) affects propagule pressure. Risk and possible mitigation measures of invasion processes will be assessed based on the results that we will obtain from our species distribution, ecological modelling, population genetics and mescocosmos studies.

Control

Control of freshwater invasive species

Here, we will overview current management strategies used to control invasive populations and discuss some potential future approaches that may be implemented for controlling invasive species. There have been several reviews addressing this, highlighting few broad categories for controlling invasive populations, and for the purpose of this report we will use the division to five basic categories: (1) mechanical; (2) physical; (3) biological, (4) biocides, and (5) autocidal methods (Gherardi et al. 2011).

(1) Mechanical methods are based on manual fishing techniques and include netting, trapping and electrofishing. Although they can effectively reduce the population densities in order to get some significant results, these methods should be conducted for an extended period of time leading to considerable costs and manpower.

(2) Physical methods are based on the temporary/permanent modification of habitats (for example drainage of ponds or sections of streams, diversion of rivers and construction of barriers) to remove non-indigenous species. This approach is considered impractical, costly and mostly ineffective (Freeman et al. 2010).

(3) Biological control methods can be broadly defined as the use of one living organism for the suppression of another (Molloy 1998). Biological control agents are natural enemies such as parasites, predators or pathogens. Successful biological control depends on the manipulation of natural enemies or on the introduction of novel ones to reduce the pest population density at a lower average than it would occur in their absence. Introducing novel alien species to a habitat as a biological control agent has led to several cases where the introduced species has become highly problematic itself (Freeman et al. 2010), so this option can be considered with extreme caution.

(4) Many effective maintenance management projects employ biocides alone or in concert with mechanical or physical methods. However, the expense for chemicals, especially when used for environmental purposes over large areas, is high (Gherardi et al. 2011). Concerns arise from the fact that there are no known biocides that are selective to crayfish, or even to crustaceans, so those with potential for use against crayfish are also toxic to other aquatic invertebrates and fish (Peay et al. 2006). Concerns are also coming from the possibility of their bioaccumulation and biomagnification in the food chain. Additionally, the evolution of resistance is frequent (Gherardi et al. 2011).

(5) Autocidal methods include the sterile male release technique (SMRT), use of and the use of sex pheromones. SMRT is based on capturing or rearing, sterilizing, and releasing large numbers of males into the wild to mate with females, which will then produce non-viable eggs. Although initially expensive, this technique causes no environmental contamination or impacts on non-target species. It is species-specific and offers the additional advantage that, at low density, sterile specimens may seek and mate with the remaining fertile individuals (Gherardi et al. 2011). Reduced competitivnes of treated males and their inability to mate in presence of wild males were highlithed as the highest risks of the method.

Use of sex pheromones, by releasing the large quantities of female sex pheromones in an area can confuse males and prevents them from finding mates, as well as pheromones may work as attractants in traps during the mating season. Once males are removed from the population, less mating might take place and a quick reduction in the size of the population is achieved (Gherardi et al. 2011). The use of sex pheromones has the advantages of having no adverse effects on both the environment and human health, being at the same time relatively species-specific (Aquiloni & Gherardi 2010).

Stebbing et al. (2014) investigated a new, alternative method of achieving functional sterilization of crayfish, which is the manual removal of the modified first ad second pleopods in males. These pairs of pleopods are used for transfering spermatophores to female during copulation. The remaining pleopods in males have no obvious function, and in females pleopods are used to carry eggs. Again, reduced competitivnes of treated males and their inability to mate in presence of wild males are the highest risks for the method success.

After reviewing all control methods, it is clear that there is no simple or unique solution for any habitat type or species population (Freeman et al. 2010). Integrated pest management (IPM) using a range of control techniques to suit specific sites would probably yield the best results (Gherardi et al. 2011). Future research on control methods by the AQUAINVAD-ED group will help in the development of novel approaches and successful management of invasive species.

Louisiana red swamp crayfish (*Procambarus clarki*)

Freshwater crayfish species are large opportunistic omnivores, and in some parts of Europe, they commonly dominate the benthic biomass. In response to their size, population density, and polytrophic links, these invasive crayfish become an important component of freshwater ecosystems (Bubb et al. 2004) and their foodwebs, and therefore, their introduction same as their elimination can have a substantial impact on the aquatic environment (changes in fish populations, losses in biodiversity).

Plenty of research was undertaken in the last couple of decades to determine the feasibility of eradicating established non-native crayfish populations from the wild, where they threaten notable habitats or important populations of native species, and over recent years, those technologies improved dramatically resulting in 86% succes in 1000 attempts of eradictions (Stebbing et al 2014).

The case of Lake Casette, where an integrated trapping-SMRT approach was used in LIFE RARITY project (Aquiloni & Zanetti 2014), showed a reduction in *P. clarkii* population at 87% in just 2 years of activity, being a great example of successful control with no obivious effect on local habitats and communities.

Mechanical methods are time-consuming and require a dedicated effort, and, in the absence of continuous catchings after some period, populations are returning to their former levels within a couple of breeding seasons. Also trapping of ovigerous big females might lead to some feedback mechanisms and crayfish may respond to low numbers in the population by reaching maturity earlier and producing more eggs (Gherardi et al. 2011). Depending on location, some of the mechanical methods proved to be more or less suitable (seine and fyke nets are efficacious in lentic waters, same as electrofishing in shallow and clear waters) and others demonstrated to be more or less effective depending on size or gender of individuals (drag netting is showing good results on small crayfish, i.e. individuals with less than 35 mm carapace length; seine and fyke nets displayed better results than trapping on juveniles and berried females; electrofishing removes crayfish of all ages and yields a more balanced sex ratio compared with trapping) (Freeman et al. 2010). Also combination of different mechanical tools with different mesh sizes is bringing better success in catching wider size ranges, as it was showed by Roqueplo et al. (1995), who used standard cylindrical traps and traps shaped like a tambourine to trap a population of *P. clarkii* from a pond, finding also that the color of the trap is important -black catching more crayfish than white. All these considerations must be taken into account when selecting methods for a certain location.

Some physical methods, for instance, drainage of the pond, cannot be an effective physical method with burrowing species, such as *P. clarkii*, which can survive out of water for long periods (Gherardi et al. 2011). But there still may be opportunities to attempt crayfish control using some physical methods, although they will remain site-specific (Freeman et al. 2010). Kerby et al. (2005) showed that in California large barriers (e.g., waterfalls and culverts) may succeed: a mark-recapture study indicated that *P. clarkii* moved both up and downstream between pools and barriers significantly reduced crayfish population.

Crayfish are susceptible to various microbial pathogens and parasites (Gherardi et al. 2011). Freeman et al. (2010) discussed the potential extensive use of biological control: viruses (the intranuclear bacilliform viruses, IBVs and the white spot syndrome virus, WSSV), bacteria, fungi (*Fusarium spp*. and the burn spot disease), oomycetes (the crayfish plague, *Aphanomyces*; *Saprolegnia* and *Achyla*), microsporidia (including *Thelohania contejeani*, *Vavraia parastacida*), Rickettsia- like organisms, and the Ichrhyosporea *Psorospermium spp*., infecting crayfish as biocontrol agents. The main problem in using most of them lies in the common lack of host-specificity. There is thus the risk that microbes and other parasites will spread to non-target organisms, including native crayfish species (Gherardi et al. 2011). The most famous crayfish parasite, mentioned above, is the oomycete *A. astaci*, the etiological agent of the most devastating disease known as crayfish plague that highly affects the European, Asian and Australian crayfish while North American species of Cambaridae family are much more resistant to infection. It was introduced into Europe from North America in 1860 and since then it devastated many native populations of crayfish in Europe, leading to the disappearance of 90% of the native *A. astacus* in Sweden (Gherardi et al. 2011).

Various fish and mammals prey on crayfish, such as otters (*Lutra lutra*), mink (*Mustela vison*), grayling (*Thymallus thymallus*), pike (*Esox lucius*), perch (*Perca fluviatilis*), Atlantic salmon (*Salmo salar*), brown trout (*Salmo trutta*) and European eel (*Anguilla anguilla*), and thus, in theory, they could be used for the biocontrol of crayfish. Fürst (1977) suggested that predation by eels and perch were the most important factors in limiting the establishment of newly introduced crayfish in 44 Swedish waters. Although fish predation has an impact on crayfish populations, conflicting opinions exist over the efficacy of predatory fish in controlling crayfish populations (Freeman et al. 2010). Sublethal effects of fish predation in the long-term may reduce crayfish growth, reproduction, and survival (Holdich et al. 1999). European eel introduced into the Rumensee in Switzerland has reduced an expanding *P. clarkii* population to less than 10% within 3 years, whereas pike, introduced at the same time, had no effect (Gherardi et al. 2011). The suitability of fish species as biological control agents will significantly vary depending on the habitat type and water body conditions in the target area (Freeman et al. 2010). Aquiloni et al. (2010) run several inclusion/exclusion experiments to investigate the impact of European eel on an invasive population of *P. clarkii* in an Italian wetland. It was shown that, even with limitations such as its low consumption rate and its predation beeing gap-size limited, *A. anguilla* is a good candidate for mitigating the damage produced by *P. clarkii* in Italy, since it has a benthonic feeding habit and is able to tolerate partially deoxygenated waters which are properties that match the lifestyle of crayfish and its typically occupied habitats. Moreover, they are able to detect crayfish by odor and could enter crayfish burrows (Gherardi et al. 2011).

Biocides such as organophosphate, organochlorine, and pyrethroid insecticides, rotenone, and surfactants, have also been used to control invasive crayfish. Since no biocides are selective to crayfish, the focus has been given to chemicals that are not persistent in the environment but are readily available and relatively inexpensive (Gherardi et al. 2011). Natural pyrethrum is the oldest known and widely used insecticide; it is primarily produced from the flowers of *Chrysanthemum cinerariaefolium* and *C. cineum* (Gherardi et al. 2011). Main advantages are low toxicity to mammals and birds, a rapid breakdown in sunlight, the absence of toxic residues, and its harmlessness to plants. The essential disadvantage is that is, however, toxic to other crustaceans, insects, and fishes (Peay et al. 2006). Biocides that were also tested in the field for use in control are including synthetic pyrethroid BETAMAX VET and organophosphate insecticides Baytex PM40. There are many other biocides whose potential efficacy in invasive crayfish control has still to be evaluated, as well as its possible impacts on non-target species (Freeman et al. 2010).

Autocidal methods used in crayfish include the sterile male release technique (SMRT) and the use of sex pheromones. Aquiloni et al. (2009) tested the potential effectiveness of ionising radiation with irradiation dosage of 20 Gy. This dosage did not alter neither the survival or mating ability of *P. clarkii* males nor significantly affected their reproductive success by reducing (by 43%) the number of hatchlings. SMRT showed perspective for control of *P. clarkii* populations, especially because it is less demanding in terms of cost and management (sterilization and release can take place on the same day). Sex pheromones are widely used to control insect pests (El-Sayed et al. 2006), but by now the use of pheromones has not been successfully applied to control crustacean decapod invaders (Aquiloni & Gherardi 2010) since to date the chemical nature of sex pheromones in crayfish is still unknown (Gherardi et al. 2011).

Preliminary trials of the manual removal of the first and second pleopods in males of signal crayfish (*Pacifastacus leniusculus*) have shown that technique could be almost 100% successful, with no impact on the males' competitivnes or in finding a mate, while signifficantly impairing spermatophore deposition (Stebbing et al. 2014). As a part of our research, we are planing to test this method also on males of red swamp crayfish.

Seeing great potential in biocontrol with the natural predators (such as the European eel, *A. anguilla*), and in Sterile Male Release Technique (SMRT) and in the manual removal of the pleopods, these are the techniques that we will be primarily oriented in the control of *P. clarkii* populations, but, depending on the situation at the selected locations, we will see which method or combination of methods will be applicable.

American bullfrog (Lithobates catesbeianus)

Even if the American bullfrog has been listed among the 100 worst alien invasive species, there have been only a few technological advances in the effective control and management of this invasive species in the last years (Orchard et al. 2011). While the eradication of isolated population is generally not cost intensive, only a few successful examples of eradications in greater wetland areas have been documented but proved to be laborious and expensive (Adams & Pearl 2007; Kraus 2009). The main approaches for bullfrog eradications have been the manual capture "electro frogger" technique for adult specimens, which can prevent reproduction if carried out in the first weeks of the year (Orchard et al. 2011) or the complete drying out of aquatic habitats.

The usage of biocontrol by artificially increasing the abundance of native predators which prey on bullfrog tadpoles has been proven as unproductive based on this species unpalatability and this species high dispersal capability. The application of pesticides to tackle bullfrog invasions is questionable and, although pesticides exist, the side effects on the amphibian population would need further investigations.

Zebra Mussel (Dreissena polymorpha)

Nowadays many strategies have been adopted for the eradication of zebra mussel but with poor results. The large majority of chemicals known to kill zebra mussels also kill other types of organisms (e.g. mussels, fish) in the surrounding environment (Cope et al. 1993). The most commonly used methods are chlorine treatments, which are effective but not selective and they can also have a considerable impact on surrounding wildlife (Trincoll Journal 1997), while manual removal with high-pressure washing is probably the safer method, but very expensive in terms of human and monetary resources (U.S. Department of the Interior, U.S. Geological Survey, 2005). Other methods including thermal changes, acoustical vibration, desiccation, ultraviolet light, and electrical current filters have been used without notable results (U.S. Department of the interior, U.S. Geological Survey, 2005).

In 2013, a new chemical has been tested, a bio-pesticide called Zequanox®. Since Zequanox® is derived from the dead cells of a naturally occurring microbe, zebra mussels sense it as non-threatening and do not close their bivalves as they do when other chemicals like chlorine are used. Once ingested, Zequanox deteriorates a mussel's digestive lining, causing death in about 30 days. Different studies demonstrated the effectiveness of this chemical and its apparent selectivity that makes it a good eradication method without negative impacts on non-target species (Meehana et al. 2014; Whitledge et al. 2015).

To have a better understanding of zebra mussel colonisation mechanism, field studies will analyze the seasonal linked relations in zebra mussel colonisation with a 1-year study aiming to describe in which period of the year (1) new colonisations are more probable and consequently (2) if more attention is needed in the activities that could facilitate zebra mussel spread (e.g. boats transfer). We will firstly analyse zebra mussel colonisation preferences in relation to substrate type (Marsden & Lansky 2000), secondly if zebra mussel colonisation shows preferences in relation to substrate colour and thirdly if colonisation preferences in relation to substrate depth exist.

Killer shrimp (Dikerogammarus villosus)

While no prevention mechanism exists for intracontinental dispersion, mandatory ballast control and ballast filtration systems are being implemented to prevent further transcontinental dispersion. Shoreline treatment plants for ballast water are also being considered, although this could be a costly option (Crosier et al. 2011). Effective chemical methods are neither known, but it has been suggested that killer shrimp can be killed by oxidizing biocides (Abdel-Fattah 2011).

Chinese mitten crab (Eriocheir sinensis)

The control of Chinese mitten crab has been a problem since its first discovery in Europe (Germany) in 1912. It is indeed difficult because of its abundance, ubiquity, high reproductive rate and a wide range of physiological tolerances (Rudnick et al. 2003). It seems that eradication programs are unsuccessful once the crab has established self-sustaining populations. Up to now, no effective management approach has been developed and all eradication efforts have shown limited efficiency (Gollasch 2006). Physical control by crabs trapping has not been found effective in reducing the damage caused to riverbanks and the feeding on trapped fish. Even the methods to minimise the future spread of the mitten crab are quite limited (Gollasch, 2006).

Since it seems to be impossible to eradicate it, some ways to take advantage of these big amounts of crabs have been found. In the past, Chinese mitten crabs have been used as bait for eel fishing, to produce fish meal, cosmetic products and for human consumption (Panning & Peters 1932; Peters et al. 1936; Leppäkoski 1991). Furthermore, since it is edible and suitable for human consumption, zoologists at the Natural History Museum of London have suggested that commercial fishermen should exploti this species and export it to China where it is considered a delicacy (Owen 2003)

Topmouth Gudgeon (Pseudorasbora parva)

Topmouth gudgeon eradication activities in UK started in 2004 and, now, a 5-year project with the aim to totally eradicate topmouth gudgeons from the UK by 2017 is ongoing (Environment agency 2014). The eradication is conducted with piscicide that could potentially threaten non-target species. This process of eradication could be considered as an indirect impact on indigenous species by topmouth gudgeons. For this reason, monitoring activities are planned before and after the eradication to study the effects of the eradication on non-target species and the recovering time of the environment.

Control of marine invasive species

Depending on the type of introduction (unintentional or intentional), different management actions need to be taken into account. In contrast to a manageable control measures in defining impact and removal of invaders in terrestrial and freshwater ecosystems, the control of IMS is based on pre-border principle: knowledge on vector/pathway introduction and likelihood of invasion coupled with IMS impact. Since data on impact of IMS are generally deficient, it is important to implement the rest of previously mentioned pre-border steps (Ojaveer et al. 2015). The most introduction of MIS is unintentional and its of great relevance to ensure their early detection, as was in a case of marine green alga *Caulerpa taxifolia*. Early identification and quick respond (including diver crew mobilization and alga chlorine treatment) started 17 day after the discovery of invasive *Caulerpa taxifolia* in one California Lagoon. It resulted in successful eradication components of *C. taxifolia* on a local scale based on knowledge in biology and ecology of the invader, characteristics of invaded field (biological, physical and sociological) and implementation of immediate action by field experts (Anderson 2005).

Molecular approach is also valuable source of information for early detection and pathway reconstruction of IMS history. However, it needs to be highlighted that although the application of genetic technology in invasion biology is improving at high rate, there is a need to feel the gap in communication steps between genetic scientists and users of genetic information (national and local authorities) (Darling 2014).

Including and raising public awareness about the invasive seaweed occurrence is important and aided by sailors, fishers and divers (Meinesz 2007). For example, the U.S. Environmental Protection Agency and the Hawai'i Department of Land and Natural Resources made an easy education tool (waterproof cards for boaters, divers and fishermen) for Hawai (available http://www3.epa.gov/region9/water/oce/seaweed/alien.html). Meinesz (2007) noted that around 2000 pressed articles related *C. taxifolia* invasion, which have been published by media, are obtained due to informed public. Also, testing of ballast water treatment methodologies and ballast water sampling may require co-operation with other stakeholders (involved in industry) (Ojaveer et al. 2015)

Convention on Biology Diversity (CBD 1992) states that prevention measure are the most cost-effective strategy for alien species management. In European strategy on invasive alien species, under section *Unintentional introductions* key actions are given for common practice: risk analyses of pathways and vectors, sectoral activities carrying out EIA (Environmental Impact Assessment) and SEA (Strategy Environmental Assessment), minimizing identified risk and the introduction of alien species due to water translocation (Genovesi & Shine 2004).

As previously mentioned, hull fouling and ballast water present one of the main vectors for AIS spreading. Thus, there are some implemented measure for their management. The coating of hull fouling can be used in one of two ways (GISP 2008):

1. The coating has biocide or non-toxic compounds which drain from the coating gradually and preventing the settlement of AIS

2. The metallic-coating that prevents attachment of AIS

Widely used effective biocide tributyltin (TBT) (GISP 2008) was banned by IMO (1990) due to its wide studied negative impact (highly toxic) on biodiversity, which in term probably increase the bioinvasion risk associated with hull fouling (Hewitt et al. 2009).

Treatment of ballast water include heat treatment, chemical treatment, ultrafiltration, ultraviolet light, etc. This treatment are used in thousands of tonnes of ballast water procedure although they have drawbacks (Bax et al. 2013). The most common treatment method in use is ballast water exchange, used by several countries to reduce the risk of alien species introductions from ballast tanks to coastal water (Casas-Monroy 2014). It is hoped that pumping 3 times the volume of ballast tank through the tank will lead to 95% exchange of the original ballast water. As a result ballast water will be replaced with oceanic ballast that poses little menace of AIS spread to coastal ecosystems (Bax et al. 2013). Risk reduction is variable for marine coastal taxa, unlike the introduction of ocean water into tanks with great success for reducing survival rates of freshwater taxa (Bailey et al. 2011).

Green sea fingers (Codium fragile subsp. *fragile*)

In the study of biological control on invasive species, Scheibling et al. (2008) noted the snail *Littorina litorea* that feeds on basal parts of green sea finger without, affecting its propagation. Control with sea urchins also turned into the favor of green sea finger: in large density conditons, sea urchins will feed on native kelp and turf algae. Once the native kelp and turf algae are overgrazed, sea urchin may consume green sea finger (Sumi & Scheibling 2005). Chemical herbicides could harm the environment and manual removal did not seem effective because *C. fragile* reproduces from fragments. The population density can be reduced just temporarily, but that is an expensive method and the population is quickly renewed. There is no other attempt of *C. fragile* removal since physiological traits would most probably make it ineffective (GISD 2014).

Japanese wireweed (Sargassum muticum)

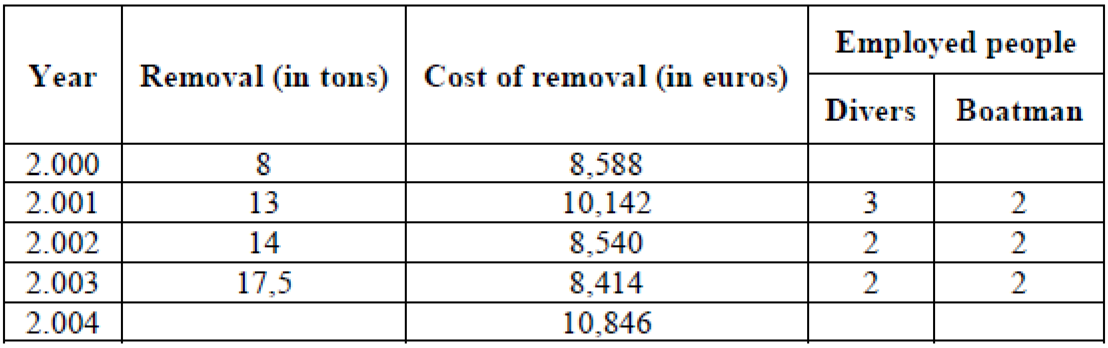
In eradication measures for *S. muticum,* putty knives were used to scrap a holdfast, but in nine months the population was again fully recovered (Smith 2015).

Critchley et al. (1986) state that in the absence of long-term chemical/biological control, mechanical control (harvesting and cutting) is proposed as mitigation measure. Mechanical removal technique of *Sargassum muticum* took place at the commercial beach in city Gijon (Asturias Region, Spain) (Figure 11.). The sweeping started in summer, during the reproductive cycle of the invader.

Figure 11. Picture of commercial beach in Gijon (by Rico, J. M.)

Opposed to expected results, manual removing caused even further spreading of embryo-carried thalli and the population of S. muticum was quickly renewed. The effort put in this removal is shown in Table 1. Divers and boatman were involved in this action, being able to recogniz S. muticum. It was recommended by experts that eradications should begin before reproduction because of the small chances of embryo spread (personal communication, Rico, J. M.).

Table 1. Effort put in removal of invasive *S. muticum*



Year	Removal (in tons)	Cost of removal (in euros)	Employed people	
			Divers	Boatman
2.000	8	8,588		
2.001	13	10,142	3	2
2.002	14	8,540	2	2
2.003	17,5	8,414	2	2
2.004		10,846		

Conclusion

The necessity for species-specific assessment studies that show technical and financial feasibility is the first step in any eradication project. For most attempts to eradicate an invasive species, the lack of species-specific eradication techniques have shown to induce significant problems (e.g. in fish: Scalera & Zaghi 2004). Moreover, prevailing studies on the impact of invasive species are short-term research. For example, 88% of the 85 quantitative studies analyzed by Scalici & Gherardi (2007) monitoring the impacts of NICS had a duration of one year or less. There is an urgent need for long-term studies, especially in multiple invaded areas with complex interactions among indigenous and non-indigenous species as a strong foundation for any further management or control measure.

Previous studies have shown an unequal distribution of scientific attention mostly after a species had already become invasive. Risk assessment and control measures for general pathways and vectors could have prevented economic and ecological damage in a variety of environments before AIS establishment. Therefore, it is necessary to 1) develop modelling tools for a broad spectrum of species to assess potential risks in the beginning of an invasion or even before; 2) quantify the impacts made by AIS to 3) better understand the effects AIS have on ecological, socio-economical and health aspects, and 4) develop existing control methods and novel approaches to provide successful future management of invasive species.

Risk assessment and modelling of potential distribution of non-indigenous species is crucial to define those likely to cause harm in some areas, and then to have the possibility to better direct monitoring programs, early detection and control efforts on prioritized areas

For that reason, partners and fellows of AQUAINVAD-ED will perform a variety of studies, focusing on impacts, risk assessments and control measures for high profile AIS, to fill current gaps in our knowledge of non-indigenous species and further fill general gaps of understudied aspects, providing useful info and developing efficacious management tecniques in accordance with the EU Regulation 1143/2014.

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7.6. Aquainvad-ED Deliverable 3.3. Risk assessmet of AIS: development of AS-ISK

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Summary

Decision-support tools are widely supporting decision-making processes in business, social sciences, medicine, politics, games, information technologies, and transport, and they are major building blocks in environmental management and science today. Decision-support tools are central to the risk analysis of alien species, assisting in the identification (screening) and assessment of risks associated with alien species as well as providing support to decision makers involved in their management (David et al. 2012). The screening of potentially invasive alien species aims at identifying which species are likely or not to be invasive.

Risk assessment is crucial for supporting many legislative tools, for example EU Regulation 1143/2014 on invasive alien species, and decision-making including prevention (to inform legislation and justify restrictions, such as on trade and/or consumer activities), early detection (warning) and rapid response (prioritizing action and guiding surveillance) and long-term control (prioritizing species for control and monitoring) (Beninde et al. 2015; Genovesi et al. 2015; Tollington et al. 2015). Additionally, risk assessments are required to justify measures that may affect trade without raising any conflict with the World Trade Organisation (WTO) (Shine et al. 2010) and for communicating with other sectors (e.g. conservation management).

RA can have a double function: prioritize alien invasive species already present in an area and predict the potential new invasive species for a certain area. RA protocols are based on the generally accepted premise that species invasive in some parts of the world have an increased chance of being invasive in other areas with similar environmental conditions. RA generally assesses and scores several elements such as the biogeography and history of the species, the presence of ‘undesirable traits’, species biology and ecology, climate match, presence of potential predators and control methods.

Many RA protocols have been developed. A recent study by Roy et al. (2018) reviewed the existing risk assessment protocols, and, with reference to the requirements of the EU Regulation on IAS (1143/2014) and international agreements including the World Trade Organisation, Convention on Biological Diversity and International Plant Protection Convention, coupled with consensus methods, identified and agreed upon 14 minimum standards (attributes) a risk assessment scheme should include. The agreed minimum standards were: 1. Basic species description; 2. Likelihood of invasion; 3. Distribution, spread and impacts; 4. Assessment of introduction pathways; 5. Assessment of impacts on biodiversity and ecosystems; 6. Assessment of impact on ecosystem services; 7. Assessment of socio-economic impacts; 8. Consideration of status (threatened or protected) of species or habitat under threat; 9. Assessment of effects of future climate change; 10. Completion possible even when there is a lack of information; 11. Documents information sources; 12. Provides a summary in a consistent and interpretable form; 13. Includes uncertainty; 14. Includes quality assurance. In deriving these minimum standards, gaps in knowledge required for completing risk assessments and the scope of existing risk assessment protocols were revealed, most notably in relation to assessing benefits, socio-economic impacts and impacts on ecosystem services but also inclusion of consideration of climate change.

Explanation of the work carried out and overview of the progress

At the beginning of Aquainvad-ED project Copp et al. (2016) developed AS-ISK (Aquatic Species Invasiveness Screening Kit), a decision-support tool for aquatic species, developed for all aquatic taxa. Elena Tricarico helped testing this new tool. It replaces former decision support tools separated per taxon, developed and adapted from Pheloung et al.'s Australian Weed Risk Assessment (WRA). AS-ISK comprises 49 basic questions on the taxon's biogeographical and historical traits and its biological and ecological interactions. It includes a preamble of general background information on the species as well as questions on its documented potential socio-economic impacts and those on ecosystem services in all the introduced range (questions 1-3; 9-14; 22-25; 28; 37-39; 44-48), a session focused on the RA area, and an additional section (six questions; questions 50-55) for the assessor to predict how forecasted changes in climate are likely to influence the risks of introduction, establishment, dispersal and impact of the species in the RA area. AS-ISK provides a quantitative output and is compliant with the "minimum requirements" (Roy et al. 2014, 2018) for assessing species, filling the gaps identified by Roy et al. (2018). It indeed provides a final score and outcome for the species based on the answers. Answers are justified by documented data from the literature and are weighed according to confidence level of the assessor based on the available data (low, medium, high, very high). When data are not available or anecdotal, the assessor can express the personal opinion, scoring low the confidence level of the answer. The full protocol is available here: https://www.cefas.co.uk/services/research-advice-and-consultancy/invasive-and-non-native-species/decision-support-tools-for-the-identification-and-management-of-invasive-non-native-aquatic-species/.

During the project, EU Commission financed a study (still ongoing, Elena Tricarico is part of the team) for the development of risk assessments to tackle priority species and enhance prevention (https://publications.europa.eu/en/publication-detail/-/publication/c01568d9-025e-11e8-b8f5-01aa75ed71a1/language-en). Risk assessments and associated management annexes were produced for ten selected species alongside tasks to develop and maintain a risk assessment template and to collect evidence on management techniques, implementation costs and cost-effectiveness. Among the species, there were assessed some aquatic already invasive/potentially invasive aliens present/absent in Europe: *Chrysemys picta* (painted turtle), *Gambusia affinis* (western mosquitofish) and *G. holbrooki* (eastern mosquitofish), *Limnoperna fortunei* (golden mussel) and *Orconectes rusticus* (rusty crayfish). EU risk assessment template is a modified version of the GB scheme and includes socio-economic impacts, impacts on ecosystem services and climate change, thus addressing the gaps found by Roy et al. (2018). The full version of the template is available at the webste of non-native species in UK: http://www.nonnativespecies.org/index.cfm?pageid=143.

Conclusions

Considering the recent advances, AS-ISK and the template approved by EU study are recommended as protocols to be used to perform a risk assessment for aquatic alien species.

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7.7. Methods for prediction of areas at risk of invasion

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Summary

The ability to predict the spatial patterns of natural phenomena, such as hazards (Brenning 2005), patterns of fires (Catry et al. 2010) or species distribution (Franklin 2010), constitutes an important tool for managers, helping to improve the effectiveness of prevention and detection, and facilitating resources allocation. Effective mangement is, indeed, required to prevent the impact caused by invasive alien species. Modeling the risk allows to identify areas that are more likely to be invaded, providing important outputs for an effective approach to developing a more focussed management strategy (Liu & Li 2009). Applying appropriate management action will have benefits on the biodiversity and the economy of these areas, considering the heavy impacts caused by invasive alien species on native biota as well as on ecosystems services and goods (Bradshaw et al. 2016; Vilà et al. 2010).

Species Distribution Models (SDMs), also known as suitable habitat models, niche models and bioclimatic envelope models, have been used to predict the potential spread of invasive alien species in new regions, under current and future climates. They are strongly recommended to guide conservation decision making (CDM) towards invasive alien species as prevention of their establishment and further spread being favored over eradication and control that may be required in the later stages of invasion (Barbet-Massin et al. 2018). In the last three decades, interest in using species distribution models has esponentially grown dramatically (Guisan & Thuiller 2005). Even though species–environment relationship analysis has always been a central issue in ecology (Guisan & Zimmermann 2000), the earliest example of using correlations between the distribution of species and environmental variables has been provided by Johnston (1924), predicting the potential spread of the invasive alien cactus species in Australia (Guisan & Thuiller 2005). The main idea behind

SDMs is that it is possible to relate and quantify environmental factors to species geographic distribution using statistical functions or algorithms to describe a species' environmental niche (Hallstan 2011).

Climate change and invasive alien species are two primary drivers of global environmental change (Rahel & Olden 2008), and their future complex interaction is not easy to predict. The impact of climate change and rising average global temperatures can have a severe influence on species' geographical ranges that are often set primarily by climate and consequently the host environment (Szyniszewska 2007). Climate change will interact with other existing stressors and affect the distribution, spread, abundance, and impact of invasive alien species (Hellmann et al. 2008). Moreover, climate change could favor and convert alien species considered benign today into the noxious pests of tomorrow (Runyon et al. 2012). Projections of the impacts of future climate change on species distribution are of major importance for biological invasions, especially given the increasing urgency to inform management authorities (Barbet-Massin et al. 2018). However, these techniques can live up to the challenge only when carefully tailored with mutual cooperation and quality information exchange between modelers and decision makers (Guisan et al. 2013).

Even though successful examples of good practice with using SDMs in CDM exist, they have been mostly hidden in the grey literature and thereby less accessible for learning (Guisan et al. 2013), making proper validation studies with focus on predictive accuracy difficult and rare to be found (Barbet-Massin et al. 2018). Two core assumptions of SDMs are often violated when modeling invasive alien species. First, assumption of niche conservatism over space and time is not always met, as the naturalized climatic niches can differ from their native climatic niches. Second, invasive alien species are not in equilibrium with their environment, so their climatic niche might be underestimated (Barbet-Massin et al. 2018; Gallien et al. 2012). Assumption that the modelled invasive species is in equilibrium or pseudo-equilibrium with its environment (Guisan & Theurillat 2000) is sometimes incorrect, because non-equilibrium concept is more realistic in ecology (Guisan & Zimmermann 2000).

To reach the equilibrium, invasive alien species should have been already colonized all suitable places and be absent from all unsuitable sites (Guisan & Thuiller 2005), thus representing a challenge when using distributional data (Gallien et al. 2012). Violating the equilibrium hypothesis when modeling species distributions can cause an underestimation of the potential climatic niche of the species, which can in turn lead to underestimation of the geographical area the species can invade (Barbet-Massin et al. 2018). However, SDMs of invasive species often violates basic SDMs assumptions (Elith et al. 2010).

The range of ecological conditions within which a species can maintain populations can be defined as its niche (Jiménez-Valverde et al. 2011). In the context of biological invasions, it is important to specify that there are two possible views of an invasive species' niche (Gallien et al. 2010). Fundamental niches represent all the environmental conditions where a species can live (i.e. the sum of ecological situations where populations of an organism can have a growth; Broennimann et al. 2007), while the realized niche is where the species actually lives (i.e. the fundamental niche constrained by biotic interactions; Broennimann et al. 2007).

To model the ecological niches of the focal species in the studied region, modelers use all occurrence data available throughout the world (for both native and invasive ranges) or data from the native or invaded range only. Occurrence records of both native and invaded regions should be considered to estimate species' global niche with full-range biotic and abiotic niche requirements (Gallien et al. 2012), while for estimation of specific potential regional niche only data from the invaded range can be use to account for the specificities of the invaded range. Models taking only data from the native range, where species is in equilibrium with environment, will predict species establishment of populations in only areas with similar climatic conditions as their native range. First empirical evidence that an invasive alien species can occupy climatically distinct niche following introduction into new area was shown in spotted knapweed, *Centaurea maculosa* L. (Broennimann et al. 2007) and, as a result, SDM failed to predict the invaded distribution of this species. Beaumont et al. (2009) quantified differences among the native and invasive realized niches of *Hieracium* species, providing further evidence that calibration of SDMs based on native region data only decreases predictive capacity of model, misrepresenting their potential invasive distribution. On the other hand, in case of the Asian hornet, *Vespa velutina nigrithorax* du Buysson, 1905 (Barbet-Massin et al. 2018), results showed better predictive accuracy when models were calibrated using only data from the invaded region which suggest the lack of niche conservatism.Once the SDMs have been considered valuable after evaluation (e.g., cross-validation), the outputs of SDMs can be used to generate **risk maps** (Figure 1). Risk maps are visual tools to describe where invasive alien species might arrive, establish, spread, or cause harmful impacts according to the variables considered in the SDMs. These maps will be used by the managers or govement agencies to inform management decisions (Jiménez-Valverde et al. 2011). All risk maps have an associated error or uncertainty which is important to account for the interpretation of the results. Also, risk maps should be interpreted with a full understanding of what the map is showing as well as the assumption in the modelling processs. Maps of the potential distribution of an invasive alien species do not indicate those areas where the species will have a higher impact than in other areas or the areas that due to suitability invasion will occur first. Thus, maps often reflect components that contribute to overall pest risk, but not the risk in its entirety (Venette et al. 2010).

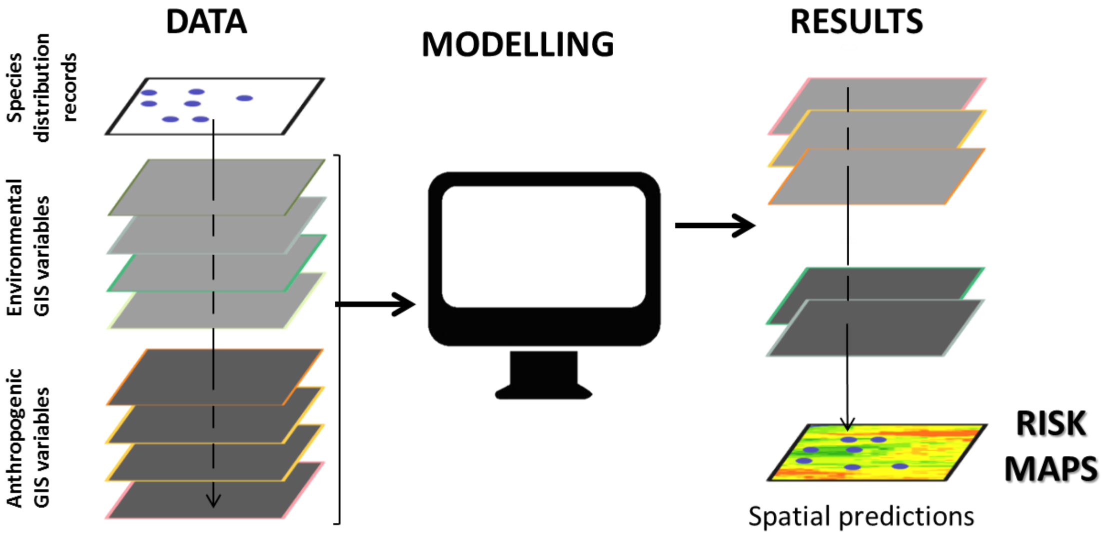

Figure 1. Risk map created by using SDMs.

Explanation of the work carried out and overview of pogress

In aquatic environments, species-occurrence records are scarce, and only a fraction of aquatic invaders are well tracked (Ready et al. 2010). The use of SDMs in aquatic environments is further limited because the predictor variables that determine species presence (e.g., water temperature, salinity, and dissolved oxygen) are often unavailable for many regions (Ready et al. 2010) or, as reported above, are not related witht the species invasion due to niche shift. In addtion, authors have suggested that human-mediated expansion might be more relevant than the expansion due to climate change, which might have important implications for management of aquatic invasive species. Hence, in this project we have implemented two studies: a first one based on the inclusion of anthropogenic variables that migh explain the spread of invasive alien species in the United Kingdom, and a second one to study the impact of climate change on the world distribution of a notable invasive alien species, i.e. the North American bullfrog, *Lithobates catesbeianus*.

The effects of different factors on invasive species occurrence can vary among ecosystems and across spatial and temporal scales. Ribeiro et al. (2008) suggested that characteristics of the recipient ecosystem are as relevant as the characteristics of the invading species. Human-related factors play a predominant role in explaining the invasion of non-native species in economically developed realms, as is the case for the UK (Gallardo & Aldridge 2013). However, in other realms, the environmental characteristics of the river basins are the ones best explaining the distribution and spread of invasive alien species. Recent studies suggest that dispersal at broad spatial scales is directly shaped by human intervention but that dispersal at smaller spatial scales is often dictated by natural phenomena (Medley et al. 2015). Therefore, one of the objectives of Aquainvad-ED project within the topic of distribution pathways in fresh waters (WP2) is a methodological study to determine the importance of different type of variables in the biological invasion of different taxa at macroecological scale. The outputs of this study can provide valuable insights for the developing better predictive models and thus more effective risk maps.

Some species might exert relevant impacts on biosecurity, economy and conservation, as it is the case for zebra mussel, *Dreissena polymorpha*, included in the lost of the 100 worst invasive species worldwide and in Europe. With the purpose of prioritizing and facilitating management, a risk map has been created using the most important variables responsible for the distribution of zebra mussel in the United Kingdom (for details of model please see D2.1). Using the same approach and the same predictor variables, our results can be extrapolated to other regions. For instance, zebra mussel distributon is mainly shaped by boat ramps. According to this, the risk map (Figure 2) shows many localities located in England as the most vulnerable to invasion. Thus, preventive measures (e.g. cleaning the boats) should be taken to avoid the arrival of this species

Predicting dispersal on amphibians presents an interesting challenge because they can uniquely use both terrestrial and aquatic systems (Peterson et al. 2013). The North American bullfrog, *Lithobates catesbeianus* (hereafter referred to as bullfrog), is one of the hundred worst invasive species in the world (Nori et al. 2011) that has been associated with declines in native amphibian populations (Peterson et al. 2013). Except for being carriers of *Batrachochytrium dendrobatidis,* a fungus that is the agent of chytridiomycosis, the large tadpoles can outcompete the larvae of native species while adults can directly prey on native amphibians (Ficetola et al. 2007). Bullfrog inhabit water bodies, such as ponds or reservoirs, and the availability of permanent water bodies is critical for bullfrog physiological and ecological requirements at all stages of their life history, and therefore for its establishment. In previous models developed for the species, human footprint and climate (minimum temperature ranging between -20 and +14 °C) were found to be positively correlated (Ficetola et al. 2007) on global level, while native frog species richness, usually taken as important for resistance to invasions, showed not negatively correlated to the likelihood of *L. catesbeianus* population establishment in China (Liu & Li 2009).

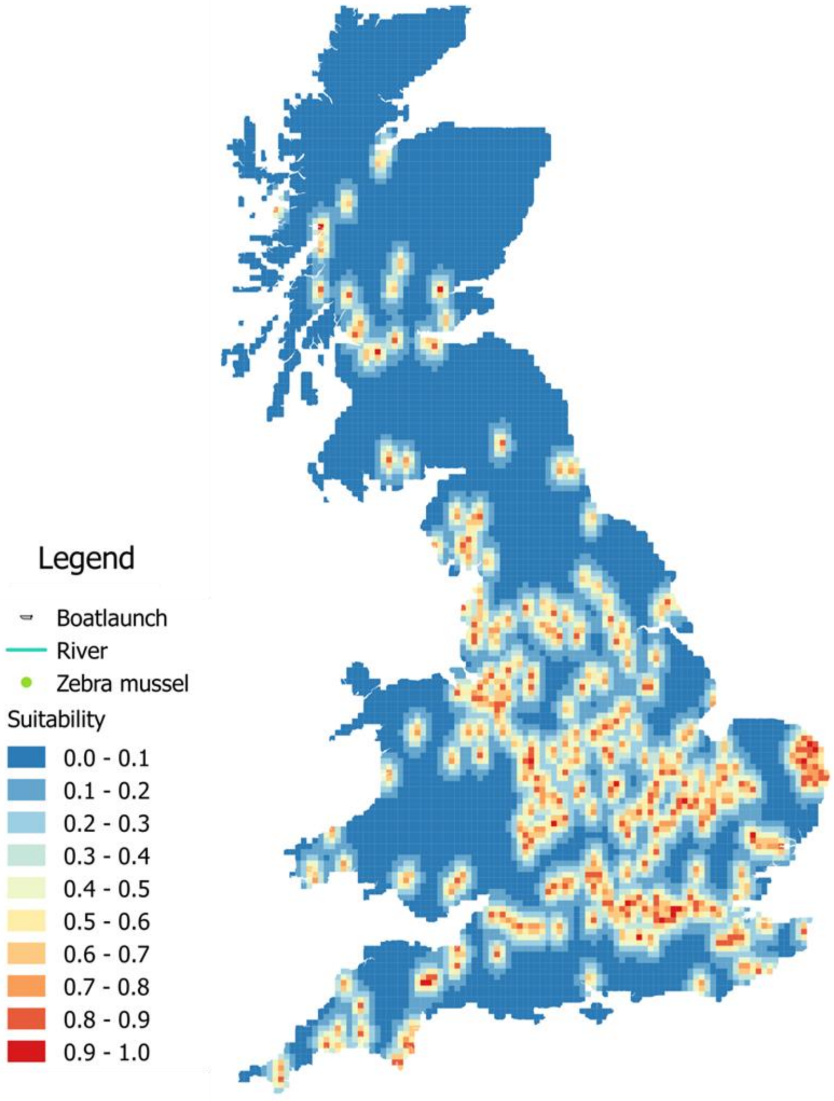

Figure 2. Risk map of zebra mussel in the United Kingdom. The most vulnerable areas are indicated in orange/red.

Climate suitability is commonly used as the most important factor for predicting the potential distribution of invasive alien species (Liu & Li 2009). For the work carried out under Aquainvad-ED project, the current and future climatic suitability areas of the invasive species *L. catesbeianus* were assessed at worldwide level. The species distribution modelling was based on nine different algorithms in the BIOMOD2 package (Gama et al. 2017; Thuiller et al. 2016), and summarized in an ensemble forecasting approach. To model the species distribution, six climatic variables ecologically meaningful for the selected species were considered: BIO4 = Temperature Seasonality, BIO5 = Max Temperature of Warmest Month, BIO6 = Min Temperature of Coldest Month, BIO13 = Precipitation of Wettest Month, BIO14 = Precipitation of Driest Month and BIO15 = Precipitation Seasonality, chosen from a set of 19 different climatic variables from the WorldClim datasets (Hijmans et al. 2005, version 1.4; http://www.worldclim.org/). Three timeframes (current, 2050 and 2070) were modeled using two increasing CO_2 emission scenarios. The performance of individual models was fair according to the area under the receiver operating characteristic curve (AUC) and useful according to true skill statistics (TSS). The individual models that best performed where random forest (RF) for both AUC and TSS. Temperature seasonality, minimum temperature of the coldest month, maximum temperature of the warmest month and precipitation of the driest month were the most important variables predicting bullfrog occurrence. Current suitability maps identified that in the future an increase in adequate areas is expected at higher latitudes, especially in North America and Central Europe and a concomitant decrease in adequate areas around the Mediterranean (Figure 3). Current adequate areas for the species represent 3.8% worldwide and these are expected to increase up to 5.2% in the future. Overall, the results indicate that climate change will favor the expansion of *L. catesbeianus* into new river basins, and conservation efforts should take place in order to minimize negative impacts of bullfrog presence and protect native amphibian species under threat.

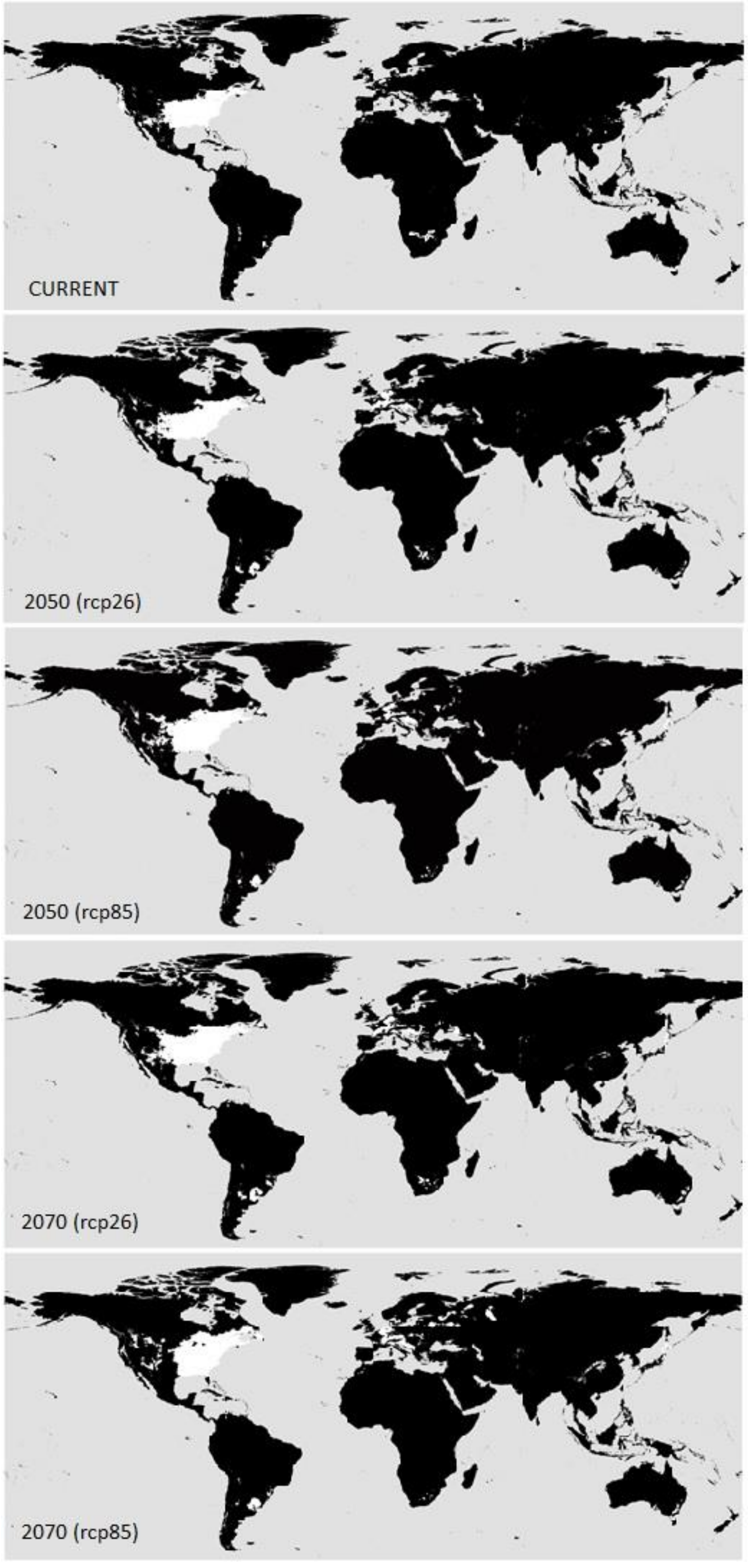

Figure 3. Binary maps (suitable/white–unsuitable/black areas) of predicted worldwide distribution for three time periods (current, 2050 and 2070) and two RCP scenarios (rcp26 and rcp85).

Conclusions

Based on previous research, we concentrated our analysis on factors that can affect the introduction and establishment of the study species. The methodologies used to identify pathways of invasion in freshwater as well as the climatic suitability provided reliable models that allowed us to produce risk maps that can be used for management actions.

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